doses. Since guanethidine treatment results in high concentrations (about 0.5 mM) in ganglia of both adult and neonatal rats [11, 12] and in adrenal medulla [13], it was expected that guanethidine would produce a significant decrease in polyamine levels in these tissues. The data in Table 1 show that treatment of neonatal animals with guanethidine for up to 4 days produces only a modest rise in putrescine and no decrease in spermidine and spermine levels in SCG. In contrast, MGBG (Table 1) produced a doubling of putrescine levels and a modest, but statistically significant, decrease in spermidine (22 per cent) and spermine (35 per cent) concentrations in ganglia. Similarly, treatment of adult rats for up to 10 days with guanethidine (40 mg/kg/day, i.p.) caused no decrease in spermidine or spermine levels in adrenal medulla (data not shown). These results suggest that inhibition of polyamine synthesis is not involved in the cytotoxic effects of guanethidine. This conclusion is also supported by our failure to overcome the cytotoxic effects of guanethidine with maximally tolerated doses of spermidine, spermine, combinations of the two, or by exogenous administration of SAM at doses which produce marked increases in SAM levels in the ganglia (data not shown). In addition, MGBG administration, at doses which decrease spermidine and spermine levels (Table 1), is not cytotoxic *in vivo* to sympathetic neurons.

It is unclear why guanethidine fails to lower polyamine levels in ganglia and adrenals. It is present in these structures at total concentrations which are far in excess of the K_i for *in vitro* inhibition. Inhibition of diamine oxidase is probably of no consequence since ganglia do not contain detectable amounts of the enzyme (data not shown). This may suggest that much of the guanethidine is bound (perhaps in storage vesicles) and that the free concentration in the cytoplasm is much smaller. Alternatively, it may indicate that polyamines turn over very slowly in sympathetic neurons, which accumulate guanethidine selectively and which are almost all post-mitotic by birth in the rat [14]. The decrease in the levels of spermidine and spermine that is produced by MGBG may result from an ability of MGBG to accumulate equally well in glial cells in the ganglia which are proliferating postnatally and which would be expected to have higher turnover rates of polyamines [4, 5]. It is possible that guanethidine might inhibit increases in polyamines after a stimulus (e.g. nerve growth factor) which increases ornithine decarboxylase and presumably polyamine biosynthesis.

In summary, guanethidine has been shown to be a potent competitive inhibitor ($K_i = 25\ \mu M$) of SAM DC from several tissues and a moderately potent non-competitive inhibitor of thymus diamine oxidase ($K_i = 90\ \mu M$) in the rat. Polyamine levels, however, are not decreased in tissues (sympathetic ganglia, adrenal medulla) in which guanethidine accumulates to a high concentration. Although it does not appear that the ability to inhibit either of these enzymes explains the cytotoxic effects of the drug on sympathetic neurons, inhibition of these enzymes may be important in other pharmacologic and toxicologic properties of this agent which is widely used clinically and as an experimental tool.

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Quantitative structure–activity relationships for the inhibition of heart and brain cyclic AMP phosphodiesterases by some phenylbutenolides

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Cyclic AMP phosphodiesterase (PDE) is ubiquitously distributed in mammalian tissues [1] and exists under a wide variety of multiple forms. This particularity partly explains the differential sensitivities to drugs of the tissues and makes phosphodiesterase a suitable target for the development of specific new drugs. The therapeutic interest of inhibiting specifically the PDE in a given tissue has been recently emphasized by Weiss and Hait [2]. We previously described a new class of synthetic potent inhibitors of phosphodiesterases acting preferentially on the heart PDE [3], and exhibiting cardiotonic properties. These compounds (Fig. 1) are phenylbutenolides differently substituted in the 4 position of the phenyl ring by complex groups of a large size (AP 10:*R* = Glucose—O—C_6H_4—CO—CH_2—; IP 24:*R* = Glucose—O—C_6H_4—CO—NH—; IP 17:*R* = HOOC—CH_2—O—C_6H_4—CO—CH_2—). The present study considers more simply substituted phenylbutenolides (Table 1) synthetized in our laboratory by Prigent *et al.* [4, 5]. The aim of this report was to answer the following questions. (i) Is the presence of a complex aromatic group in the 4 position a prerequisite for the inhibition of PDE? (ii) Does the nature of substituent *R*

