where $DF_{octanol}$ and $DF_{aqueous}$ are the respective octanol and aqueous dilution factors.

Partition Coefficients: Hydrophilic Compounds ($P < 50$). The inhibitor (0.3–0.5 mg) was dissolved in each buffer (2 mL, vide supra), octanol (2.00 mL) was added, and the mixture was shaken and centrifuged as described above. A portion of the octanol phase (50.0 μL) was evaporated at 40 °C under a stream of dry, filtered air and was reconstituted in the appropriate mobile phase (800 μL). The remaining octanol phase was removed by aspiration and the aqueous phase (50.0 μL) was diluted with the HPLC mobile phase (950 μL).

In Vitro Enzyme Assays. Assays of purified human renal renin,[18] bovine cathepsin D,[18] pepsin,[18] and human plasma renin[2b] were performed as previously described. Inhibition of monkey plasma renin was measured by the same procedure as for human plasma renin except that only one-half of the incubation volume was assayed.

Monkey Experiments. Monkeys were dosed intravenously ($n = 5$) and intraduodenally ($n = 2$) with compound **21** as described previously.[2b] Blood samples were withdrawn for measurement of plasma renin activity.[19] Plasma levels (P_L) of **21** were determined in the blood samples from two of the iv monkeys (5, 15, 30, 60, 120, and 180 min samples) by a renin inhibition assay as described previously for id rat experiments.[2d] The data were fitted to a bis-exponential decay model:

$$\text{monkey 1} \quad P_L = 289.1e^{-0.1991T} + 4.964e^{-0.02385T}$$

$$R = 0.99967$$

$$\text{monkey 2} \quad P_L = 1009e^{-0.2270T} + 8.581e^{-0.02093T}$$

$$R = 0.99996$$

Drug Metabolism Studies. Compound **21** was labeled with ^{14}C at the oxazolidinone carbonyl[13] and had a specific activity of 57 μCi/mg. Male Sprague–Dawley rats, weighing 180–300 g, were dosed at 1 mg/kg either orally by gavage or intravenously into the femoral vein. Urine and feces were collected over 3 days following drug administration. Bile was collected from another two rats (one each dosing procedure) after surgical implantation of a bile duct cannula under diethyl ether anesthesia. The feces were homogenized in 70% aqueous ethanol and aliquots were burned in a sample oxidizer. All samples were assayed for total radioactivity by liquid-scintillation spectrometry and corrected for quenching with an internal standard.

Metabolic patterns is urine, bile, and fecal samples were determined by HPLC on a C-18 column with a linear 15–50% aqueous acetonitrile gradient containing 0.1% trifluoroacetic acid. Some of the radioactive peaks in the samples were tentatively identified by comparison of their retention times with those of authentic reference standards, based on absorbance at 215 nm or radioactivity. A second set of HPLC conditions using 41.5% aqueous acetonitrile containing 0.01 M tetramethylammonium perchlorate, 0.01 M dodecylsulfate disodium salt, and 0.01 M sodium phosphate was employed to demonstrate the absence of the free amino compound resulting from cleavage between histidine and the benzylsuccinate residue.

Intraduodenal Rat Experiments. Rats were dosed id with compound **21** and plasma drug levels were determined by a renin inhibition assay as described previously.[2d]

Acknowledgment. The assistance of Jill Kadam and Gary Young with iv monkey experiments, of Jeff Elst with partition coefficient determinations, of Gary Rotert with the preparation of [^{14}C]-**21**, and of Mary Jo Leveque with solubility determinations is gratefully acknowledged.

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Inhibition of Ornithine Decarboxylase by the Isomers of 1,4-Dimethylputrescine

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1,4-Dimethylputrescine (2,5-hexanediamine) was separated into its racemic and meso isomers by fractional crystallization of its dibenzoyl derivative. The racemic form was resolved into its (+)- and (−)-isomers with (+)- and (−)-dibenzoyltartaric acids. None of the three isomers (meso, +, and −) inhibited ornithine decarboxylase (ODC) activity in vitro, while all the three were strongly inhibitory of ODC when assayed in vivo in rats or in H-35 hepatoma cells. In rat liver the three isomers also decreased the putrescine pool while only the (+)-isomer decreased spermidine content. In the H-35 cells the (−)- and (+)-isomers decreased the spermidine and spermine content. When ODC was induced in the latter by insulin it was found that the (−)-isomer strongly inhibited protein and ODC synthesis, while the (+)-isomer and the meso isomer were less inhibitory. The meso isomer was a good inducer of ODC antizyme in rat liver, while the (+)- and (−)-isomers were poor inducers of the former.

