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Activities and some Properties of 5′-Nucleotidase, Adenosine Kinase and Adenosine Deaminase in Tissues from Vertebrates and Invertebrates in Relation to the Control of the Concentration and the Physiological Role of Adenosine

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1. The maximal activities of 5′-nucleotidase, adenosine kinase and adenosine deaminase together with the K_m values for their respective substrates were measured in muscle, nervous tissue and liver from a large range of animals to provide information on the mechanism of control of adenosine concentration in the tissues. 2. Detailed evidence that the methods used were optimal for the extraction and assay of these enzymes has been deposited as Supplementary Publication SUP 50088 (16pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K.,from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978), **169**, 5. This evidence includes the effects of pH and temperature on the activities of the enzymes. 3. In many tissues, the activities of 5′-nucleotidase were considerably higher than the sum of the activities of adenosine kinase and deaminase, which suggests that the activity of the nucleotidase must be markedly inhibited *in vivo* so that adenosine does not accumulate. In the tissues in which comparison is possible, the K_m of the nucleotidase is higher than the AMP content of the tissue, and, since some of the latter may be bound within the cell, the low concentration of substrate may, in part, be responsible for a low activity *in vivo*. 4. In most tissues and animals investigated, the values of the K_m of adenosine kinase for adenosine are between one and two orders of magnitude lower than those for the deaminase. It is suggested that 5′-nucleotidase and adenosine kinase are simultaneously active so that a substrate cycle between AMP and adenosine is produced: the difference in K_m values between kinase and deaminase indicates that, via the cycle, small changes in activity of kinase or nucleotidase produce large changes in adenosine concentration. 5. The activities of adenosine kinase or deaminase from vertebrate muscles are inversely correlated with the activities of phosphorylase in these muscles. Since the magnitude of the latter activities are indicative of the anaerobic nature of muscles, this negative correlation supports the hypothesis that an important role of adenosine is the regulation of blood flow in the aerobic muscles.

Changes in the concentration of adenosine are considered to regulate a number of important physiological processes [e.g. rate of blood flow in heart, skeletal muscle and brain, rate of lipolysis in adipose tissue and neurotransmission in brain (Berne *et al.*, 1971; Rubio *et al.*, 1975; Schwabe *et al.*, 1975; Fain & Wieser, 1975; Daly, 1976; Phillis & Edstrom, 1976; Schubert *et al.*, 1976; Kuroda *et al.*, 1976)]. In most, if not all, tissues, adenosine is produced from AMP by the action of 5′-nucleotidase (EC 3.1.3.5) and is converted into inosine or back into AMP via the reactions catalysed by adenosine deaminase (EC 3.5.4.4) and adenosine kinase (EC 2.7.1.20) respectively. [In general, non-specific phosphatases and purine nucleoside phosphorylase are considered not to be involved in adenosine metabolism (Murray *et al.*, 1970; Parks & Agarwal, 1972; Sullivan & Alpers, 1971; Fernley, 1971; Hollander, 1971).] The steady-state concentration of adenosine will be maintained by the activities of these three enzymes and changes in its concentration may be brought about by modifications in the activities of one or more of the enzymes. This system for controlling the concentration of adenosine is, in principle, similar to that controlling the concentration of cyclic AMP. Since a comparative study of the maximum activities and properties of adenylate cyclase and phosphodiesterase provided useful information about the enzymic basis for control of the changes in the concentration of cyclic AMP (Arch & Newsholme, 1976), it was considered that a comparative study of the enzymes involved in adenosine metabolism would provide similar information on adenosine. Consequently,

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a systematic comparative study of the activities and properties of these three enzymes has been undertaken and the results are presented and interpreted in this paper.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.; Triton X-100, dithiothreitol, propan-2-ol and ethyl acetate were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.; EDTA and all inorganic chemicals were obtained from Fisons Scientific Apparatus, Loughborough, Leics. LE11 0RG, U.K. Silica-coated plastic sheets (Polygram SIL N-HR/UV_{254}) were obtained from Camlab Ltd., Cambridge CB4 1TH, U.K.

Sources of animals

Mature animals were obtained from sources described previously (Newsholme & Taylor, 1969; Sugden & Newsholme, 1973; Arch & Newsholme, 1976). Apart from the rats, which were male, and the bees, which were workers, male and female animals were used indiscriminately. All the animals had access to food and water for at least 24h before being killed.

Preparation of homogenates

Tissues were removed from animals as soon as possible after death. Scallops, lobsters, bees and blowflies were cooled on ice before they were killed. The tissues were homogenized in ground-glass homogenizers with 10–50vol. of extraction medium at 0°C. Some vertebrate muscles were first homogenized in a Silverson homogenizer (Silverson Machines Ltd., Chesham, Bucks. HP5 1PQ, U.K.) before the use of the ground-glass homogenizer. For the assay of adenosine kinase and adenosine deaminase, the extraction medium consisted of 1mM-$MgCl_2$, 1mM-EDTA and 0.1% (v/v) Triton X-100 at pH7.0. A buffer was not used routinely, since the assays were frequently performed at different pH values with the same homogenates. Moreover, preliminary experiments established that the inclusion of buffer in this extraction medium did not affect the activities of the enzymes or the stability of the activities in homogenates (Arch, 1974). Glycerol (20%, v/v) was included in the extraction medium for livers, lobster tissues and some insect flight muscle (see the Results section). Since adenosine may accumulate in ischaemic tissues and in tissue homogenates, the assays for adenosine kinase and adenosine deaminase were usually carried out as soon as possible after death of the animal (see the Results section). For the assay of 5′-nucleotidase, the extraction medium consisted of 10mM-Tris/maleate, 1mM-EDTA and 0.1% (v/v) Triton X-100 at pH7.0.

