Pflügers Arch. 387, 245–251 (1980)

Pflügers Archiv
European Journal of Physiology

Increase of Adenosine Content in Cerebral Cortex of the Cat During Bicuculline-Induced Seizure*

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Abstract. In order to elucidate whether adenosine may be involved in the increase in cerebral blood flow (CBF) during functional hyperemia, cortical tissue levels of adenosine, inosine and hypoxanthine were measured in the cat following bicuculline (3 mg/kg) – induced seizure. In addition, the subcellular distribution of 5′-nucleotidase in the cortex was determined by histochemical techniques. Experiments were performed on anaesthetized and immobilized cats and tissue samples to be analyzed for the different purine compounds were obtained by freezing through the trepanized and non-trepanized skull. Control values for adenosine, inosine and hypoxanthine on the trepanized side were 1.31, 1.12 and 3.79 nmoles/g, respectively. The cortical content of adenosine increased to 3.85 nmoles/g, 15 s after beginning of seizure activity and remained elevated for 20 min. Inosine and hypoxanthine also increased, exhibiting highest values after 20 min. Values for adenosine and lactate were found to be generally higher when analysis was performed from tissue frozen through the intact skull bone. In all experiments the adenosine content correlated with lactate levels. From measurements of plasma concentrations of adenosine and inosine in the sinus sagittalis superior it is concluded that seizure is also accompanied by an enhanced release of adenosine from the brain. Localization of 5′-nucleotidase by electron-microscopic cytochemistry revealed that this enzyme is associated almost exclusively with plasma membranes of glial cell membranes including perivascular astrocytes. Thus high concentration of adenosine can be expected to accumulate locally in the brain cortex. In view of the well known increase in CBF during onset of seizure and the time course of adenosine formation, our findings support the view that adenosine may be involved in the initial phase of seizure-induced functional hyperemia of the brain.

Key words: Adenosine – Inosine – Hypoxanthine – Lactate – 5′-Nucleotidase – Seizure – Cerebral blood flow.

Introduction

Coupling between cerebral activity and blood flow is accomplished by strong intrinsic regulatory mechanisms (metabolic factors), but also may involve neurogenic and humoral mechanisms. Factors such as K^+, H^+, Ca^{2+} and adenosine (for references see Kuschinsky and Wahl 1978) have been proposed in the past to participate in the functional hyperemia associated with enhanced neuronal activity. The evidence available indicates that most likely multiple metabolites rather than a single factor may be responsible for the observed vascular adjustments.

Among the different substances proposed, adenosine has attracted much interest in recent years. This nucleoside was shown to be a potent vasodilator when topically applied to pial vessels (Berne et al. 1974; Wahl and Kuschinsky 1976). Furthermore, evidence is accumulating, that adenosine can also modulate neural signal transmission (for references see McIlwain 1979). On the other hand, adenosine has been demonstrated to be formed at an enhanced rate from nervous tissue under conditions such as electrical stimulation (Pull and McIlwain 1972, 1973; Rubio et al. 1975; Schubert et al. 1976; Schultz and Lowenstein 1978), and brain hypoxia (Pull and McIlwain 1972; Rubio et al. 1975; Nordström et al. 1977).

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* A preliminary report of this investigation was presented at the 49th Meeting of the German Physiological Society at Göttingen (Pflügers Archiv 373: 74, 1978) and at the first conference of the studygroup for neurochemistry (Hoppe-Seyler's Z. Physiol. Chemie 359: 461–462, 1978)

0031-6768/80/0387/0245/$01.40

In order to determine whether formation of adenosine in the brain is related to changes in vascular conductance, we measured adenosine levels in the cerebral cortex during generalized seizure. This experimental condition has recently been described to be associated with an increase of cerebral metabolism and blood flow (Chapman et al. 1977; Meldrum and Nilsson 1976; Mueller et al. 1979) and an immediate increase in the diameter of pial vessels (Kuschinsky and Wahl 1979). Seizure was induced by bicuculline, a plant alkaloid that blocks the inhibitory action of γ-aminobutyric acid (GABA) at postsynaptic sites (Curtis and Johnston 1974).