Ornithine decarboxylase (ODC, L-ornithine decarboxylase, EC 4.1.1.17) is a permanent target for studies on the inhibition of the proliferative and neoplastic processes.[1,2] The inhibitors include ornithine and polyamine (putrescine, spermidine, and spermine) derivatives and are usually of three types. To the first type belong the "suicide" or mechanism-based enzyme inhibitors such as the substance analogue (difluoromethyl)ornithine (DFMO), and the product analogues of the alkyne 1,4-diaminobutane type.[3] DFMO was found to be useful in the treatment of human parasitic diseases.[4] To the second type belong polyamine analogues such as alkylspermidines which do not directly inhibit the enzyme but exert a feed-back repression.[5] Finally, the third type of ODC inhibitors are the product analogues which are competitive inhibitors of ODC such as the *N*-alkyl, 1-alkyl, and 2-alkylputrescines.[6,7] It was shown that among the latter the methylputrescines are the best in vivo inhibitors of liver ODC in rats treated with thioacetamide or dexa-

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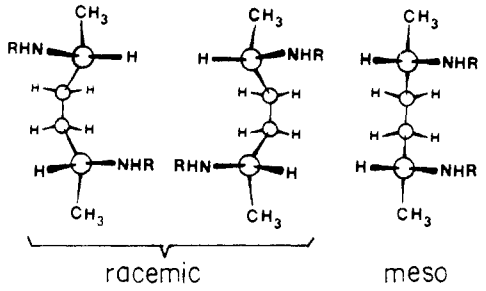


Figure 1. Racemic and meso isomers of 1,4-dimethylputrescine (R = H) and *N,N'*-dibenzoyl-1,4-dimethylputrescine (R = COC_6H_5).

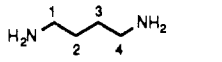
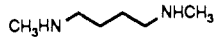
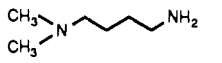
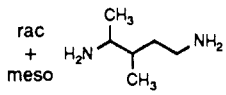
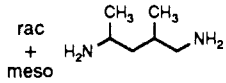
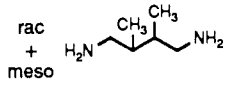
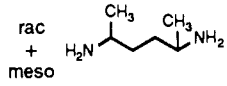
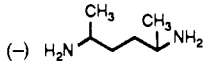
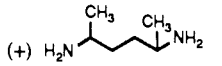
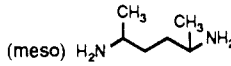
methasone.[6,7] They also inhibit de novo ODC synthesis in H-35 hepatoma cells.[8] It was therefore of interest to analyze the in vivo and in vitro effects of dimethylputrescines on ODC activity in order to define which of their structural requirements are necessary for the inhibition of ODC activity.

It has been shown that 1,4-dimethylputrescine (1,4-DMP) is not inhibitory of ODC activity when assayed directly on the enzyme but strongly inhibits the enzyme when assayed in vivo.[8,9] This diamine is not degraded by the diamine oxidases which oxidize putrescine[10] and therefore decrease the intracellular levels of pharmacologically active diamines. Enzyme inhibition is usually related to the diastereoisomerism of the inhibitor, although there are examples where a lack of stereospecificity was found.[11] Hence, 1,4-dimethylputrescine was resolved into its meso-, (+)-, and (−)-isomers (Figure 1, R = H) and the in vivo inhibitory effect on ODC activity of each of the isomers was examined in order to establish if the inhibition by this diamine is stereospecific.

Results and Discussion

Resolution of 1,4-Dimethylputrescine into Its Isomers. 1,4-Dimethylputrescine (2,5-hexanediamine) was prepared by reduction of 2,5-hexanedione dioxime with sodium in ethanol following the original method[12] rather than the newer one which made use of Raney Ni.[13] Separation of the racemic and meso isomers was achieved following an early lead[14] which reported that *N,N'*-dibenzoyl-2,5-hexanediamine (Figure 1, R = PhCO) can be separated by fractional crystallization from absolute ethanol into a so-called ϕ-isomer and a κ-isomer. We found that the more insoluble ϕ-isomer is *meso-N,N'*-dibenzoyl-2,5-hexanediamine, while the more soluble κ-isomer is the racemic derivative. While the ^{13}C NMR spectrum of 2,5-hexanediamine does not differentiate between the meso and the racemic isomers, the spectrum of the dibenzoyl derivative clearly shows the mixture of both isomers. It also allows the monitoring of their separation, and both isomers can also be differentiated by TLC analysis. Hydrolysis with concentrated hydrochloric acid of the *meso*-dibenzoyl derivative afforded *meso*-2,5-hexanediamine dihydrochloride.

Table I. Effect of Dimethylputrescines on the in Vitro and in Vivo Activity of Ornithine Decarboxylase

structure	inhibition of ODC activity: in vitro K_i, mM	in vivo[a] rat liver (time, h)	% H-35 cells
	3.3	55 (1)	90
	no inhibn	30 (1)	72
	3.0	60 (1)	68
rac + meso 	3.8	70 (1)	75
rac + meso 	2.9	76 (1)	85
rac + meso 	1.0	ND[b]	ND
rac + meso 	no inhibn	96 (1), 70 (3)	91
(−) 	no inhibn	97 (1), 90 (3)	97
(+) 	no inhibn	98 (1), 70 (3)	96
(meso) 	no inhibn	93 (1), 65 (3)	90

[a] For the in vivo studies 75 μmol/100 g of rat weight of the diamine were injected intraperitoneally to thioacetamide-treated rats either 1 or 3 h before sacrifice. The rat liver enzyme had a specific activity of 4.7 ± 0.3 units/mg of protein, while the hepatoma cell line had an activity 3.8 ± 0.4 units/mg of protein. [b] ND = none detected.

