The Effect of Structural Modifications of the Isoproterenol Molecule on the Stimulation of Deoxyribonucleic Acid Synthesis in Mouse Salivary Glands

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SUMMARY

A single injection of *dl*-isoproterenol causes, after a lag period of 20 hr, a marked stimulation of DNA synthesis and cell division in the salivary glands of rodents. The ability to stimulate DNA synthesis can be altered by modifications in the isoproterenol molecule as follows. (a) Replacement of the isopropyl group at the end of the side chain by a $-CH_2CH_3$ group results in an almost inactive molecule. In contrast, bulkier groups, such as $-C(CH_3)_3$, do not greatly alter the capacity to stimulate DNA synthesis. (b) Substituents on the α- and β-carbon atoms of the side chain have little or no effect on the ability of the parent molecule to stimulate DNA synthesis. (c) At least one of the two —OH groups on the phenyl ring is necessary for full activity. When both —OH groups are absent, the compound is totally inactive in stimulating DNA synthesis. (d) The *d*- and *l*-isomers are equally active in stimulating DNA synthesis.

The ability to stimulate DNA synthesis in the salivary gland is usually correlated with the capacity to stimulate α-amylase secretion in the same organ and to cause glycogenolysis in the liver. However, there are a number of exceptions: for instance, (a) when both —OH groups of the phenyl rings are absent, the compound is still capable of eliciting salivary gland secretion; (b) *d*-isoproterenol, although quite effective in stimulating DNA synthesis, does not produce glycogenolysis in the liver.

INTRODUCTION

A single intraperitoneal injection of *dl*-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol (isoproterenol) causes a marked stimulation of DNA synthesis and cell proliferation in the salivary glands of both rats (1) and mice (2). The stimulation of DNA synthesis begins after a lag period of 20 hr and reaches a peak at 28–30 hr. Among the various metabolic changes that have been reported to occur in the salivary glands after the administration of *dl*-isoproterenol (3–8) is a decrease in α-amylase activity in both rats (9) and mice (8), the decrease being most pronounced 2 hr after administration of the drug.

Because of the interesting possibilities disclosed by the use of a purified chemical compound for stimulating DNA synthesis in the intact animal, we have investigated the effect of structural modifications of the isoproterenol molecule on its capacity to stimulate DNA synthesis. In addition, we have compared the ability of an isoproterenol analogue to stimulate DNA synthesis in the

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salivary gland with its ability to stimulate α-amylase secretion in the same organ and to cause glycogenolysis in the liver (10).

METHODS AND MATERIALS

General Methods

Fels A male mice, bred in our laboratory, were used when about 4 months of age and weighing 28–31 g. The animals were kept on a 12-hr light and dark schedule (no light from 6:00 p.m. to 6:00 a.m.) and fed ad libitum until 2 hr before the experiment, when their food, but not their water, was withdrawn. All the experiments were started at 10:00 a.m.

The animals were treated by intraperitoneal injection with the compounds described below. For the determination of DNA synthesis, the animals received ^{3}H-thymidine 27.5 hr later; for measurements of α-amylase or liver glycogen, the mice were killed 2 hr after the injection of the compound to be tested.

In all cases the animals were killed by cervical dislocation and the salivary glands or liver was removed promptly. The three major salivary glands (submaxillary, parotid, and sublingual) were used for determination of the specific activity of DNA, while for α-amylase measurements only the parotid was used, since the other salivary glands contain very little α-amylase activity.

Determination of DNA specific activity. The animals were given a subcutaneous injection of ^{3}H-thymidine (New England Nuclear Corporation, 6.7 Ci/mmole), 0.3 μCi/g of body weight, and were killed after 30 min. The salivary glands were homogenized in 0.25 M sucrose containing 5% citric acid, and the specific activity of DNA was determined by the method of Scott *et al.* (11), as described previously (12).

Determination of α-amylase activity. Parotid glands were homogenized in 5 ml of distilled water, and α-amylase activity was determined by the method of Bernfeld (13). The incubation mixture contained the following, in a final volume of 1 ml: 10 μmoles of Na_2PO_4 (pH 6.9), 3 μmoles of NaCl, 5 mg of soluble starch, and 0.5 ml of homogenate. The reaction was terminated after 3 min at 25°. One unit of enzyme activity liberated 1.0 μmole of β-maltose per minute.

