The binding of [^3H]adenosine to synaptosomal and other preparations from the mammalian brain

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1. A high-affinity adenosine-binding site with K_d (adenosine) 0.5–1.3 μM was demonstrated in particulate and synaptosomal fractions isolated from the cerebral cortex of guinea pig, rat and ox. 2. Binding of [^3H]adenosine to this site was inhibited by theophylline and by 2-chloroadenosine, but not by four other adenosine analogues. 3. Endogenous adenosine, found to be present in some preparations at approx. 1 pmol/mg of protein, diminished the binding capacity of the preparations for [^3H]adenosine. 4. Addition of the adenosine deaminase inhibitor *erythro*-9-[1-(1-hydroxyethyl)heptyl]-adenine revealed the presence of a second lower affinity binding site with K_d (adenosine) 5–9 μM and a higher maximal adenosine-binding capacity. The inhibitor partially blocked binding to the high-affinity site in preparations from which adenosine deaminase had been removed by washing. 5. To preparations of particulate fractions maintained under iso-osmotic conditions, adenosine attachment was non-saturable and temperature-dependent, indicating the existence of an active uptake process. 6. The location and binding constant of the high-affinity adenosine-binding site suggest that it corresponds to the receptor site for adenosine-activated adenylate cyclase.

Physiological and regulatory responses to adenosine are widespread in biological systems, in a few of which adenosine receptors have received initial characterization (see Baer & Drummond, 1979, and the Discussion section of the present paper), by binding studies and by observation of adenosine-activated adenylate cyclases. We recently reported high-affinity binding of adenosine to preparations from the brain (Newman *et al.*, 1980) and now examine the binding in greater detail to link it with previous findings (Kakiuchi *et al.*, 1969; Sattin & Rall, 1970; McIlwain, 1972; Daly, 1977; Newman & McIlwain, 1977, 1978) on adenylate cyclases and other enzymes of the brain, and with the observations of Schwabe *et al.* (1979) on adenosine binding to a differently derived cerebral membrane preparation.

Experimental

Materials

[2- ^3H]Adenosine (sp. radioactivity 21 Ci/mmol) was obtained from The Radiochemical Centre,

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Amersham, Bucks., U.K. Adenosine deaminase, adenosine, adenine, inosine and cyclic AMP were from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.; 2-chloroadenosine was from Sigma, Kingston-upon-Thames, Surrey, U.K.; theophylline hydrate was from BDH, Poole, Dorset, U.K.; Carb-O-Sil was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; t.l.c. plates (Polygram, Sil G) were from Macherey Nagel and Co., 5160 Duren, Postfach 307, Germany; glass microfibre filters (GF/B) were from Whatman, Maidstone, Kent, U.K.; and membrane filters (Nulflow) were from Oxoid, London S.E.1, U.K. The adenosine deaminase inhibitor *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine was a kind gift from Burroughs Wellcome Co., Research Division, Triangle Park, NC, U.S.A.

Subcellular fractionation

Cerebral cortex from guinea-pig, rat and ox brain was fractionated as described previously (Newman & McIlwain, 1978), except that an SS-34 rotor in a Sorvall RC-5B centrifuge was used to prepare the primary fractions. Recovery of protein [as a percentage ($n = 4$) of that in the whole homogenate] was $22.8 \pm 4.4\%$ in the nuclear fraction, which was washed once with a volume of 0.32 M-sucrose/0.5 mM-EDTA equal to that of the homogenate,

$44.2 \pm 7.9\%$ in the primary mitochondrial fraction, and $33.8 \pm 4.3\%$ in the supernatant fraction. In some experiments the supernatant was further fractionated by centrifugation at $105\,000g$ for 1 h in a type 40 rotor with a Spinco model L ultracentrifuge to yield a microsomal supernatant. The primary mitochondrial fraction was separated into myelin, synaptosomal and mitochondrial fractions by sucrose-density-gradient centrifugation at $95\,000g$ for 1.5 h in an SW-27 rotor, whereas when fractions were prepared from slices that had been superfused as described previously (Newman & McIlwain, 1978), an SW-50 rotor was used and only the synaptosomal fraction was collected. In some experiments the fractions were washed by dilution in 4–30 vol. of water, centrifugation at $10\,000g$ for 20 min and resuspension of the pellet in 10 mM-Tris/HCl/5 mM-MgCl₂, pH 7.4. Recovery of protein from the density gradient [as a percentage ($n = 4$) of that in the primary mitochondrial fraction] was $14.7 \pm 2.4\%$ in the myelin fraction, $62.7 \pm 7.2\%$ in the synaptosomal fraction and $21.1 \pm 6.6\%$ in the mitochondrial fraction. Further characterization of fractions prepared in this manner has been given by Marchbanks (1975). The recovery of protein in the primary and synaptosomal fractions was not affected by incubation of slices or the various agents used for superfusion.

