ruled out. Such interactions are not likely to happen in therapeutics because cyclohexanol, as well as cefotiam, has subsaturating plasma levels. On the other hand, neither cyclohexanol nor cefotiam could displace drugs bound to site I or II because of their weak affinities; saturation of HSA binding sites could only occur with cefotiam or cyclohexanol concentrations above 1 mM greatly in excess of therapeutic plasma levels.

The binding of cyclohexanol and cefotiam to RBCs follows a non-saturable process within the therapeutic range. The erythrocyte bound fraction is higher in phosphate buffer than in plasma (Table 3) indicating a retention of both compounds by the plasma proteins. Intra-erythrocyte fraction of the two drugs is low (Table 3). This would tend to indicate that they cannot easily penetrate RBC membranes by passive diffusion.

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Receptor binding at two different temperatures to discriminate agonist and antagonist behaviour of adenosine A_1 receptor ligands in rat brain

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Abstract—The inhibitory binding constants, K_i, at the adenosine A_1 receptor in rat brain have been measured at 0 and 25°C for 25 typical ligands. The K_i ratios at the two temperatures are greater and smaller than unity for adenosine agonists and xanthine antagonists, respectively. These results suggest that two-temperature measurements of in-vitro K_i constants represent a simple method of discriminating between in-vivo agonistic and antagonistic behaviour of A_1 adenosine receptor ligands.

In general, receptor binding measurements give accurate information on drug receptor affinities but little or none about the nature of the effect which therefore has to be determined by independent in-vitro and in-vivo experiments. However, drug design and testing would be expedited by a method able to discriminate between agonist and antagonist properties by simple affinity constant measurements. This has been proved possible in some cases by adding specific substances able to modulate in different ways the binding of drugs which produce different effects. For instance, it is known that prior incubation of brain membranes with γ-aminobutyric acid (GABA) modulates the binding of benzodiazepine receptor ligands in such a way that the ligand agonistic, antagonistic and inverse agonistic behaviour can be discriminated (Möhler & Richards 1981). Moreover, it is generally recognized that the presence of certain ions, e.g. Na^+ or Mg^{2+}, can modulate in different ways the binding of agonists and antagonists in several receptor systems (Creese 1985). As far as the adenosine A_1 receptor is concerned, it has been reported (Goodman et al 1982) that guanine nucleotides and divalent cations affect agonist and antagonist binding differently; in particular, GTP (guanosine-5′-triphosphate) is currently employed for its property of reducing agonist but not antagonist affinity at this receptor.

A different way of modulating the recognition process of

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drugs by receptors can be identified by changing the temperature of the binding experiment. Weiland et al (1979) studied the specific binding of a series of ligands to the β-adrenergic receptor of turkey erythrocyte membranes in the range of temperatures 1–37°C and observed that full agonists display a large affinity decrease with increasing temperature, whereas the affinities of antagonists remain essentially unaffected.

Murphy & Snyder (1982) investigated three agonists cyclohexyl adenosine, (−)-N^6-phenylisopropyladenosine and (+)-N^6-phenylisopropyladenosine and two antagonists 3-isobutyl-1-methylxanthine and 1,3-diethyl-8-phenylxanthine at the A_1 adenosine receptor of bovine brain tissue in a temperature range of 0–30°C and found that agonist affinity increases while antagonist affinity decreases with increasing temperature.

We report here preliminary results assessing the reliability of the A_1 agonist/antagonist discrimination by simple two-temperature (0 and 25°C) binding experiments.

Materials and methods

Male Wistar rats, 150–200 g, were decapitated and the whole brain (minus striatum) was dissected on ice. The tissue was homogenized in a Polytron (setting 5) in 20 vol of 50 mM Tris-HCl buffer pH 7·4. The homogenate was centrifuged at 48 000 *g* for 10 min and the pellet was suspended in buffer, centrifuged and resuspended in Tris-HCl containing 2 int. units mL^{-1} of adenosine deaminase (Sigma, type VI). After 30 min incubation at 37°C the membranes were centrifuged and the pellet was stored at −70°C.