Assay of enzyme activities

Adenosine kinase and adenosine deaminase were assayed simultaneously in one incubation tube by following the conversion of radioactive adenosine into AMP and inosine respectively. In order to lower the rate of breakdown of radioactive AMP by 5′-nucleotidase and/or the inhibition of adenosine kinase by AMP or ADP (Meyskens & Williams, 1971), adenylate kinase, creatine phosphokinase and phosphocreatine were included in the assay medium. Thus the final product of the adenosine kinase reaction was ATP (radioactively labelled). The incubation medium consisted of (final concentrations) 3.3mM-citric acid, 77mM-Na_2HPO_4, 4mM-$MgCl_2$, 0.4mM-EDTA, 8mM-phosphocreatine, 0.2mg of creatine phosphokinase/ml (EC 2.7.3.2), 0.03mg of adenylate kinase/ml (EC 2.7.4.3), 4mM-ATP and various concentrations (0·5–200μM) of [G-^{3}H]adenosine (0.2–0.8μCi) or [U-^{14}C]adenosine (0.006–0.1μCi) at pH7.5. The concentrations of citric acid and Na_2HPO_4 were varied for assays conducted at other pH values. The reaction was started by addition of a volume (6μl) of homogenate to the incubation medium in a small tube so that the total volume was 20μl. The mixture was incubated for 3–15min before addition of 3.5μl of 2M-$HClO_4$ containing adenine, adenosine, hypoxanthine, inosine, ADP and AMP at approx. 5mM concentrations. (Shorter incubation times were necessary when the concentration of the substrate was low.) Control tubes were incubated for the same period of time, but $HClO_4$ was added before the tissue homogenate. The tubes were centrifuged at 2000*g* for 5min and the supernatant was used subsequently for chromatography (see below). Preliminary experiments established that the rate of accumulation of radioactivity in adenine nucleotides, and inosine plus hypoxanthine was linear during the assay, provided that not more than 25% of the adenosine was converted into products.

5′-Nucleotidase was assayed by measuring the conversion of [^{14}C]AMP into [^{14}C]adenosine. Unlabelled adenosine was included in the incubation medium, since it decreased the conversion of [^{14}C]-adenosine into [^{14}C]inosine. Since adenosine is a competitive inhibitor of nucleotidase from some vertebrate tissues (M. N. Fisher & E. A. Newsholme, unpublished observations), a low concentration (0.3mM) was included in the assay medium for assay of the enzyme from vertebrate tissues, but 3mM was included in the assay medium for the enzyme from

invertebrate tissues (see the Results section). The incubation medium consisted of 50mM-Tris/maleate, 10mM-$MgCl_2$, 0.4mM-EDTA, 1mM-dithiothreitol, 0.3 or 3mM-adenosine and various concentrations (25–800μM) of [8-^{14}C]AMP (0.03μCi). In all other respects, the method was similar to that described above for adenosine kinase and adenosine deaminase, except that the reaction was usually terminated after 10min and adenosine was omitted from the $HClO_4$ used to stop the reaction. The percentage conversion of [^{14}C]AMP into adenosine was kept low (below 10%) for those tissues in which the activity of AMP deaminase is high (i.e. anaerobic vertebrate muscles) so that a linear time course for the reaction was obtained.

Adenine, adenosine, hypoxanthine, inosine and adenine nucleotides present in the terminated assay solutions were separated from each other by t.l.c. on silica-coated plastic sheets, as described previously (Arch & Newsholme, 1976). A portion (10μl) of the supernatant of the terminated assay solution was applied to the chromatogram, which was developed with propan-2-ol/ethyl acetate/8M-NH_3 (9:4:3, by vol.).

Expression of results

All enzyme activities are expressed as nmol of product formed/min per g of fresh tissue at 30°C. For the comparative study, the activities reported are the means of a number of determinations on tissues from different animals of each species. These activities are mean values of $V_{max.}$, which were obtained by extrapolating the linear portion of a kinetic plot (Hofstee, 1952) to infinite substrate concentrations. Any substrate inhibition of adenosine kinase at pH7.5 was ignored, but the measurable maximal activity was never less than 80% of the value of $V_{max.}$. Since, in the present work, no account has been taken of such factors as season, diet, size, age and sex of the animals, and since precise quantitative interpretations are not made from these activities, a conventional statistical presentation was not meaningful. The number of determinations of each activity together with the range of activities is given in parentheses in Tables 2–6. Standard deviations can be calculated from the range of activities. However, any use of the reported activities for precise quantitative analysis must be made with caution.

Results

Control experiments on conditions of extraction and assay

A comparative study of maximum enzyme activities and K_m values is only of value if possible variations in properties of the enzyme from different animals are taken into account. Ideally, the study should include a detailed analysis of optimal extraction conditions and properties for each enzyme from each tissue. However, the amount of work involved would restrict the accumulation of data to a very few animals. Therefore, in order to minimize the possibility that variations in enzyme properties were overlooked in the detailed comparative study reported in this paper, the effects of extraction conditions, pH and temperature were investigated with tissues from selected animals representing the major classes investigated. The results of these experiments and reasoning supporting the claim that the methods used were optimal for the extraction and assay of the enzymes have been deposited with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K. (Supplementary Publication SUP 50088). The results are summarized below.

(1) The inclusion of Triton X-100 (0.1%, v/v) in the extraction media increased the activities of 5′-nucleotidase and adenosine kinase from all tissues and the activity of adenosine deaminase from lobster tissues and mouse brain.

(2) The inclusion of glycerol (20%, v/v) in the extraction medium increased the activity of adenosine kinase from vertebrate livers, lobster tissues and some insect flight muscles.

(3) The activity of adenosine kinase from mouse brain was low in Tris/maleate buffer, possibly owing to the low concentration of Na^+ in this buffer (Arch, 1974). The mouse brain enzyme, assayed in citrate/sodium phosphate buffer, had a sharp pH optimum at 5.3 when the concentration of adenosine was 10μM. However, when the concentration of adenosine was 0.5μM, it had a broader pH optimum at 6.5. The reasons for this difference are substrate inhibition by 10μM-adenosine at the higher pH values and a high K_m value for adenosine at the lower pH values (see Table 6). The activity of adenosine kinase from locust tissues varied little over the pH range 5–10.

(4) Adenosine deaminase from mouse brain had a broad pH optimum between 6 and 7, whereas the locust flight-muscle enzyme had a well-defined optimum at pH8.

