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Studies on 3,7-Dimethyl-1-(5-oxo-hexyl)-xanthine(BL 191). I. Cyclic 3′,5′-Nucleotide Phosphodiesterase (PDE) and the Inhibitory Effect of BL 191 on PDE in Rat Brain and Heart[1]

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Some properties of cyclic 3′,5′-nucleotide phosphodiesterase (PDE) in the rat cerebral cortex and heart muscle and inhibitory effect of 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191) on PDE have been investigated by comparison with theophylline and caffeine as PDE inhibitor.

1) Cyclic adenosine monophosphate (AMP) hydrolysis in the supernate of the cerebral cortex and heart muscle is linear with respect to time (up to 30 min) and protein concentration (up to 150 μg and 400 μg in the incubation medium on the brain and heart respectively).

2) PDE activity obtained in the absence of Mg^{2+} could be increased up to 70 per cent by adding Mg^{2+} with the supernate of the cerebral cortex as the enzyme.

3) Substantial activity was present in all of the fraction although most was contained in the supernate.

4) From typical Lineweaver-Burk plots, K_m values for the supernate of the cerebral cortex and heart muscle were found to be 130 μM and 74 μM respectively.

5) Inhibitory effect of BL 191 on PDE from the rat cerebral cortex supernate was similar to the action of theophylline and was slightly more potent than that of caffeine. The inhibition of BL 191, theophylline and caffeine was noncompetitive; the K_i was calculated to be 3.0 mM, 3.1 mM and 5.1 mM respectively. The K_i values of 1.65 mM, 1.80 mM and 1.55 mM were found in the heart muscle supernate treated with BL 191, theophylline and caffeine respectively: the type of inhibition was competitive.

Sutherland and Rall[3] have reported that cyclic adenosine 3′,5′-monophosphate (cyclic AMP) was hydrolyzed to 5′-AMP by tissue extracts from several sources and that this hydrolysis was inhibited by caffeine. These results were confirmed and extended by Butcher and Sutherland,[4] who demonstrated that methyl xanthines inhibited cyclic 3′,5′-nucleotide phosphodiesterase (PDE); the most potent inhibitor was theophylline. Since then, theophylline has been used by a number of workers as a reference inhibitor of PDE.[5] Potentiation of a hormonally stimulated cellular response by inhibitors of phosphodiesterase has been proposed as one of four criteria for proving that this response is mediated by intracellular increase in cyclic AMP.[6] Recently, however, theophylline has been shown to have a number of actions unrelated to phosphodiesterase inhibition. McNeill, *et al.*[7] showed that theophylline had direct effects on cardiac contractility, apparently independently of inhibition of phosphodiesterase. Furthermore, lipolytic concentrations of both theophylline[8] and caffeine[9] could

1) Meeting of Tohoku Branch, Pharmaceutical Society of Japan, Sendai, May, 1973.
2) Location: *Aobayama, Sendai.*
3) E.W. Sutherland and T.W. Rall, *J. Biol. Chem.*, **232**, 1077 (1958).
4) R.W. Butcher and E.W. Sutherland, *J. Biol. Chem.*, **237**, 1244 (1962).
5) F. Honda and H. Imamura, *Biochem. Biophys. Acta.*, **161**, 267 (1968); A.R. Somervill, M.L. Raboughans and A.A. Smith, *Biochem. J.*, **120**, 11P (1970); E. Robert and D.G. Simonsen, *Brain Res.*, **24**, 91 (1970); H. Sheppard and G. Wiggan, *Molec. Pharmacol.*, **7**, 111 (1971).
6) E.W. Sutherland, G.A. Robison and R.W. Butcher, *Circulation*, **37**, 279 (1968).
7) J.H. McNeill, M. Nassar and T.M. Brody, *J. Pharmacol. Exptl. Therap.*, **165**, 234 (1969).
8) J.F. Kuo and E.C. Derenzo, *J. Biol. Chem.*, **244**, 2252 (1969).
9) R.W. Butcher, C.E. Baird and E.W. Sutherland, *J. Biol. Chem.*, **243**, 1705 (1968).

not be demonstrated to elevate the levels of cyclic AMP in isolated lipocytes. Dalton, *et al.*[10] have reported that theophylline probably has several activities in lipocytes, only one of which is inhibition of phosphodiesterase. Finally, Sheppard[11] has reported that theophylline can inhibit norepinephrine-stimulated adenyl cyclase in ghosts of rat erythrocytes. A number of xanthine derivatives[12] have also been shown to inhibit the high *K*m PDE, with *K*i values between 10^{-5} and 10^{-2} M.

