and shown to be homogeneous by glc on a diethylene glycol succinate column at 117°.

3-Methoxy-4-methylphenol was prepd by diazotization and acid hydrolysis of 3-methoxy-4-methylaniline,[22] crystd from C_6H_6 and ligroin, mp 59.5–61.5°, lit.[22] bp 136–137° (20 mm). *Anal.* ($C_8H_{10}O_2$) C, H. 2,4-Dimethoxyphenol was prepd by Baeyer-Villiger oxidn of 2,4-dimethoxyacetophenone,[23] mp 25–28°, lit.[24] 28°.

Substituted Ph Ethers of *N*-Acetyl-3,5-dinitro-L-tyrosine Et Ester (Table I, 12–16).—A 3-fold excess of the appropriate substituted phenol was condensed with *N*-acetyl-3,5-dinitro-L-tyrosine Et ester by the Meltzer[14] modification of the method of Barnes[13] to form the dinitrodiphenyl ethers **12–16**. Crystd from EtOH.

Substituted Ph Ethers of *N*-Acetyl-3,5-diiodo-L-tyrosine Et Ester (Table I, 17–21).—The dinitro compds **12–16**) were hydrogenated, tetrazotized, and decompd in aq I_3^- soln by standard methods.[8,13,14] Compds **19–21** were formed in aq I_3^- soln which contd twice as much H_2O as in the standard procedure in order to prevent the formation of side products with an extraneous ir peak at 2100 cm^{-1} in **20**, or with high I analyses in **19** and **21**. The crude product was dissolved in C_6H_6 and chromatogd on acid-washed alumina, using increasing concns of $CHCl_3$ or of Et_2O in C_6H_6. Fractions eluting with 10–25% $CHCl_3$ in C_6H_6 were crystd from acetone (**17**) or EtOH (**18**). Compds **19–21** were eluted with 20–50% Et_2O in C_6H_6 and crystd from EtOH.

Substituted Ph Ethers of 3,5-Diiodo-L-tyrosine (Table II, 6–10).—The diiodo methoxyphenyl ethers (**17–21**, 1 g) were hydrolyzed to the amino acids **6–10** by heating in const-boiling HI (6 ml) and AcOH (20 ml) under reflux for 6 hr, except compd **21** which was heated under reflux for 8 hr. The soln was evapd to dryness at 60–70° under reduced pressure. The residue was dissolved in a small amt of H_2O. The soln was filtered, aq AcONa was added to pH 4.9, and the soln was refrigerated overnight. The ppt was collected and dissolved in HCl, and aq AcONa was added to pH 4.9, yielding a granular solid.

3-[4-(2-Hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-L-alanine (*o*-L-thyroxine, 3).—The procedure of Niemann and Mead[3] for the synthesis of *o*-DL-thyroxine was used. A soln of I_2 (140 mg, 0.55 mmole) in 6 ml of 1 *M* KI was added dropwise during 7 min to a well-stirred soln of *o*-3,5-diiodo-L-thyronine (**6**, 139 mg, 0.27 mmole) in 5 ml of 7 *N* NH_4OH and cooled in an ice bath. Stirring was contd for 30 min, then $NaHSO_3$ was added to reduce unreacted I_2. Dil aq HCl was added until the soln was at pH 3.0. After refrigeration overnight the ppt was collected by filtration, dissolved in 7 *N* NH_4OH, reptd at pH 3.0, washed with cold H_2O, and dried at 100° (1 mm) over P_2O_5 to yield 124 mg (60%) of *o*-L-thyroxine (**3**) (see Table I).

3-[4-(3-Hydroxy-4,6-diiodophenoxy)-3,5-diiodophenyl]-L-alanine (*m*-L-thyroxine, 4b).—*m*-3,5-Diiodo-L-thyronine (**7**, 278 mg, 0.53 mmole) was iodinated as described for the prepn of **3** to yield 331 mg (81%) of **4b**.

Acknowledgments.—One of the authors (E.C.J.) is grateful to Professor Robert Schwyzer for his warm hospitality at the Institute for Molecular Biology and Biophysics, Swiss Federal University, Zürich, during the preparation of this manuscript.