The resolution of the *rac*-2,5-hexanediamine was best achieved by treatment of the synthetic base (the mixture of the meso and racemic isomers) with either (−)-dibenzoyltartaric acid, which allowed the isolation of the corresponding (−)-diastereoisomer, or with (+)-dibenzoyltartaric acid, which afforded the (+)-diastereoisomer. The corresponding (−)- and (+)-bases were isolated from the tartrates by treatment with alkali and extraction of the base from the aqueous solutions with chloroform, followed by a final purification using a distillation step. (−)-2,5-Hexanediamine had $[\alpha]_D = -6.1°$ and the (+)-isomer had $[\alpha]_D = +6.1°$. The corresponding hydrochlorides could be prepared from the tartrates by treatment with hydrochloric acid and had opposite rotations to those shown by the respective bases.

The dibenzoyl derivatives of the (−)- and (+)-2,5-hexanediamines were identical with those obtained by resolution of the κ-dibenzoyldiamine. Hydrolysis of the latter followed by resolution of the free base with the optically active tartaric acids afforded the optically active tartrates. Decomposition of the latter with alkali, followed by benzoylation of the (−)- and (+)-diamines, established that the κ-isomer is identical with the racemic isomer.

Effect of Dimethylputrescines on the in Vitro and in Vivo Activity of ODC. Putrescine is a weak inhibitor of ODC. Its K_i toward the enzyme isolated from the livers of thioacetamide-treated rats is shown in Table I. Alkylation of both NH_2 groups with methyl residues abolished its inhibitory effect, while dimethylation of only one

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(10) Frydman, R. B.; Ruiz, O.; Kreisel, M.; Bachrach, U. *FEBS Lett.* **1987**, *219*, 380–386.
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(12) Ciamician, G.; Zanetti, C. U. *Ber. Deutsch. Chem. Ges.* **1889**, *22*, 1968.
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(14) Günther, H.; Tafel, J. *Ber. Deutsch. Chem. Ges.* **1895**, *28*, 328.

Table II. Effect of the Isomers of 1,4-Dimethylputrescine on the Polyamine Content of Dexamethasone- and Thioacetamide-Treated Rats

treatment	time, h	polyamine content, nmol/g of liver			
		putrescine	spermidine	spermine	1,4-dimethylputrescine
dexamethasone		980 ± 70 (100)[a]	2300 ± 200 (100)	2170 ± 200 (100)	
thioacetamide		1370 ± 100 (100)	2680 ± 250 (100)	2400 ± 200 (100)	
(−)-isomer dexamethasone	1	100 ± 10 (10)	1280 ± 150 (8)	2240 ± 200 (100)	1640 ± 150 (18)[b]
	4	580 ± 30 (59)	2080 ± 180 (90)	2220 ± 250 (100)	40 ± 5 (0.5)
thioacetamide	1	55 ± 10 (4)	1870 ± 180 (70)	2600 ± 300 (107)	2700 ± 300 (27)
	3	290 ± 20 (21)	2400 ± 200 (90)	3000 ± 200 (120)	1550 ± 18 (15)
(+)-isomer dexamethasone	1	60 ± 5 (6)	2180 ± 200 (95)	1930 ± 200 (90)	1800 ± 100 (20)
	4	660 ± 50 (67)	850 ± 100 (37)	1800 ± 150 (83)	400 ± 50 (4)
thioacetamide	1	50 ± 10 (4)	1950 ± 200 (73)	2500 ± 180 (103)	1850 ± 180 (18)
	3	510 ± 50 (37)	1475 ± 100 (55)	2600 ± 200 (107)	900 ± 100 (9)
meso isomer dexamethasone	1	160 ± 5 (16)	1920 ± 200 (83)	2060 ± 180 (95)	1640 ± 180 (18)
	4	890 ± 100 (91)	2280 ± 200 (98)	2250 ± 250 (104)	50 ± 5 (0.5)
thioacetamide	1	360 ± 50 (26)	2800 ± 200 (104)	2350 ± 200 (97)	2800 ± 300 (28)
	3	410 ± 50 (30)	2500 ± 230 (98)	2350 ± 250 (97)	1150 ± 150 (12)

[a] The values in parentheses indicate the percent of polyamine content; the values of the polyamine content in the livers treated with dexamethasone or thioacetamide are taken as 100%. The results are the mean ± SD of three experiments in duplicate. [b] The percentage is calculated with respect to the total analogue injected/g of liver (10 ± 0.5 μmol).