Adenosine binding

The assay medium for determination of adenosine binding routinely consisted of 20 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂, 0.25 mM-EDTA, $0.1\ \mu\text{M}$ -[³H]-adenosine and $25\ \mu\text{M}$ -erythro-9-[1-(1-hydroxyethyl)-heptyl]adenine in a total volume of 0.2 ml. For specific experiments, the [³H]adenosine concentration was varied more widely (see Table 3). The reaction was started by adding the tissue preparation to be assayed, containing about $100\ \mu\text{g}$ of protein. After 90 min at 0°C , 2.5 ml of 10 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂ was added and the contents of each incubation tube were then immediately filtered by vacuum through a Whatman GF/B filter, diameter 2.5 cm, or in some cases a Millipore HA filter, pore size $0.45\ \mu\text{m}$. The tubes were washed out twice with an equal volume of the same buffer and the filters were then washed a further three times and dried in an oven at 80°C before being placed in vials containing 4 ml of toluene/Synperonic NXP (2:1, v/v) scintillation fluid containing 2,5-diphenyloxazole (0.55%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%, w/v).

Non-specific binding was determined (see Bennett, 1979) in a reaction mixture of the composition specified above, but containing in addition non-radioactive 0.1 mM-adenosine. Samples of the same tissue preparations were added to this reaction

mixture, and the radioactivity bound to the filters in the presence of the excess of unlabelled adenosine was subtracted from the total radioactivity retained. The value obtained was converted into the amount of adenosine specifically bound by using the known specific radioactivity of [³H]adenosine. Of 3.5×10^5 c.p.m. present in each incubation tube, approx. 3×10^3 c.p.m. were retained on each type of filter, and of this retention 1×10^3 c.p.m. were contributed by labelled adenosine bound non-specifically. Each determination was performed in triplicate, with additional tubes in duplicate for determination of non-specific binding.

For determination of affinity constants, 1 ml of the $1\ \mu\text{M}$ -[³H]adenosine solution obtained by approx. 50-fold dilution of the stock solution was mixed with 0.6 ml of unlabelled $105.5\ \mu\text{M}$ -adenosine to give a final concentration of $40.2\ \mu\text{M}$. Then 5–100 μl volumes of both $1\ \mu\text{M}$ and $40.2\ \mu\text{M}$ solutions were used to give a final adenosine concentration range of 0.05 – $16\ \mu\text{M}$ in the incubation volume of 0.2 ml.

The extent of loss of bound [³H]adenosine during the filtering and washing procedure was examined with the synaptosomal fraction in the experiments of Fig. 1 (see Bennett, 1979). The fraction ($95\ \mu\text{g}$ of

Fig. 1. Dissociation of bound adenosine in relation to assay conditions

Binding assays were carried out with $3\ \mu\text{M}$ -[³H]-adenosine in the absence and presence of unlabelled 1.5 mM-adenosine, but reaction mixtures were diluted 250-fold at specified times before filtration (see text) with wash buffer at 0°C . The main Figure shows retained binding on the filter of $0.063\ \text{pmol}$ of [³H]adenosine/mg of protein (r). Points give mean values for three results, and the vertical lines show the S.E.M. The inset is a logarithmic plot of initial binding minus r , with extrapolation for the 30 s occupied by filtration.

protein) was kept for 90 min at 0°C in 0.2 ml of 20 mM-Tris-HCl (pH 7.4)/0.5 mM-EDTA/5 mM-MgCl₂/0.3 μ M-[2-³H]adenosine, with and without unlabelled 1.5 mM-adenosine. At various times before filtration, the reaction mixture was diluted 250-fold with ice-cold 10 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂, kept at 0°C and filtered. The volume of diluting fluid was chosen to be equal to or greater than the volumes of fluid used in the filtering and washing procedure, which in the routine assay occupied about 30 s. The extrapolation of Fig. 1 suggests that some 7% of bound [³H]adenosine is lost during the washing of the routine assay, but no correction for this has been applied in reporting data.

Identification of material bound by t.l.c.

Synaptosomal material (390 μ g of protein) was incubated with [³H]adenosine at a final concentration of 0.68 μ M in a total volume of 0.4 ml, with and without excess non-radioactive adenosine. After 90 min at 0°C the mixtures were filtered through 0.45 μ m Millipore filters, which were thoroughly washed and then eluted with 0.25 ml of 10% (w/v) trichloroacetic acid containing 0.9 mM-adenosine. The filters were dried by centrifugation and 100 μ l of the eluate corresponding to each original incubation tube was applied to a Polygram Cel 300 polyethyleneimine-cellulose thin-layer plate containing fluorescent indicator, which was developed for 3 h in butanol/ethyl acetate/methanol/NH₃ (sp.gr. 0.88) (7:4:3:4, by vol.). Standard solutions were applied to the plate as 50 μ l spots in 10% trichloroacetic acid and detected after development under u.v. light. The separation gave *R_F* values for adenosine, hypoxanthine and inosine of 0.42, 0.35 and 0.23 respectively, and the areas of the plate corresponding to these compounds were scraped off and counted for radioactivity in Triton/toluene scintillant containing 3% Carb-O-Sil.