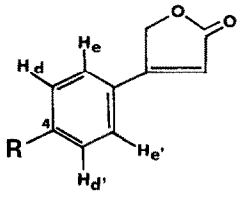

Fig. 1. General formula of the phenylbutenolides studied. The NMR chemical shifts δ of meta H_eH_e', protons are given in Table 1.

affect the heart PDE and the brain enzyme in the same way? (iii) Is it possible to modify the tissue-specificity of the inhibition by changing the nature of the substituent?

Inhibitory potencies were investigated both on cyclic AMP phosphodiesterase of a rat brain preparation, and on a beef heart commercial enzyme.

Compounds tested. The synthesis of compounds 1–11 (Fig. 1, Table 1) studied in this report and the related NMR data were already reported [4, 5]. H_e and H_e' protons (Fig. 1) were found to be equivalent and gave a single signal.

Materials. [8-^{3}H]Adenosine 3′,5′ monophosphate was supplied by the Radiochemical Centre, Amersham. Unlabeled cyclic AMP, 5′ nucleotidase from *Ophiophagus hannah* venom were purchased from Sigma Chemical Co. Anion-exchange resin Bio-Rad AG 1 × 2 (200–400 mesh) was obtained from Bio-Rad Labs.

Enzyme preparations. The beef heart PDE (Sigma Chemical Co) was a lyophilized preparation. The rat brain PDE was prepared as described by Brooker *et al.* [6].

Enzyme assay. Cyclic AMP phosphodiesterase activity was assayed by the two step radio-isotopic procedure of Thompson and Appleman [7]. Substrate cyclic AMP was 0.25 μM. All test compounds were solubilized in dimethylsulfoxide (Me_2SO) and brought to the adequate concentration with the incubation buffer. Final Me_2SO concentration was 12.5 per cent. This Me_2SO amount slightly inhibited PDE activity, but did not modify the percentuel inhibition found with water-soluble drugs. Test compounds and Me_2SO were examined to ensure that they did not interfere with the assay. The biological activities of the test compounds on heart and brain enzymes were expressed as the logarithm of per cent inhibition at 1 mM concentration.

Physico-chemical parameters and biological activities relationships. The lipophilic parameter of the compounds used in this study is the logarithm of *n*-octanol–water partition coefficient (log P) calculated by means of Rekker's hydrophobic fragmental constant *f* [8]. The electronic effects of the substituents *R* were characterized by the Hammet constant σm [9], which traduces the electronic influence of *R* on the meta position (with respect to this substituent) of the phenyl ring. Some σm values were taken from literature [9], (*R* = —H, —NO_2, —NH_2, —CN, —COOH and —NH—CO—CH_3). The lacking data were calculated as follows (i) We found a close correlation (equation 1, Table 2) between δ (chemical shift of the H_e proton (Fig. 1) in the NMR spectrum) and σm for the above substituents; so, an experimental δ permitted the calculation of a lacking σm value from equation 1 (data obtained for the carboxylic substituents concerned their protonated forms). (ii) σm for carboxylic substituents in their anionic form were approximated by supposing that the same deviation exists between protonated and anionic forms as between the σm of —COOH (0.37) and —COO^- (0.10) taken from [9]. Graphical determination of the pK_a of compounds 2, 3, 7, 10 from titration curves in the presence of 12.5% Me_2SO, and examination of u.v. spectra of compound 9 at different pH, established that, in the assay medium (pH 8), compounds 7, 9, 10 are in the non-ionic form.