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8-Substituted Theophyllines. *In Vitro* Inhibition of 3′,5′-Cyclic Adenosine Monophosphate Phosphodiesterase and Pharmacological Spectrum in Mice

ELIZABETH B. GOODSELL,* HERMAN H. STEIN, AND KATHLEEN J. WENZKE

Abbott Laboratories, North Chicago, Illinois

Received May 29, 1971

A series of 8-substituted theophylline derivatives was tested for *in vitro* 3′,5′-cyclic AMP phosphodiesterase (PDE) inhibition. The unbranched derivatives, Me to *n*-Hex (**2–7**), cyclopropyl (**12**), cyclobutyl (**13**), cyclopentyl (**14**), *i*-Pr (**10**), and *i*-Bu (**11**) were more potent inhibitors than theophylline (T). The *n*-heptyl (**8**) and 4-hydroxybutyl (**9**) were inactive. *In vitro* activity was influenced by H_2O and lipid solubility as well as by the size and configuration of the substituent. The symptomatology pattern in mice changed from CNS stimulation (T) to pronounced depression (**7**). Compds **4**, **5**, **12**, and **14** were active antidepressants; **4**, **7**, and **10** were effective against pentylenetetrazole-induced mortality. Antiedema activity was present in **4**, **5**, **6**, **11**, **12**, and **13**. Marked hyperglycemic responses were induced by **5**, **6**, and **7**. CNS depression and hyperglycemia appear to be directly related to *in vitro* PDE inhibition. The other activities are not readily explained, but an involvement in some aspect of the cyclic AMP–adenyl cyclase–PDE–Ca^{2+} system is suggested.

The methylxanthines (caffeine and theophylline) are established cyclic AMP phosphodiesterase (PDE) inhibitors. As such they are capable of exerting characteristic effects in certain *in vitro* systems, *e.g.*, stimulate exocrine pancreas[1] and gastric[2] secretion, increase lipolysis,[3] produce a negative inotropic effect on rat portal vein,[4] simulate the effect of vasopressin in the toad bladder,[5] etc.

To date their proven *in vivo* effects pertinent to PDE inhibition have been limited. Hynie, *et al.*,[3] have established the lipolytic action of theophylline (T) in rats, and Malamud[6] obtained a stimulation of DNA synthesis in the parotid gland of the mouse. It is fairly well ascertained that insulin release induced in the rat by T is the result of increased cyclic AMP levels.[7] Otherwise, the majority of their pharmacological effects has been secondary, *i.e.*, potentiation of the activity of a primary compound such as the hormone epinephrine. Potentiation of this nature has been accepted as evidence of cyclic AMP involvement.[8]

Cyclic AMP has been implicated in a wide variety of physiological processes, as has been indicated to some extent above. To explain the manner in which these different effects result from intracellular production

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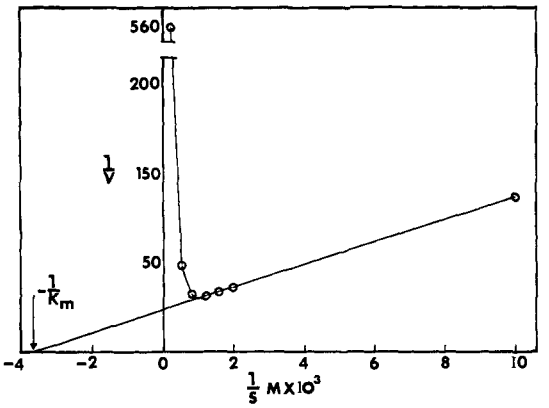

Figure 1.—Lineweaver–Burk plot of hydrolysis of cyclic AMP by PDE illustrating substrate inhibition; $K_m = 2.6 \times 10^{-4}$ *M*; the basal incubation mixture contained 80 m*M* Tris buffer, pH 7.5, 3.3 m*M* $MgSO_4$, 0.15 mg of alkaline phosphatase (*E. coli*), and appropriate amount of enzyme (approximately 3.5 mg of protein) in a total volume of 1.5 ml. The reaction was initiated by the addition of 0.3 ml of 4 m*M* cyclic AMP and terminated 15 min later by 0.2 ml of 55% TCA.

of one substance (cyclic AMP) Robison and Sutherland's[8] discerning second messenger concept has been invoked. In brief, the first messenger is the compound (hormone) which travels from its site of origin to the target tissue, stimulates the membrane-bound adenyl cyclase which in turn produces an alteration in the intracellular level of a second messenger, cyclic AMP. The ultimate response depends upon the environment of the particular tissue. In addition to the physiological stimulation of the synthesizing hormone, cyclic AMP levels can also be affected by changing the activity of PDE, the enzyme which hydrolyzes cyclic AMP.