Results

Subcellular distribution and compounds bound

Measurements with subcellular fractions isolated from guinea-pig neocortex and resuspended in 0.32 M-sucrose with 0.1 μ M-[³H]adenosine in the presence of the adenosine deaminase inhibitor erythro-9-[1-(1-hydroxyethyl)heptyl]adenine showed the greatest binding per unit of protein to occur in the microsomal and myelin fractions (Table 1). There was little binding in the soluble as opposed to the particulate fractions.

Of the total labelled adenosine added to the incubation tubes for the synaptosomal fraction, 76% was recovered as adenosine after correction for recovery of the label applied to the t.l.c. plate (72.6%). When radioactivity bound in the additional presence of excess unlabelled adenosine was

Table 1. Adenosine binding to subcellular fractions of guinea pig cerebral cortex

Fractions were obtained as described in the Experimental section and samples kept for 90 min at 0°C with [³H]adenosine at a final concentration of 0.1 μ M in the presence of 25 μ M-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine. Radioactivity bound was corrected for non-specific binding determined in the presence of unlabelled 0.1 M-adenosine. Filtration was performed by using Whatman GF/B glass-fibre filters unless otherwise stated. Mean values are given $\pm$ s.d. with the numbers of observations in parentheses. The relatively high s.d. values are attributed to the presence of variable amounts of endogenous adenosine (see text).

Preparation	[³ H]Adenosine bound (pmol/mg of protein)
Homogenate	0.16 $\pm$ 0.02 (5)
Nuclear	0.21 $\pm$ 0.09 (3)
Crude mitochondrial	0.21 $\pm$ 0.08 (8)
10000 g Supernatant	0.15 $\pm$ 0.08 (10)
Microsomal	0.38 $\pm$ 0.19 (6)
105000 g Supernatant, 0.45 μ m filters	0.17 $\pm$ 0.06 (3)
Myelin	0.43 $\pm$ 0.17 (3)
Synaptosomal	0.13 $\pm$ 0.04 (4)
Mitochondrial	0.17 $\pm$ 0.09 (4)

subtracted, however, 92% of the labelled material adsorbed to the filters was adenosine, with only minor components corresponding to inosine and hypoxanthine.

Properties of adenosine-binding sites in the primary mitochondrial and the synaptosomal fractions

Adenosine binding to these fractions was linear with protein concentration (Fig. 2b) and showed a pH optimum at 7.4 (Fig. 2d). Binding was increased in the presence of Mg²⁺ (Fig. 2c), but reached a plateau at 5 mM. When the material was stored in iso-osmotic solution so that osmotic lysis took place only during the exposure period, binding reached equilibrium around 90 min (Fig. 2a). Routine assays were therefore performed for 90 min at 0°C, at pH 7.4 and in the presence of 5 mM-MgCl₂.

The effects of various compounds structurally related to adenosine on binding are shown in Fig. 3. Adenine had virtually no effect, whereas inosine, AMP and cyclic AMP produced dose-dependent inhibition of [³H]adenosine binding, although not to as great an extent as inhibition by adenosine itself or by its analogue 2-chloroadenosine. The effect of various treatments of the primary mitochondrial fraction was examined. Storage at 4°C, washing, and deliberate disruption of the structure by osmotic lysis resulted in increased [³H]adenosine binding measured at 0.1 μ M-adenosine concentration from 0.22 $\pm$ 0.07 to respectively (mean $\pm$ s.d. of three to

Fig. 2. Factors affecting adenosine binding

In all experiments [³H]adenosine was present at 0.1 μM and non-specific binding was determined in the presence of unlabelled 0.1 mM-adenosine. (a) Time course of [³H]adenosine binding in initially intact and in lysed synaptosomal preparations. Synaptosomes (80–100 μg of protein) were prepared as described in the Experimental section and added directly to a solution of 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA (●), or were lysed by diluting in 10 vol. of water and being left for 1 h in ice before assay (○). (b) Dependence of adenosine binding on the quantity of primary mitochondrial preparation used. Various amounts of the mitochondrial fraction (expressed as protein) were kept with 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA for 90 min at 0°C. (c) Mg²⁺-dependence of adenosine binding to the synaptosomal fraction. Synaptosomes (103–145 μg of protein) were kept for 90 min at 0°C in buffer containing 20 mM-Tris/HCl, pH 7.4, 0.5 mM-EDTA and various amounts of MgCl₂ to give the final Mg²⁺ concentrations shown on the abscissa. (d) pH-dependence of adenosine binding to the crude mitochondrial fraction. A primary mitochondrial preparation (100 μg of protein) was kept for 90 min at 0°C in 5 mM-MgCl₂ with 20 mM-sodium phosphate buffers over the pH range 5.5–9.