This paper presents some properties of phosphodiesterase and evidence of the inhibitory action of 3,7-dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191) on PDE in the rat cerebral cortex and heart muscle.

Experimental

Preparation of Subcellular Fractions——Sprague-Dawley JCL male rats weighing 250 to 300 g were guillotined, brain and heart muscle were isolated and chilled in ice-cold solution containing 0.32 M sucrose and 50 μM Tris at a final pH of 7.4. Subsequently, the cerebral cortex was isolated by the method of Growinski, *et al.*[13] One part of the cerebral cortex and heart muscle was homogenized in 9 parts of the same isotonic cold solution. A glass homogenizer with a loose Tefron pestle was used. The homogenization was divided into two 1-min periods with an interval for cooling between them. The homogenate was centrifuged for 10 min at $1000\times g$ at 0°. The precipitate was washed twice by brief homogenization in the sucrose medium and centrifuged to discard the nuclei fraction. The supernatant fluid was pooled and centrifuged at $10000\times g$ for 20 min at 0°. The precipitate was diluted in the same sucrose medium and was noted as the mitochondrial fraction. This supernatant fluid was centrifuged at $105000\times g$ for 60 min at 0°. The precipitate was designated as the microsomal fraction and the supernatant fluid as the supernatant fraction.

Assay Procedure——All cyclic 3′,5′-nucleotide phosphodiesterase assays were by the method of Pöch[14] with a cyclic AMP concentration of 3.6×10^{-4} M, unless otherwise indicated. Enzymatic activity was measured as the rate of hydrolyzing cyclic AMP in a standard reaction medium (final volume 500 μl) containing cyclic 3′,5′-^{14}C-AMP (0.05 μCi), 3 mM Mg-acetate, 2 mM 5′-AMP, 100 mM Tris-HCl, pH 7.5, enzyme at 100 μl of 0.4—3 mg protein/ml. Incubations were run at 37° for 15 min and were stopped by 200 μl of 0.17 M $ZnSO_4$ and 200 μl of 0.15 M $Ba(OH)_2$.

After centrifugation (3000 rpm, 10 min), 300 μl of the clear supernate was transferred into counting vials with 10 ml of scintilating fluid (4 g PPO, 100 mg dimethyl POPOP, 1000 ml toluene, 99.5% of 400 ml ethanol and 100 ml dioxane) and was counted with a liquid scintillation spectrometer. The results were corrected for quenching by the use of internal standards. PDE-activity was determined after incubation with ^{14}C-3′,5′-AMP as substrate by the decrease in radioactivity after removal of the reaction product (^{14}C-5′-AMP) by $ZnSO_4$ and $Ba(OH)_2$-precipitation.

Protein Determination——All protein determinations were by the method of Lowry, *et al.*[15] with the use of serum albumin as standard.

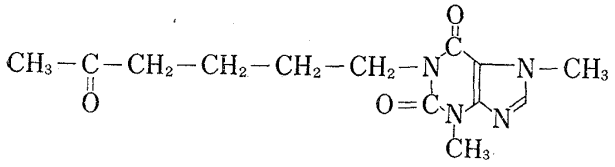

Fig. 1. Structure of BL 191

Preparations of PDE-inhibitors——BL 191 (Fig. 1), a vasoactive agent which was investigated by Farbwerke Hoechst AG.,[16] was used compared with theophylline and caffeine as well-known PDE inhibitors. BL 191 has approximately 10 and 4 times the solubility in water of theophylline and caffeine respectively.