Methods

Experiments were performed on 31 cats of both sexes which were anaesthetized with glucochloralose (40–50 mg/kg i.v.) and immobilized with gallamine (15 mg/kg), i.v. Seizure was induced with bicuculline (3 mg/kg, i.v.). All animals were artificially ventilated by use of a Bird Mark 8 respirator, their body temperature was maintained between 37 and 38° C, and arterial blood pH, pCO_2 and pO_2 were measured at 38° C using Radiometer equipment. The respective values obtained in the control animals and in the preseizure phase of the experimental group were: pH 7.37 ± 0.03, pCO_2 29.8 ± 1.4 (SD) mm Hg, pO_2 130 ± 18.0 (SD) mm Hg. During the first 2 min after onset of seizure the values were: pH 7.34 ± 0.04 (SD), pCO_2 30 ± 2.3 (SD) mm Hg, pO_2 132 ± 10.3 (SD) mm Hg. After 20 min of seizure some metabolic acidosis developed as is evident from the decrease in pH and the increase in lactate: pH 7.12 ± 0.09, pCO_2 32.2 ± 4.0 (SD) mm Hg, pO_2 133 ± 20 (SD) mm Hg, lactate 2.89 ± 1.69 (SD) mM (control: 0.79 ± 0.24 mM). In addition, arterial blood pressure and endtidal CO_2 were continuously monitored by use of a Statham pressure transducer connected to a Hellige amplifier and an Uras analyzer (Hartmann u. Braun), respectively. Mean arterial blood pressure was 134 ± 18 (SD) mm Hg in the control phase and 251 ± 16 (SD) mm Hg during the first 2 min of seizure. Thereafter, blood pressure declined slowly and approximated control values after 20 min of seizure.

Trepanation (1.5 × 2 cm) of the bone over the left parietal lobe was carried out under microscopic control using a dental drill, which was continuously cooled with isotonic saline (22° C) in order to avoid traumatization of the underlying tissue. After removal of the dura, the brain surface was continuously superfused with warmed (38° C) mock spinal fluid [composition (mM): Na^+ 156; K^+ 3; Ca^{2+} 1.5; Cl^- 151; HCO_3^- 11; 305 mosmol; pH 7.16] which was equilibrated with 5% CO_2–95% N_2 and which formed a fluid layer of 1–2 cm. By this procedure constancy of CO_2 in mock spinal fluid covering the brain surface was assured. Mock spinal fluid by itself was previously shown not to induce any changes in vascular diameter (Wahl and Kuschinsky 1976). The skull bone of the contralateral side was left intact and only freed from muscle and connective tissue.

The EEG was recorded from screw electrodes inserted into the frontal bone and displayed on a Tektronix 5111 storage oscilloscope.

Analytical Procedures. Brain was frozen by superfusing the trepanized and non-trepanized side of the skull with isopenthane, precooled to its melting point (−156° C), for 5 min. Freezing was continued with liquid nitrogen until the whole head of the animal had attained the temperature of −180° C. Tissue to be analyzed was then removed in the frozen state with a saw and freeze-dried. This procedure made it possible to selectively remove (razor blade) only cortical tissue for subsequent analyses.

Procedures for the determination of adenosine, inosine and hypoxanthine have been described in detail previously (Schrader et al. 1976). In brief, tissue was extracted with perchloric acid and the KOH neutralized extract was treated with charcoal. Following thin layer chromatography of the charcoal eluate, adenosine and its degradatives were quantitated spectrophotometrically by enzymatic degradation of all purine compounds to uric acid. Using a dual wavelength double beam spectrophotometer (Perkin-Elmer, Model 356) as little as 50–100 pmoles of each purine compound could be detected. The tissue lactate content and lactate concentration in the arterial blood were determined according to the method of Gutmann and Wahlefeld (Gutmann and Wahlefeld 1974). For calculation of tissue levels of all metabolites, dry weight was taken to be 20% of wet weight (Katzman and Pappius 1973).