NH_2 did not (Table I). The presence of one methyl residue at any position of the butane chain (except for the 1,2-dimethyl analogue) increased the inhibitory effect of the diamine. Substitution by two methyl residues decreased the inhibitory effect, which was entirely abolished in 1,4-dimethylputrescine (Table I). Hence, for the in vitro inhibitory activity it is necessary that either the C_1 or C_4 methylene should be unsubstituted. 1,4-Dimethylputrescine was however a strong in vivo inhibitor of ODC when assayed in rats or in H-35 hepatoma cells (Table I). When the meso-, (+)-, and (−)-isomers of 1,4-dimethylputrescine were assayed, similar results were obtained with each of them (Table I). None of them affected ODC activity in vitro, while all the three were inhibitory in vivo. However, when the isomers were examined as a function of concentration, the meso isomer was the weakest inhibitor at lower doses while the (+)- and (−)-isomers showed similar inhibitory effect (Figure 2A). The ED_{50} (half maximal effective values) were calculated from the dose–response curves and were found to be 12.5 ± 1.5 μmol/100 g of rat weight for the (+)- and (−)-isomers and 40 ± 2.7 μmol/100 g of rat weight for the meso isomer. When their effect was examined as a function of time, the meso isomer showed the least lasting effect (Figure 2B and Table I). At higher doses and shorter times the three isomers showed similar inhibitory effects, suggesting a lack of strict stereospecificity.

These results led to a search for a possible differential clearing of the three isomers from the livers of the treated rats, as well as of their effect on the free polyamine pools. No significant differences were found among the three isomers when their concentrations in the liver were measured 3–4 h after their administration to the animals (Table II). In thioacetamide-treated rats a slower disappearance of the isomers from the livers was found than in the dexamethasone-treated animals. Since 1,4-dimethylputrescine was not oxidized by oxidases[10,15] and since it was not converted into its aminopropylated or aminoacetylated derivatives,[15] the disappearance of the isomers from the liver should be due either to excretion or to binding to macromolecules. The three isomers strongly decreased the putrescine pool 1 h after administration, while at longer periods (3–4 h) this pool increased again but never reached the level of those of the untreated animals (Table II). It has been reported that 1,4-dimethylputrescine (the mixture of the racemic and meso forms) decreased spermidine content in chick embryos probably through inhibition of the aminopropyl transferases.[15] We found that only the (+)-isomer decreased spermidine content in rat liver (Table II).

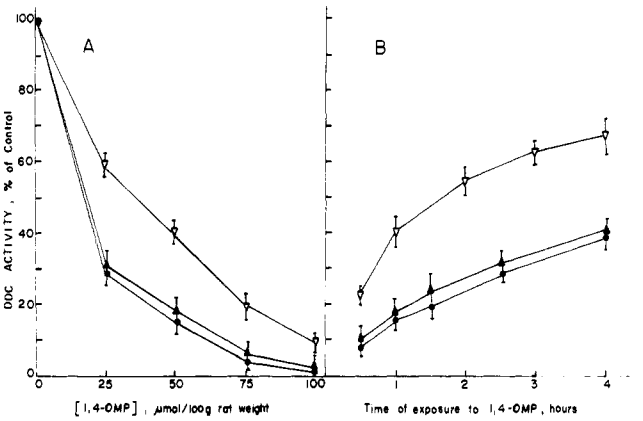

Figure 2. (A) Dose response of increasing concentrations of 1,4-dimethylputrescine (−)-isomer (●), (+)-isomer (▲), and meso isomer (▽) on the liver ODC activity of dexamethasone-treated rats. (B) Effect of the isomers as a function of time. The putrescine analogues were injected at a 50 μmol/100 g of rat weight dose. Values represent the mean ± SD of four experiments with three rats each.

In cells treated with DFMO the uptake of chemicals such as MGBG and polyamines was highly enhanced.[16] In the aforementioned cases the polyamine pools were deprived by the DFMO treatment. When the isomers of 1,4-dimethylputrescines were administered together with DFMO to thioacetamide-treated rats, the concentration of the meso isomer was greater (after 1 h) by about 130% as compared to those of the controls, where the DFMO was omitted. The concentrations of the (−)- and (+)-isomers were greater by 65%. Therefore, DFMO apparently facilitates the transport of the diamines into the liver, although the combined action of DFMO and 1,4-dimethylputrescine was not additive in decreasing the endogenous polyamine pools, which were similar to those given in Table II for the thioacetamide-treated rats 1 h after administration.

When assayed on the H-35 cell line, the three isomers of 1,4-dimethylputrescine were found to decrease the ODC

(15) Sarhan, S.; Dezeure, F.; Seiler, N. *Int. J. Biochem.* **1987**, *19*, 1037–1047.

(16) Alhonen-Hongisto, L.; Seppanen, P.; Jänne, J. *Biochem. J.* **1980**, *192*, 941–945.

Table III. Effect of the 1,4-Dimethylputrescine Isomers on H-35 Hepatoma Cell ODC Activity and Polyamine Pools[a]

treatment	ODC activity, units/mg of protein	putrescine[d]	spermidine[d]	spermine[d]
DMEM[b]	1.30 ± 0.05	29 (47)	190 (57)	165 (37)
insulin	6.20 ± 0.8	62 (100)	335 (100)	452 (100)
insulin + (−)-1,4-DMP	0.78 ± 0.04	43 (69)	139 (41)	218 (48)
insulin + (+)-1,4-DMP[c]	0.78 ± 0.06	15 (24)	183 (55)	270 (60)
insulin + *meso*-1,4-DMP	1.05 ± 0.13	49 (80)	183 (55)	300 (66)

[a] The results are the mean of four experiments in duplicate for the ODC activity determinations and of three experiments in duplicate for the polyamine determinations. [b] Dulbecco's minimal essential medium. [c] The isomers (0.1 mM) were administered to the cell together with the inducer and incubated for 4 h. The values in parentheses indicate the percent of polyamine content compared to the polyamine content of the insulin cells, which is taken as 100%. [d] nmol/10^7 cells.