All the data (log P, σm, δ) used in the correlations are presented in Table 1. Assignments of NMR signals for lactonic or aromatic H_d protons, and electronic effect contants for substituents *R* such as σp (commonly used for para or ortho electronic effects), $\mathcal{F}$ and $\mathcal{R}$, taken from [9], were also used in the research of structure–activity relationships, but without significant correlations, however.

Simplified phenylbutenolides taken into account in this report were found inhibitors of both heart and brain cyclic AMP phosphodiesterases (Table 1). They were less potent than the parent compounds which inhibited bovine heart enzyme by more than 90 per cent at 1 mM [3]. As parent compounds, they were more efficient on the heart enzyme than on the brain enzyme, with the exception of compound 5.

For the heart enzyme, the inhibitory potency of the test compounds showed a correlation with the electronic effect constant σm of substituent *R* (equation 2, Table 2). This relation, however, only explained 67 per cent of the variations in activity of all the test compounds. Equation 3, which does not include compound 9 was able to explain 85

Table 1. Experimental biochemical activities and physico-chemical parameters of phenylbutenolides

Compound No.	Nature of substituent *R*	σm*	δ†	log P‡	Experimental biochemical activity (log per cent inhibition of PDE) ± S.D. (n = 3) Bovine heart	Rat brain
1	—H	0.00	7.64	1.49	1.45 ± 0.08	1.18 ± 0.04
2	—CH_2—COOH	−0.64	7.50	−3.18	1.30 ± 0.05	0.95 ± 0.09
3	CH_3—CH_2—CH(COOH)—	−0.22	7.72	−2.14	1.41 ± 0.08	1.00 ± 0.06
4	—CH_2—COO—CH_3	0.31	7.75	1.28	1.60 ± 0.01	1.52 ± 0.05
5	CH_3—CH_2—CH(COO—CH_3)—	0.25	7.72	2.32	1.65 ± 0.01	1.75 ± 0.02
6	—NH—CO—CH_3	0.21	7.70	0.72	1.59 ± 0.02	1.43 ± 0.08
7	—NH—CO—CH_2—CH_2—COOH	0.15	7.67	0.70	1.72 ± 0.03	1.34 ± 0.02
8	—NO_2	0.71	7.90	1.26	1.85 ± 0.01	1.52 ± 0.01
9	—NH_2	−0.16	7.46	0.47	1.76 ± 0.08	1.41 ± 0.04
10	—COOH	0.37	7.78	1.24	1.81 ± 0.01	1.56 ± 0.04
11	—CN	0.56	7.94	1.14	1.89 ± 0.01	1.53 ± 0.03

* σm: electronic effect constant, taken from reference 9 or calculated as indicated under experimental section.

† δ: NMR chemical shift of proton e, taken from [4, 5].

‡ P: *n*-octanol–water partition coefficient, calculated by means of Rekker fragmental constants [8].

Table 2. Correlation between two physico-chemical parameters, and correlations between biochemical activities and physico-chemical parameters of phenylbutenolides

No.	Equation	n*	r†	s‡	F§	α¶
1	$\delta = 7.59 + 0.52 \pm 0.07\ \sigma m$	6	0.962	0.054	49.97	<0.01
2	A‖ (Bov. heart) = $1.58 + 0.41 \pm 0.10\ \sigma m$	11	0.817	0.116	18.07	<0.005
3	A (Bov. heart) = $1.55 + 0.47 \pm 0.07\ \sigma m$	10	0.924	0.080	46.65	<0.001
4	A (rat brain) = $1.32 + 0.13 \pm 0.02$ logP	11	0.867	0.129	27.31	<0.001
5	A (rat brain) = $1.35 + 0.14 \pm 0.01$ logP	10	0.979	0.055	180.75	≪0.001

* Number of compounds considered.
† Correlation coefficient.
‡ Standard error of the estimate.
§ Fischer–Snedecor criterion observed value.
¶ Level of significance.
‖ Biochemical activity.

per cent of the variations in activity of the ten other compounds. From these relationships it appears clearly that the biochemical activity of substituted phenylbutenolides is directly dependent upon the influence of the electronic effects of the substituent *R* on the meta position of the phenyl ring. The inhibitory potency of these drugs increases with increasing electron withdrawing potency of *R*. Nevertheless, a clear-cut explanation of all the variations in biochemical activity would require an additional hitherto unidentified physico-chemical parameter of non-electronic and non lipophilic nature, since the introduction of σp or log P failed to provide any statistical significance to the correlation.