It may be argued, therefore, that a nonhormonal substance as well could alter intracellular cyclic AMP levels in a discrete area, depending upon its ability to penetrate to a particular site and to fit the enzyme receptor.

Many methylxanthines have been examined for pharmacological activity. The tendency was to make a bulkier, more complex molecule. When one considers the 6-fold increase in PDE inhibition resulting from the removal of a Me group from caffeine to form T, it appears that, at least with respect to PDE inhibition, major structural changes are not essential.

Only one series of methylxanthines has been reported for its effect on PDE.[9] Various side chains were introduced on the 7-N while a CH_2-3-pyridyl substituent was maintained in the 8 position. None of these derivatives was as potent an inhibitor as T.

We are reporting a series of simple 8-substituted T derivatives which are potent PDE inhibitors and which exhibit pharmacological spectra significantly different from T.

Experimental Section

Enzyme Studies.—The enzyme was prepd from beef heart according to Butcher and Sutherland[10] and carried through one $(NH_4)_2SO_4$ fractionation prior to dialysis. The incubation mixt contd Tris buffer (80 m*M*), pH 7.5, $MgSO_4$ (3.3 m*M*), alkaline phosphatase, *Escherichia coli* (0.15 mg), PDE (about 3.5 mg of protein), and either the test compd or buffer in a total vol of 1.5 ml. The mixt was equilibrated at 37° for 10 min and the reaction was initiated by the addn of 0.3 ml of cyclic AMP (4 m*M*). It was terminated after 15 min with 0.2 ml of 55% trichloroacetic acid. The supernatant was treated with Norit to remove interfering substances. Inorg phosphate formed from 5'-AMP by the nucleotidase was measured by the method of Fiske and SubbaRow.[11]

Pharmacological Tests.—The compds were evaluated in mice of the Charles River strain using an established battery of tests. Besides acute ip toxicity and gross symptomatology, the following tests were performed; modified dopa,[12] protection against pentylenetetrazole-induced fatality, effect on hexobarbital sleeping time, antiedema effect using the mouse paw carrageenin test,[13] and estimation of blood glucose values. Other activities investigated were local anesthesia, ganglionic blockade, analgesia, parasympathomimetic properties, adrenergic and cholinergic blockade, and anticoagulant activity.

8-Substituted Theophyllines.—All of these compds were synthesized from 5,6-diamino-1,3-dimethyluracil and the appropriate carboxylic acid by the general procedure of Hager, *et al.*,[14] except that the decolorization step with charcoal was omitted. δ-Valerolactone was utilized for the prepn of 8-(4-hydroxybutyl)-T. Dry EtOH was used to recryst the final products. Yields ranged from 15 to 30%, the latter values being attained with the longer, unbranched derivs.

Results and Discussion

Enzyme Studies.—The K_m value obtained (2.6×10^{-4} *M*) using cyclic AMP as the substrate was in accordance with that reported in the literature.[10,15] In addition, substrate inhibition was repeatedly observed at concns above 1.75×10^{-3} *M* (Figure 1). It is unlikely that this phenomenon was the result of 5'-AMP (product) inhibition, since the presence of alk phosphatase prevented its accumulation. As a conquence of this latter enzymatic activity, P_i and adenosine levels increased throughout the incubation period. Separation of the two enzyme reactions, *i.e.*, hydrolysis of cyclic AMP by PDE and dephosphorylation of 5'-AMP formed by phosphatase, did not alter the results; the curves obtained were superimposable on those shown in Figure 1. It was further determined that adenosine (1×10^{-2} *M*) was not an inhibitor of either system nor did high concns of cyclic AMP (6×10^{-3} *M*) have any effect on the activity of the phosphatase. Although this enzyme is subject to substrate inhibition (5'-AMP at 4×10^{-3} *M*) such levels were not reached during the experimental conditions outlined above. Thus, the substrate inhibition observed is attributed to the enzymatic activity in this particular PDE preparation. Further substantiation of substrate inhibition is obtained by noting that in some methods of assay for adenyl cyclase[16] where T is required to inhibit PDE, cyclic AMP can substitute effectively for the methylxanthine.