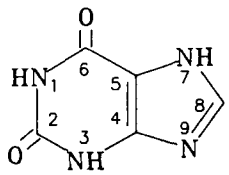
Binding experiments with [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA, New England Nuclear, 13·5 Ci $mmol^{-1}$) (Bruns et al 1980) were performed in 1 mL of buffer containing 1 nM [^{3}H]CHA, membranes from 15 mg (wet weight) of tissue and the compounds to be tested. After 150 min incubation at 0°C or 120 min at 25°C, separation of bound from free ligand was performed by rapid filtration through Whatman GF/B filters which were washed three times with ice-cold buffer, dried and counted in 5 mL of acidified Instagel (Packard, Downers Grove, USA). The incubation times were chosen according to the results of previous time-course experiments and were similar to those used by other authors (Murphy & Snyder 1982). Non-specific binding was defined as binding in the presence of 10 μM (−)-N^6-phenylisopropyl adenosine and was ≤ 10% of total binding. To determine IC50 values, the test compounds were added in triplicate to the binding assay at, at least, six concentrations and the IC50 values were calculated by means of the IBM computer program LIGAND (Munson & Rodbard 1980). K_i values were calculated from the Cheng-Prusoff equation (Cheng & Prusoff 1973).

All adenosine and xanthine compounds used in the work were obtained from Research Biochemical Incorporated (Natick, MA, USA).

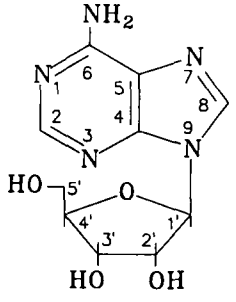
Results and discussion

Final values of K_i (25°C), K_i (0°C) and of the ratios K_i (0°C)/K_i (25°C) are reported in Tables 1 and 2 for agonists and antagonists, respectively. Fig. 1 shows two typical [^{3}H]CHA displacement curves carried out at 25°C for a characteristic agonist, (+)-N^6-phenylisopropyladenosine, and a characteristic xanthine antagonist, 1,3-dipropyl-8-cyclopentyl xanthine. The Hill coefficients, nH, are also reported for the two temperatures in Tables 1 and 2 and are not significantly different from unity for either class of compounds suggesting the presence, at least in our experimental conditions, of a single class of binding site. On average, agonists appear to be rather stronger ligands than antagonists but the most significant difference between these two classes of drugs is given by the ratio K_i (0°C)/(K_i(25°C), which is greater (2·0–5·9) or smaller (0·15–0·80) than unity for agonists and antagonists, respectively. The two distributions of the ratio have average and standard deviation of 3·2 ± 1·1 for agonists and 0·38 ± 0·17 for antagonists and are different at the significance level of $P \leq 0{\cdot}01$ (Wilcoxon-Mann-Whitney test).

Present findings suggest that antagonistic binding is always an exothermic process while that of agonists is inevitably endothermic and therefore must be driven by an increase of entropy. This result is somewhat surprising considering the chemical structure of these drugs where the agonists are characterized by a ribose moiety which is missing in antagonists (Schemes I, II).



SCHEME I. Structure of adenosine.



SCHEME II. Structure of xanthine.

Rational considerations would suggest that molecules having a larger number of ether or hydroxyl oxygens (in this case the agonists carrying the ribose moiety) are more efficient hydrogen

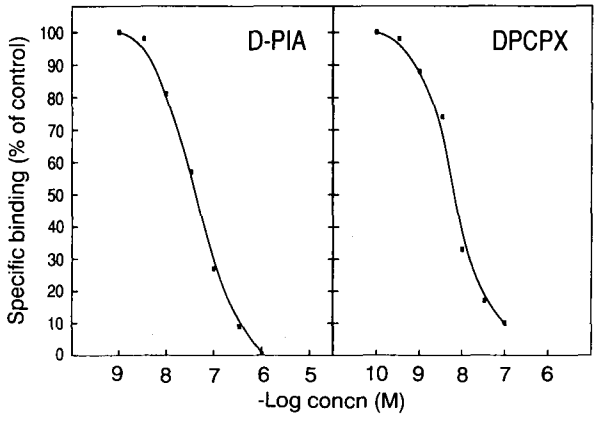


FIG. 1. Inhibition of binding, at 25°C, of 1 nM [^{3}H]CHA by a typical agonist, (+)-N^6-phenylisopropyladenosine (on the left), and a typical antagonist, 1,3-dipropyl-8-cyclopentylxanthine (on the right), in membranes from rat brain. Points are means of three or more samples. Curves are the best-fit solutions to the one-site Langmuir model (Munson & Rodbard 1980). The two-site model did not converge.