In 5 animals which were heparinized with 1,000 U.S.P. heparin/kg, the plasma-concentrations of adenosine and inosine were determined in blood samples from the abdominal aorta and the superior sagittal sinus. The superior sagittal sinus was exposed through a medium burr hole at the cranial end of the sagittal crest. Usually, 10 ml blood were drawn into a syringe containing 10 ml of icecold Locke-solution (NaCl 150, KCl 5, $NaHCO_3$ 6, $CaCl_2$ 2.2, Na_2HPO_4 0.9, NaH_2PO_4 0.1, Glucose 5 mM, pH 7.4) in which also 2×10^{-4} M dipyridamole was present. Addition of dipyridamole, a known inhibitor of cellular uptake and deamination of adenosine (Schrader et al. 1972) proved to effectively prevent changes of the plasma concentration of adenosine during and after sampling. Following immediate centrifugation in the cold to remove the erythrocytes, an aliquot of the supernatant was extracted with 2 N perchloric acid and neutralized with KOH. The neutralized acid extract was processed as already described for tissue samples. The concentration of adenosine and inosine in the plasma (C) was calculated according to the following formula:

$$C = c' \cdot \left[a + b - \left(\frac{a \cdot \mathrm{Hk}}{100} \right) \right] \cdot d^{-1}$$

where (a) represents the volume of blood drawn, (b) the volume of Locke-solution the blood was diluted with, (d) the volume of the supernatant after removal of erythrocytes, which is analyzed for adenosine and inosine and (c') the amount of adenosine and inosine present in (d). Hk: hematocrit on the venous or arterial side, respectively.

Cytochemistry of 5′-nucleotidase was performed on the brain of three cats according to the method described previously (Kreutzberg et al. 1978). Animals were killed by intracranial perfusion with cold Karlsson-Schultz-formalin (15 min). After quick removal of the brains they were fixed en bloc for another 15 min in Karlsson-Schultz-formalin, then rinsed and cut. Vibratome sections were incubated for 60 min in a medium containing AMP as substrate (pH 6.8). After incubation the sections were postfixed with glutaraldehyde, osmicated, dehydrated, block stained with uranyl acetate and embedded in Durcopan®. Thin sections were treated with Reynold's leadcitrate and then viewed in a Zeiss EM 10 electron microscope. For control some sections were incubated in the presence of *Naja-naja* antiserum or of an antiserum against human placental 5′-nucleotidase or in the absence of the substrate AMP. These treatments resulted in a strong or complete suppression of the appearance of enzyme reaction products on the ultrastructural level.

Results

Figure 1 summarizes our data on the cortical levels of adenosine, inosine and hypoxanthine measured after different periods of seizure. Seizure activity occurred 12 to 20 s after the injection of bicuculline as judged by the isolated spike activity in the EEG which precedes the

tonic discharge (Chapman et al. 1977). It can be seen that 15 s after beginning of seizure activity the tissue content of adenosine increased about threefold from a control value of 1.3 to 3.8 nmoles/g. Under these conditions values for inosine also increased slightly, while no significant changes were found for hypoxanthine. Analysis of tissue samples taken 30 s and 20 min after onset of seizure revealed that the cortical content of adenosine was still increased; levels of inosine and hypoxanthine after 20 min of seizure were elevated even more than adenosine.

The seizure-induced increase in the formation of adenosine was paralleled by an enhanced formation of lactate (Table 1). Tissue levels of adenosine correlated with respective values for lactate during the first 30 s of seizure. These data can be represented by a straight line: lactate $= 0.277 \cdot$ adenosine $+ 0.98$, $r = 0.583$, $n = 25$; i.e. adenosine tends to accumulate 1.3 times faster in the cortex than lactate. As is also evident from data given in Table 1, formation of lactate exceeds by far that of adenosine after 20 min of seizure.