Glycogen concentration. Glycogen concentration in the liver was measured by the method of Roe and Dailey (14), as described previously (6).

Compounds Tested

The structural formulas of the compounds used in the experiments are shown in Fig. 1. All compounds were racemic unless otherwise stated. The following were purchased: *dl*-isoproterenol-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride(I) (Winthrop Laboratories, New York); 1-(3,4-dihydroxyphenyl)-2-*n*-butylaminoethanol(IV) (Frinton Laboratories, South Vineland, New Jersey); and 1-(3-hydroxyphenyl)-2-isopropylaminoethanol hydrochloride (XIV), 1-(4-hydroxyphenyl)-2-methyl-2-methylaminoethanol hydrochloride(XVII), 1-(3-hydroxyphenyl)-2-ethylaminoethanol hydrochloride(XVIII), and 1-(4-hydroxyphenyl)-2-methylaminoethanol(XIX) from the Aldrich Chemical Company, Milwaukee.

The following compounds were kindly donated through the courtesy of Drs. Sydney Archer and Frederick Nachod of the Sterling-Winthrop Research Institute, Rensselaer, New York: 1-(3,4-dihydroxyphenyl)-2-ethylaminoethanol(V), 1-(3,4-dihydroxyphenyl)-2-*tert*-butylaminoethanol(VI), 1-(3,4-dihydroxyphenyl)-2-*sec*-butylaminoethanol (VII), 1-[3,4-dihydroxyphenyl]-2-(1,3-dimethylbutylamino)ethanol(VIII), 1-(3,4-dihydroxyphenyl)-2-methyl-2-methylaminoethanol(IX), 1-(3,4-dihydroxyphenyl)-2-methyl-2-isopropylaminoethanol(X), 1-(3,4-dihydroxyphenyl)-2-methyl-2-cyclohexylaminoethanol(XI), *l*-isoproterenol bitartrate dihydrate(III), *d*-isoproterenol bitartrate (II), and *d*-1-(4-hydroxyphenyl)-2-isopropylaminoethanol tartrate(XIVa).

Also kindly supplied without charge were L(+)-*threo*-1-phenyl-2-amino-1,3-propanediol(XXI) (Parke Davis and Company, Detroit) and 5-(1-hydroxy-2-isopropylaminoethyl)-8-hydroxyquinoline(XX) (Charles Pfizer).

Analytical results obtained for those elements indicated were within ±0.3% of the calculated values. Melting points were