Table 1. Inhibitory binding constants, K_i (M), of a series of agonists at the A_1 adenosine receptor in rat brain tissue at 0 and 25°C and their K_i (0°C)/K_i (25°C) ratios. Values represent means from at least three separate experiments. (nH = Hill coefficient; e.s.d.s on equilibrium constants and on Hill coefficients are in the range 2–10%)

Agonists	K_i (0°C)	nH	K_i (25°C)	nH	K_i Ratio
5′-(N^6-Cyclopropyl)carboxamidoadenosine	2.4×10^{-9}	0.95	4.1×10^{-10}	0.93	5.9
N^6-Cyclopentyladenosine	1.5×10^{-10}	0.98	3.2×10^{-11}	0.99	4.7
N^6-Cyclohexyladenosine	4.2×10^{-9}	0.97	1.0×10^{-9}	0.97	4.2
2-Phenylaminoadenosine	4.4×10^{-6}	1.01	1.2×10^{-6}	0.98	3.7
5′-(*N*-(Methyl)carboxamidoadenosine	2.1×10^{-7}	0.91	6.5×10^{-8}	0.94	3.2
(−)-N^6-Phenylisopropyladenosine	1.8×10^{-9}	0.93	6.0×10^{-10}	0.95	3.0
2-Chloroadenosine	1.2×10^{-8}	0.89	4.3×10^{-9}	0.93	2.8
N^6-Phenyladenosine	1.1×10^{-8}	1.00	4.1×10^{-9}	0.97	2.7
N^6-Benzyladenosine	1.5×10^{-7}	1.05	5.8×10^{-8}	1.00	2.6
N^6-Phenylethyladenosine	2.6×10^{-8}	0.91	1.1×10^{-8}	0.95	2.4
N^6-Methyladenosine	2.9×10^{-7}	0.96	1.3×10^{-7}	0.93	2.2
(+)-N^6-Phenylisopropyladenosine	2.5×10^{-8}	0.96	1.2×10^{-8}	0.98	2.1
5′-(*N*-Ethyl)carboxamidoadenosine	1.7×10^{-8}	0.98	8.6×10^{-9}	0.96	2.0

Table 2. Inhibitory binding constants, K_i (M), of a series of antagonists at the A_1 adenosine receptor in rat brain tissue at 0 and 25°C and their K_i (0°C)/K_i (25°C) ratios. Values represent means from at least three separate experiments. (nH = Hill coefficient; e.s.d.s on equilibrium constants and on Hill coefficients are in the range 2–10%)

Antagonists	K_i (0°C)	nH	K_i (25°C)	nH	K_i Ratio
3-n-Propylxanthine	2.0×10^{-5}	0.89	2.5×10^{-5}	0.91	0.80
Theobromine	3.3×10^{-5}	0.93	5.2×10^{-5}	0.95	0.63
1,3-Dipropyl-8-*p*-sulphophenylxanthine	9.4×10^{-8}	0.91	2.1×10^{-7}	0.93	0.45
8-Phenyltheophylline	3.6×10^{-8}	1.01	8.1×10^{-8}	1.00	0.44
Caffeine	1.9×10^{-5}	0.98	4.5×10^{-5}	0.96	0.42
7-n-Propylxanthine	4.6×10^{-6}	0.95	1.4×10^{-5}	0.94	0.33
Theophylline	3.8×10^{-6}	0.97	1.2×10^{-5}	0.94	0.32
7-(β-Chlorethyl) theophylline	4.3×10^{-6}	0.94	1.4×10^{-5}	0.96	0.31
1,3-Dipropyl-8-cyclopentylxanthine	6.4×10^{-10}	0.95	2.2×10^{-9}	0.95	0.29
3-Isobutyl-1-methylxanthine	1.5×10^{-6}	0.91	5.9×10^{-6}	0.93	0.25
1,3-Diethyl-8-phenylxanthine	2.0×10^{-8}	1.01	1.1×10^{-7}	1.00	0.18
8-Cyclopentyltheophylline	1.9×10^{-9}	1.00	1.3×10^{-8}	0.97	0.15

bonding donors or acceptors and that their binding should become more exothermic than that of molecules lacking such a hydrogen bonding group (here the antagonists). A possible interpretation of the present results arises, however, from considering that the drug molecule is completely hydrated in solution, forming the maximum number of hydrogen bonds compatible with its chemical structure and that a part of such energetically favourable interactions can be lost during the binding process owing to a mismatch between drug and receptor hydrogen bond donor-acceptor groups.

From a more practical point of view present findings are consistent with the proposal that two-temperature measurements of in-vitro inhibitory binding constants can be a simple method of discriminating between in-vivo agonistic and antagonistic behaviour of A_1 adenosine receptor ligands, agonists and antagonists being characterized by K_i (0°C)/K_i (25°C) ratios respectively greater or less than unity.

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