Result and Discussion

Time-course and Protein Dependence

Cyclic AMP hydrolysis in $105000\times g$ supernate of both the rat cerebral cortex and heart muscle linearly increased with time (up to 30 min) and protein concentration (up to

10) C. Dalton, J.B. Quinn, C.R. Burghardt and H. Sheppard, *J. Pharmacol. Exptl. Therap.*, **173**, 270 (1970).
11) H. Sheppard, *Nature*, **228**, 567 (1970).
12) J.A. Beavo, **N.L.** Rogers, O.B. Crofford, J.G. Hardman, E.W. Sutherland and E.V. Newanm, *Molec. Pharmacol.*, **6**, 597 (1970).
13) J. Growinski and L. Iverson, *J. Neurochem.*, **13**, 655 (1966).
14) W.R. Kukovetz and G. Pöch, *Naunyn-Schmiedebergs Arch. Pharmak.*, **272**, 268 (1971).
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
16) K. Popendiker, I. Boksay and V. Bollmann, *Arzneimittel-Forsch.*, **21**, 1160 (1971).

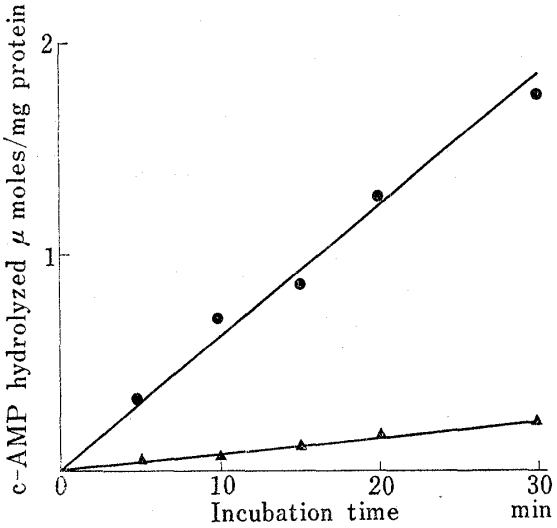

Fig. 2. Rate of Cyclic AMP Hydrolysis in Supernatant Fraction of the Rat Cerebral Cortex and Heart Muscle

Assays were performed as described in the text. Incubations were run at 37° in the assay medium containing 86.7 μg and 288.0 μg protein from the cerebral cortex and heart muscle respectively.
—●—: PDE-activity from the supernate of the cerebral cortex
—▲—: PDE-activity from the supernate of the heart muscle

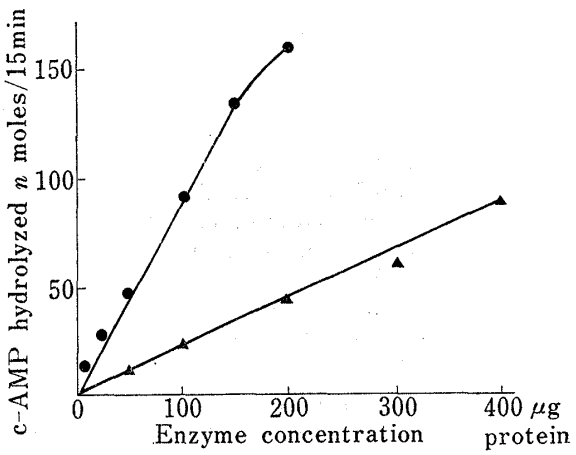

Fig. 3. Cyclic 3′,5′-Nucleotide Phosphodiesterase Activity in Supernatant Fraction of the Rat Cerebral Cortex and Heart Muscle as a Function of Enzyme Concentration

Conditions are as described in the text. Incubations were run at 37° for 15 min at 3.6×10^{-4}M substrate levels.
—●—: PDE-activity from the supernate of the cerebral cortex
—▲—: PDE-activity from the supernate of the heart muscle

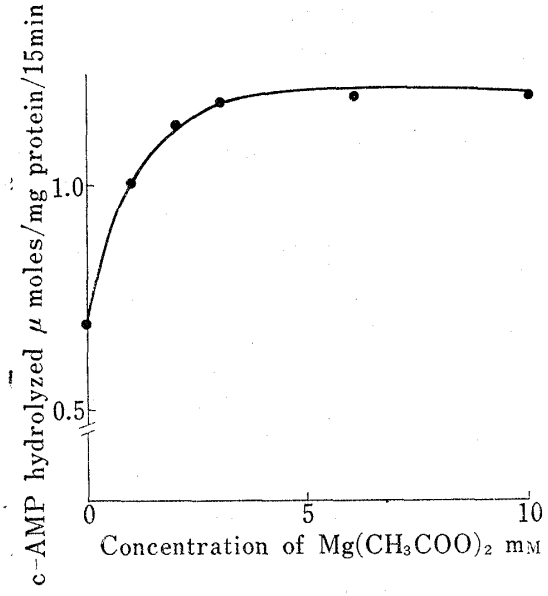

Fig. 4. Influence of Mg^{2+} Concentration on PDE from the Rat Cerebral Cortex Supernate

Conditions are as described in the text with varying concentrations of $Mg(CH_3COO)_2$.

approx. 150 μg and 400 μg in the incubation medium on the cerebral cortex and heart muscle respectively) (Fig. 2 and 3).