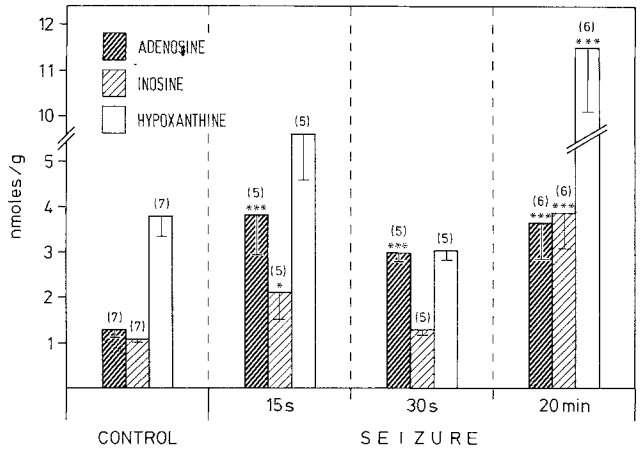

Fig. 1. Changes in the brain cortex of the content of adenosine, inosine and hypoxanthine following seizure induced by bicuculline. Corical tissue was frozen through the trepanized skull (for details see methods) after different periods following beginning of seizure activity. Asterisks indicate significant differences by *t*-test analysis from control values (*, $P < 0.025$; **, $P < 0.01$; ***, $P < 0.005$) mean values ± SEM

In order to clarify to what extent trepanation of the skull might have influenced our results, analysis of the cortical tissue content of the contralateral side, covered by the intact skull bone, was performed in most of the experiments. As can be seen from the data summarized in Table 1, there is no significant difference between trepanized and non-trepanized side when the purine compounds and lactate were analyzed under control conditions. However, during bicuculline-induced seizure tissue samples of the non-trepanized side generally exhibited higher values compared with respective values of the trepanized side. This difference was most pronounced in the adenosine and inosine fraction analyzed 20 min after onset of seizure. It should be noted, however, that values for lactate were not different at this time interval.

In an additional experimental series we investigated whether the enhanced formation of adenosine in cortical tissue is reflected by an augmented release of this nucleoside into the cerebral circulation. As is evident from data given in Table 2, low levels of adenosine and inosine are constantly present in the arterial blood and there is a significant release ($P < 0.05$) of both purine compounds as judged from the respective arterial venous differences. During bicuculline-induced seizure, known to be associated with a 7-fold increase in blood flow to the gray matter (Mueller et al. 1979), the venous concentrations of adenosine and inosine were not significantly changed.

The site of adenosine formation in the brain is determined by the cellular localization of 5′-nucleotidase catalyzing the hydrolysis of AMP. We therefore determined by use of cytochemical techniques the

Table 1. Cortical tissue content of lactate, adenosine, inosine and hypoxanthine following bicuculline-induced seizure. Tissue was either frozen on the trepanized skull after removal of the dura (○) or through the intact skull bone (●). (For details see Methods.) Asterisks indicate significant differences by paired *t*-test analysis between trepanized and non-trepanized side (*, $P < 0.025$; **, $P < 0.005$). Mean values ± SEM

			Lactate µmoles/g	*n*	Adenosine nmoles/g	*n*	Inosine nmoles/g	*n*	Hypoxanthine nmoles/g	*n*
Control		○	0.97 ± 0.14	(7)	1.31 ± 0.18	(7)	1.12 ± 0.08	(7)	3.79 ± 0.54	(7)
		●	1.15 ± 0.25	(4)	1.20 ± 0.09	(4)	0.97 ± 0.17	(4)	3.56 ± 0.52	(4)
Seizure	15 s	○	2.27 ± 0.25	(5)	3.85 ± 0.98	(5)	2.10 ± 0.55	(5)	5.62 ± 1.0	(5)
		●	–		–		–		–	
	30 s	○	1.70 ± 0.2	(5)	3.0 ± 0.21	(5)	1.31 ± 0.09	(5)	3.18 ± 0.31	(5)
		●	2.87 ± 0.38**	(4)	4.4 ± 1.08	(4)	2.12 ± 0.59	(4)	3.27 ± 0.32	(4)
	20 min	○	11.49 ± 1.51	(6)	3.65 ± 0.72	(6)	3.89 ± 0.81	(6)	11.59 ± 1.91	(6)
		●	10.97 ± 1.42	(4)	13.34 ± 6.59*	(4)	7.39 ± 2.42*	(4)	14.75 ± 3.43	(3)