(5) Most values of the temperature coefficient, Q_{10} (activity at $T+10$°C/activity at T°C) were between 2 and 3 for 5′-nucleotidase, 2.1 and 5.4 for adenosine kinase and 1.1 and 2.3 for adenosine deaminase.

The preliminary control experiments demonstrated an effect of univalent cations on adenosine kinase activity which could be important *in vivo*. The activity of adenosine kinase from a number of vertebrate species was inhibited by a low concentration of K^+ (Table 1). A detailed study of the mouse brain showed that the activity fell sharply when the composition of the assay medium was changed from 140mM-Na^+

Table 1. *Effect of Na^+ and K^+ on the activity of adenosine kinase from various animal tissues*
The activity of adenosine kinase was measured at pH 7.5 in citrate/sodium phosphate and citrate/potassium phosphate buffers at concentrations that gave 120mM- and 20mM- or 140mM- and 0mM-Na^+ and -K^+ respectively in the incubation medium. The concentration of adenosine was 7μM. Further details of the assay are described in the Materials and Methods section.

Animal	Tissue	Concn. of Na^+ (mM) ... / Concn. of K^+ (mM) ...	Adenosine kinase activity (nmol/min per g fresh wt.) 140 / 0	120 / 20	Ratio of activities in 0mM-K^+/20mM-K^+
Earthworm (*Allolobophora longa*)	Nerve cord		66	62	1.06
Locust (*Schistocerca gregaria*)	Cerebral ganglion		122	121	1.01
	Flight muscle		109	106	1.03
Frog (*Rana temporaria*)	Liver		639	546	1.17
	Heart		35	28	1.25
Domestic pigeon (*Columba livia*)	Brain		19	11	1.72
	Heart		22	12	1.83
	Pectoral muscle		30	16	1.88
Domestic fowl (*Gallus gallus*)	Liver		901	563	1.60
	Heart		18	13	1.38
Laboratory mouse	Brain		65	27	2.41
	Heart		193	90	2.14
	Liver		1263	579	2.18

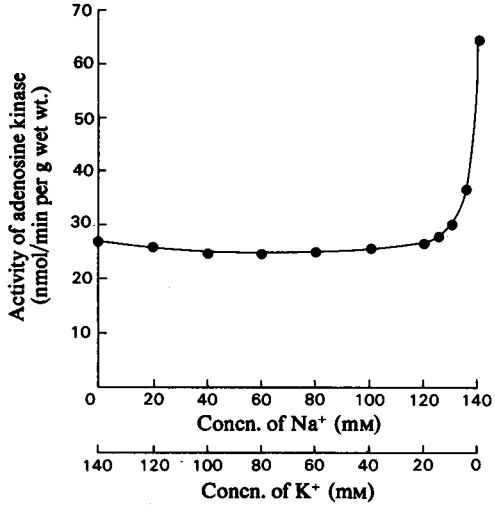

Fig. 1. *Effect of different proportions of Na^+ and K^+ on the activity of adenosine kinase from mouse brain*
The activity of adenosine kinase from mouse brain was measured at pH 7.5 in the presence of different proportions of citrate/sodium phosphate and citrate/potassium phosphate buffers, mixed so that the concentration of Na^+ plus K^+ in the incubation medium was 140mM. The concentration of adenosine in the incubation medium was 7μM. Further details of the assay are described in the Materials and Methods section.

to 130mM-Na^+ and 10mM-K^+ (Fig. 1) (see also Arch, 1974).

Activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase

The mean values of $V_{max.}$ for 5'-nucleotidase in muscle tissue range from 1.6 to 4530nmol/min per g fresh wt. of tissue (hind-leg femoral muscle of locust and cardiac muscle of rat respectively; see Table 2). The activity was higher in the heart than in skeletal muscle from each vertebrate studied. The lowest activity in heart or skeletal muscle of the vertebrates was observed in pigeon (see also Reis, 1940). The mean values of $V_{max.}$ for adenosine kinase in muscle tissue range from 7.2 to 1410nmol/min per g (pectoral muscle of pheasant and flight muscle of honey bee respectively). The activities were highest in insect flight muscles and vertebrate hearts and lowest in vertebrate anaerobic skeletal muscles (Table 2). The mean values of $V_{max.}$ for adenosine deaminase in muscle tissues range from 43 to 1500nmol/min per g (gastrocnemius muscle of mouse and catch adductor muscle of scallop respectively). In the vertebrates, the activity was higher in heart than in skeletal muscle (Table 2). The activities of adenosine deaminase were higher than those of adenosine kinase in muscles of lobster, scallop and of all the vertebrates except the mouse. However, the opposite situation applied in a number of insect flight muscles (Table 2).

The activities of these enzymes in nervous tissues are presented in Table 3. The mean values of $V_{max.}$ for 5'-nucleotidase range from 71 to 5280nmol/min per g (brain of the frog and cerebral ganglion of the cockroach respectively). The mean values of $V_{max.}$ for adenosine kinase, at pH7.5, range from 18 to 189nmol/min per g (brain of the frog and pterothoracic ganglion of the waterbug respectively). The mean values of $V_{max.}$ for adenosine deaminase range from <5 to 500nmol/min per g (nerve cord of the earthworm or pterothoracic ganglion of the waterbug and brain of the trout respectively). Thus, in nervous tissues, the range of activities for adenosine kinase was small compared with the ranges for 5'-nucleotidase and adenosine deaminase.

The activities of all these enzymes, especially adenosine kinase, in vertebrate livers were high and in general were similar to one another (Table 4). The mean values of $V_{max.}$ for 5'-nucleotidase ranged from 511 to 8230nmol/min per g (frog and rat respectively). The mean values of $V_{max.}$ for adenosine kinase, at pH7.5, ranged from 690 to 10500nmol/min per g (frog and trout respectively). The mean values of $V_{max.}$ for adenosine deaminase ranged from 318 to 1830nmol/min per g (domestic fowl and frog respectively).