The Lineweaver–Burk plots in Figure 2 show the inhibition to be competitive. Derivatives with 8-substituted chains longer than 5 were not sol at 5×10^{-4} *M* and were excluded from the graph. Studies at lower concns show that they, as well as the branched

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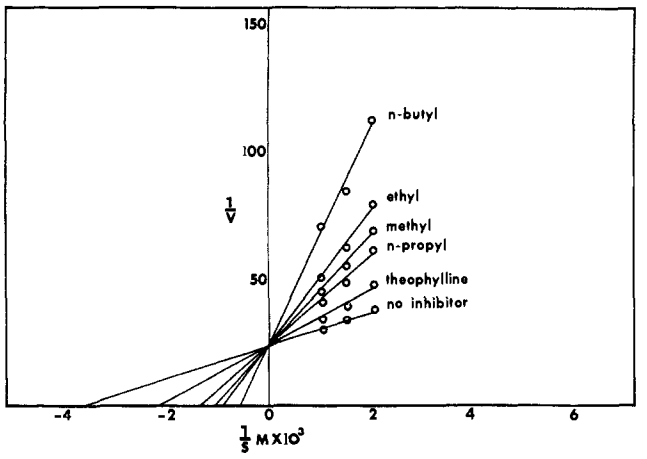
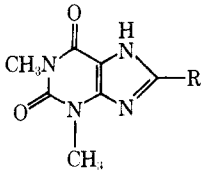


Figure 2.—Lineweaver-Burk plot illustrating the competitive nature of PDE inhibition by the 8-substituted theophylline derivatives (5×10^{-4} *M*). Incubation mixture as in Figure 1 with the exception that the enzyme was preincubated for 10 min in the presence of the inhibitor.

and cyclic compounds, were competitive inhibitors. The calcd inhibition constants are listed in Table I.

TABLE I

8-SUBSTITUTED THEOPHYLLINE DERIVATIVES



Compd	R[a]	Mp, °C[d]	Water solubility,[e] mg/ml, 24°	log *P*[f]	K_i,[h] *M* × 10^{-4}	$\lambda_{max\ H_2O}$ (ε), mμ
1	H		6.7	0.117	4.2	272 (10,700)
2	CH_3	324–326	4.3	0.304	1.6	274 (12,500)
3	C_2H_5	268–271	2.6	0.802	1.0	275 (12,000)
4	*n*-C_3H_7[b]	259–261	0.7	1.248	2.1	275 (14,600)
5	*n*-C_4H_9	233–235	0.4	1.848	0.7	275 (13,000)
6	*n*-C_5H_{11}	224–225	0.09	2.368[g]	0.4	275 (13,600)
7	*n*-C_6H_{13}	199–201	0.02	2.888[g]	0.2	275 (12,600)
8	*n*-C_7H_{15}[c]	196–198	0.006	3.408[g]	N.I.[i]	
9	4-OHC_4H_8[c]	218–220	>15.0	0.127	N.I.[i]	275 (13,800)
10	*i*-C_3H_7	274–275	0.4	1.164	0.5	275 (13,300)
11	*i*-C_4H_9	227–230	0.4	1.576	1.5	275 (15,100)
12	*c*-C_3H_5[c]	283–285	0.4	1.159	0.6	280 (16,100)
13	*c*-C_4H_7[c]	298–301	0.08	1.499	0.9	276 (14,600)
14	*c*-C_5H_9[c]	247–249	0.13	1.928	2.1	276 (13,900)

[a] All compds were analyzed for C, H, N, O. Anal. results were within ±0.4% of the theor values. [b] Mass and nmr spectra were consistent with the proposed structure. [c] Compds previously unreported. Nmr and mass (only **8**, **9**, and **14** measured) spectra were consistent with the proposed structure. [d] Thomas-Hoover capillary mp apparatus; values uncorrected. [e] An excess of the compd was added to 10 ml of triple-distd H_2O in a screw top test tube sealed with paraffin. The tubes were placed on a reciprocating shaker for 24 hr at room temp (24°); after filtration the absorbance was measured with a Coleman Model 124 spectrophotometer. [f] Method of T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.*, **86**, 5175 (1964). [g] By calculation: 0.52 added for each addnl CH_2. [h] Inhibition intercept = $1/(K_s[1 + (I/K_I)])$ from Lineweaver-Burk plot. [i] No inhibition.