Table 2. Concentrations of adenosine and inosine in the plasma of blood drawn from the abdominal aorta and the superior sagittal sinus before and after bicuculline-induced seizure. Venous blood was analyzed 20–60 s (1. venous) and 5–6 min (2. venous) after beginning of seizure activity

		Adenosine		Inosine	
		(nM)	*n*	(nM)	*n*
Control	Arterial	74 ± 13	(4)	104 ± 38	(3)
	Venous	144 ± 37	(5)	578 ± 372	(3)
Bicuculline	Arterial	57	(2)	180	(2)
	1. Venous	172 ± 88	(4)	572 ± 354	(4)
	2. Venous	146 ± 78	(4)	564 ± 293	(4)

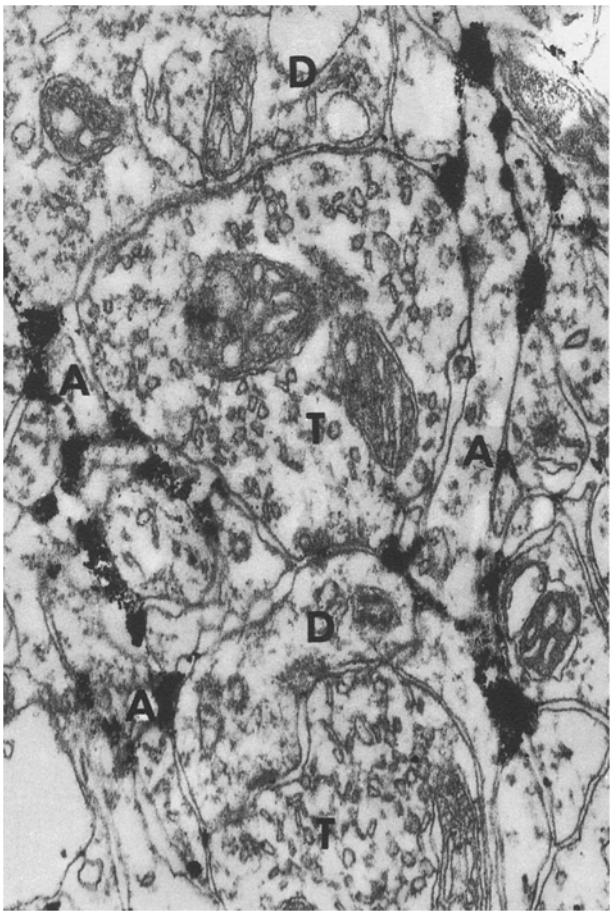

Fig. 2. Distribution of 5′-nucleotidase-activity in the frontal cortex of the cat. Deposits of electron dense reaction product demonstrate activity of 5′-nucleotidase. Strong activity is seen in plasma membranes of astrocytes (*A*) in close vicinity of synapses formed by axon terminals (*T*) and dendrites (*D*). Magnification ×50,000

cellular and subcellular distribution of this enzyme in the cerebral cortex of cats. Localization of 5′-nucleotidase as revealed by electron-microscopic cytochemistry is shown in Fig. 2 and 3. The reaction product,