The enzyme activities were measured in four further tissues from the mouse (Table 5). The jejunum contained a very high activity of the deaminase. The kidney cortex and the lung were similar to the liver in that they contained high activities of both the deaminase and the kinase. None of the enzyme activities was high in mouse blood, so that any blood present in the other tissues did not significantly affect the measured activities.

Where comparison is possible (mostly for rat and mouse tissues), there is good agreement between the enzyme activities measured in the present study and previously reported activities (Brady & O'Donovan, 1965; Baer *et al.*, 1966; Reimer & Widnell, 1975; Bosmann & Pike, 1971; Gibson & Drummond, 1972; De Jong & Kalkman, 1973). Higher activities of 5'-nucleotidase than those measured in the present study have been reported for pigeon heart (Gibson & Drummond, 1972) and perfused rat tissues (Frick & Lowenstein, 1976). However, in both studies, the activity of an enzyme with a high K_m value (0.9–12mM) was measured. When they were measured at much lower AMP concentrations, the reported activities were similar to the present values.

Values of K_m for the three enzymes and substrate inhibition of adenosine kinase

Each enzyme was assayed at a number of concentrations of substrate (i.e. AMP or adenosine) in order to be certain that maximal activities (i.e. $V_{max.}$) were measured. From these data (plotted according to Hofstee) K_m values were obtained: they are reported in Table 6. The K_m values of 5'-nucleotidase for AMP range from 31 to 980μM (liver of the frog and flight muscle of the locust respectively). The K_m values of adenosine kinase for adenosine (at pH7.5) range from <0.4 to 10μM (various tissues of the frog, pigeon and mouse, and phasic adductor muscle of the scallop respectively). The K_m values of adenosine deaminase for adenosine range from 6.6 to 54 (cardiac muscle of the domestic fowl and flight muscle of the locust respectively). It is of importance for later discussion to note that, in each tissue, the K_m value of the deaminase was at least 9-fold higher than that of the kinase. The K_m values for each enzyme, in particular adenosine deaminase, are, in general, similar for the tissues from any one species (see also Brady & O'Donovan, 1965; Ma & Fisher, 1966). The K_m values measured in the present study are similar to many of the values that have been reported previously (see Drummond & Yamamoto, 1971; Ma & Fisher, 1966; Zielke & Suetter, 1971; Anderson, 1973), but some values outside the present range have been reported for 5'-nucleotidase in partially purified preparations (see Drummond & Yamamoto, 1971; Van den Berghe *et al.*, 1977) or perfused intact tissues (Frick & Lowenstein, 1976). In addition, the K_m value of adenosine kinase for adenosine in rat heart is less than the reported value of 5.9 (De Jong & Kalkman, 1973) and the value did not approach 20μM, which was reported by Shimizu *et al.* (1972) for guinea-pig brain, in any vertebrate tissue.

Adenosine kinase at pH5.3, 5'-nucleotidase and adenosine deaminase conformed to Michaelis–Menten kinetics. However, at pH7.5, adenosine kinase from a number of tissues was inhibited by a high concentration of substrate (see also Divekar & Hakala, 1971). If the kinase from one tissue of a species was inhibited, the kinases from the other tissues of that species were also inhibited. Substrate inhibition was most marked for tissues from the rat, mouse, chicken and pigeon and, in these tissues, the optimal concentration of adenosine for adenosine kinase activity was 2–5μM. Less marked inhibition was found for tissues from the cockroach (both species) and rosechafer, for which the optimal concentration of adenosine was 10–20μM, and for tissues from the trout, for which the optimal concentration was 50–100μM.

Effect of pH on the maximum activity of adenosine kinase

The value of $V_{max.}$ for adenosine kinase from vertebrate tissues was usually higher at pH5.3 than at pH7.5 by 1.3–2.5-fold (Tables 2–4), but the kinases from trout tissues were more than twice as active at pH7.5. Since the K_m values of adenosine kinases from vertebrate tissues were greater at pH5.3 than at

Table 2. *Maximal activities of 5′-nucleotidase, adenosine kinase and adenosine deaminase in muscles from vertebrates and invertebrates*

The enzyme assays are described in the Materials and Methods section. Adenosine deaminase was assayed at pH 7.5, 5′-nucleotidase was assayed at pH 7.0 and adenosine kinase was assayed, for invertebrates, at pH 7.5 but, for vertebrates, at both pH 5.3 and 7.5. Activities are presented as mean values of $V_{max.}$ with the range of activities and the numbers of animals used given in parentheses.

		Enzyme activities (nmol/min per g of fresh muscle)			
			Adenosine kinase		
Animal	Muscle	5′-Nucleotidase	pH 7.5	pH 5.3	Adenosine deaminase
Scallop (*Pecten maximus*)	Phasic adductor	—	27 (25–30) (3)	—	640 (513–849) (3)
	Catch adductor	—	14 (13–15) (3)	—	1500 (1340–1630) (3)
Lobster (*Homarus vulgaris*)	Abdominal flexor	—	14 (11–16) (3)	—	218 (202–229) (3)
	Chela adductor	—	7.3 (6.3–8.5) (3)	—	290 (270–318) (3)
Locust (*Schistocerca gregaria*)	Flight	11 (7.4–16) (4)	138 (102–165) (6)	—	124 (107–140) (5)
	Hind-leg femoral	1.6 (1.5–1.8) (3)	46 (34–56) (3)	—	56 (35–68) (4)
Cockroach (*Periplaneta americana*)	Flight	—	42 (34–49) (3)	—	—
Cockroach (*Blaberus discoidalis*)	Flight	3620 (2930–3980) (3)	155 (136–189) (3)	—	376 (2)
Waterbug (*Lethocerus cordofanus*)	Flight	—	455 (382–528) (2)	—	<100 (2)
Rosechafer (*Pachnoda ephippiata*)	Flight	—	64 (55–80) (4)	—	113 (96–131) (3)
Honey bee (*Apismellifera*)	Flight	—	1410 (1310–1520) (3)	—	<100 (2)
Bumble bee (*Bombus* sp.)	Flight	171 (147–194) (2)	1110 (990–1240) (2)	—	55 (50–60) (2)
Solitary bee (*Colletes cunicularius*)	Flight	—	964 (882–1060) (3)	—	<100 (3)
Blowfly (*Calliphora erythroencephala*)	Flight	199 (159–220) (3)	306 (274–369) (5)	—	164 (131–189) (3)
Rainbow trout (*Salmo gairdneri*)	Red muscle	—	101 (56–147) (2)	—	780 (625–932) (2)
	White abdominal	—	32 (25–38) (2)	—	146 (143–148) (2)
Frog (*Rana temporaria*)	Heart	4440 (3930–5350) (5)	27 (22–29) (5)	47 (35–59) (3)	703 (659–758) (3)
	Gastrocnemius	29 (24–33) (3)	19 (13–28) (3)	38 (35–42) (3)	211 (175–259) (3)
Domestic pigeon (*Columba livia*)	Heart	63 (42–98) (4)	41 (36–46) (3)	63 (58–67) (3)	589 (440–659) (4)
	Pectoral	9.7 (9.0–11.0) (3)	51 (40–60) (4)	61 (36–75) (4)	231 (202–273) (4)
Domestic fowl (*Gallus gallus*)	Heart	2950 (1460–3880) (3)	27 (22–32) (4)	39 (36–42) (3)	228 (187–246) (4)
	Pectoral	187 (159–209) (3)	12 (10–15) (3)	17 (16–19) (3)	48 (41–60) (4)
	Sartorius	—	21 (14–25) (3)	27 (23–34) (3)	102 (91–108) (3)