For the brain enzyme, the inhibitory potency was in a very good correlation with the lipophilicity of the test compounds. Equation 4 (Table 2) explained 75 per cent of the variations in biochemical activity, but did not reflect the activity of compound 1. Equation 5, which does not include compound 1, entirely described the variations in activity of the ten other compounds ($r^2 = 0.96$). Thus, the inhibitory potency of the investigated drugs increases with increasing lipophilicity of *R*. An additional parameter seems to be required for a complete description of all observed variations. This parameter is not dependent on the electronic effects of the substituents *R* since the introduction of σm or σp in the correlation abolished its statistical significance. A lateral incumbrance of substituents, expressed in Å by Verloop index B [10] might be involved. Unfortunately, the Verloop table does not give all data required to test such an hypothesis.

It should be noted that a non-negligible correlation exists between σm and log P for the substituents considered in this report. So, though the regression analysis of the data did not allow the simultaneous introduction of the two parameters in the same equation, we cannot exclude that the unconsidered parameter may occur, but at a lesser extent, in the explanation of a given enzyme inhibition.

From our result, it appears firstly that the replacement of the large substituent (*R* = Glucose—O—C_6H_4—CO—CH_2—) of AP 10 by smaller groups does not suppress PDE inhibiting potency. The most efficient simplified phenylbutenolides are in the range of potency of theophylline and pentifylline [11]. The interest of such molecules is that they are simple enough to permit a quantification of their physico-chemical parameters and a better analysis of the structural requirements for PDE inhibition. Thus, the substituent *R* seems to be a factor influencing in a large way the affinity of the molecule for the enzyme without being prerequisite for this affinity. Furthermore, the H_e proton of the phenyl ring seems to be especially involved in drug-enzyme interaction.

An important point is the difference in structural requirements for the inhibition of the two considered PDE preparations. The rat brain enzyme inhibition is mainly dependent on the lipophilicity of substituent *R*, and in contrast, the main parameter responsible for the bovine heart enzyme inhibition is the electronic influence of this substituent on the meta position of the phenyl ring. This difference in sensitivity might be explained by species difference, tissue difference and by differences in the procedure of preparation. According to Thompson and Appleman [12], commercially available PDE prepared from beef heart [13] is mainly representative of one of the predominant forms of PDE, soluble and of cytoplasmic origin, referred to as the "high K_m" form, with a greater affinity for cGMP than for cAMP. In contrast, crude brain preparations contain a large proportion of a particulate form and also a soluble form with an affinity for the membrane, both exhibiting a low K_m for cAMP [12]. So, brain enzyme which seems, in this preparation, to be more involved with the membrane lipidic environment is more sensitive to the lipophilicity of the substituent than to its electronic effects. The reverse is true for the heart enzyme. Although the preparations studied are not representative of a single purified isoenzyme, this study constitutes an approach of the problem. Until now, several authors varied substitutions in different series of xanthine derivatives [14–16]. From their results, no clear relationship shows up between the various physico-chemical parameters of considered substituents and PDE inhibition. In some cases, however, the selectivity towards one isoenzyme was enhanced [15, 16]. Our results raise the possibility to design the substituents in such a way that the resultant structure will be able to act more specifically on a PDE form. This aim is partially reached with compound 5; it is more active on brain enzyme than on heart enzyme, as contrasted with all other compounds tested. Furthermore some of these phenylbutenolides present a potential additional interest for the purification of PDE:compounds with structural functional groups such as —COOH, —NH_2, —CN, could be useful ligands for covalent coupling to an insoluble matrix. Such columns of immobilized inhibitors might allow affinity chromatography of PDE (this work is in progress in our laboratory).