five values) 1.44 ± 0.20 , 1.15 ± 0.58 and 0.54 ± 0.02 pmol/mg of protein. This could be due either to increased availability of binding sites or to release of endogenous bound adenosine during the various treatments. Since less than the 90 min allowed for incubation was required for an initially intact preparation to undergo lysis and achieve saturation of the maximum available number of binding sites at 0°C (Fig. 2a), it was considered that endogenous adenosine was responsible for the lower binding of added [³H]adenosine in the untreated preparation. Further evidence for the role of endogenous adenosine is given in Table 2, which shows adenosine binding by fractions obtained from slices of guinea-pig neocortex that had been incubated and superfused with various added agents. Synaptosomal fractions derived from slices that had been incubated with adenosine showed decreases in adenosine binding, whereas in slices treated with adenosine deaminase there were significant in-

creases in both the primary mitochondrial and synaptosomal fractions. The difference between adenosine bound to these fractions in control and deaminase-treated slices (0.8–0.9 pmol/mg of protein) can be taken as a measure of endogenous adenosine bound under control conditions.

Use of adenosine deaminase inhibitor

The influence of adenosine deaminase on binding is shown by the results in Fig. 4(a) in which the effect of including various concentrations of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine in the binding-assay medium at 0.1 μM-adenosine was studied in lysed preparations before and after washing to remove cytoplasmic material associated with the particulate fractions. In unwashed fractions binding increased with increasing erythro-9-[1-(1-hydroxyethyl)heptyl]adenine concentration, owing to progressive inhibition of deaminase, whereas after

washing of the preparation to remove the deaminase there was a decrease to concentrations reached in the unwashed preparation at higher inhibitor concentrations. This could be explained by an interaction of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine with the adenosine receptor (see the Discussion section). At 30 μ M-adenosine, binding increased with *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine concentration, reaching a maximum at 25 μ M, and declined at higher concentrations (Fig. 4b). Saturation analyses were therefore conducted in the

absence and presence of 25 μ M-*erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine to determine whether inhibition of adenosine deaminase affected the results.

Fig. 3. Diminution of [³H]adenosine binding at 0.1 μ M to washed synaptosomal fractions, by structural analogues of adenosine

Washed synaptosomal material (52–108 μ g of protein) was kept at 0°C for 90 min with 0.1 μ M-³H]adenosine and unlabelled compounds as shown below at concentrations given on the abscissa. Results are calculated as percentage of radioactivity bound in the absence of added agents after correction for radioactivity adsorbed to the filter in the absence of the tissue fraction. The initial (100%) values for binding to the synaptosomal fractions were 0.47 $\pm$ 0.28 (9) pmol/mg of protein. Binding is shown in the presence of unlabelled adenosine ($\square$), inosine ($\blacktriangle$), AMP ($\circ$), cyclic AMP ($\triangle$), 2-chloro-adenosine ($\bullet$) and adenosine ($\blacksquare$).

Fig. 4. Effect of *erythro*-9-[1-(1-hydroxyethyl)heptyl]-adenine on [³H]adenosine binding to washed and to unwashed synaptosomal preparations

(a) The synaptosomal fraction (26–65 μ g of protein) was kept for 90 min at 0°C with 0.1 μ M-³H]adenosine and various concentrations of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine. The fraction was added either as a suspension in sucrose ($\bullet$) or after washing and resuspension in 10 mM-Tris/5 mM-MgCl₂ as described in the Experimental section ($\circ$). The binding reaction was carried out for 90 min at 0°C and non-specific binding was determined in the presence of 0.1 mM-adenosine. (b) The synaptosomal fraction (68–145 μ g of protein) was kept with 30 μ M-³H]adenosine and various concentrations of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine for 90 min at 0°C. Non-specific binding was determined in the presence of 4 mM-adenosine. Vertical bars from points corresponding to S.D. values ($n = 4$).

Table 2. Adenosine binding to subcellular fractions: effects of prior tissue incubation with adenosine and adenosine deaminase

Tissue slices were incubated for 30 min with glucose/bicarbonate saline (Kakiuchi *et al.*, 1969) and superfused for 20 min with glucose/bicarbonate saline containing agents specified below, before homogenization in 0.32 M-sucrose and preparation of subcellular fractions. Mean values are quoted $\pm$ S.D., with numbers of observations in parentheses.

