metabolites and the sulfhydryl group, and that the water-soluble conjugates formed were excreted in the urine. We have already reported that the activated material(s) of BHT is bound to the protein and nucleic acid [2–4]. Therefore, it is suggested that a part of quinone methide or BHT-alcohol is also covalently bound to the sulfhydryl group in protein *in vivo*.

The present study, carried out with the methods of organic solvent-extraction and Amberlite chromatography-t.l.c., supports our earlier speculation [3]: the activated material(s) of BHT reacts with the thiol compound, and the binding sites of protein for such material(s) may be the sulfhydryl group. The structure of the cysteine conjugate of quinone methide or BHT-alcohol is to be investigated in future work.

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REFERENCES

1. D. E. Hathway, *Adv. Fd Res.* **15**, 1 (1966).
2. Y. Nakagawa, K. Hiraga and T. Suga. *Chem. pharm. Bull., Tokyo* **27**, 442 (1979).
3. Y. Nakagawa, K. Hiraga and T. Suga, *Chem. pharm. Bull., Tokyo* **27**, 480 (1979).
4. Y. Nakagawa, K. Hiraga and T. Suga, *Biochem. Pharmac.* **29**, 1304 (1980).
5. M. S. Kharasch and B. S. Joshi, *J. org. Chem.* **22**, 1439 (1957).
6. J. Filar and S. Winstein, *Tetrahedron Lett.* No. 25, 9 (1960).
7. O. Takahashi and K. Hiraga, *Fd Cosmet. Toxic.* **17**, 451 (1979).
8. M. S. Patterson and R. C. Green, *Analyt. Chem.* **37**, 854 (1965).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. J. W. Daniel, J. C. Gage and D. I. Jones, *Biochem. J.* **106**, 783 (1968).
11. C. Chen and Y. Shaw, *Biochem. J.* **144**, 497 (1974).

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Lack of uptake or degradation of adenosine in the termination of its action in the beating carp atrium

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Adenosine and some of its phosphorylated derivatives are known to reduce the force and rate of contraction of atrial muscle in various species [1–4]. This effect has been attributed to a direct action on atrial muscle and also on the sino-atrial pacemaker. More recently [5], adenosine was reported to cause a slow, long-lasting hyperpolarization of cells in the sinus venosus of the frog, which is the primary pacemaker of the region. In most tissues, however, the response to adenosine is brief: isolated heart preparations exposed to adenosine recover spontaneously their original rate and force of contraction. This fade or decay of adenosine effect, observed also in smooth muscle, has been ascribed to the disappearance of adenosine from the bathing fluid through uptake into the tissue, or deamination to inactive inosine [6–8]. Proof of this rested on a demonstration that radiolabeled adenosine added to the bathing medium was gradually taken-up by the tissue where it underwent partial metabolism, and that dipyridamole, a known coronary vasodilator agent, effectively inhibited this uptake [6, 9]. Thus, a given concentration of adenosine seems to be more effective in the presence of dipyridamole, presumably because its effective bath concentration persists longer. Our recent experience with the spontaneously beating carp atrium, however, suggests that other mechanisms may be operative in the fade or potentiation of adenosine action. In this preparation, adenosine induces a dose-dependent, transient loss of inotropicity, followed by recovery. We now show evidence that (1) spontaneous recovery of force of contraction by the muscle occurs in the presence of a persisting bath concentration of adenosine, (2) the rate of recovery is inversely related to adenosine concentration, and (3) dipyridamole still exerts potentiation of the adenosine effect even though blockade of adenosine transport may not be its major contribution.