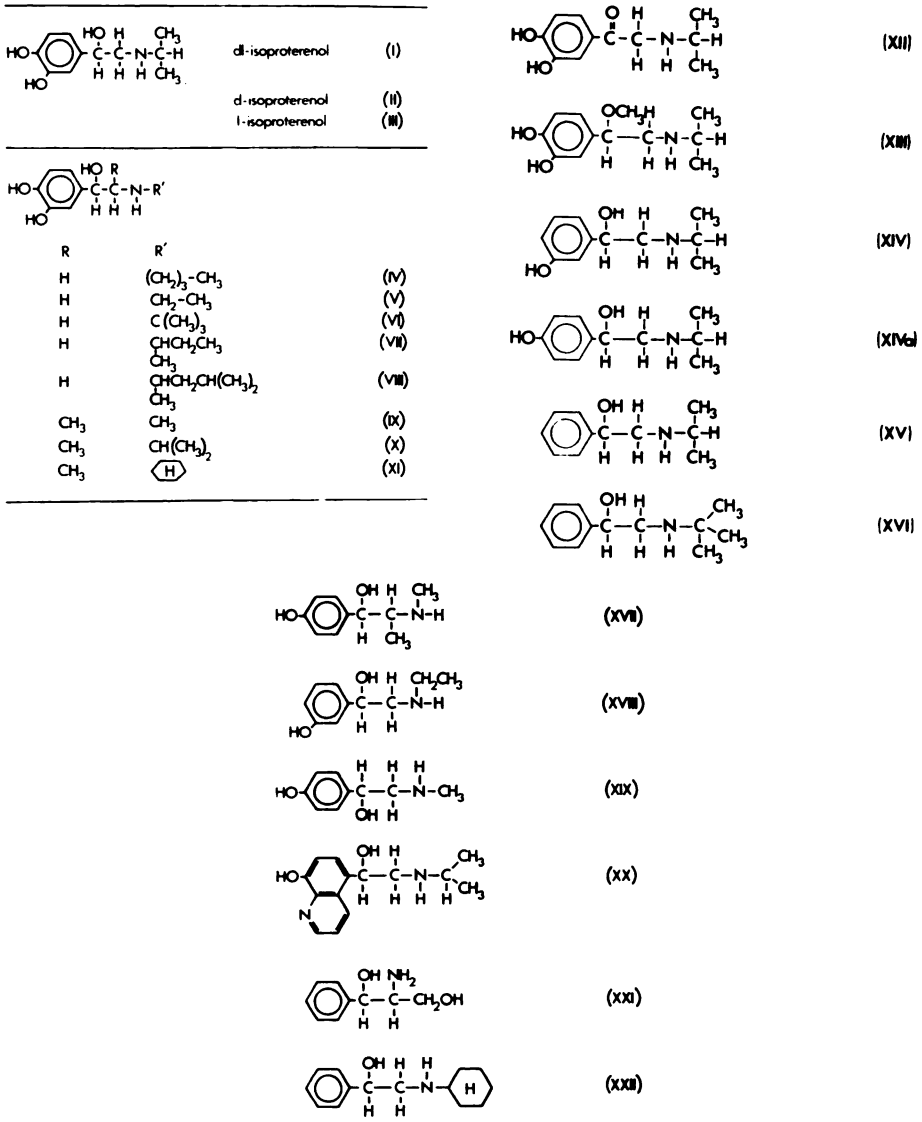

FIG. 1. *Structural formulas of isoproterenol and its analogues used in the experiments described in the text*

measured with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. NMR spectra were determined with a Varian A-60A spectrometer (tetramethylsilane as internal standard) with $CDCl_3$ (deuteriochloroform) as solvent unless otherwise noted.

Resolution of dl-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol. Commercially obtained *dl*-1-(3,4-dihydroxyphenyl-2-isopropylaminoethanol (isoproterenol) was resolved by fractional crystallization of the bitartrate salt according to the method of Beccari *et al.* (15). The *d*-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol bitartrate had $[\alpha]_D$ +31.8° (recorded, +35.9°). The *l*-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride had $[\alpha]_D$ −12.1° (recorded, −50°) (optical purity, 63%).

3,4-Dihydroxy-α-isopropylaminoacetophenone sulfate(XII). 3,4-Dihydroxy-α-chloroacetophenone (3.8 g, 0.02 mole) and isopropylamine (25 ml) were heated in refluxing absolute ethanol for 2 hr according to the method of Scheuing and Thomä (16), fol-

lowed by acidification with dilute H_2SO_4 to give XII (0.97 g, 0.0019 mole, 9.5%), m.p. 242–244° (recorded, 245°).

$C_{22}H_{32}N_2O_{10}S$
Calculated: C 51.16, H 6.30
Found: C 50.87, H 6.20

1-(3,4-Dihydroxyphenyl)-1-methoxy-2-isopropylaminoethane(XIII). The procedure for the methylation of arterenol was used (17). Dry HCl was bubbled into a suspension of 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol (3.9 g, 0.018 mole) in methanol (100 ml) until a clear solution resulted. Most of the solvent was removed on a rotary evaporator, and acetone was added. Further concentration resulted in the precipitation of XIII (3.6 g, 0.014 mole, 78%, m.p. 168° with decomposition). The NMR spectrum (D_2O) was consistent with the proposed structure.

$C_{12}H_{20}ClNO_3$
Calculated: C 55.10, H 7.65
Found: C 54.99, H 7.73

Addition of Alkylamines to Styrene Oxide: General Procedure (18)

1-Phenyl-2-cyclohexylaminoethanol(XXII). Styrene oxide (12.0 g, 0.10 mole) and cyclohexylamine (10.9 g, 0.11 mole) were stirred at room temperature for 12 days. The resulting white semisolid was triturated with petroleum ether and filtered to give 1-phenyl-2-cyclohexylaminoethanol (12.4 g, 0.057 mole, 57%), m.p. 91–93°. The melting point was unchanged after one recrystallization from acetone. The NMR spectrum exhibited peaks consistent with the proposed structure.