Preparation examined	[³ H]Adenosine bound (pmol/mg of protein)		
	No addition	Adenosine (0.1 mM) (4)	Adenosine deaminase (10 μ g/ml) (3)
Homogenate	0.21 $\pm$ 0.10 (8)	0.19 $\pm$ 0.02	0.57 $\pm$ 0.24
Nuclear fraction	0.35 $\pm$ 0.18 (4)	0.42 $\pm$ 0.25	0.51 $\pm$ 0.16
Mitochondrial fraction	0.23 $\pm$ 0.13 (7)	0.16 $\pm$ 0.10	1.21 $\pm$ 0.49
Supernatant	0.34 $\pm$ 0.15 (4)	0.16 $\pm$ 0.09	0.11 $\pm$ 0.03
Synaptosomal fraction	0.41 $\pm$ 0.25 (8)	0.08 $\pm$ 0.06	1.15 $\pm$ 0.20

Saturation analysis

Saturation analysis of adenosine binding to a primary mitochondrial preparation from guinea-pig cerebral cortex in the absence of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine showed the existence of a single high-affinity binding site, whereas addition of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine revealed a second lower-affinity binding site (Table 3). The same pattern of activity was present in the primary mitochondrial fractions isolated from rat or bovine neocortex, although the maximal binding capacity of the high-affinity receptor in these cases was significantly lower than that in guinea pig. Synaptosomes prepared from guinea-pig cerebral cortex showed a similar pattern when incubated with hypo-osmotic buffer so that complete lysis occurred. In these respects, therefore, we detect no differences between the mitochondria of the primary mitochondrial fraction and of the synaptosomes, despite their differences in cytological origin. The experiment illustrated in Fig. 5 shows that the high-affinity binding site gave similar K_d values ($1.67\mu\text{M}$ and $1.11\mu\text{M}$ respectively) and similar maximal binding capacities (B_{max} ; 6.2 and 4.7pmol/mg of protein respectively) when determined in the absence and presence of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine, whereas a lower-affinity binding site with K_d $20.2\mu\text{M}$ and B_{max} 28.7pmol/mg of protein was only observed in the presence of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine. After washing the preparation, both high- and low-affinity sites were observed in both the presence and absence of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine, whereas when the osmotic strength of the synaptosome

suspension was maintained by inclusion of sucrose in the binding-assay medium to give a final concentration of 0.32M , no saturation was evident, with binding increasing linearly with increasing adenosine concentration to reach values of 50pmol/mg of protein (Fig. 6). A similar pattern of behaviour was shown by the mitochondrial (P_2C) fraction obtained from the bottom of the sucrose gradient, where very high values of binding were obtained both with and without added inhibitor. The difference in binding between intact and lysed preparations can thus be interpreted in terms of adenosine uptake by the intact preparation.

An independent estimate of the lower-affinity binding constant for attachment of the greater proportion of adenosine to intact synaptosomal preparations was obtained from the ratio of the [^3H]adenosine-dissociation rate constant (0.113min^{-1}) from the values of Fig. 1(b), and an association rate constant ($14560\mu^{-1}\cdot\text{min}^{-1}$) derived from the values of Fig. 2(a). This estimate for the binding constant, $7.8\mu\text{M}$, was reasonably close to the value of $5.4 \pm 3.5\mu\text{M}$ given in Table 3.

Both myelin and microsomal fractions (Table 3) showed a single adenosine-binding site and addition of *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine made little difference to this finding. In the presence of theophylline, binding of $0.1\mu\text{M}$ -[^3H]adenosine to the synaptosomal fraction was significantly inhibited in a dose-dependent manner (Fig. 6), whereas binding to the myelin fraction was little affected.

Relationship to adenosine uptake

To distinguish the binding of adenosine to the high-affinity theophylline-sensitive site from its bind-

Table 3. *Affinity constants and maximal binding capacities of cerebral-cortical (unless specified) preparations*
Binding was carried out at 0°C for 90 min with $0.05\text{--}16\mu\text{M}$ -[^3H]adenosine, and radioactivity bound was corrected for non-specific binding by subtraction of radioactivity bound in the presence of a 500-fold excess of unlabelled adenosine. Values are means $\pm$ S.D. for the numbers of experiments shown in parentheses, and were derived from Scatchard (1949) transformations of plots of adenosine bound against total adenosine concentration.

Species	Preparation	Higher-affinity binding		Lower-affinity binding	
		K_d (μM)	B_{max} (pmol/mg of protein)	K_d (μM)	B_{max} (pmol/mg of protein)
Guinea pig	Primary mitochondrial	0.53 ± 0.18 (4)	14.3 ± 6.2 (4)	8.1 ± 1.4 (3)	87.0 ± 7.6 (3)
Rat	Primary mitochondrial	0.59 ± 0.14 (2)	2.1 ± 0.9 (2)	—	—
Ox	Primary mitochondrial	1.28 ± 0.18 (2)	4.7 ± 0.4 (2)	—	—
Guinea pig	Synaptosomal	0.67 ± 0.50 (7)	4.5 ± 2.8 (7)	5.4 ± 3.5 (6)	28.3 ± 9.4 (6)
Guinea pig	Synaptosomal (cerebellum)	0.44	1.7	9.0	30.5
Guinea pig	Myelin	3.3 ± 1.5 (3)	13.3 ± 6.5 (3)	—	—
Guinea pig	Microsomal	1.9 ± 0.2 (4)	9.8 ± 3.6 (4)	—	—

Fig. 5. Saturation analysis of [³H]adenosine binding to guinea-pig cortex synaptosomal fraction