The spontaneously beating carp atrium preparation, reported earlier [10], lends itself remarkably well to prolonged time–course studies. It delivers about 30,000 contractions over a period of 10 hr without significant change in base-line and responds only to acetylcholine and adenosine and their congeners, but not to noradrenaline, adren-

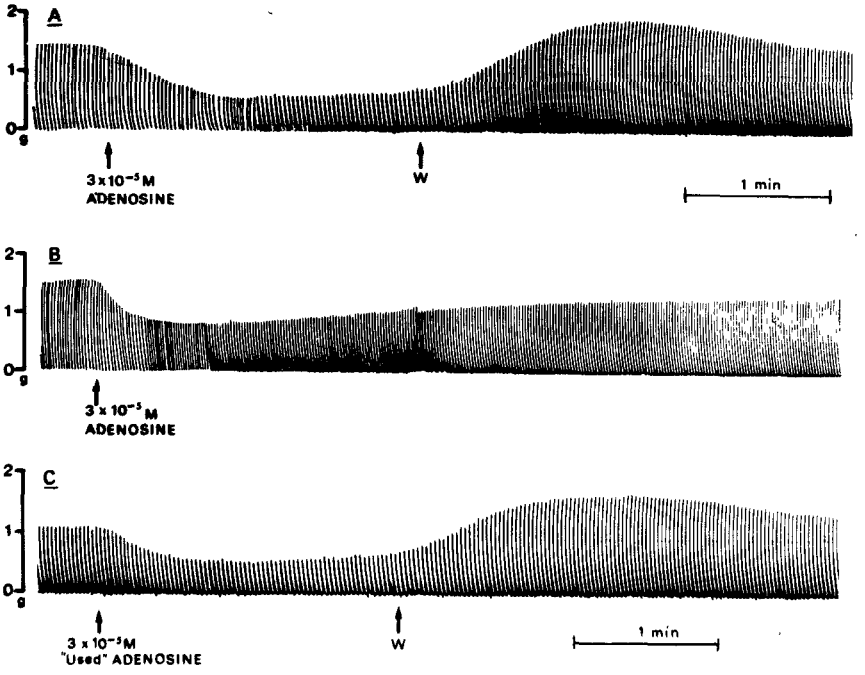

Fig. 1. Time–course of adenosine action in the isolated carp atrium. Panel A shows the rebound effect after washout (W); panel B shows spontaneous recovery of force of contraction without washout: and panel C shows the effect of "used" adenosine solution.

aline, isoprenaline, histamine, serotonin, gamma-aminobutyric acid, 3′,5′-cyclic AMP or its dibutyryl derivative. Atria were removed from live fish, *Cyprinus carpi,* immediately before use. After careful removal of connective tissue, atrial muscle bands (0.1 g each) were suspended in a 5-ml organ bath containing carp–Ringer solution (NaCl, 102 mM; KCl, 2.7 mM; $CaCl_2$, 2.9 mM; $MgCl_2$, 100 mM; and $NaHCO_3$, 10.0 mM; pH 7.8) at room temperature, vigorously aerated with a stream of compressed air which also insured adequate stirring. A starting tension of 1 g was applied to the tissue, and contractile performance was monitored with a Grass FTO 3C force displacement transducer connected to a Grass 79 polygraph. The preparation was left to equilibrate for 40 min during which the bathing solution was changed every 10 min. Following that, prolonged time–course studies were possible without changing the bathing solution for as long as 1 hr. Drugs were introduced by lateral injection of their solution in carp–Ringer.

The time–course of adenosine action is shown in Fig. 1. If, at the peak of adenosine effect, this compound was washed out with normal carp–Ringer, then a "rebound" phenomenon was observed in which the original level of force of contraction was exceeded before return to the initial base-line. On the other hand, if the muscle continued to be exposed to adenosine, then spontaneous recovery of most or all of its original inotropicity occurred within 1–14 min, the rate and extent of recovery depending on adenosine concentration. Here, the "rebound" phenomenon was not observed unless washout with carp–Ringer was performed. The relative rate of fade of adenosine effect was inversely related to concentration (Fig. 2). This inverse relationship seemed to favor an uptake or degradative