Requirement for Mg^{2+}

By adding Mg^{2+} of varying concentrations cyclic 3′,5′-nucleotide phosphodiesterase activity was increased up to about 170% of the basal activity as obtained in the absence of $Mg(CH_3COO)_2$ added (Fig. 4).

Distribution among Subcellular Fractions

Cyclic 3′,5′-nucleotide phosphodiesterase activity in all fractions was measured at 3.6×10^{-4}M substrate levels. The subcellular distribution was shown in Table I. Substantial activity was present in all of the fractions although most was contained in the supernate and 13% of activity was contained in the mitochondrial fraction of the cerebral cortex. The small amounts of activity in the $105000\times g$ particulate fractions from both the cerebral cortex and heart muscle were at least in part due to the contaminating supernatant fluid since these fractions were not washed. These results coincide with those of Menahan, *et al.*[17] and Beavo, *et al.*[18] in the point that most of

17) L.A. Menahan, K.D. Hepp and O. Wieland, *Eur. J. Biochem.*, **8**, 435 (1969).
18) J.A. Beavo, J.G. Hardman and E.W. Sutherland, *J. Biol. Chem.*, **245**, 5649 (1970).

Table I. Distribution of Cyclic 3′,5′-Nucleotide Phosphodiesterase in Crude Subcellular Fractions of the Rat Cerebral Cortex and Heart Muscle

	Protein mg/g tissue	Enzyme c-AMP hydrolyzed μ moles/g tissue	Specific activity c-AMP hydrolyzed μ moles/mg protein
Rat cerebral cortex			
mitochondrial fraction	23.9	10.3(13)[a]	0.43
microsomal fraction	9.1	2.5(3)	0.28
supernatant fraction	59.0	67.3(84)	1.14
total	92.0	80.1(100)	
starting material (1000×*g*, super.)	98.8	82.0	0.83
recovery	93%	98%	
Rat heart muscle			
mitochondrial fraction	17.4	0.02(2)	0.013
microsomal fraction	9.9	0.01(1)	0.008
supernatant fraction	50.4	1.22(98)	0.242
total	77.7	1.25(100)	
starting material (1000×*g*, super.)	80.6	1.33	0.165
recovery	96%	94%	

Experimental methods were described in the text with a cyclic AMP concentration of 3.6×10^{-4}M.
a) Parenthesized numbers indicate the percentage of each fraction in the total.

the total phosphodiesterase activity was contained in the soluble fraction in our experiment, however, no apparent activity for hydrolyzing cyclic guanosine monophosphate was found in the 1000×*g* particulate fraction, whereas these workers have noticed the activity.

Velocity-substrate Concentration Relationship

When cyclic 3′,5′-nucleotide phosphodiesterase activity of 105000×*g* supernate in the rat cerebral cortex and heart muscle was studied at varying substrate concentration, the results shown in Fig. 5 could be discerned from typical Lineweaver-Burk plots. Apparent *K*m values for the 105000×*g* supernate of the rat cerebral cortex and heart muscle were found to be 130 μM and 74 μM, respectively. These results coincide with studies of Thompson and Appleman,[19] who demonstrated that the PDE activity in the rat brain and heart muscle had two different types, and that *K*m of fraction II were found to be 104 μM and 87 μM, respectively.