(a) The synaptosomal fraction (97 μg of protein) was kept for 90 min at 0°C with 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA and various concentrations of [³H]adenosine in the absence (○) and presence (●) of 25 μM-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine. (b) Transformation of data, including those of (a), by the method of Scatchard (1949). Lines were computed by linear regression analysis and gave in the absence of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine (○) $r=0.90$, $K_d=1.67$ μM, $B_{max}=6.2$ pmol/mg of protein, and in the presence of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine (●) $r=0.90$, $K_d=1.11$ μM, $B_{max}=4.7$ pmol/mg of protein, and $r=0.87$, $K_d=20.2$ μM, $B_{max}=28.7$ pmol/mg of protein.

ing to components of the adenosine uptake system, known to be active in neural systems, the temperature-dependence of the process was studied (Table 4). Binding of [³H]adenosine to intact synaptosomes at both 0.1 μM and 30 μM was increased (18.5- and 7.3-fold respectively; $P < 0.01$) when incubation was carried out at 37°C instead of at 0°C, owing to its uptake and further metabolism, whereas in lysed synaptosomes there was no increase in binding at 0.1 μM on increasing the temperature. At 30 μM-

Fig. 6. Effect of lysis (a) and theophylline (b) on adenosine binding

(a) The synaptosome fraction (80 μg of protein) was kept for 90 min at 0°C with 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA and 25 μM-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine (○) or with the same buffer containing 25 μl of 2.3 M-sucrose to make the final concentration 0.32 M (●) and various concentrations of [³H]adenosine. (b) About 100 μg of protein from synaptosomal (●) or myelin (○) fractions was kept with 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA/25 μM-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine/0.1 μM-[³H]adenosine and various concentrations of theophylline for 90 min at 0°C. Non-specific binding was determined in the presence of unlabelled 0.1 mM-adenosine. Results are expressed as percentages of the amount of adenosine specifically bound in the absence of theophylline. The initial (100%) values for binding to the synaptosomal fraction were 0.47 ± 0.28 (9) pmol/mg of protein, and to the myelin fraction were 0.43 ± 0.24 (4).

adenosine, binding increased to values of 27 pmol/mg of protein, corresponding to the maximal binding capacity of the preparation as measured by Scatchard (1949) analysis (Table 3).

Discussion

Adenosine deaminase in interpreting binding

Demonstration of high-affinity binding in the present experiments did not require the presence of an adenosine deaminase inhibitor, unlike situations observed with fat-cells or with cerebral membranes (Malbon *et al.*, 1978; Schwabe *et al.*, 1979). To demonstrate activation of the adenosine-sensitive adenylate cyclase in cell-free systems (Prémont *et al.*, 1977; Kuroda & Kobayashi, 1978) it was necessary to incubate the homogenate or synaptosomal preparation with adenosine deaminase to remove endogenous adenosine, indicating this to be tightly bound to the high-affinity binding site. The increased binding observed on storage or washing of the preparation in the present work presumably has a similar basis (see Fig. 6a).

Table 4. *Temperature-dependence of adenosine binding to intact and lysed synaptosomal preparations*
 Synaptosomes (41–50 µg of protein) were kept at 0°C or incubated at 37°C for 90 min with 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂/0.5 mM-EDTA/25 µM-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine and [³H]adenosine at concentrations as stated above (lysed), or with the same buffer containing 25 µl of 2.3 M-sucrose to make the final concentration 0.32 M (intact). Non-specific binding was determined in the presence of 0.1 mM-adenosine for incubations with 0.1 µM-[³H]adenosine, and with 4 mM-adenosine for incubation with 30 µM-[³H]adenosine. Results are means ± S.D., for the numbers of experiments shown in parentheses.

Preparation	Temperature (°C)	Adenosine bound (pmol/mg of protein)	
		At 0.1 µM	At 30 µM
Intact	0	0.43 ± 0.18 (3)	16.1 ± 13.2 (4)
Intact	37	7.96 ± 0.86 (3)	117.3 ± 32.6 (6)
Lysed	0	1.12 ± 0.41 (7)	8.1 ± 7.9 (5)
Lysed	37	1.65 ± 0.25 (6)	26.9 ± 12.4 (6)

Results in Figs. 4 and 5 also indicate the need to consider endogenous adenosine deaminase in interpretation of adenosine binding. In the primary mitochondrial and the synaptosomal preparations, inhibition of the deaminase led to a much increased binding, especially at high adenosine concentrations, but this effect disappeared on washing to remove adherent cytoplasm. A largely soluble location for adenosine deaminase in brain has been shown by Pull & McIlwain (1974) and by Phillips & Newsholme (1979). These authors determined the K_d of the enzyme for adenosine to be 54–57 µM and 17 µM respectively, so that competition for added adenosine between the high-affinity receptor and the deaminase would only be significant over the higher range of concentrations used in the present studies.