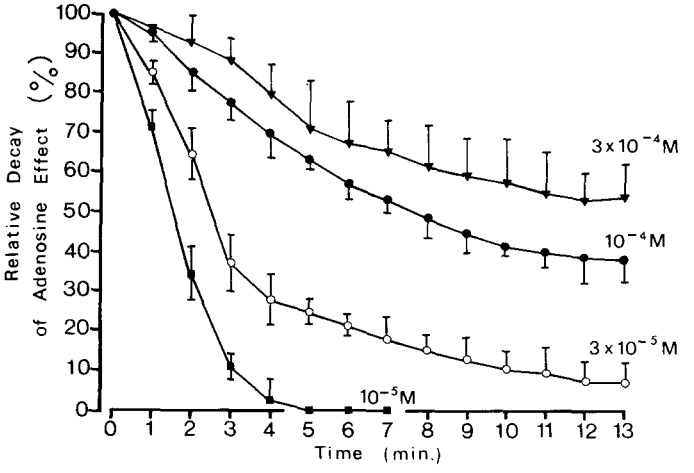

Fig. 2. Relative decay of adenosine effect with time. The maximum depression of force of contraction at a given concentration was taken as 100 per cent effect. Relative decay is

$$\frac{\text{maximum contraction amplitude—amplitude at time } t}{\text{maximum contraction amplitude}} \times 100.$$

Vertical bars indicate S.E.M. for N = 6.

Table 1. Analysis of "used" adenosine solution (30 μM) after 90 per cent fade of adenosine effect in carp atrium (10 min), compared to controls*

	λ_{max} (nm)	Absorbance units (at 260 nm)	R_f value	Bioassay (% response)
"Used" 30 μM adenosine in carp-Ringer	260†	450†	0.82‡ 0.34§	71
Fresh 30 μM adenosine in carp-Ringer	260	460	0.82 0.34	71
"Used" carp-Ringer	250–260	80–120		0

* Data are from six separate determinations.

† Measured against a blank consisting of "used" carp solution that had been in contact with the same preparation for the same length of time. Initial multiple bathing and washing of the atrial strip were found to reduce the absorbance of the "used" carp solution at 260 nm from an initial value of about 220 to 100. λ_{max} for ATP was 260 nm, and for inosine, 248 nm.

‡ Isopropanol, acetic acid, water (4:2:2, by vol.) on Kieselgel thin layer; R_f for ATP, 0.05; inosine, 0.77; and AMP, 0.42.

§ *n*-Butanol, ammonia 25% (86:20, v/v) on Keiselgel thin layer; R_f for ATP, 0.0; AMP, 0.0; and inosine, 0.12.

process over one involving desensitization of adenosine-binding sites, but, we could show by three independent procedures that the original adenosine concentration had not materially changed throughout the fade process and after its completion. This was achieved by (1) spectrophotometric analysis of the bathing medium which showed almost the original ultraviolet absorption spectrum of adenosine, (2) thin-layer chromatography which confirmed the identity of the absorbing species, and (3) a bioassay where the contents of one bath, after fade, were used to treat the same preparation, after brief washout with carp–Ringer, or a different preparation set in a parallel bath and for which a dose–response relationship had been established previously. The "used" solution evoked in each case a response anticipated from the original adenosine concentration (Table 1). Thus, fade occurred in the presence of a persisting concentration of adenosine. Conceivably, some uptake might have still occurred, but, clearly, it cannot account for a 90 per cent fade of effect in a situation where the greater part of adenosine was still external to the tissue and presumably available to the binding sites. Indeed, from the dose–response curve of adenosine (Fig. 3), there ought to have occurred a decrease in concentration from 30 μM to 1 μM if a 90 per cent fade were to be attributed entirely to uptake. Since this obviously was not the case, desensitization remains to be considered as a likely mechanism of fade. The inverse relationship between concentration and rate of fade (Fig. 2) can be reconciled with desensitization if one assumes that the observed rates are composite functions resulting from two opposing trends: (1) concentration-independent, leading to desensitization, and (2) concentration-dependent, leading to potentiation of adenosine effect. In this sense, the action of adenosine in the carp atrium was mediated by two sets of binding sites that exhibited positive cooperativity. Thus, increasing adenosine concentration must result