Table 2.—*continued*

		Enzyme activities (nmol/min per g fresh muscle)			
			Adenosine kinase		
		5′-Nucleotide	pH 5.7	pH 5.3	Adenosine deaminase
Pheasant (*Phasianus colchicas*)	Heart	—	34 (25–59) (3)	74 (50–76) (3)	191 (119–296) (3)
	Pectoral	—	7.2 (6.5–8.4) (3)	23 (18–29) (2)	97 (89–103) (3)
	Sartorius	—	14 (12–17) (3)	23 (18–36) (3)	107 (95–114) (3)
Laboratory mouse	Heart	602 (547–661) (4)	429 (364–466) (4)	500 (473–515) (4)	145 (108–188) (5)
	Gastrocnemius	79 (68–91) (4)	45 (31–63) (3)	66 (46–79) (4)	43 (39–46) (5)
Laboratory rat	Heart	4530 (3550–6200) (4)	158 (104–221) (7)	199 (98–266) (4)	1430 (1262–1700) (7)
	Gastrocnemius	176 (127–242) (5)	60 (46–70) (6)	80 (67–94) (3)	499 (420–614) (6)

Table 3. *Maximal activities of 5′-nucleotidase, adenosine kinase and adenosine deaminase in nervous tissues from vertebrates and invertebrates*

Details of presentation are as described in Table 2.

		Enzyme activities (nmol/min per g of fresh tissue)			
			Adenosine kinase		
Animal	Nervous tissue	5′-Nucleotidase	pH 7.5	pH 5.3	Adenosine deaminase
Earthworm (*Allolobophora longa*)	Nerve cord	194 (138–250) (3)	148 (126–166) (4)	—	<5 (4)
Lobster (*Homarus vulgaris*)	Cerebral ganglion	—	24 (19–29) (3)	—	296 (291–300) (3)
Locust (*Schistocerca gregaria*)	Cerebral ganglion	721 (547–948) (3)	140 (99–187) (4)	—	123 (94–154) (5)
Cockroach (*Blaberus discoidalis*)	Cerebral ganglion	5280 (4060–6650) (3)	70 (67–74) (3)	—	311 (306–317) (3)
Waterbug (*Lethocerus cordofanus*)	Pterothoracic ganglion	—	189 (175–203) (2)	—	<5 (2)
Rainbow trout (*Salmo gairdneri*)	Whole brain	—	62 (48–75) (2)	—	500 (426–565) (2)
Frog (*Rana temporaria*)	Whole brain	71 (62–81) (4)	18 (13–24) (4)	39 (35–48) (3)	216 (173–258) (4)
Domestic pigeon (*Columba livia*)	Whole brain	1020 (710–1390) (4)	30 (25–38) (3)	46 (37–58) (3)	129 (102–175) (4)
Domestic fowl (*Gallus gallus*)	Whole brain	1160 (868–1610) (4)	30 (24–33) (3)	53 (38–62) (3)	57 (51–60) (3)
Laboratory mouse	Whole brain	606 (511–801) (8)	156 (114–190) (6)	204 (174–243) (6)	85 (58–125) (6)
Laboratory rat	Whole brain	2420 (1480–3230) (7)	136 (105–200) (6)	233 (187–293) (4)	174 (109–218) (6)

pH 7.5 (Table 6), the activities of the kinases from all tissues were greater at pH 7.5 than at pH 5.3 when the concentration of adenosine was low compared with the K_m values. Adenosine kinase from invertebrates and adenosine deaminase from vertebrates and invertebrates were frequently assayed at pH 5.3, but,

Table 4. *Maximal activities of 5'-nucleotidase, adenosine kinase and adensoine deaminase in livers from vertebrates*

Details of presentation are as described in Table 2.

	Enzyme activities (nmol/min per g of fresh liver)			
		Adenosine kinase		
	5'-Nucleotidase	pH 7.5	pH 5.3	Adenosine deaminase
Rainbow trout (*Salmo gairdneri*)	—	10500 (10200–10800) (2)	—	—
Frog (*Rana temporaria*)	511 (504–523) (3)	690 (634–720) (3)	1030 (985–1120) (3)	1830 (1600–2080) (3)
Domestic pigeon (*Columba livia*)	2490 (1710–3000) (3)	898 (725–1050) (3)	1040 (730–1220) (3)	527 (344–635) (3)
Domestic fowl (*Gallus gallus*)	974 (857–1010) (3)	1690 (1180–2600) (3)	1990 (1300–2990) (3)	318 (283–363) (3)
Laboratory mouse	2160 (1590–3470) (4)	2770 (1940–3520) (3)	3600 (3500–3880) (3)	1740 (1210–2400) (5)
Laboratory rat	8230 (6440–11980) (4)	2810 (2210–3730) (5)	4090 (2860–7060) (4)	1090 (725–1590) (4)

Table 5. *Maximal activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase in some mouse tissues*

Blood was collected in a heparinized tube from the neck after decapitation. Details of the assays and presentation of data are described in the legend to Table 2.