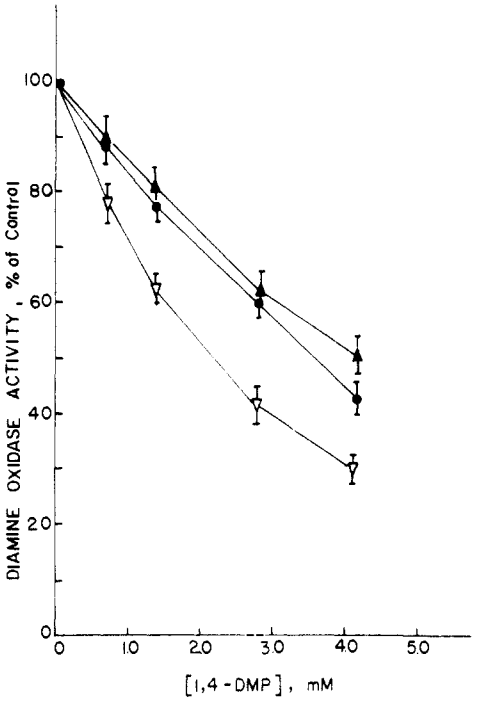

Figure 3. Effect of increasing concentrations of 1,4-DPM (−)-isomer (●), (+)-isomer (▲), and meso isomer (▽), on the oxidation of putrescine by hog kidney diamine oxidase. Values represent the mean ± SD of three experiments.

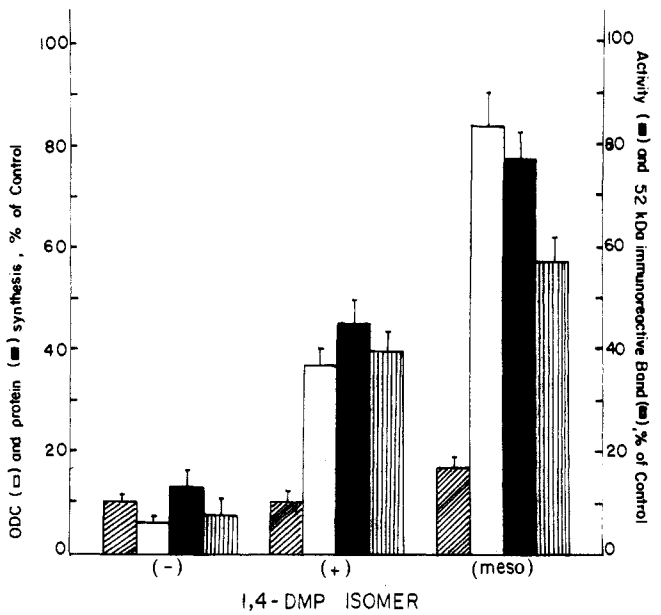

Figure 4. Effect in H-35 cells of the 1,4-dimethylputrescine isomers on the ODC activity (diagonal stripes), protein synthesis (solid), ODC synthesis (open), and 52-kDa ODC band (verticle stripes). The results are the mean ± SD of three experiments in duplicate. The ^{35}S incorporation into total proteins was 108 ± 7.2 × 10^4 dpm/mg of protein; incorporation into ODC in the control: 720 ± 36 dpm/mg protein.

activity below its level in the noninduced cells (Table II). They also reduced the free polyamine pools in the cells and [at variance with the results obtained in rat liver (Table I)] the (−)- and (+)-isomers markedly decreased the spermidine and spermine content of the cells. Both in rat liver and in H-35 cells, the meso isomer decreased the putrescence pool by less than the (+)- and (−)-isomers.

Putrescine was oxidized by plant and mammalian diamine oxidases, while 1,4-dimethylputrescine was not.[10,15] The effect of the three isomers of the latter on the activity of the oxidases of both origins was examined and it was found that the three isomers inhibit the mammalian oxidase more than the plant oxidase. The meso isomer was the strongest inhibitor (Figure 3). Hence, the weaker inhibitory effect found for the meso isomer on the putrescine pool (Table II) could in part be explained by its higher inhibition of the oxidase which degrades putrescine. The aforementioned results suggest that there is no strict stereospecific interaction between the diamine oxidase and the optical isomers of 1,4-dimethylputrescine.