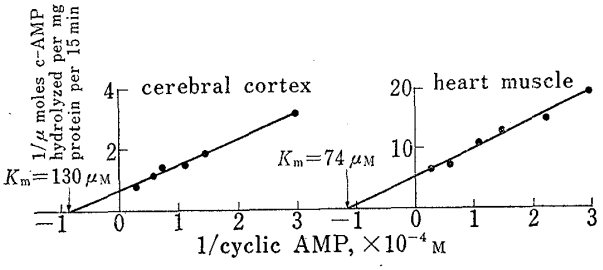

Fig. 5. Double-reciprocal Plot of the Hydrolysis of Cyclic AMP by the Rat Cerebral Cortex and Heart Muscle

Assays were performed as described in experimental. The *K*m for cyclic AMP on PDE from the rat cerebral cortex and heart muscle was about 130 μM and 74 μM, respcteively. The unit of velocity is μ moles per mg of protein per 15 min. Substrate concentrations ranged from 33.75 μM to 360 μM for cyclic AMP.

Effect of 3,7-Dimethyl-1-(5-oxo-hexyl)-xanthine (BL 191)

Fig. 6 demonstrates the inhibition of cyclic 3′,5′-nucleotide phosphodiesterase in the rat cerebral cortex and heart muscle produced by various concentrations of BL 191. In the supernate of both the cerebral cortex and heart muscle examined, BL 191 exerted marked and dose-dependent inhibitory effects on PDE activity. Using the drug concentration that produced 50% inhibition of

19) W.J. Thompson and M.M. Appleman, *J. Biol. Chem.*, **246**, 3145 (1971).

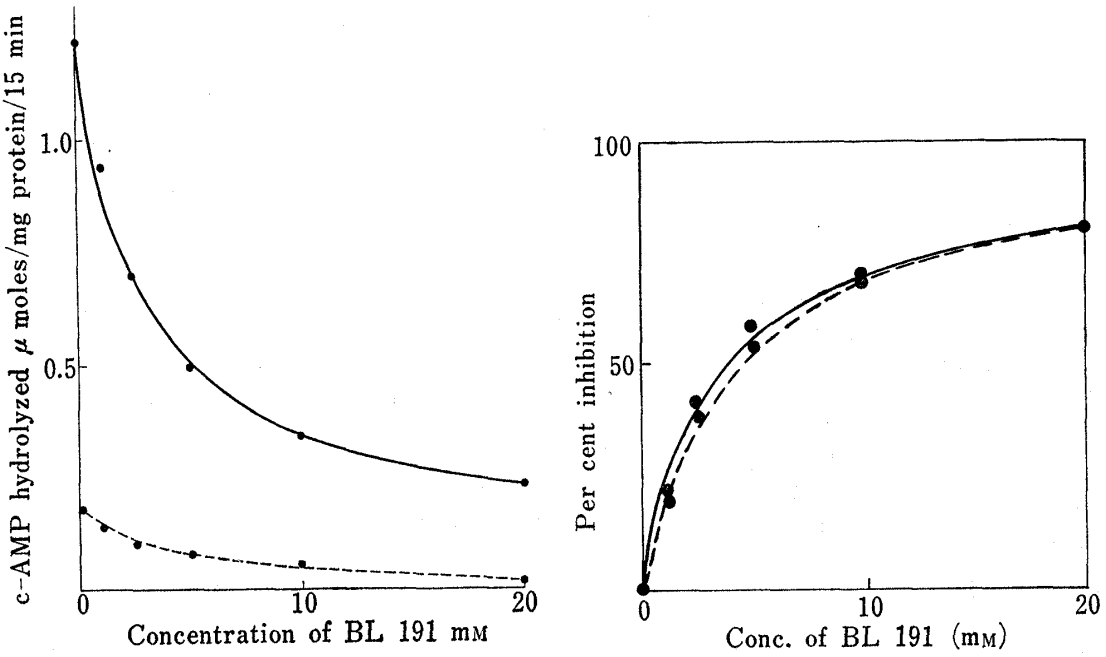

Fig. 6. Inhibitory Effect on Cyclic 3′,5′-Nucleotide Phosphodiesterase by BL 191

Assays were performed as described in experimental.
—●—: PDE-activity from the supernate in the cerebral cortex
--●--: PDE-activity from the supernate in the heart muscle

the enzymatic activity (ID_{50}), when the concentration of cyclic AMP was 3.6×10^{-4}M, it may be seen that BL 191 showed approximately 4 mM of ID_{50} in both the cerebral cortex and heart muscle.