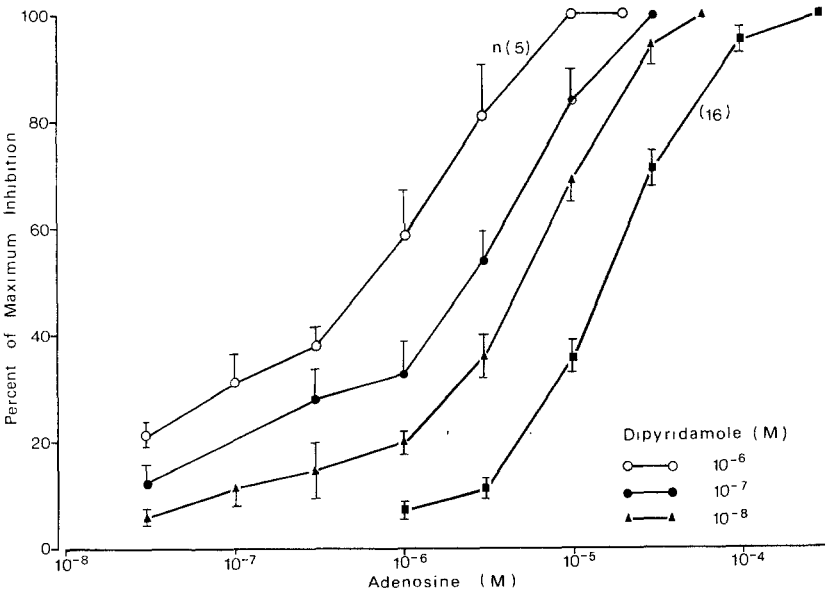

Fig. 3. Dose–response curve of adenosine alone (■) and in the presence of the indicated concentrations of dipyridamole; (n) are replications; vertical bars are S.E.M. The EC_{50} values are: adenosine, 16 μM; (+ dipyridamole, 0.01 μM), 5 μM; (+ dipyridamole, 0.1 μM), 2.4 μM; and (+ dipyridamole, 1 μM), 0.68 μM. Dipyridamole alone (1 μM) had no effect on the contractility of the muscle.

in a progressive delay of fade, until the potentiation process itself becomes saturated.

The potentiating effect of dipyridamole (Fig. 3), which cannot be ascribed in the present case to blockade of adenosine uptake, may have resulted from the sensitization of one set of adenosine-binding sites, but with the distinction that dipyridamole was more potent in this respect than adenosine. We have no proof that they share the same binding site that is involved in sensitizing the muscle, but this is not unlikely in view of their structural similarity and the documented evidence on the affinity of dipyridamole to the adenosine carrier in cell membranes.

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REFERENCES

1. A. M. Drury and H. J. Szent-Gyorgi, *J. Physiol., Lond.* **68**, 213 (1929).
2. P. B. Hollander and J. L. Webb, *Circulation Res.* **5**, 349 (1957).
3. T. N. James, *J. Pharmac. exp. Ther.* **149**, 233 (1965).
4. M. Goto, A. Yatani and Y. Tsuda, *Jap. J. Physiol.* **27**, 81 (1977).
5. H. C. Hartzell, *J. Physiol., Lond.* **293**, 23 (1979).
6. S. V. Hopkins, *Biochem. Pharmac.* **22**, 341 (1973).
7. M. I. Jacob and R. M. Berne, *Am. J. Physiol.* **198**, 322 (1960).
8. H. P. Baer and G. I. Drummond, *Proc. Soc. exp. Biol. Med.* **127**, 33 (1968).
9. N. Kolassa, K. Pfleger and M. Tram, *Eur. J. Pharmac.* **13**, 320 (1971).
10. Y. Lass, S. Akselrod, B. Gavish, S. Cohen and A. Fisher, *Experientia* **35**, 650 (1979).