Effect of *meso*-, (+)-, and (−)-1,4-Dimethylputrescines on the Modulation of ODC Activity. The molecular mechanism by which the three isomers of 1,4-dimethylputrescine exert their inhibitory effect on ODC activity was explored in the H-35 cells and in rat liver. In the H-35 cells they inhibited the enzyme to a similar extent when added together with insulin (Table III). The possibility that this similarity could result from an averaging of different inhibitory molecular mechanisms led us to examine the effect of each of the three isomers on protein synthesis and ODC synthesis in relation to ODC activity. [^{35}S]Methionine was added to the cell cultures 30 min before harvest and 3.5 h after administration of the insulin (see the Experimental Section). The labeled ODC was immunoprecipitated with an excess of ODC–polyclonal antibodies. The radioactive immunoprecipitated enzyme was subjected to SDS-PAGE and the labeled immunoreactive ODC bands were cut out and counted for radioactivity. The effects of the three isomers on ODC activity, protein synthesis, and ODC synthesis are shown in Figure 4. The (−)-isomer strongly inhibited both protein and ODC synthesis, the (+)-isomer was less inhibitory of both, while the meso isomer affected very little protein and total ODC synthesis. The decrease in the total ODC synthesis was coincident with the decrease in the 52-kDa ODC band when the (−)- and (+)-isomers were assayed. For the meso isomer the decrease of the 52-kDa ODC band was higher than the decrease of the total ODC synthesis (Figure 4) due to the formation of labeled proteins of smaller molecular weights (ca. 10, 23, and 36 kDa). These smaller proteins were also immunoreactive with the ODC–polyclonal antibodies and they are very likely ODC degradation products. Therefore it is conceivable that while the (−)-isomer completely inhibited ODC synthesis; the meso

isomer did not affect the latter but strongly increased its degradation.

The effect of each isomer on the induction of antizyme[17] in rat liver was examined after an intraperitoneal injection of each of them (75 μmol/100 g of rat weight) into dexamethasone-treated rats 1 h before sacrifice. At this concentration ODC activity was inhibited to a similar degree (95 ± 5% inhibition) by all the three isomers. The 150000*g* supernatant fractions of the liver extracts were filtered through a Sephadex G-75 column in the presence of 250 mM NaCl and the eluates were assayed for antizyme activity. It was found that the meso isomer was a good inducer of the antizyme (6 units/mg of protein in the 150000*g* supernatant), while the (+)- and (−)-isomers induced only about 25% of the amount of antizyme induced by the meso isomer. Since it is known that antizyme will interact with ODC to give an inactive antizyme–ODC complex which is degraded faster than free ODC,[18] the induction of antizyme by the meso isomer could lead to an increased degradation of ODC. Therefore, although the three isomers apparently showed little overall stereospecificity in their inhibitory effect on ODC activity both on hepatoma cells and rat liver, there seems to be a higher selectivity when the mechanism by which they exert their inhibition is more closely examined.

Conclusions

ODC is inhibited by its reaction product: 1,4-diaminobutane (putrescine). Alkylation of one of the two NH_2 groups did not abolish this inhibitory effect, which was only lost if both NH_2 groups were alkylated. Introduction of methyl residues at any position of the butane chain resulted in more active inhibitors of ODC. 2,3-Dimethylputrescine and 2-methylputrescine have similar K_i's and are more efficient inhibitors than putrescine itself, while 1,2-dimethylputrescine and 1,3-dimethylputrescine are weaker inhibitors than 1-methyl- and 2-methylputrescines. The latter are better inhibitors than putrescine.

Substitution of both the C_1 and C_4 methylenes of the butane chain resulted in an inactive diamine. Either the meso, the (+)-, or the (−)-1,4-dimethylputrescines were devoid of inhibitory effects on ODC. They were, however, strong inhibitors of rat liver ODC or of ODC from hepatoma cells when assayed in vivo. Synthetic 1,4-dimethylputrescine (the mixture of the meso and racemic isomers) was found to inhibit ODC activity in the Ehrlich ascites tumor cells.[9] There were no significant differences in the in vivo inhibitory effect of the aforementioned three isomers of 1,4-dimethylputrescine which could suggest an absence of strict stereospecificity. However, a closer examination at the molecular level revealed significative differences in their mode of action. While the (−)-isomer almost completely abolished de novo protein and ODC synthesis, the meso isomer did not affect the latter but strongly increased ODC degradation. In rat liver this isomer strongly induced antizyme formation.

It was known that 1,4-dimethylputrescine inhibited the diamine oxidase from mammalian origin.[10] The three isomers of the diamine were found to be inhibitory, although the meso isomer exerted a greater inhibition. This lack of stereospecificity could be expected from what is known about the broad substrate specificity of oxidases. The isomers were not inhibitory of the plant diamine oxidase, suggesting that the architecture of the latter is different from that of the mammalian enzyme. All the three isomers of 1,4-dimethylputrescine strongly decreased the putrescine content in rat liver and in the H-35 cells, but only the (+)-isomer decreased the liver spermidine content, probably due to a specific inhibition of the aminopropyl transferase. This result should be confirmed by a direct assay of the isomers on the aminopropyl transferases.

Experimental Section

Melting points were determined on a Kofler melting point apparatus and are uncorrected. ^{13}C NMR spectra were recorded on a FT-80A spectrometer. Optical activities were measured with a Perkin-Elmer Model 10141 automatic polarimeter. Microanalysis were performed by UMYMFOR (University of Buenos Aires-CONICET). TLC was performed either on silica gel F-254 plates (Merck, 0.25 mm layer thickness) or on precoated cellulose plates (Merck, 0.1 mm layer thickness). Dioximes were spotted with a 5% ferric chloride aqueous solution, and diamines were spotted by spraying with a ninhydrin solution (0.5% ninhydrin, 0.4% acetic acid, 5% 2,6-lutidine in acetone) followed by heat (100 °C).

