The finding that a correlation between brain S_2-receptor affinity and serotonin antagonist activity *in vivo* exists in the vascular system of the pithed rat makes it likely that serotonin receptors mediating contraction of the resistance vessels belong to the S_2-subtype. This study is, however, not decisive on this point. The assumption that increases in diastolic pressure reflect increases in peripheral resistance (and thereby constriction of the arteriolae) is only allowed when cardiac output is constant. This criterion is not met in this study, since it is as yet unknown how different doses of a serotonin antagonist affect cardiac output changes to a single serotonin dose and whether different antagonists behave in the same way in this respect. What can be concluded from this study is that the receptors mediating the hypertensive response to serotonin in the vascular system of the rat belong to the S_2-subclass. Thus, affinity for brain S_2-receptors can be used to describe the serotonin antagonist potency of a drug towards the hypertensive response to this autocoïd *in vivo*.

In summary, the relationship between binding affinity for S_2-receptors in rat frontal cortex tissue assayed *in vitro* using [^{3}H]mianserin and functional antagonism towards pressor effects *in vivo* in the vascular system of the pithed normotensive rat was investigated. A close correlation ($r = 0.89$) was found to exist between both parameters. The vasopressor response to serotonin in the pithed rat is therefore mediated via S_2-serotonergic receptors.

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Selective mitochondrial activity distinguishes aspirin from salicylate and benzoate in yeast cells

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There are numerous reports of the ability of salicylate drugs to inhibit a wide range of metabolic activities in mammalian systems, and attempts are made to correlate these effects with therapeutic properties. Of particular interest to us are the claims that this group of drugs depresses mitochondrial ATP synthesis [1, 2]. It is difficult to establish whether or not the mitochondrial system is solely or selectively affected by a drug in animal cells, but in yeast cells this point can be readily ascertained [3]. The procedure exploits the ability of yeast cells to grow and divide in the absence of mitochondrial respiratory activity (e.g. under anaerobiosis), meeting energy requirements solely from glycolysis. This necessitates the presence of a fermentable energy source such as glucose but if the substrate is non-fermentable (e.g. a Krebs cycle intermediate), then in the absence of respiration, growth would be precluded. By the same token, selective or specific inhibition, either of mitochondrial function or biogenesis, by a drug would not significantly affect growth in glucose medium but would arrest growth in non-fermentable medium. If the anti-mitochondrial activity of the drug in question were due to interaction with the mitochondrial genome (mtDNA), this could induce the mitochondrial mutation known as *petite colonie*. Scoring this condition is greatly facilitated by its exceptionally high spontaneous mutation rate [about 1% of cells of most yeast strains (*Saccharomyces*), give rise to *petite* colonies on plating] and its easily recognisable phenotype of small white colony and respiratory deficiency (for a detailed account of the *petite* mutation, see [4]). Using this yeast system, a comparative study was made of the activities of the chemically related compounds acetyl salicylate (aspirin), salicylic acid and benzoic acid.

Materials and methods

Aspirin (*o*-acetylsalicylic acid) and benzoic acid were obtained from BDH (Dorset, U.K.), and salicylic acid from Sigma Chemical Co. (St. Louis, MO). Eighteen haploid yeast strains of this laboratory were used and culture medium contained 1% yeast extract with 2% glucose (YED) or 4% glycerol (YEG) as fermentable and non-fermentable carbon and energy sources, respectively. Effects on growth by the drugs were assessed first on agar

medium by drop-inoculation of strains onto drug-containing medium as described in [3]. For tests in liquid medium, cells were pre-grown in YED for 16 hr and inoculated into shake flasks (with side arms to facilitate O.D. reading) to a concentration of 10^6/ml and incubated at 30°.

The respiration rate of cells was measured polarographically in a Clark-type oxygen electrode coupled to a pen recorder. Cells were washed twice in distilled water and resuspended in 2 ml HEPES buffer, pH 6.5, in the electrode chamber. The effect of aspirin at concentrations of 0, 0.05, 0.1, 0.5 and 1 mg/ml was measured after 26 hr culture in 15 ml YEG initially with 1.5×10^7 cells.

Petite colonies were scored firstly on their small size and secondly on their inability to grow when transferred to YEG. In assessing *petite* induction by aspirin, cells were grown to stationary phase in YED in 2 mg/ml of the drug, sampled and plated.

Results and discussion

In all strains, aspirin was more inhibitory to growth on solid YEG than on YED, m.i.c.s (concentrations totally inhibiting growth) ranging from about 1.5 to 2.5 mg/ml on YEG and from 2.5 to 3.5 mg/ml on YED depending on the strain. (A 3-fold difference was the highest noted.) These results indicated that aspirin at high concentrations was, on the whole, more inhibitory to the mitochondrial system than to other cellular processes. Salicylate and benzoate were more potent inhibitors of growth than aspirin but they showed little or no discrimination against the mitochondrion (i.e. m.i.c.s in YEG = YED). The results also indicated that aspirin had a different mode of action from benzoate and salicylate since there was no clear cross-correlation in the inhibition of strains: strains that were relatively sensitive to benzoate tended to be sensitive to salicylate but not to aspirin.