	Enzyme activities (nmol/min per g of fresh tissue)		
Mouse tissue	5'-Nucleotidase	Adenosine kinase	Adenosine deaminase
Whole blood	41 (29–52) (3)	88 (78–95) (3)	140 (82–184) (3)
Kidney cortex	3390 (2810–3830) (3)	784 (549–1080) (3)	1060 (880–1350) (4)
Lung	570 (552–593) (3)	607 (587–632) (3)	662 (439–980) (3)
Jejunum	—	224 (210–242) (3)	12200 (10400–15000) (3)

since the activities were never greater than those measured at pH7.5, they are not reported.

Discussion

There is evidence that the concentration of adenosine is maintained in a steady state. Thus, although the concentration of adenosine in well-oxygenated tissues remains constant, adenosine is continuously released (Pull & McIlwain, 1972; Berne & Rubio, 1974) and, at the same time, labelled adenosine may be rapidly metabolized (Berne & Rubio, 1974; Berne *et al.*, 1974; Newman & McIlwain, 1977). The three quantitatively important reactions involved in the maintenance of the steady-state concentration are those catalysed by 5'-nucleotidase, adenosine kinase and deaminase as follows:

$$\text{AMP} \xrightarrow{\text{Nucleotidase}} \text{adenosine} \underset{\text{Kinase}}{\overset{\text{Deaminase}}{\rightleftarrows}} \text{Inosine} \quad \text{AMP}$$

It is possible that the rate of adenosine efflux from the tissue could be important in the removal of adenosine, but the measured rates of this process are low in comparison with the values of $V_{max.}$ for adenosine kinase and deaminase (Pull & McIlwain, 1972; Bockman *et al.*, 1975; Schwabe *et al.*, 1975; Frick & Lowenstein, 1976).] Although the concentration of adenosine is maintained in a steady-state condition, the maximum activity of 5'-nucleotidase is often much greater than the sum of those of adenosine kinase and deaminase (see Tables 2–4). Consequently, the activity of the nucleotidase *in vivo* must be considerably lower than maximal. One mechanism for lowering the activity of 5'-nucleotidase *in vivo* may

be that the concentration of substrate (i.e. intracellular AMP) is well below the K_m value of the enzyme. Thus the concentration of AMP in a number of tissues is similar to or below the K_m value of the nucleotidase (Table 7). Moreover, much of this AMP may be bound to proteins and/or localized within the mitochondrion (Elbers *et al.*, 1974; Kohn *et al.*, 1977). This could play an important role not only in decreasing the activity of this enzyme *in vivo*, but also in modifying the activity of nucleotidase under certain physiological conditions, in order to produce a change in the steady-state concentration of adenosine. Indeed, it is established that, in some tissues, hypoxia or electrical stimulation increases the tissue concentration of both AMP and adenosine and, furthermore, the rate of efflux of adenosine from the cell is increased (Pull & McIlwain, 1972; Rubio *et al.*, 1973; Berne & Rubio, 1974; Mentzer *et al.*, 1975). The activity of 5'-nucleotidase may also be inhibited and regulated by free ATP or ADP (i.e. not complexed with Mg^{2+}) (see Ipata, 1968; Kluge *et al.*, 1972; Sullivan & Alpers, 1971; Nakatsu & Drummond, 1972; Gibson & Drummond, 1972), but this property has not been investigated further in the present work.

In the discussions in this paper, it is assumed that most of the activity of the nucleotidase resides intracellularly. There is some suggestion that 5'-nucleotidase is responsible for the hydrolysis of extracellular AMP (see, for example, Trams & Lauter, 1974; Newby *et al.*, 1975; Frick & Lowenstein, 1976; Gorin & Brenner, 1976). If this is indeed the case for muscle, liver and brain, the interpretations in this paper in relation to the control of the rate of production of adenosine need to be modified.

Properties of adenosine kinase and adenosine deaminase in relation to the control of adenosine concentration

There is considerable evidence to support the view that the activities and K_m properties of adenosine kinase and adenosine deaminase that are reported in the present work are meaningful *in vivo*. For example, labelled adenosine, injected into the guinea pig, is taken up rapidly by the liver and lungs (Pfleger *et al.*, 1969), which contain high activities of adenosine kinase (see Tables 4 and 5). Furthermore, after intraperitoneal injection of a labelled adenosine analogue, 6-(methylmercapto)purine ribonucleoside, which can be phosphorylated but not deaminated (Caldwell *et al.*, 1966; Schnebli *et al.*, 1967), the compound is distributed (presumably in the phosphorylated form) in mouse tissues roughly in proportion to the activity of adenosine kinase [see Caldwell *et al.* (1966) and Tables 2–5]. The rather low concentration of this analogue in the brain in relation to the activity of adenosine kinase may indicate the presence of a blood–brain barrier to the compound [and to adenosine (Berne *et al.*, 1974)].

The fate of exogenously added adenosine in various tissues depends on the concentration of adenosine: at low concentrations ($<5\mu M$ for mammalian tissues), adenosine is mainly phosphorylated, but deamination predominates at higher concentrations (Santos *et al.*, 1968; Liu & Feinberg, 1971; Meyskens & Williams, 1971; Schrader *et al.*, 1972; Mustafa *et al.*, 1975; Woo *et al.*, 1977). This latter result is predicted from the properties of the two enzymes (Parks *et al.*, 1975), since the K_m values of the deaminase are considerably higher than those of the kinase (see Table 6), and since in some species the kinase is inhibited at concentrations of adenosine between the two K_m values. The present results also predict that the route of metabolism of adenosine varies between species and tissues. For example, at high substrate concentration the ratio of activities for the two enzymes (i.e. $V_{max.\ (kinase)}/V_{max.\ (deaminase)}$) ranges from 0.009 to >38 (catch adductor muscle of the scallop and pterothoracic ganglion of the waterbug respectively; Tables 2 and 3).