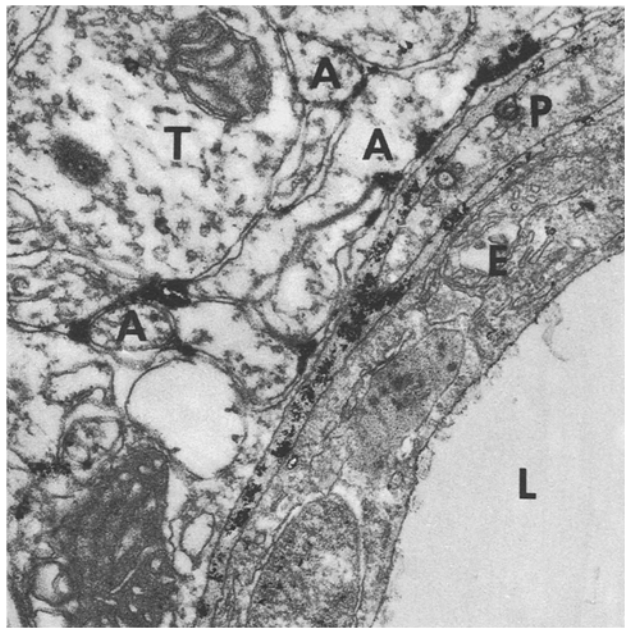

Fig. 3. 5′-nucleotidase-activity in plasma membranes of perivascular astrocytes (*A*) partially touching basement membranes which cover pericytes (*P*) and endothelial cells (*E*). *L:* capillary lumen; *T:* Synaptic terminal. Cat frontal cortex. Magnification ×40,000

lead orthophosphate, is electron dense and appears as black deposits in the electron micrographs. 5′-nucleotidase was found to be associated almost exclusively with plasma membranes of glial cells. In the gray matter of the cortex, astrocytes are the most prominent carriers of 5′-nucleotidase. Enzyme activity can be located in the plasma membrane of astroglial cell processes, especially as they cover synapses (Fig. 2). It is of particular interest that enzyme activity can also be demonstrated in plasma membranes of perivascular astrocytes, the processes of which touch the basement membrane of endothelial cells (Fig. 3).

Discussion

Our results clearly indicate that in the cat, enhanced neuronal activity during bicuculline-induced seizure is associated with an accelerated formation of adenosine in the cerebral cortex. Adenosine accumulated rapidly, reaching maximal values within about 15 s. It is well documented that cerebral blood flow also rapidly increases during seizure (Meldrum and Nilsson 1976; Mueller et al. 1979); vascular diameter of the pial vessels was shown to increase by about 26% above control values only 15 s after seizure activity started (Kuschinsky and Wahl 1979). Thus, it is conceivable that adenosine may participate in the adaption of

cerebral vascular resistance to the seizure-induced enhanced metabolic demands of the brain. This possible regulatory role of adenosine is further emphasized by the subcellular distribution of 5′-nucleotidase, the enzyme which catalyzes the formation of adenosine from AMP. Similar to previous results obtained in the rat (Kreutzberg et al. 1978), a close relationship was found to exist between the location of 5′-nucleotidase on membranes of perivascular astrocytes and the site of adenosine action at the vessel wall. It therefore appears likely that adenosine may reach by far higher concentrations locally than would be expected from our values which are average figures for the whole cortex.

No estimate can be made at present as to the extent adenosine may contribute to the increase in cerebral blood flow during seizure. Factors other than adenosine must also be considered to be involved. A decrease in perivascular pH (Kuschinsky and Wahl 1979) as well as an increase in the extracellular potassium concentration (Pedley et al. 1976) was observed immediately after the onset of an epileptic seizure. Since H^+- and K^+-ions are known to be potent vasodilators in the cerebral circulation, it is likely that these local factors act in concert with adenosine in setting the tone of the cerebral smooth muscle cells. In this respect it should be noted that the dilatory action of adenosine on pial vessels can be attenuated by simultaneous increase of K^+ or by lowering the pH (Wahl and Kuschinsky 1977).

Our findings are not in accordance with a recent report in the literature, demonstrating that in the rat cortex adenosine, which was measured by a newly elaborated HPLC-technique, did not change during seizure elicited by bicuculline (Rehncrona et al. 1978). The reason for this discrepancy is not apparent at present. Since similar to our results, adenosine has recently been reported to increase in rat brain also during bicuculline-induced seizure (Winn et al. 1978, 1979), species differences can be excluded as underlying cause. It shall be emphasized, however, that data reported by Winn et al. are for whole brain, while in the present study only cortical tissue was analyzed. Also differences in the technique of tissue sampling appear to be unlikely to explain the above discrepancy (Marshall et al. 1975). We have analyzed adenosine and its degradatives in cortical tissue frozen either rapidly after removal of the dura or more slowly through the intact skull bone as suggested by Nordström (Nordström et al. 1978). Both methods gave comparable values for all metabolites. During seizure, tissue levels of adenosine were always increased; they were, however, generally lower on the trepanized side. Whether this difference is due to washout of adenosine into the mock spinal fluid, covering the exposed brain, or to a more rapid freezing of the cortical tissue is not known.