Further examination of Table I permits speculation about some of the physical characteristics upon which *in vitro* PDE inhibition depends; *e.g.*, size of the 8 substituent, configuration, partition coefficient, and H_2O solubility. With the exception of **4** and **8**, PDE inhibition was directly related to the length of the substituent chain. The low H_2O solubility (1.5×10^{-4} *M*) of **8** might initially be proposed to explain its lack of activity. However, had it followed the established sequence, the solubility would have been sufficient to indicate inhibition. The behavior of **4** cannot be explained at this time.

Conversely, the inhibitory properties of the cyclic and branched derivatives were inversely related to the size of the substituent so that **10** > **11**; **12** > **13** > **14**.

Only a few comments can be made interrelating the configuration of the substituent. Cyclizing the chain decreased the potency so that **5** > **13**; **6** > **14**. Due to the unorthodox behavior of **4**, the Pr analogs cannot be compared.

The influence of the partition coefficient paralleled that of the size of the substituent discussed above, including the exceptions noted for **4** and **8** as well as the inverse relationship with the branched and cyclic derivatives.

The results obtained with **9** indicate, however, that the partition coefficient is not the prime factor governing *in vitro* inhibition, inasmuch as both **9** and T have essentially the same log *P* but only T is an inhibitor. This difference may be due to either the greater H_2O solubility of **9** or to some specific effect of the OH group.

Pharmacological Studies.—The toxicity changes observed were biphasic (Table II). Initially, the introduction of a small substituent, *e.g.*, **2** or **3**, increased toxicity. From **4** through **8** the toxicity decreased with each additional CH_2. Branching or cyclization of the particular substituent produced compounds of greater toxicity, *i.e.*, **14** was more toxic than **6**.

In-depth investigation of the symptomatology was not practical since the behavior was found to be dose dependent. Some degree of standardization was achieved by using 0.25 of the ip LD_{50} as the observational dose. The pattern was evaluated visually as well as by motor activity chambers equipped with photocells; both methods yielded similar results.

The symptomatology changed from potent CNS stimulation with T to marked sedation with **7**; **8** retained attenuated depressant activity. A transitional group was formed by **2**, **3**, and **4**; both stimulation and depression occurred within the 1-hr observation time span. The cyclic and branched derivatives were all depressants with the exception of **13** which was a stimulant.

An analogous reversal of symptomatology by modification of 6-thio-T in the 8 position has been reported.[17] A hypnotic effect in rats equivalent to thiopental was elicited with 8-ethylthio-6-thio-T while 8-(2-*N*,*N*-diethylaminoethyl)thio-6-thio-T was a CNS stimulant. Unfortunately, no PDE studies were carried out.

Potentiation of the hypnotic activity of hexobarbital by those compounds with depressant symptomatology was probably of a nonspecific additive nature.

Among the unbranched analogs, the hyperglycemic effect was related to chain length (as well as to log *P*), reaching a maximum with **5**, plateauing with **6** and **7**. Cyclization or branching diminished the response.

Marked activity in the modified dopa test, when observed, resided in compounds with odd-number **8** substituents: **6**, **12**, **14**.

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Table II
Pharmacological Activity of 8-Substituted Theophyllines

Compd	R	ip LD_{50}, mg/kg	Symptomatology[a]	Hexobarbital test[b]	Hyperglycemia[c]	Modified[d] dopa test	Effect *vs.*[e] pentylene tetrazole	Antiedema[f]
1	H	200	S	0	0	++	−	+[d]
2	CH_3	130	D/S	0	±	0	−	0
3	C_2H_5	175	D	+	+	0	0	0
4	*n*-C_3H_7	250	S/D	0	++	+++	++	+
5	*n*-C_4H_9	250	D	+	+++	0	±	+
6	*n*-C_5H_{11}	400	D	+	+++	+++	+	+
7	*n*-C_6H_{13}	500	D	+	+++	0	++	0
8	*n*-C_7H_{15}	>1000	D±	+	+	0	0	0
9	$4OHC_4H_9$	750	S	0	−	0	0	0
10	*i*-C_3H_7	150	D	0	±	0	++	0
11	*i*-C_4H_9	250	D	+	+	0	+	+
12	*c*-C_3H_5	175	D	+	++	+++	+	+
13	*c*-C_4H_7	200	S	0	+	0	+	+
14	*c*-C_5H_9	200	D±	0	±	+++	0	0