The large difference in the K_m values for adenosine for these two enzymes in the large number of animals investigated (see Table 6) is similar to the system involved in the utilization of cyclic AMP (i.e. the difference in K_m values between the high- and low-K_m phosphodiesterases is greater than 10-fold). Some of the advantages of such a dual system for utilization of metabolic messengers have been described previously (Arch & Newsholme, 1976).

The concentrations of adenosine reported for mammalian muscle and brain (with freeze-clamped tissue) range from 0.6 to $30\mu mol/g$ of fresh tissue, although higher concentrations have been reported for tissues left anaerobic for some time (Rubio *et al.*, 1973, 1975; Berne & Rubio, 1974; Mentzer *et al.*, 1975; Bockman *et al.*, 1976; Newman & McIlwain, 1977). In general, these concentrations are greater than the K_m values for adenosine kinase, but less than those for adenosine deaminase, which suggests that only the kinase is saturated with substrate *in vivo*.

Role of the AMP–adenosine substrate cycle

An important aspect of the enzyme system for the control of adenosine concentration is that one reaction that destroys adenosine (i.e. adenosine kinase) opposes the reaction that produces adenosine (i.e. 5'-nucleotidase). Since it is very likely that both these enzymes are simultaneously active in the cell, this results in substrate cycle between AMP and adenosine, as follows:

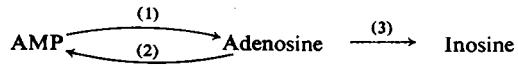

Table 6. *K_m values of 5'-nucleotidase for AMP and of adenosine kinase and adenosine deaminase for adenosine in tissues from vertebrates and invertebrates*

The K_m values for adenosine kinase and adenosine deaminase from invertebrates were measured at pH 7.5. For vertebrates, K_m values for adenosine kinase were measured at both pH 7.5 and 5.3. Further details of their measurement are described in the Materials and Methods section. Mean values are presented, with the range and number of animals used given in parentheses. See Tables 2–4 for systematic names.

		K_m values (μM)			
			Adenosine kinase		
Animal	Tissue	5'-Nucleotidase	pH 7.5	pH 5.3	Adenosine deaminase
Earthworm	Nerve cord	107 (87–132) (3)	8.5 (7.0–10) (3)	—	—
Scallop	Phasic adductor muscle	—	10 (6.6–13) (2)	—	27 (18–34) (3)
	Catch adductor muscle	—	6.9 (5.1–8.7) (2)	—	23 (18–27) (2)
Lobster	Cerebral ganglion	—	1.2 (0.8–1.5) (3)	—	—
	Claw adductor muscle	—	3.2 (1.8–3.9) (3)	—	48 (46–50) (3)
Locust	Cerebral ganglion	850 (619–987) (3)	3.9 (3.3–5.1) (3)	—	—
	Flight muscle	980 (640–1570) (3)	3.2 (2.4–3.7) (3)	—	54 (45–62) (3)
	Leg muscle	176 (165–184) (3)	2.2 (2.1–2.3) (3)	—	48 (35–57) (3)
Cockroach (*Blaberus discoidalis*)	Cerebral ganglion	223 (194–244) (3)	—	—	—
Honey bee	Flight muscle	—	3.4 (3.1–3.6) (3)	—	—
Bumble bee	Flight muscle	—	6.3 (5.3–7.2) (3)	—	—
Blowfly	Flight muscle	556 (470–612) (3)	—	—	—
Rainbow trout	Brain	—	5.2 (5.2) (2)	—	47 (40–54) (2)
	Liver	—	4.3 (3.4–5.2) (2)	—	—
	White abdominal muscle	—	2.7 (2.5–2.9) (2)	—	28 (27–28) (2)
Frog	Brain	154 (120–213) (3)	<0.4 (3)	0.9 (0.6–1.2) (3)	18 (16–21) (3)
	Liver	31 (31–32) (3)	<0.4 (3)	1.0 (0.8–1.1) (3)	21 (18–23) (3)
	Heart	75 (70–78) (3)	<0.4 (3)	1.5 (1.2–1.9) (3)	20 (18–23) (3)
	Gastrocnemius muscle	61 (59–64) (3)	<0.4 (3)	1.4 (1.2–1.6) (3)	12 (11–14) (3)
Domestic pigeon	Brain	85 (83–88) (3)	<0.4 (3)	0.8 (0.6–1.1) (3)	14 (10–20) (3)
	Liver	42 (39–46) (3)	<0.4 (3)	1.2 (0.9–1.4) (3)	—
	Heart	76 (49–94) (3)	<0.4 (3)	1.3 (1.0–2.0) (3)	16 (10–20) (3)
	Pectoral muscle	65 (52–74) (3)	<0.4 (3)	0.9 (0.5–1.1) (3)	12 (10–14) (3)
Domestic fowl	Brain	83 (62–106) (3)	0.7 (0.5–0.8) (3)	1.3 (1.1–1.4) (3)	8.4 (7.6–8.8) (3)
	Liver	60 (53–65) (3)	0.5 (0.4–0.6) (3)	1.2 (1.1–1.2) (3)	8.4 (7.7–8.9) (3)

Table 6—*continued*

		K_m values (μM)			
			Adenosine kinase		
		5′-Nucleoside	pH 7.5	pH 5.3	Adenosine deaminase
Domestic fowl—*continued*					
	Heart	55 (42–65) (3)	<0.4 (3)	1.1 (0.9–1.3) (3)	6.6 (6.1–7.0) (3)
	Pectoral muscle	49 (47–53) (3)	<0.4 (3)	1.3 (1.1–1.6) (3)	7.8 (7.5–7.9) (3)
Laboratory mouse	Brain	139 (128–150) (4)	0.7 (0.5–0.9) (4)	1.7 (1.3–1.9) (4)	16 (10–19) (4)
	Liver	105 (102–110) (3)	<0.4 (3)	—	18 (15–19) (3)
	Heart	115 (100–144) (4)	1.1 (0.9–1.4) (3)	1.8 (1.6–1.9) (3)	23 (15–27) (4)
	Gastrocnemius muscle	182 (127–215) (3)	<0.4 (3)	—	24 (22–26) (3)
Laboratory rat	Brain	245 (205–304) (4)	1.5 (1.4–1.5) (3)	2.4 (2.1–2.8) (3)	34 (30–39) (3)
	Liver	175 (155–192) (4)	0.5 (0.4–0.6) (3)	2.4 (2.1–2.8) (3)	46 (34–58) (3)
	Heart	137 (132–140) (3)	0.7 (0.5–0.9) (3)	1.8 (1.2–2.4) (3)	32 (28–39) (3)
	Gastrocnemius muscle	83 (74–95) (4)	0.8 (0.4–1.5) (4)	1.9 (1.7–2.1) (4)	32 (25–39) (3)