Preparation of *rac*- and *meso*-2,5-Hexanediamine (1,4-Diamineputrescine). Sodium (40 g) was added in small chips to a solution of 20 g of 2,5-hexanedione dioxime in 500 mL of ethanol. The mixture was heated under reflux during 4 h while magnetic stirring was kept throughout the reaction. It was then cooled, 400 mL of ice–water were added, the solution was extracted with chloroform (6 × 150 mL), the extracts were pooled and evaporated to dryness in vacuo at 40 °C, and the hexanediamine was distilled at 86 °C (30 mmHg) [lit.[12] bp 175 °C (753 mmHg)]: 8.2 g (51%); ^1H NMR (Cl_3CD) δ 2.86 (m, 2, CH), 1.35 (m, 8, CH_2, NH_2), 1.05 (d, 6, CH_3). ^{13}C NMR (Cl_3CD) δ 45.20 (CH), 35.20 (CH_2), 22.40 (CH_3).

Separation of *rac*-*N,N'*-Dibenzoyl-2,5-hexanediamine and *meso*-*N,N'*-Dibenzoyl-2,5-hexanediamine. 2,5-Hexanediamine (4.2 g of the rac and meso mixture) was dissolved in 8 mL of water, 4.2 g of Na_2CO_3 were added, followed by 8 g of benzoyl chloride which were added in 4 portions to the cooled (5 °C) solution over a 30-min period while the mixture was constantly stirred. After the addition was completed, the mixture was further stirred at 20 °C during 30 min, 15 mL of a concentrated NaOH solution was then added, the mixture was stirred for 5 min, and the precipitate was filtered, washed with cold water, and dried. It was crystallized twice by dissolution in the minimum amount of boiling absolute ethanol. The *meso*-*N,N'*-dibenzoyl-2,5-hexanediamine crystallized on cooling (5 °C) of the ethanol solution: 5.1 g (44%); mp 244 °C (lit.[14] mp 238 °C for the *N,N*-dibenzoyl-φ-2,5-hexanediamine); ^{13}C NMR (TFA) δ 174.73 (CO), 135.95, 130.30, 128.80, 128.42 (C_6H_5), 50.83 (CH), 32.89 (CH_2), 19.16 (CH_3). Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

The filtrates were concentrated to a small volume and the *rac*-*N,N'*-dibenzoyl-2,5-hexanediamine which precipitated was filtered and recrystallized from absolute ethanol: 5.0 g (43%); mp 203–205 °C (lit.[14] 193–198 °C for the *N,N'*-dibenzoyl-κ-2,5-hexanediamine); ^{13}C NMR (TFA) δ 174.70 (CO), 135.92, 130.28, 128.83, 128.37 (C_6H_5), 51.17 (CH), 32.94 (CH_2), 19.18 (CH_3); TLC (3% MeOH in $CHCl_3$) *meso*-dibenzoyl R_f 0.63; *rac*-dibenzoyl R_f 0.59. Anal. ($C_{26}H_{24}N_2O_2$) C, H, N.

***meso*-2,5-Hexanediamine Dihydrochloride.** *meso*-*N,N'*-Dibenzoyl-2,5-hexanediamine (2 g) dissolved in 35 mL of concentrated hydrochloric acid was placed in heavy-walled glass tube which was sealed under vacuum and then heated at 140 °C for 20 h. The tube was then cooled to 5 °C before opening, the precipitated benzoic acid was filtered, and the filtrate was evaporated to dryness in vacuo. The solid dihydrochloride thus obtained was recrystallized from anhydrous methanol–ether: 1 g (86%); mp > 300 °C. Anal. ($C_6H_{18}N_2Cl_2$) C, H, N.

Resolution of *rac*-2,5-Hexanediamine. (±)-2,5-Hexanediamine (3 g, 26 mmol) dissolved in 28 mL of ethanol was dropwise added to a stirred solution of 9.7 g (26 mmol) of (−)-dibenzoyltartaric acid monohydrate in 95 mL of ethanol kept at 50 °C. The solution was then left at 20 °C overnight and filtered, and the precipitate was recrystallized three

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times from a mixture of ethanol–water 1:3 (v/v): 2 g; $[\alpha]_D = -86.2°$ (1% in water). Anal. ($C_{24}H_{32}N_2O_9$ for the monohydrate) C, H, N. The tartrate (2 g) was suspended in 4 mL of water, the suspension was adjusted to pH 10 with a sodium hydroxide solution, and the (−)-2,5-hexanediamine was extracted from the aqueous solution with chloroform (5 × 5 mL). The extracts were evaporated in vacuo at 40 °C, and the residual base was distilled at 85 °C (30 mmHg): 0.32 g (71%); $[\alpha]_D = -6.1°$ (2% in water).