[a] S, stimulant which implies one or all of the following: increased activity, irritability, grooming, squealing, hyperreflexia, running, tremors, preconvulsiveness, convulsions; D, depressant: decreased motor activity, ataxia, postural changes, prostration, loss of righting reflex. Dose was 0.25 LD_{50}. [b] Hexobarbital Na (100 mg/kg ip) 1 hr after test compd (25 mg/kg ip) (+) statistically significant potentiation. [c] Mice fasted 2 hr prior to drug administration (ip); 1 hr after drug, blood was drawn from the tail and glucose estimated with Dextrostix; (0) no significant change from control (±) 40–60% increase (+) 75–100% increase; (++) 130% increase (+++) 160–200% increase (−) 25% decrease. [d] Amitriptyline (25 mg/kg po) used as a positive control yielded (++) response. Test compds 50 mg/kg po; (++) salivation, piloerection, increased activity, occasional squealing, some fighting; (+++) as above plus blood drawn. [e] Pentylenetetrazole (120 mg/kg sc) 30 min after test compd (1/2 LD_{50} ip); (−) potentiation, (0) no significant difference, (±) 40–60% protection, (+) 61–80%, (++) 81–100%. [f] Mice sacrificed 4 hr after drug administration (50 mg/kg po), (d) diuretic activity, (+) approximately 50% inhibition of edema formation.

The sedative effect of **7** is probably the reason for the protection afforded against pentylenetetrazole, similar to the activity of pentobarbital in this regard. The activity of **4** and **10** cannot be explained in this manner and may be more specific.

Although the diuretic properties of T contributed significantly to its antiedema activity, secondary testing of this series as antiinflammatory agents did not reveal any significant diuretic activity in the other active compounds; namely **4**, **5**, **6**, **11**, **12**, **13**.

Introduction of the OH group on **5** to form **9** dramatically changed its properties. The H_2O solubility increased, the log *P* decreased and the ability to inhibit PDE *in vitro* was lost, as mentioned above. Toxicity was greatly reduced; symptomatology reverted back to stimulation, while the only pharmacological effect obtained was a slight but definite hypoglycemia. It is probable that the 40-fold increase in H_2O solubility was responsible for the dramatic reversal of the pharmacological effects so that **9** had properties more in common with T than with **5**, the parent compound.

The series was devoid of activity in the following areas: local anesthesia, ganglionic blockade, analgesia, anticoagulation, adrenergic blockade, cholinergic stimulation and blockade.

The two striking correlations between pharmacological activity and *in vitro* PDE inhibition, namely, CNS depression and hyperglycemia, are clearly evident in the series of unbranched substituted compounds. As the chain was lengthened, the potency as a PDE inhibitor increased and the CNS depression became more profound. Investigations in our laboratory as well as recent literature reports, have shown that specific groups of pharmacological agents which produce depression in animals are potent *in vitro* PDE inhibitors. These include the tranquilizers (thioridazine, chlorpromazine, perphenazine, mepazine),[18,19] the sedative–antidepressants (amitriptyline, nortriptyline, imipramine),[20] the antihistamines (thephorin, diphenhydramine),[18] and the α-blocking agent, phenoxybenzamine.[18] Barbiturates did not share this *in vitro* enzymatic activity.[18]

This presents an anomaly which remains to be resolved. Does PDE inhibition result in stimulation (theophylline and caffeine) as has so long been presumed, or in depression as has been shown above?

The role of cyclic AMP in promoting liver glycogenolysis has been well documented so that the hyperglycemia noted can be hypothesized as the result of PDE inhibition in the liver. The blood glucose values increased with chain length and with the ability to inhibit PDE *in vitro*, maximally plateauing with **5**, **6**, and **7**.

Determination of *in vivo* PDE activity after administration of these compounds would contribute significantly to the elucidation of the role played by the enzyme.

Future research may very likely explain the diverse effects of the methylxanthines by their participation in some phase of the biochemical system involving cyclic AMP–adenylcyclase–PDE–Ca^{2+}. Caffeine has long been known to influence Ca^{2+} mobilization in skeletal muscle and recently Kakiuchi and Yamazaki[21] have demonstrated the presence of two different activities of PDE in rat brain, one of which is Ca^{2+} dependent. Sattin and Rall[22] reported a block of the cyclic AMP elevating effect of adenosine in guinea pig cerebral cortex slices by the methylxanthines, a property independent of PDE activity but intimately associated with the system proposed above.

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