More detailed assessment of the effects of these drugs on the growth of cells was obtained from growth curves in liquid medium. The first strain to be tested was B/B and results are shown in Fig. 1. It can be seen that growth in YEG was stimulated by aspirin at concentrations ranging from 50 to 1000 μg/ml (concentrations of 10 and 25 μg/ml had no detectable effect). This stimulatory effect was a consistent feature of aspirin seen in a number of repeat experiments both with B/B and other strains. At concentrations greater than 1 mg/ml, aspirin depressed growth rate in YEG and arrested growth at about 2 mg/ml.

Growth in YED medium shows two phases (diauxy): initially, glucose represses the synthesis of the mitochondrial respiratory system (Crabtree effect) and growth proceeds by glycolysis (first phase). When glucose is depleted, adaption to the respiratory (second) phase proceeds following a short lag during which the respiratory chain and

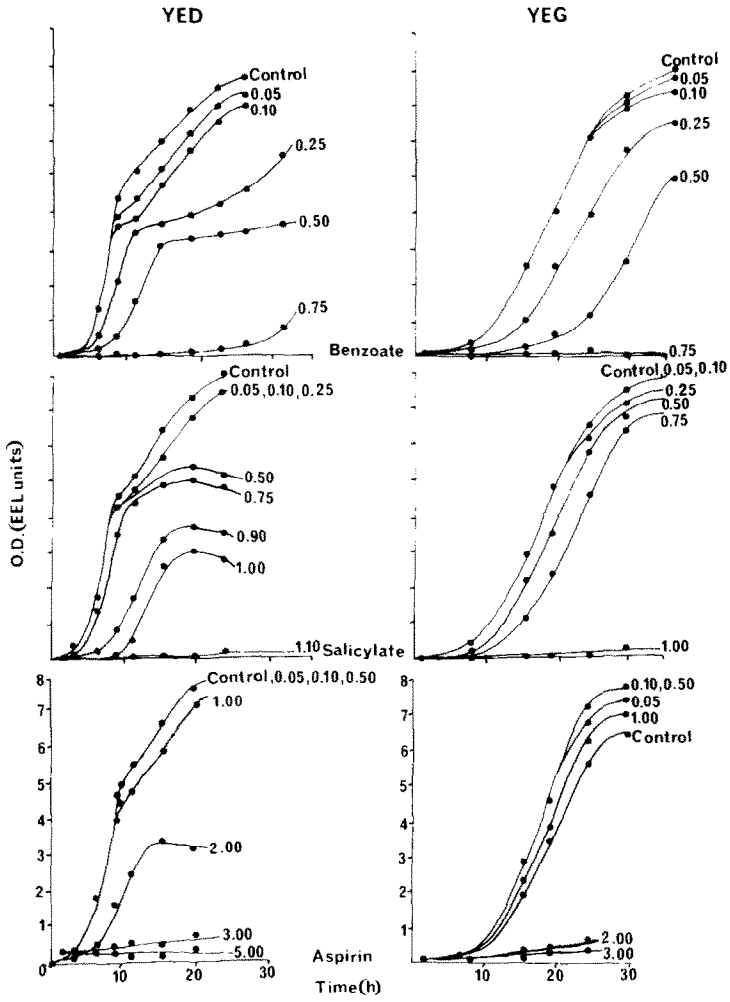

Fig. 1. Effects of aspirin, salicylate and benzoate on the growth of yeast cells (haploid strain B/B) in glucose (YED) and glycerol (YEG) media. Amounts in mg/ml. O.D., optical density measured in the EEL colorimiter (O.D. is closely correlated with density of cells).

associated enzymes are synthesised. It can be seen that aspirin had no stimulatory effect on the growth of cells during the glycolytic phase. This indicates that the increased growth rate in YEG medium is a specific effect on the mitochondrial system and not stimulation of cellular metabolism in general. At 2 mg/ml in the YED culture, aspirin caused a small reduction in growth rate during the glycolytic phase while adaptation to respiration was blocked at this concentration. At a concentration of 3 mg/ml, aspirin totally inhibited growth. It can also be seen that the drug had an effect on the timing of the change-over to the respiratory phase, which was advanced at a concentration of 1 mg/ml. Once respiration was established, there was little or no effect on growth rate at this concentration. This was also true for the lower concentrations.

