amino-4-nitrotoluene (Fig. 1). In the case of 4-acetylamino-2-aminotoluene, the unknown metabolite was produced to the extent of approximately 50 per cent of the 2,4-diacetylaminotoluene at each incubation period.

The results indicate that 2,4-toluenediamine is mostly *N*-acetylated by hamster liver cytosol to 4-acetylamino-2-aminotoluene and to a much lesser extent to 2-acetylamino-4-aminotoluene. Indirect evidence is furnished by production of 2-acetylamino-4-nitrotoluene from 2-amino-4-nitrotoluene. Both 4-acetylamino-2-aminotoluene and 2-acetylamino-4-aminotoluene then became substrates for *N*-acetyltransferases and were further *N*-acetylated to 2,4-diacetylaminotoluene. Since only a small quantity of 2-acetylamino-4-aminotoluene was formed from 2,4-toluenediamine and in turn was readily *N*-acetylated to 2,4-diacetylaminotoluene, therefore only a trace of 2-acetylamino-4-aminotoluene could be detected *in vitro*. However, it is a urinary metabolite of 2,4-toluenediamine in rats, although to a much lesser extent than 4-acetylamino-2-aminotoluene and 2,4-diacetylaminotoluene.

The major unknown product from using 4-acetylamino-2-aminotoluene as a substrate is not 2,4-diacetylaminotoluene or 4-diacetylamino-2-aminotoluene because it differs in the R_f values and gas chromatographic retention times from the authentic substances. It may be ^{14}C-labeled 4-acetylamino-2-aminotoluene which may have occurred through *N*-deacetylation of the substrate, 4-acetylamino-2-aminotoluene, with subsequent *N*-acetylation with the acetyl-^{14}C moiety from acetyl-[1-^{14}C]CoA. It has been shown that acetylaminoaryl compounds are readily deacetylated by liver and other tissue preparations from several mammalian species [8, 9]. However, since the *N*-deacetyltransferases are located in the microsomal fraction [8], this should not be the case for 4-acetylamino-2-aminotoluene which was incubated with liver cytosol. Another possibility is that a diacetylamino-aminotoluene may be a product, since the mass spectrum had a parent peak of mass 206 which points toward such a substance. Previously, it had been reported that 2-diacetylaminofluorene was formed in small amounts from 2-aminofluorene by rat liver slices [10], supporting our finding. However, the identity of this product requires further investigation.

We conclude that 2,4-toluenediamine is metabolized by hamster liver cytosol to three acetylaminotoluene metabolites, mostly 4-acetylamino-2-aminotoluene, of which a small amount is further metabolized to 2,4-diacetylaminotoluene, and 2-acetylamino-4-aminotoluene which is very readily further metabolized to 2,4-diacetylaminotoluene.

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Inhibition of a high molecular weight cyclic 3′,5′-nucleotide phosphodiesterase isolated from rat liver

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Butcher and Sutherland [1] first described the relative inhibitory potency of three methylxanthines on a partially purified cyclic 3′,5′-nucleotide phosphodiesterase of beef heart. More recently, a number of substituted xanthines were found to inhibit cyclic AMP phosphodiesterase of epididymal fat pad of rat in a manner corresponding closely to their lipolytic activity [12]. The most active substituted xanthine, 1-methyl-3-isobutylxanthine, was 15-fold more inhibitory than theophylline. A similar order of relative inhibitory potency has also been observed for the highly purified enzyme from beef heart [3].

Several of the cyclic 3′,5′-purine nucleotides and their analogs have also been found to inhibit phosphodiesterase from a number of sources [3, 4]. In most instances, the inhibitory activity of these compounds correlates with their ability to serve as alternate substrates for cyclic 3′,5′-nucleotide phosphodiesterase. In the present study, a soluble cyclic 3′,5′-nucleotide phosphodiesterase with a high degree of specificity for cyclic 3′,5′-purine nucleotide monophosphates was isolated from the soluble fraction of rat liver. Since the molecular weight of the liver enzyme was estimated by gel filtration chromatography to be 380,000 daltons, approximately 3-fold greater than the highly purified phosphodiesterase of beef heart [3, 5], it was of interest to determine the response of this isolated enzyme to substituted xanthines and several cyclic 3′,5′-nucleotide monophosphates and their analogs.