The kinetics of the hydrolysis of cyclic AMP by PDE in the rat cerebral cortex and heart muscle and the inhibition of this hydrolysis by BL 191, theophylline and caffeine were investigated by Dixon plots and are presented in Fig. 7 and 8. As can be seen, the inhibition of BL 191 was non-competitive; the *Ki* was calculated to be 3.0 mM in the case of $105000\times g$ supernate of the rat cerebral cortex. The inhibitory effect of BL 191 on PDE from the rat cerebral cortex, therefore, was similar to the action of theophylline and was slightly more potent than that of caffeine (*Ki* value; 3.1 mM of theophylline, 5.1 mM of caffeine) (Fig. 7). The *Ki* value of 1.65 mM was found in the rat heart muscle supernate where the type of inhibition was competitive (Fig. 8). With theophylline and caffeine, the same competitive type of inhibition as in BL 191 with *Ki* values of 1.80 mM and 1.55 mM was obtained respectively. In this respect, it is noticeable that caffeine and theophylline were competitive inhibitors of the beef heart PDE[4] while caffeine inhibited the dog heart enzyme noncompetitively,[20] suggesting that PDE differed with different tissues.

The pharmacodynamic actions of papaverine as a potent inhibitor of PDE are much more limited than those of the much weaker PDE-inhibitor theophylline. Whereas theophylline induces most of the effects of adrenergic beta receptor stimulation[21] or the effects of increased formation of cyclic AMP in various tissues, the actions of papaverine are mainly confined to the smooth muscle. While it is known there is no evidence that papaverine markedly stimulates cardiac contraction and rate glycogenolysis in liver or lipolysis in adipose tissue to some extent, which would be due to its inhibitory effect on PDE in the supernates of these tissues. The most likely explanation at present would be that methyl xanthine is able to penetrate the cellular membranes of many tissues more easily than papaverine.

20) K.G. Nair, *Biochemistry*, **5**, 150 (1966).
21) G.A. Robison, R.W. Butcher and E.W. Sutherland, *Ann. N. Y. Acad. Sci.*, **139**, 703 (1967).

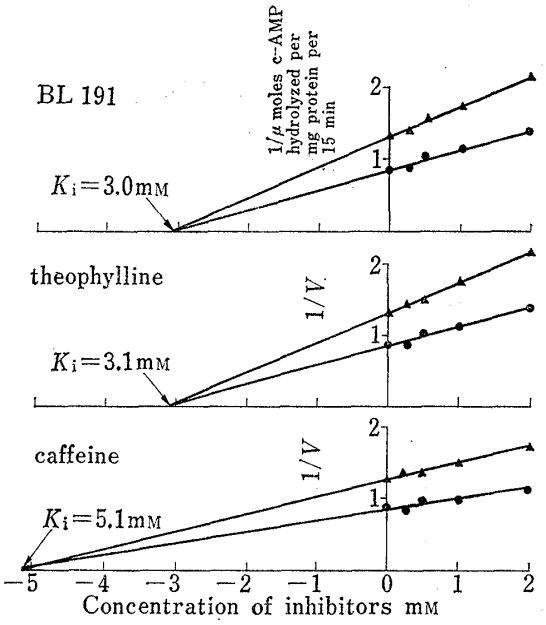

Fig. 7. Inhibitory Effect of PDE by BL 191 compared with Theophylline and Caffeine in the 105000×*g* Supernate of the Rat Cerebral Cortex Measured at Two Substrate Concentrations and Determined from Dixon Plots

—▲—: [S]=1.8×10^{-4}M. —●—: [S]=3.6×10^{-4}M. Abszssa(1/*V*): control rate of hydrolysis (15 min) with the higher [S] was 1.14 μ moles/mg protein.