The concentration of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine used to inhibit the deaminase (25 µM) is far in excess of the K_i (erythro-9-[1-(1-hydroxyethyl)heptyl]adenine) value of 75 nM reported for the enzyme by Skolnick *et al.* (1978), but was that found to give maximum adenosine binding at an adenosine concentration of 30 µM (Fig. 3b). The adenosine-binding site shown in its presence to have K_d values of 5–9 µM in synaptosomal fractions may correspond to adenosine kinase, for which K_m values of 20 µM and 2 µM for adenosine were reported by Shimizu *et al.* (1972) and by Phillips & Newsholme (1979) in guinea-pig and rat brain homogenates respectively.

Inhibition of adenosine binding by the higher concentrations of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine can be ascribed to its having an antagonistic action at an adenosine receptor, as found by Ueland (1978) for the 'adenosine-analogue-binding protein' from mouse liver. At 30 µM, erythro-9-[1-(1-hydroxyethyl)heptyl]adenine was found to halve the amount of 0.1 µM-adenosine bound to this protein, although erythro-9-[1-(1-hydroxyethyl)heptyl]adenine had only a small effect at 5 µM-adenosine and no effect at all on the binding of cyclic AMP. In the present work, the amounts of

adenosine bound to a washed preparation in the presence of 25 µM-erythro-9-[1-(1-hydroxyethyl)heptyl]adenine were only one-half those bound to the corresponding unwashed preparation in the absence of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine (Fig. 4a). The relationship between binding and erythro-9-[1-(1-hydroxyethyl)heptyl]adenine concentration therefore represents the joint effect of erythro-9-[1-(1-hydroxyethyl)heptyl]adenine on the two processes: of adenosine deaminase and of adenosine binding.

Affinity and specificity of adenosine binding

The affinity constants for adenosine binding to guinea-pig synaptosomal preparations, evaluated here as 0.67 and 5.4 µM, are significantly lower than those found for adenosine binding to myelin and microsomal fractions (Table 3). The values of 1.7 and 13.6 µM found by Schwabe *et al.* (1979) for an unfractionated particulate preparation from rat brain are compatible with microsomal and myelin-derived material being its major components. The microsomal and myelin fractions of guinea-pig neocortex also carry its greatest adenosine-binding capacity (Table 1).

It is, however, in the synaptosomal fraction now examined that adenosine binding is most sensitive to a pharmacologically active agonist and an antagonist: 2-chloroadenosine inhibited with K_i 3 µM, and theophylline with K_i 11 µM. By contrast, the preparation of Schwabe *et al.* (1979) gave data from which K_i values of 84 and 2137 µM for the two compounds can be calculated.

Adenosine-uptake systems

An uptake system for adenosine has been shown to be present in incubated cerebral tissues from guinea pigs (Shimizu *et al.*, 1972), for which a K_m of 20 µM for adenosine was calculated. Comparable systems were shown in cultured mouse astrocytes (K_m 3.4 µM; Hertz, 1978) and in synaptosomes isolated from the *Torpedo* electric organ (K_m 2 µM;

Meunier & Morel, 1978; Zimmerman *et al.*, 1979). The latter authors showed uptake to be decreased by 70% at 0°C compared with that at room temperature, corresponding to a temperature coefficient, Q_{10} , for uptake of 1.8, whereas the present results (Table 4) can be used to calculate a Q_{10} of 3.1 for apparent binding of 0.1 μM -adenosine and a Q_{10} of 2.1 for 30 μM -adenosine. The non-saturable nature of adenosine binding to synaptosomes in iso-osmotic solutions (Fig. 4) shows that the present preparations are capable of adenosine uptake, but, since there was no significant increase in adenosine binding at 0.1 μM to lysed synaptosomes when the temperature was increased (Table 4), the majority of the binding results in the present report can be taken to refer to a site distinct from that involved in the uptake process.

Adenylate cyclase and the binding sites

The existence of a specific high-affinity theophylline-sensitive adenosine-binding site in plasma membranes obtained from the primary mitochondrial and the synaptosomal fractions isolated from both cerebral and cerebellar cortex of guinea pig and rat suggests that this binding site may mediate the activation of adenylate cyclase by adenosine. The K_a for adenosine binding to this site closely corresponds to the value of 0.5 μM found by Prémont *et al.* (1979) for the concentration of adenosine giving half-maximal stimulation of adenylate cyclase in rat striatal homogenates, the lower affinity of 30 μM found in intact tissue (Sattin & Rall, 1979) probably being accounted for by restricted access of adenosine to its receptor. The data concerning 2-chloro-adenosine and theophylline, quoted above, are also compatible with values for activation or inhibition of cerebral adenylate cyclase (Daly, 1977; Prémont *et al.*, 1977).