Cyclic 3′,5′-nucleotide phosphodiesterase activity was determined using three assay procedures. Assay procedure I consisted of measuring the release of orthophosphate

after a secondary incubation period employing *Crotalus atrox* venom [1]. This assay was conducted at a high substrate concentration (1 mM) under previously described conditions [6]. Assay procedure II was conducted according to the method of Thompson and Appleman [7] involving batch use of Bio-Rad AG-1-×2 resin (200–400 mesh). Radioactivity in a 0·5-ml aliquot of the assay supernatant was counted in 10 ml Insta-Gel (Packard Instrument Co., Downers Grove, Ill.) using a Beckman LS-255 liquid scintillation spectrometer. Counting efficiency was 42 per cent. The radioisotopes, [8-^{3}H]cyclic AMP (sp. act., 28 Ci/m-mole) and [^{3}H]cyclic GMP (sp. act., 0·65 Ci/m-mole), were obtained from Schwarz/Mann (Orangeburg, N.Y.). The [8-^{3}H]cyclic AMP was purified by repetitive chromatography on AG-50W-×4 resin and the [^{3}H]cyclic CMP was used directly without purification. Assay procedure III was a spectrophotometric determination carried out according to Hrapchak and Rasmussen [5] at 100 μM cyclic AMP concentration. This assay provided the necessary sensitivity and stability to directly monitor the complete NaCl-gradient elution of cyclic AMP phosphodiesterase activity during DEAE-cellulose chromatography.

The kinetic parameters were obtained from the intercepts of double reciprocal plots according to Lineweaver and Burk [8] by linear regression. For the determination of kinetic constants, the concentration of cyclic nucleotides was varied between 0·6 and 100 μM. In all instances, total hydrolysis of substrate was restricted to less than 20 per cent. Enzyme activity is expressed as nmoles cyclic nucleotide hydrolyzed/min (munits)/mg of protein. Protein was determined by the method of Lowry *et al.* [9]. Enzyme activity was determined in triplicate and all compounds were examined for potential interference with the 5′-nucleotidase-catalyzed conversion of 5′-AMP to adenosine. Alkylated xanthines were obtained from G. D. Searle & Co. (Chicago, Ill.). The 5′-methylene phosphonate analog of cyclic AMP was provided by Dr. J. G. Moffatt of the Syntex Institute of Molecular Biology (Palo Alto, Calif.).

The enzyme was extracted from the liver of Buffalo strain rats by homogenization in 0·25 M sucrose containing 1 mM $MgCl_2$ (20% w/v) using a glass Teflon pestle homogenizer. The homogenate was centrifuged at 12,000 *g* for 15 min. The supernatant was decanted and centrifuged at 105,000 *g* for 30 min. All operations throughout the isolation were performed at 4°. Prior to isolation, the soluble fraction of rat liver was subjected to DEAE-cellulose chromatography to determine the number of cyclic AMP hydrolytic activities resolved by this technique. The supernatant of rat liver was rapidly equilibrated with buffer A (25 mM Tris HCl, pH 7·4, 0·5 mM dithiothreitol and 1 mM $MgCl_2$) containing 30 mM NaCl on a Sephadex G-25 column (2·6 × 60 cm) and applied to a microgranular DEAE-cellulose column. Under these conditions, only one peak of cyclic AMP phosphodiesterase activity was identified (Fig. 1). This is in agreement with observations by other investigators [10, 11] who have indicated that the predominant form of phosphodiesterase activity occurring in the cytosol of rat liver is a cyclic 3′,5′-nucleotide phosphodiesterase catalyzing both the hydrolysis of cyclic AMP and cyclic GMP. No attempt was made to confirm the presence of a cyclic GMP-specific phosphodiesterase activity also found in the cytosol of rat liver [11].