If aspirin stimulates mitochondrial activity, it might be expected that the presence of the drug in appropriate amounts would lead to an increase in growth rate during the respiratory phase. The absence of any such effect could be explained by the fact that at an optical density of about 4.5 units (the start of the respiratory phase), the density of cells is high ($\sim 10^8$/ml) and this could dilute out the effects of the drug. Indeed, experiments were carried out in which inoculum size was varied and they showed that stimulatory and inhibitory effects of aspirin were greatly influenced by cell numbers, activity being much reduced at high cell density.

From these growth studies, the tentative conclusion was drawn that aspirin selectively reacts with mitochondria in yeast cells, that at lower concentrations this has a stimulatory effect on the organelle but high concentrations inhibit its respiratory function. Some support for these conclusions came from a study of the effects of aspirin on the respiratory activity of cells. Respiring cells of strain B/B were placed in the oxygen electrode and the rate of oxygen uptake measured. Addition of aspirin to the system had no detectable, immediate effect up to 2 mg/ml, but concentrations higher than this directly depressed oxygen uptake and at 5 mg/ml, respiratory activity was totally blocked. Although aspirin at the lower doses had no immediate stimulatory effect on respiratory rate, it was found that cells pre-grown in aspirin (0.25–1 mg/ml) for 16 hr in YEG medium had a significantly greater rate of oxygen uptake than cells in control cultures (~20% increase). Thus, the stimulatory effect was somewhat delayed and possibly required mitochondrial biosynthesis for its manifestation. The other drugs did not affect oxygen uptake. It is of interest that Hial and associates [5] found that salicylate drugs including aspirin, stimulated growth, protein and nucleic acid synthesis in two mammalian cell lines at low concentrations but at high doses (> 1 mM), these processes were inhibited. It is feasible that the stimulation of biosynthesis resulted from increased energy metabolism and that inhibition could be attributed in part to depressed respiration in these mammalian cells. Reports of inhibition of respiratory activity are already available in the literature: in animal cells, aspirin causes a decrease in ATP formation [1] and can uncouple oxidative phosphorylation [2].

Unlike aspirin, benzoate and salicylate showed no growth stimulatory properties but depressed growth in both YEG and YED at concentrations in excess of 100 μg/ml in the case of benzoate and 250 μg/ml for salicylate. Benzoate showed a dose-dependent foreshortening of the glycolytic phase in YED culture while m.i.c.s for both drugs were about 1 mg/ml in YED and YEG, although there was a possible selective mitochondrial activity of salicylate in that some growth was achieved at 1 mg/ml in YED but not in YEG. It was concluded from these results that benzoate and salicylate had little, if any, preferential mitochondrial activity but showed a general inhibitory effect in which salicylate was marginally less potent than benzoate. Both compounds were two to three times as inhibitory as aspirin to the growth of yeast cells at the comparatively high concentrations.

Petite *induction*. Aspirin induced the *petite* mutation in all strains tested in doses in excess of 1 mg/ml (Table 1). This was found to be a consistent feature of aspirin in a number of independent experiments in which the extent of the induction was strain-dependent. The increase in *petite* mutants was shown to be induction and not selection since the relative growth rates of *petite* cells and normal cells in the presence of aspirin were not detectably different from corresponding control cultures assessed from growth curves.

Benzoate and salicylate at a concentration of 1 mg/ml had no effect on the frequency of the *petite* mutation in any of the five strains used in these tests (Table 1).

In summary, it may be said that the results with yeast cells corroborate the findings with mammalian systems in which aspirin was found to inhibit respiratory activity. However, the reactivity of aspirin with mitochondria showed two other aspects in yeast cells, namely mitochondrial stimulation at low concentrations and mitochondrial mutagenesis at high concentrations, neither of which would be readily detected in mammalian systems. Since the main chemical difference between aspirin and the other two compounds is the presence of an acetyl group in the molecule, it is possible that aspirin acts as an acetylating agent, acetylation of mtDNA leading to mutagenesis, and of membrane proteins [6, 7] to inhibition of mitochondrial function. The mechansim of stimulation is more difficult to visualise. Extrapolating to mammalian cells, there may be a connection between the increase in the rate of biosynthesis as reported by Hial *et al.* [5] and stimulation of respiration as already mentioned and this is open to investigation, but mitochondrial mutagenesis as exemplified by the *petite*

Table 1. Effect of aspirin on the mitochondrial mutation *petite* (ρ^-) and viability in various strains of yeast

Treatment	Strain	No. of colonies	Cell viability*(%)	No. ρ^-	ρ^- (%)
Controls	B/B	456	100	3	0.66
	188	1068	100	1	0.09
	D6	801	94	5	0.66
	A30	1084	100	9	0.93
	D18	709	100	4	0.56
Aspirin (2 mg/ml)	B/B	2060	23	58	2.80 (4)†
	188	1493	10	23	1.54 (18)
	D6	1424	14	45	3.16 (5)
	A30	300	4	24	8.0 (9)
	D18	1217	19	32	2.63 (5)

* Ratio of colonies to cells plated.