Interactions of theophylline and adenosine at phosphodiesterases (see Bonnafous *et al.*, 1979) appear to be distinct from the present binding phenomena at synaptosomes (IC_{50} values, about 20 μM ; Newman *et al.*, 1980), for IC_{50} values for inhibition by theophylline of phosphodiesterases from various cerebral sources are 120–800 μM (Daly, 1977). The greater incidence of binding sites in the brain of the guinea pig, in contrast with that of the rat (Newman *et al.*, 1980), may account for the greater sensitivity of the cyclic AMP-generating system of the guinea pig to adenosine when compared with that of the rat (see Traversa & Newman, 1979).

So far, a permeant coupling between the adenosine receptor and adenylate cyclase has been shown only for turkey erythrocytes (Braun & Levitzki, 1979). Londos & Wolff (1977) proposed the existence of two distinct adenosine-sensitive sites on adenylate cyclase, an intracellular P-site requiring

integrity of the purine ring mediating inhibition, and an extracellular R-site requiring integrity of the ribose ring mediating activation of the enzyme. A biphasic action for adenosine has been found in human and pig platelets (Jakobs *et al.*, 1979), micromolar concentrations mediating stimulation and higher concentrations inhibition. In mouse brain cultures sub-micromolar concentrations mediate inhibition of the increase in cyclic AMP produced by adrenergic agents, whereas higher concentrations mediate stimulation (Van Calcar *et al.*, 1979). These authors conclude that both actions occur via R receptors, classified as A1 (inhibition) or A2 (activation), and on this basis the high-affinity binding site determined in the present work may represent the A2 site.

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References

- Baer, H. P. & Drummond, G. I. (eds.) (1979) *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*, Raven Press, New York
- Bennett, J. P. (1979) in *Neurotransmitter Receptor Binding* (Yamamura, H. I., Enna, S. J. & Kuhar, M. J., eds.), pp. 57–90, Raven Press, New York
- Bonnafous, J. C., Dornand, J. & Mani, J. C. (1979) *FEBS Lett.* **107**, 95–99
- Braun, S. & Levitzki, A. (1979) *Biochemistry* **18**, 2134–2138
- Daly, J. (1977) *Cyclic Nucleotides in the Nervous System*, Plenum Press, New York
- Hertz, L. (1978) *J. Neurochem.* **31**, 55–62
- Jakobs, K. H., Saur, W. & Johnson, R. A. (1979) *Biochim. Biophys. Acta* **583**, 409–421
- Kakiuchi, S., Rall, T. W. & McIlwain, H. (1969) *J. Neurochem.* **16**, 485–491
- Kuroda, Y. & Kobayashi, K. (1978) *Proc. Jpn. Acad. Ser. B* **54**, 243–247
- Londos, C. & Wolff, J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5482–5486
- Malbon, C. C., Hert, R. C. & Fain, J. N. (1978) *J. Biol. Chem.* **253**, 3114–3122
- Marchbanks, R. M. (1975) in *Practical Neurochemistry* (McIlwain, H., ed.), pp. 208–242, Churchill Livingstone, London
- McIlwain, H. (1972) *Biochem. Soc. Symp.* **36**, 69–85
- Meunier, F. M. & Morel, N. (1978) *J. Neurochem.* **31**, 845–851
- Newman, M. E. & McIlwain, H. (1977) *Biochem. J.* **164**, 131–137
- Newman, M. E. & McIlwain, H. (1978) *Biochem. J.* **170**, 73–79
- Newman, M. E., de Lucia, R., Patel, J. & McIlwain, H. (1980) *Biochem. Soc. Trans.* **8**, 141–142

- Phillips, E. & Newsholme, E. A. (1979) *J. Neurochem.* **33**, 553–558
- Prémont, J., Perez, M. & Bockaert, J. (1977) *Mol. Pharmacol.* **13**, 662–670
- Prémont, J., Tassin, J.-P., Blanc, G. & Bockaert, J. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Baer, H. P. & Drummond, G. I., eds.), pp. 259–269, Raven Press, New York
- Pull, I. & McIlwain, H. (1974) *Biochem. J.* **144**, 37–41
- Sattin, A. & Rall, T. W. (1979) *Mol. Pharmacol.* **6**, 13–23
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Schwabe, U., Kiffe, H., Puchstein, C. & Trost, T. (1979) *Naunyn-Schmiedebergs Arch. Pharmacol.* **310**, 59–67
- Shimizu, H., Tanaka, S. & Kodama, T. (1972) *J. Neurochem.* **19**, 687–698
- Skolnick, P., Nimitkitpaisan, Y., Stalvey, L. & Daly, J. W. (1978) *J. Neurochem.* **30**, 1579–1582
- Traversa, U. & Newman, M. E. (1979) *Biochem. Pharmacol.* **28**, 2363–2365
- Ueland, P. M. (1978) *Eur. J. Biochem.* **86**, 27–34
- Van Calker, D., Miller, M. & Hamprecht, B. (1979) *J. Neurochem.* **33**, 999–1005
- Zimmermann, H., Dowdall, M. J. & Lane, D. A. (1979) *Neuroscience* **4**, 979–993