$C_{14}H_{21}NO$
Calculated: C 76.71, H 9.59
Found: C 76.76, H 9.67

1-Phenyl-2-isopropylaminoethanol(XV). This was prepared from styrene oxide (6.0 g, 0.05 mole) and isopropylamine (4.0 g, 0.055 mole) in 1.2% yield (0.289 g, 0.00062 mole), m.p. 90°, as described above (reaction time, 5 days).

$C_{11}H_{17}NO$
Calculated: C 73.74, H 9.50
Found: C 73.86, H 9.58

1-Phenyl-2-tert-butylaminoethanol(XVI). This was prepared from styrene oxide (12.0 g, 0.10 mole) and *tert*-butylamine (8.0 g, 0.11 mole) in 25% yield (4.9 g, 0.025 mole), m.p. 92–94.5°, as described above (reaction time, 13 days).

$C_{12}H_{19}NO$
Calculated: C 74.61, H 9.84
Found: C 74.87, H 10.06

RESULTS

Some of the compounds investigated were lethal when given at a dose of 0.8 μmole/g of body weight. These compounds were then used at a dose level of 0.25 μmole/g of body weight. At this dose, isoproterenol still possesses considerable activity in stimulating DNA synthesis in the salivary glands of mice (19). There were no deaths among the mice treated with the compounds at the doses given in Tables 1 and 2. Toxic signs were present in the first 2–4 hr after injection of isoproterenol or some of its analogues, but the animals seemed to be in good health by the following day.

The results are given in Table 1 for the compounds used at a dose of 0.8 μmole/g of body weight, and in Table 2 for the compounds used at a dose of 0.25 μmole/g. In both tables, the control value for α-amylase activity is given by the activity of parotid glands from untreated mice, which was equal to 12.6 ± 1.8 μmoles of β-maltose liberated per minute per milligram of protein.

The control value for liver glycogen concentration is also given in both tables for the glycogen concentration of livers from untreated mice, which was equal to 1546 ± 250 mg/100 g of fresh tissue.

For the specific activity of salivary gland DNA, the control values are given for mice that received no treatment. Values for isoproterenol-treated mice differ in the two tables. In Table 1, the value is given for mice killed 28 hr after a single injection of 0.8 μmole of isoproterenol per gram of body weight; in Table 2, for mice killed 28 hr after a single injection of 0.25 μmole of isoproterenol per gram of body weight. On the basis of these figures, an increase in the specific activity of DNA above 1200 dpm/mg

TABLE 1

Effect of isoproterenol and its analogues on DNA synthesis and α-amylase activity of salivary glands and glycogen concentration of liver in mice

Isoproterenol, 0.8 μmole/g of body weight, was injected intraperitoneally. Mice were killed 2 hr afterward for determination of α-amylase activity and glycogen concentration, and 28 hr after isoproterenol administration (30 min after receiving ^{3}H-thymidine) for the specific activity of DNA. There were three animals per group, except for the two groups for which standard deviations are given, which contained at least 12 animals.

Group	Compound administered	Specific activity of DNA	α-Amylase activity	Glycogen concentration
		dpm/mg DNA	*μmoles/min/mg*	*mg/100 g fresh wt*
A	I[a]	6,700 ± 300	2.0 (1.2–3.0)	10 (0–15)
	II	6,050 (4,900–6,900)	2.9 (2.5–3.2)	1,330 (1,100–1,700)
	III	6,350 (5,100–7,100)	2.4 (2.4–2.5)	150 (0–500)
B	IV	4,350 (2,950–5,200)	2.3 (0.9–3.3)	30 (0–70)
	VI	4,500 (2,100–9,000)	2.0 (1.4–2.8)	160 (15–300)
	VII	4,450 (1,600–10,100)	1.7 (1.0–2.3)	170 (10–400)
	X	1,400 (500–2,000)	1.9 (1.4–2.5)	1,300 (1,200–1,350)
C	XII	2,500 (2,000–3,100)		
	XIII	3,300 (2,700–4,400)	3.5 (2.6–4.6)	700 (500–900)
D	XIV	8,000 (5,100–14,800)	2.0 (1.6–2.2)	500 (50–800)
	XIVa	3,500 (2,500–4,000)	1.7 (0.9–2.6)	400 (150–700)
E	XIX	1,840 (900–2,800)	2.6 (2.4–2.7)	850 (700–950)
	XX	4,000 (3,100–4,600)	6.5 (5.0–8.7)	590 (50–1,050)
	XXI	270 (180–400)	13.2 (10.5–15.7)	650 (400–800)
No treatment		650 ± 150	12.6 ± 1.8	1,546 ± 250

[a] Isoproterenol.