(−)-2,5-Hexanediamine dihydrochloride was obtained by dissolving 0.5 g of the (−)-tartrate in hot water (1.2 mL), followed by addition of a mixture 0.3 mL of concentrated HCl and 3 mL of ethanol. The mixture was cooled and ether (4 mL) was slowly added at 5 °C. After several days, the dihydrochloride was filtered off and crystallized from methanol–ether: 0.13 g (70%); mp 291–293 °C; $[\alpha]_D = +13.2°$ (1% in methanol). Anal. ($C_6H_{18}N_2Cl_2$) C, H, N.

(−)-*N*,*N*′-Dibenzoyl-2,5-hexanediamine was obtained from (−)-2,5-hexanediamine by following the procedure described above; mp 203–205 °C (mixed melting point with *rac*-*N*,*N*′-dibenzoyl-2,5-hexanediamine, 202–204 °C); ^{13}C NMR (TFA) δ 174.72 (CO), 135.91, 130.29, 28.88, 128.35 (C_6H_5), 51.15 (CH), 32.95 (CH_2), 19.20 (CH_3). Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

(+)-2,5-Hexanediamine was obtained from 2,5-hexanediamine by precipitation of the diastereomer (+)-*N*,*N*′-dibenzoyltartaric acid monohydrate; $[\alpha]_D = +86.5°$ (1% in water). The base was liberated from the tartrate as described; $[\alpha]_D = +6.1°$ (2% in water). The dihydrochloride was obtained from the (+)-tartrate as described for the diastereomer: mp 291–293 °C; $[\alpha]_D = -11.4°$ (2% in methanol). The dibenzoyl derivative was obtained as described; mp 203–205 °C. Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

Enzyme Preparations and Inhibition Studies. ODC was obtained from the livers of rats which had been injected with thioacetamide (100 mg/kg of body weight) and assayed essentially as described elsewhere.[7] The specific activity of the preparation was 0.2 nmol/min per mg of protein. The K_i constants were calculated from the data obtained by replotting of the $K_{m,app}$ vs inhibitor concentrations. Soya diamine oxidase (prepared as described[10]) and kidney diamine oxidase (Sigma, St. Louis, MO) were assayed as described.[10]

ODC–antizyme activity was assayed by adding known amounts of a partially purified ODC (2.5 nmol of ODC activity corresponding to 2500 dpm $^{14}CO_2$) to aliquots of the Sephadex G-75-eluated fractions and by measuring the amount of the added activity as suggested by Heller et al.[9] One unit of ODC–antizyme activity is the amount of extract necessary to decrease the ODC activity by 1 nmol of $^{14}CO_2$/1 h.

Rat Treatment. Wistar female rats (100–130 g) were treated as described.[7] The putrescine analogues tested were administered to the rats intraperitoneally in a saline-buffered solution. The livers were excised and the enzyme was prepared as described elsewhere.[7]

Cell Culture. Reuber H-35 hepatoma cells were maintained in Dulbecco's minimal essential medium containing 10 mM Hepes, glutamine (0.3 g/L), sodium bicarbonate (0.9 g/L), penicilline (2.5 × 10^5 UI/L), streptomycine (0.05 g/L), and 10% fetal bovine serum. In order to study ODC induction, the serum-deprived cultures were refurbished with fresh serum-free medium containing insulin at a 10^{-6} M concentration. The polyamine analogues were added to the cultures together with the inducer at a 0.1 mM concentration and incubated for 4 h. The cells were rinsed with PBS, harvested with 0.4 mL of the buffer, disrupted by sonication, and centrifuged at 5000*g*. The supernatants were used after dialysis.

Polyamine analyses of both the livers and the cells were done on perchloric extracts and analyzed as described elsewhere.[7] The rate of ODC synthesis as well as the effect of the diamines on the total protein synthesis was measured in the hepatoma cell line by the incorporation of [^{35}S]methionine. The labeled aminoacid (50 μCi) was added to the induction medium (4 mL) which was incubated for 30 min at 37 °C under 5% CO_2. The cells were then harvested, washed with an ice-cold buffer solution [25 mM Tris-HCl, (pH 7.5), 10 mM L-methionine, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.2 M sucrose], suspended in ODC buffer,[7] sonicated, and the radioactivity incorporated into the cellular soluble proteins was measured. Newly synthesized ODC was determined by incubating an aliquot of the cell extracts with 1 μg of anti-ODC immunoglobin and then by precipitating the immunoreactive enzyme with formalin-fixed *Staphylococcus aureus* strain Cowan I cells. The pellets were dissolved in cracking buffer, and the labeled immunoreactive enzyme was analyzed by SDS-PAGE according to the method of Laemmli.[20] The gels were impregnated with diphenyloxazole and dried under vacuum. The ODC was visualized by fluorography. Otherwise, an electrophoretic transfer of the proteins from the gels to nitrocellulose sheets using an LKB Transblot apparatus was made. The efficiency of the transfer was confirmed by staining the polyacrylamide gels with silver nitrate after autoradiography. The labeled ODC bands were excised from the nitrocellulose sheets and the ^{35}S content of each band was measured in a liquid scintillator.

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