Table 7. *K_m values of 5′-nucleotidase and concentrations of AMP in some tissues*

The K_m values for 5′-nucleotidase are taken from Table 6. Concentrations of AMP are taken from Lowry *et al.* (1964) (mouse brain), Veech *et al.* (1973) (rat brain), Faupel *et al.* (1972) (rat liver), Opie *et al.* (1971) (rat heart) and Beis & Newsholme (1975) (other tissues). The muscles were at rest, except for the rat heart, which was perfused and beating.

Animal	Tissue	K_m of 5′-nucleotidase (μM)	Content of AMP (nmol/g of wet tissue)
Locust	Flight muscle	980	50
Blowfly	Flight muscle	556	60
Pigeon	Pectoral muscle	65	50
Domestic fowl	Pectoral muscle	49	20
Laboratory mouse	Brain	139	200
	Skeletal muscle	182	30
Laboratory rat	Brain	245	41
	Liver	175	15
	Heart	137	60
	Skeletal muscle	83	50

in which (1) represents 5′-nucleotidase, (2) represents adenosine kinase and (3) represents adenosine deaminase. Evidence has been presented (see above) that, under basal conditions (i.e. when the concentration of adenosine is low), most of the adenosine produced in reaction (1) will proceed through reaction (2). In other words, the ratio cycling rate/flux will be large. This is precisely the condition that produces a high sensitivity in metabolic control (Newsholme & Crabtree, 1976; Newsholme, 1978). However, the above system is such that, under all conditions, adenosine kinase approaches saturation with substrate, whereas adenosine deaminase is not saturated and the substrate concentration may be below the K_m of this enzyme for adenosine. Consequently, an increase in the activity of the nucleotidase or a decrease in that of the kinase will markedly increase the rate of conversion of AMP into inosine. This increased rate of conversion occurs only because there has been a large increase in the concentration of adenosine. An even larger increase in the concentration of adenosine will result if adenosine kinase is inhibited by adenosine. [It should be emphasized that this proposed large increase in adenosine concentration in response to a small change in one of the enzyme activities is only possible because of the

near-saturation of adenosine kinase with adenosine. If this were not the case, the cycle would produce no increase in sensitivity in relation to the change in enzyme activity and the change in the adenosine concentration; see Crabtree (1976) for discussion.]

Enzyme activities and the physiological role of adenosine

It has been suggested that a reduction in oxygen tension in muscle (e.g. due to increased mechanical activity) increases the extracellular concentration of adenosine, which results in arteriolar vasodilation, and this increases blood flow to the muscle (Berne *et al.*, 1971). The activities of the adenosine-metabolizing enzymes reported in the present paper are consistent with this theory. Thus the enzyme activities are higher in the more aerobic muscles (e.g. the activities are higher in heart than skeletal muscle of each vertebrate species investigated: Table 2). Furthermore, the activities of adenosine kinase or deaminase are inversely correlated with the activities of glycogen phosphorylase (EC 2.4.1.1) reported previously (Crabtree & Newsholme, 1972, 1975). The correlation coefficients for log–log plots are −0.87 ($P < 0.001$) and −0.70 ($P < 0.05$) for the kinase and deaminase respectively. Since the activity of phosphorylase in muscle provides a good indication of the dependence of the muscle on anaerobic rather than aerobic metabolism for energy production, this supports the view that adenosine plays a role in regulation of blood flow in vertebrate muscle, in general, as well as in mammalian muscles.

The similarity between the activities of adenosine kinase in heart and brains of the same animal (Tables 2 and 3) and the high activity of 5′-nucleotidase in vertebrate brains (except for the frog: see Table 3) supports the view that adenosine is an important regulator of blood flow in brain (Berne *et al.*, 1974; Rubio *et al.*, 1975). Since the brain of the newborn mammal can withstand prolonged periods of hypoxia (Bullough, 1958; Mott, 1961; Krebs, 1972), it is consistent with this view that the activities of 5′-nucleotidase and adenosine kinase in the brain of the newborn mouse are low (Arch, 1974).

Role of adenosine-metabolizing enzymes in liver, lung, kidney and blood

In general, the activities of adenosine kinase are considerably lower than those of 5′-nucleotidase in muscle and brain (see above), but the opposite situation is found in liver, lung and blood (Tables 4 and 5). The high activity of the kinase in the livers of vertebrates may play a role in removal of adenosine from blood that is derived from the muscles of the lower limbs and the gut. The activity of adenosine deaminase in the absorptive area of the gut (i.e. jejunum) is high and this will provide the initial mechanism for removal of adenosine entering the body from the gut. The liver could then lower the blood concentration of adenosine to non-physiological levels, so that other tissues, later in the circulatory system (e.g. heart), would not be influenced by adenosine produced earlier in the circulation. Adenosine kinase in the lung and erythrocytes may play a similar role. The lung, in particular, is placed in an anatomical position where it can play an important role in lowering the concentration of blood adenosine before it enters the left ventricle of the heart and hence the coronary and peripheral circulation. In this case, the removal of adenosine by adenosine kinase in the lung could be similar to the role of this tissue in metabolizing other important physiological regulators, such as prostaglandins, biogenic amines and vasoactive peptides (Heinemann & Fishman, 1969; Ferreira & Vane, 1967; Gillis & Roth, 1976).

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