† The numbers in parentheses represent X spontaneous rate.

mutation is likely to be peculiar to yeast. The possibility remains that high concentrations of aspirin may affect mtDNA adversely in mammalian cells which could be a source of toxicity.

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Inhibition of agonist-induced hydrolysis of phosphatidylinositol and muscarinic receptor binding by the calcium antagonist 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethopybenzoate-HCl (TMB-8)

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Agonist-stimulated hydrolysis of phosphatidylinositol (PI) may be an essential step leading to an increase in intracellular Ca^{2+} concentration [1], although in some cells the hydrolysis apparently follows rather than precedes Ca^{2+} entry [2, 3]. The breakdown of PI in the exocrine pancreas is stimulated by agonists in the absence of extracellular Ca^{2+} [4] but, since these cells have intracellular stores from which Ca^{2+} can be released by agonists [5], this result does not eliminate a role for Ca^{2+} as the activator of PI breakdown in the pancreas. 8-(*N*,*N*-Diethylamino)-octyl-3,4,5-trimethoxybenzoate-HCl (TMB-8) has been used as an intracellular Ca^{2+} antagonist [6–8]. The mode and site of action of this agent have not been defined, although it has been proposed to immbolise Ca^{2+} at the membrane storage sites [7]. We used TMB-8 in an attempt to study the role of intracellular stores of Ca^{2+} in the activation of PI hydrolysis in the pancreas and report the results of these studies here.

The hydrolysis of PI in mouse pancreas was measured *in vitro*, as described previously [4], using pancreatic slices in which the PI had been prelabelled by injection of the mice with *myo*-[2-^{3}H]inositol. The pancreas slices were washed and incubated in Ca^{2+}-free Krebs, pH 7.4, at 37°. Tissue was preincubated with TMB-8 for 5 min prior to a 30-min incubation with agonist.

The Ca^{2+}-free EGTA Krebs solution consisted of (mmoles/l): NaCl, 126; KCl, 4.7; glucose, 2.8; Na fumarate, 2.7; Na glutamate, 4.9; Na pyruvate, 4.9; Tris (hydroxymethyl) aminomethane (Tris), 3; $MgCl_2$, 1.13; ethyleneglycol-bis-(β-amino-ethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 0.1; adjusted with HCl to pH 7.4 at 37° and bubbled with O_2.

The effect of TMB-8 on muscarinic receptor binding was investigated using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to mouse pancreatic acini rather than to pancreas slices due to difficulties encountered with estimating [^{3}H]QNB binding in pancreas slices (our results, unpublished; [9]). Similar binding studies were carried out on a preparation from submandibular gland.

Mouse pancreatic acini were isolated using the method of Williams *et al.* [10]. The acini were resuspended in a buffer consisting of (mmoles/l): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.2; glucose, 14; $CaCl_2$, 2.5; and which also contained soybean trypsin inhibitor (0.1 mg/ml) and essential and non-essential minimal Eagle's medium amino acid supplement (1%, final concentration); equilibrated with 95% O_2/5% CO_2.

Mouse submandibular glands were homogenized in 10 vol. of buffer consisting of (mmoles/l): NaCl, 137; KCl, 5.4; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 5.6; Tris, 20; adjusted to pH 7.4 with HCl.

Muscarinic receptor binding was measured in isolated pancreatic acini and in submandibular gland homogenates incubated in the presence or absence of TMB-8 (2×10^{-5} and 2×10^{-4} moles/l) with [^{3}H]QNB (6×10^{-10} moles/l) in a volume of 2 ml. Submandibular gland homogenates were incubated for 60 min at room temperature; pancreatic acini were incubated at 37° for 120 min. TMB-8 was added 5 min before the addition of [^{3}H]QNB. Bound [^{3}H]QNB was separated by rapid filtration through Whatman GF/B glass fibre filters followed by two washes with 4 ml of ice-cold phosphate buffer (0.1 mole/l, pH 7.4). Non-specific binding was estimated from ^{3}H bound in the presence of atropine (10^{-5} moles/l) and represented 35 and 10% of total bound ^{3}H in pancreatic acini and submandibular gland homogenates respectively. Total ^{3}H bound represented less than 5% of ^{3}H present in the incubation medium.

The DNA content of the pancreatic acini was measured using the fluorescent dye Hoechst 33258 as described by Labarca and Paigen [11].

An unpaired *t*-test was used to determine the significance of differences in two group comparisons. For multiple group comparisons, results were analysed using a two-way analysis of variance to determine the significance between groups. To test if there was an interaction between the agonist and TMB-8, factorial analysis [12] was used.

Activation of muscarinic receptors or cholecystokinin (CCK) receptors produces secretion by the exocrine pan-