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Type B monoamine oxidase activities toward β-phenylethylamine in discrete hypothalamic and circumventricular nuclei of the rat

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Monoamine oxidase (MAO; EC1.4.3.4) plays an important role in brain function by catalyzing the deamination of various monoamines, and it is designated type A and type B MAO based on substrate specificity and inhibitor sensitivity [1, 2]. Some investigators have demonstrated the differential effects of hormonal manipulations [3, 4] and emotional behaviors [5, 6] on each type of MAO, which led us to postulate that the two types of MAO may play different physiological roles in the regulation of several neuroendocrine secretions and some emotional behaviors. It was, therefore, of interest to study further the physiological roles of these enzymes in discrete brain nuclei.

We first showed that MAO catalyzing serotonin oxidation (5-HT–MAO; type A MAO) and MAO catalyzing tyramine oxidation (type A + B MAO) were unevenly distributed in various brain nuclei [7, 8] and that high proportions of type B MAO were found in discrete circumventricular regions of the rat when using the specific type A MAO inhibitor clorgyline [9].

It was shown recently by several authors [10–12] that the substrate specificity of β-phenylethylamine (PEA), which has long been regarded as a specific substrate for type B MAO [13], changed dramatically with changes in PEA concentration and pH of the reaction medium and that PEA was oxidized by either type of MAO at relatively high concentrations, based on inhibition studies with clorgyline and the specific type B MAO inhibitor deprenyl. On the basis of these findings, it was suggested that a low concentration of PEA as substrate is needed to assay type B MAO activities exclusively. In the present experiment, therfore, we determined MAO activities toward a low concentration of PEA (10 μM; PEA–MAO; type B MAO) in discrete hypothalamic nuclei and in some circumventricular regions of the rat by applying radiochemical micromethods to freeze-dried sections [14].

Male Wistar–King rats weighing 250–350 g were used. The animals were decapitated, and the brains were removed rapidly and placed on ice. The parts containing the preoptic area and hypothalamus and that of the lower brain stem were isolated and frozen in liquid nitrogen. Frontal sections of 200 μm thickness were made in a cryostat at −13°. The sections were freeze-dried overnight at −30° and 10^{-3} mm Hg and stored in evacuated tubes at −20° until use.

The individual preoptic and hypothalamic nuclei, or areas, were dissected carefully freehand under a steromicroscope, according to the atlas of König and Klippel [15]. The area postrema (coordinate, p 7.0 mm) was dissected also, according to the atlas of Palkovits and Jacobowitz [16]. The schematic drawings of the dissected nuclei are shown elsewhere except for the area postrema [7, 8]. Each sample (2–8 μg) was weighed using an electronic microbalance (Type EO-12, Eto. Co.). The sensitivity of this balance is 0.1 μg.

MAO activity was determined by a modification of the radiochemical methods of Wurtman and Axelrod [17] and White and Wu [18]. Ice-cold buffer–substrate (15 μl) was added to each tube containing tissue samples in an ice-bath (final concentration, 0.05 M sodium phosphate buffer (pH 7.4) and 10 μM β-[ethyl-1-^{14}C]phenylethylamine hydrochloride, 64.16 mCi/mmole, New England Nuclear Corp., Boston, MA). After preincubation for 15 min at 0°, the experimental and blank (containing no enzyme) tubes were mixed without warming and incubated at 37° for 10 min. The reaction was stopped by the addition of 3 N HCl. The radiolabeled products were extracted by 50 μl of toluene, and the toluene layer was washed once with 30 μl of 0.3 N HCl. After centrifugation, 20 μl of the organic layer was transferred to a counting vial, to which 10 ml of scintillator toluene solution was added. Radioactivity was determined by liquid scintillation spectrometry. The counting efficiency was 78 per cent. The preliminary experiments demonstrated that the reaction was linear with respect to