TABLE 2

Effect of isoproterenol and its analogues on DNA synthesis and α-amylase activity of salivary glands and glycogen concentration of liver in mice

Isoproterenol, 0.25 μmole/g of body weight, was injected intraperitoneally. Mice were killed 2 hr afterward for determination of α-amylase activity and glycogen concentration, and 28 hr after isoproterenol administration (30 min after receiving ^{3}H-thymidine) for the specific activity of DNA. There were three animals per group, except for the two groups for which standard deviations are given, which contained at least 12 animals.

Group	Compound administered	Specific activity of DNA	α-Amylase activity	Glycogen concentration
		dpm/mg/DNA	*μmoles/min/mg*	*mg/100 g fresh wt*
A	I[a]	5,000 ± 260	2.2 (1.4–3.0)	180 (10–300)
B	V	240 (60–480)	11.1 (10.0–12.1)	20 (0–50)
	VIII	3,400 (1,800–7,700)	2.6 (2.2–3.2)	1,400 (1,000–2,000)
	IX	2,000 (600–3,000)	5.9 (4.6–7.5)	300 (70–500)
	XI	1,500 (1,150–2,050)	5.4 (4.3–6.0)	900 (600–1,200)
D	XV	100 (50–150)	3.6 (2.2–4.7)	1,610 (900–2,100)
	XVI	100 (50–150)	6.8 (3.1–9.9)	1,750 (800–3,200)
E	XVII	1,300 (950–1,500)	1.3 (0.9–1.7)	1,800 (1,500–2,200)
	XVIII	1,250 (150–1,950)	2.8 (2.6–3.0)	1,600 (1,000–2,200)
	XXII	75 (30–100)	14.2 (13.9–14.4)	1,545 (1,300–2,050)
No treatment		650 ± 150	12.6 ± 1.8	1,546 ± 250

[a] Isoproterenol.

of DNA constitutes a modest but statistically significant stimulation of DNA synthesis. The results in each group were reasonably consistent, but there were exceptions; for instance, some animals were not stimulated by compounds IX, X, and XVIII, while others were stimulated slightly.

Effect of structural modifications of isoproterenol on stimulation of DNA synthesis in salivary glands. Tables 1 and 2 show that both the *d*- and *l*-isomers of the drug are quite active in stimulating DNA synthesis. This was true whether we used the *d*- and *l*-isoproterenol resolved in our laboratory or the stereoisomers supplied by Sterling-Winthrop. Changes within the side chain, but exclusive of the end group (compounds X, XII, and XIII), may decrease but do not abolish the ability of an analogue to stimulate DNA synthesis. In particular, the replacement of H by CH_3 on the β-carbon results in markedly decreased activity.

The end group must be bulky (at least 3 carbon atoms) for the structure to be active in stimulating DNA synthesis. Replacement of the isopropyl group by an ethyl group (V) results in an inactive compound. Also inactive are compounds XVII, XVIII, and XIX.

Most interesting are the results obtained when the phenyl ring is modified. When one of the OH groups remains, the compound is active. In fact, when only the 3-OH group is retained (XIV), the compound is even more active than isoproterenol itself. However, when both OH groups are removed, the compounds become totally inactive in stimulating DNA synthesis (XV and XVI).

Effect of structural modifications of isoproterenol on α-amylase secretion in salivary glands. Only three compounds failed to cause a significant decrease in α-amylase activity in the salivary gland: V, XXI, and XXII. In general, there was a correlation between stimulation of DNA synthesis and decrease in α-amylase activity. There were two exceptions, however, XV and XVI, both of which decreased α-amylase activity but failed to stimulate DNA synthesis. XV is identical with isoproterenol except for the absence of OH groups on the aromatic ring.