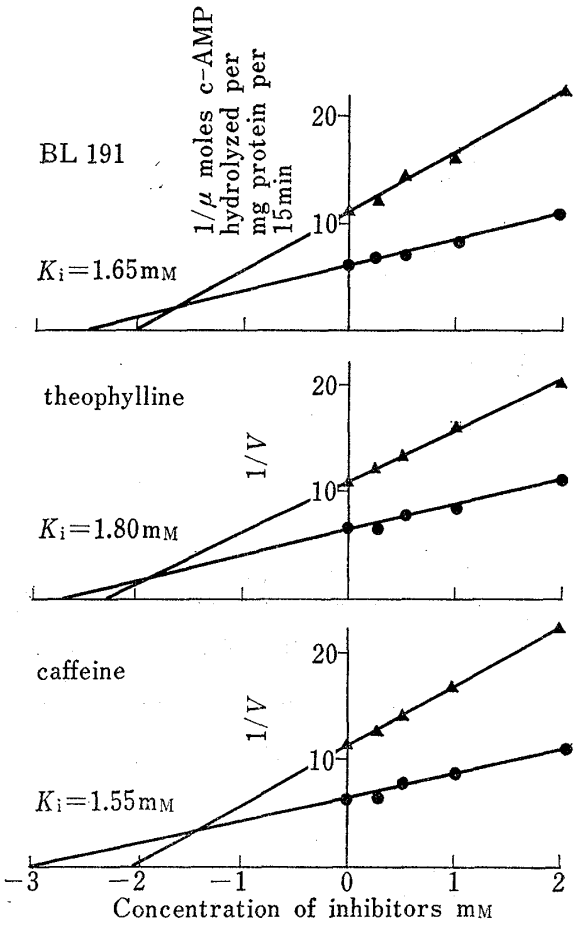

Fig. 8. Inhibitory Effect of PDE by BL 191 Compared with Theophylline and Caffeine in the Supernate of the Rat Heart Muscle Measured at Two Substrate Concentrations and Determined from Dixon Plots

—▲—: [S]=0.9×10^{-4}M. —●—: [S]=3.6×10^{-4}M. Abszssa(1/*V*): control rate of hydrolysis (15 min) with the higher [S] was 0.16 μ moles/mg protein.

Furthermore, Rall and West[22] demonstrated that the inotropic response to norepinephrine by segments of electrically driven rabbit atria was increased in the presence of 0.2 mM to 1 mM theophylline. In our experiments, using the atrial preparation in guinea pigs, the potentiation of cardiac inotropic responses to norepinephrine was increased in the concentration of 0.1 mM BL 191, although the cardiac inotropic effects were not seen in the same concentration of BL 191 alone. The results of these investigations are consistent with the hypothesis that the inotropic actions of catecholamine in the cardiac muscle involve the formation and subsequent action of cyclic AMP. Thus the potentiating effect of BL 191 and theophylline reported would be due to the inhibition of cyclic 3′,5′-nucleotide phosphodiesterase, which, in turn, would lead to a greater accumulation of cyclic AMP at any effective concentration of catecholamines. Extending this interpretation further, the inotropic actions of higher concentration of methyl xanthine alone would be attributed primarily to the accumulation of cyclic AMP to an effective level; the formation of the 5′-AMP in the absence of added catecholamine would be postulated to occur as the results of the "basal" activity

22) T.W. Rall and T.C. West, *J. Pharmacol. Exptl. Therap.*, **139**, 269 (1963).

of adenyl cyclase apparently not dependent upon the presence of catecholamine.[23]

In conclusion, BL 191 has been shown to be a potent noncompetitive inhibitor of PDE prepared from the rat cerebral cortex and a competitive type of inhibitor of the enzyme prepared from the rat heart muscle. Evidence has been presented to suggest that PDE would considerably differ with tissues and animals in the type of inhibition by a number of inhibitors.

Other workers[24] have reached similar conclusions based on differential inhibition of a variety of cyclic 3′,5′-nucleotide phosphodiesterases by several reagents.

23) F. Murad, Y.M. Chi, T.W. Rall and E.W. Sutherland, *J. Biol. Chem.*, **237**, 1233 (1962); L.M. Klainer, Y.M. Chi, S.L. Freidberg, T.W. Rall and E.W. Sutherland, *ibid.*, **237**, 1239 (1962).

24) J. Vernikos-Danellis and C.G. Harris, *Proc. Soc. Exptl. Biol. Med.*, **128**, 1016 (1968); J.B. Smith and D.C.B. Mills, *Biochem., J.* **120**, 20P (1970); M.S. Amer and G.R. Mckinney, *Pharmacologist*, **12**, 291 (1970); M. Chsin, D.N. Harris, M.B. Phillips and S.M. Hess, *Biochem. Pharmacol.*, **21**, 2433 (1972).