Different correlations between physico-chemical parameters of substituted phenylbutenolides and phosphodiesterase inhibition have been found with bovine heart and rat brain enzymes. These different structural requirements for drug affinity raise a possibility of improving the specificity of drug action.

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3,4-Dihydroxyphenylacetic acid content of sympathetic ganglia as a possible biochemical indicator of small intensely fluorescent cell participation in ganglionic transmission

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Dopamine (DA) in the small intensely fluorescent (SIF) cells of sympathetic ganglia [1], is thought to be released onto sympathetic neurons as a result of stimulation of muscarinic receptors on the SIF cells by preganglionic cholinergic neurons [2]. The concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) within the ganglia is a relative measure of DA formation and metabolism [3]. Presently there is no biochemical technique to evaluate sympathetic ganglionic transmission in freely moving rats. It may be possible, however, to monitor the participation of the SIF cells in ganglionic transmission by analyzing the content of DOPAC in the ganglion. In a model proposed by Libet [4], DA released from ganglionic SIF cells in response to preganglionic cholinergic neuronal activation might produce at least three actions: (a) elicit a direct hyperpolarization of the principal neurons; (b) produce a long-lasting modulatory change in the muscarinic response of the principal neurons to acetylcholine; and (c) reduce the release of acetylcholine from preganglionic neurons. In this report we demonstrate that drugs that interact with cholinergic, adrenergic and dopaminergic receptors alter the metabolism of DA in the celiac ganglion of the rat. The celiac ganglion was chosen for study because it apparently has the highest rate of DA metabolism of the rat sympathetic ganglia [5].

DA and DOPAC were analyzed in a single celiac ganglion by gas chromatography–mass spectrometry, as described previously [3]. The doses of the drugs and the time of killing are shown in Table 1. Our previous studies have shown that the doses of the drugs administered altered brain biogenic amine metabolism. Celiac ganglia were removed under a dissecting microscope after rats (male, Sprague–Dawley, 150–180 g, obtained from Zivic Miller Laboratories, Allison Parks, PA) were decapitated.

The content of DA in the ganglion remained essentially normal after each of the drug treatments (Table 1). In contrast, DOPAC content changed in a manner which suggested that the drugs had activated or inhibited specific receptors. As reported previously, treatment with the muscarinic agonist oxotremorine induced a rise of DOPAC in the ganglion, a rise that was antagonized by atropine pretreatment [5]. Atropine treatment alone resulted in a fall of DOPAC content, suggesting that normally there is tonic activation of SIF cell muscarinic receptors by preganglionic cholinergic neurons.

The β-adrenergic receptor agonist isoproterenol had no effect on the content of DOPAC in the celiac ganglion. In contrast, the α-adrenergic receptor agonist phenylephrine decreased the content of DOPAC, and prior treatment with phenoxybenzamine prevented the fall of DOPAC. Moreover, phenylephrine could partially antagonize the rise of DOPAC after oxotremorine treatment.

The administration of the DA agonist apomorphine had not effect on the content of DOPAC, but the DA antagonist haloperidol, administered alone, significantly increased DOPAC. The increase was not blocked by administering haloperidol together with apomorphine, but it appeared to be blocked by treatment with atropine. The apparent blockade, however, may be the algebraic sum of the fall normally induced by atropine and the rise induced by haloperidol. Phenylephrine treatment reversed the elevated levels of DOPAC induced by haloperidol as well. The blockade of the haloperidol-induced rise of DOPAC by atropine and phenylephrine probably represents physiological antagonism rather than competitive blockade at a common receptor. Whether the haloperidol-induced increase of DOPAC in the ganglia is the consequence of release of DA metabolism from the constraints of a negative