The formation of adenosine appears to be closely related to the generation of neuronal action potentials. Synaptic activation in vivo (Schubert et al. 1976) and electrical stimulation of brain cortical slices (Pull and McIlwain 1972, 1973) was shown to be associated with the release of ^{3}H-adenosine and this effect could be blocked by tetrodotoxin (Pull and McIlwain, 1973). Concerning the mechanism for formation of adenosine it has been suggested that this nucleoside is derived from ATP which may be released together with transmitter substances in the course of synaptic stimulation (Barberis and McIlwain 1976; Schubert et al. 1976, 1979). This hypothesis requires ATP to be degraded to AMP in the extracellular space, thereby providing the substrate for 5′-nucleotidase, known to be an ectoenzyme (Kreutzberg et al. 1978; Schubert et al. 1979).

Besides its action on cerebral vascular resistance, adenosine is known to increase cAMP levels in the brain by a direct stimulatory action on adenylate cyclase (Daly 1979). It appears possible that adenosine formed during seizure may be responsible for the elevated cAMP levels also observed under this condition (Folbergrova 1977). In support of this view is the observation that theophylline, a well known inhibitor of the actions of adenosine, antagonized the seizure-induced increase in cAMP levels (Sattin 1971).

Adenosine and inosine appear to be constantly released from the normal brain as judged from the AV-differences for both purine compounds under control conditions. Taking the cortical blood flow to be 130 $ml \cdot min^{-1} \cdot 100\ g^{-1}$ (Sakurada et al. 1979), about 70 $nmoles \cdot min^{-1} \cdot 100\ g^{-1}$ purine compounds in the form of adenosine and inosine are released. Interestingly, this value is about one fifth of the amount which is released from the heart (Schrader et al. 1977, 1979). Despite the increase in tissue levels of adenosine and inosine during seizure, the concentration of these purine compounds did not change in the superior sagittal sinus. However, cerebral blood flow – though not measured in our experiments – is known to increase after bicuculline about 5- to 7-fold (Meldrum and Nilsson 1976; Mueller et al. 1979), so that the rate of release of adenosine may be augmented by the same factor. Such an increment in the release of adenosine appears to be relatively small when compared with the myocardium, but may reside in the fact that in the brain only relatively small amounts of adenosine can cross the blood brain barrier and reach the intravascular space (Berne et al. 1974). Whether an increased leakiness of the blood brain barrier – known to occur during seizure (Johansson 1977; Johansson und Lund 1978; Mueller et al. 1979) – might contribute to the release of adenosine under our experimental conditions, cannot be determined from the results presented. Nevertheless, it is possible that changes of

vascular permeability might account for the secondary decrease of tissue adenosine and its degradatives determined after 30 s of seizure (Fig. 1).

Concerning the metabolism of adenosine in the brain, recent evidence suggests that adenosine can also be formed from S-adenosylhomocysteine (SAH) by action of the SAH-hydrolase (Schatz et al. 1978). To what extent this cytosolic enzyme contributes to the formation of adenosine under different physiological conditions is not known. It should be noted, however, that SAH-hydrolase was demonstrated to tightly bind adenosine (Hershfield 1978). Thus, formation of adenosine at the cell membrane and also within the cell as well as binding of adenosine to intracellular proteins will considerably complicate any further attempts to extrapolate from tissue levels of adenosine to the concentrations of this nucleoside at the site of cerebral resistance vessels.

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Received November 19, 1979/Accepted June 18, 1980