Effect of structural modifications of isoproterenol on liver glycogenolysis. Some of the analogues that caused salivary gland secretion also produced liver glycogenolysis. There were several exceptions, such as compounds II, VIII, X, and XV–XVIII, which do not produce glycogenolysis, and, conversely, compound XXI, which caused glycogenolysis but no salivary gland secretion. Most interesting is *d*-isoproterenol(II), which does not produce glycogenolysis, although it can stimulate α-amylase secretion and DNA synthesis in the salivary gland. Again, similar results were obtained whether we used our own *d*- and *l*-isoproterenol or the stereoisomers supplied by Sterling-Winthrop.

DISCUSSION

It is now well established that a single intraperitoneal administration of isoproterenol causes a burst of cellular proliferation in the salivary glands of rodents (1, 2). The wave of mitoses is preceded by a marked increase in DNA synthesis, which in mice begins at 20 hr and reaches a peak 28–30 hr after the administration of the drug (2). The dose of isoproterenol used is admittedly high and far beyond the physiological range. However, the object of these studies is not the pharmacological action of isoproterenol, but its use as a chromatographically pure chemical compound that can stimulate DNA synthesis and cell division in the intact animal. In this respect, it is no more unphysiological than other stimuli used for the study of induced DNA synthesis and cell division, such as partial hepatectomy (20, 21), high doses of folic acid (22), mercuric chloride necrosis (23), or explants *in vitro* [see review by Baserga (24)].

As a model of stimulated DNA synthesis, the isoproterenol-stimulated salivary gland offers some unique features, and for this reason we thought it worthwhile to investigate the effect of simple changes in the isoproterenol molecule upon its ability to stimulate DNA synthesis. Since this drug is also well known as a powerful stimulant of salivary gland secretion (9) and liver glycogenolysis (10), we have also studied the effects of structural changes on these two

properties, in an attempt to correlate them with the ability to stimulate DNA synthesis.

Some of the isoproterenol analogues were too toxic at a dose of 0.8 μmole/g of body weight, and were used at a dose of 0.25 μmole/g, which still causes a marked stimulation of DNA synthesis.

The optically active *d*- and *l*-isomers of isoproterenol (II and III) are both capable of stimulating DNA synthesis and α-amylase secretion, although *d*-isoproterenol(II) has little or no effect on liver glycogenolysis. According to Houssay and co-workers (25), *l*-isoproterenol is much more effective than the *d*-isomer in causing enlargement of the parotid gland after chronic administration. Since chronic administration of isoproterenol produces hypertrophy as well as hyperplasia of the salivary glands, one is tempted to hypothesize that both isomers can cause hyperplasia (as shown in the present experiments), but that only *l*-isoproterenol can produce hypertrophy. If this is true, a powerful tool for separating the molecular events of hyperplasia from those of hypertrophy will be available.

The end group of the side chain cannot be less bulky than the isopropyl radical; otherwise the ability to stimulate DNA synthesis decreases markedly (compound V). This may be due to the fact that compounds with less bulky end groups have more affinity for *alpha*-receptors than does isoproterenol (26) and may not be available in sufficient concentrations for *beta*-receptors or other activities. Groups bulkier than isopropyl do not interfere to any large extent with the DNA-stimulating activity.

Changes on the α- and β-carbons on the side chain have little effect on the DNA-stimulating capacity. There is one exception, compound X, in which a CH_3 replacing an H atom on the β-carbon causes almost total disappearance of the activity.

More interesting, however, are modifications on the phenyl ring. When one —OH group is present, the compound (XIV) may be even more active than isoproterenol in stimulating DNA synthesis. If both —OH groups are absent, the compound (XV) is totally inactive, although it is still quite capable of stimulating salivary gland secretion, suggesting that it is taken up by cells. This is not surprising, since removal of —OH groups should not decrease the affinity of a compound toward cell membranes. It is also important to compare V and XVIII, both with a $—CH_2CH_3$ at the end of the lateral chain, but XVIII having only one —OH on the phenyl ring. Although XVIII cannot be classified as very active in stimulating DNA synthesis, it is certainly more active than V, suggesting that the less bulky CH_2CH_3 group is not really inactive but probably causes more binding to *alpha*-receptors. Compound XVI also causes α-amylase secretion without stimulating DNA synthesis, but otherwise the correlation is fairly close. We have not yet found a compound that will stimulate DNA synthesis *without* stimulating salivary gland secretion.

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