The enzyme was routinely isolated from the 78,000 *g* supernatant by 50% saturation with solid $(NH_4)_2SO_4$ at neutral pH. The protein pellet was desalted and equilibrated with buffer A on Sephadex G-25. Calcium phosphate gel [12] was added to the protein solution (30–45 mg/ml) in a ratio of 2 g gel/g of protein. The mixture was centrifuged after 10 min, and the pellet sequentially eluted with 1 vol. of 0·348 M and 0·803 M $(NH_4)_2SO_4$ in buffer A/35 mg of gel. The 0·803 M $(NH_4)_2SO_4$ fraction was re-equilibrated with buffer A containing 30 mM NaCl on a Sephadex G-25 and applied to a DEAE-cellulose column. The column was eluted as previously described and the cyclic AMP hydrolytic activity emerged as a single peak at the same point in the gradient as shown for the supernatant fraction in Fig. 1. The pooled fractions were concentrated by ultrafiltration and applied to a Sephadex G-200 column (2·6 × 84 cm). The column was eluted with buffer A. Using the more sensitive radio-metric assay (procedure II), cyclic AMP phosphodiesterase activity was eluted as a single peak which coincided with cyclic GMP phosphodiesterase activity. Similar results were obtained when chromatography was carried out on a Sepharose 6B column (2·6 × 90 cm). In practice, the enzyme as measured by orthophosphate release was purified 35-fold (138 munits/mg of protein) through the DEAE-cellulose step and 50-fold (210 munits/mg of protein) through the G-200 step. Analytical poly-acrylamide gel electrophoresis [13] of the concentrated enzyme from Sephadex G-200 indicated that the enzyme preparation was still heterogenous with four protein-staining bands.

During the earlier stages of isolation, the enzyme was quite labile and could only be stored in a lyophilized state. In contrast to phosphodiesterases of other tissues [3, 14], the enzyme was not activated by any other fractions from DEAE-cellulose or Sephadex columns, suggesting either the absence of an endogenous protein activator or a tightly bound protein activator.

At 1 mM substrate concentration the enzyme catalyzed the hydrolysis of cyclic GMP and cyclic IMP at essentially the same rate as cyclic AMP. Cyclic 3′,5′-tubercidin monophosphate was hydrolyzed at 87 per cent of the rate of cyclic AMP. The enzyme did not catalyze the hydrolysis of cyclic 3′,5′-pyrimidine ribonucleotides. The apparent Michaelis constants ± S. E. of 42 ± 4·8 and 51 ± 4·6 μM were determined for cyclic AMP and cyclic GMP respectively. The enzyme exhibited normal hyperbolic kinetic behavior for both substrates from 0·6 to 100 μM.

As shown in Table 1, 1-methyl-3-isobutyl- and 1-ethyl-3-propyl-xanthine were the most effective inhibitors of the liver enzyme. Although not identical, a similar order of relative inhibitory potency for substituted xanthines has been observed for the enzyme from beef heart [3] and rat epididymal adipose tissue [2]. Theophylline was only moderately active compared to the other derivatives and approximately the same order of relative inhibitory activity

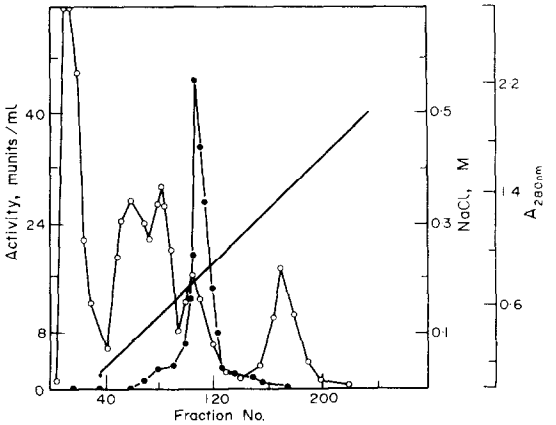

Fig. 1. DEAE-cellulose chromatography of the 105,000 *g* supernatant of rat liver. Protein (701 mg) was applied to a 2 × 10 cm column and washed with 100 ml of buffer A containing 30 mM NaCl. The column was eluted with a linear gradient formed with 400 ml of both 30 mM and 500 mM NaCl in buffer A. Fractions of 4 ml were collected at a flow rate of 30 ml/hr and their absorbance (–○–○–) at 280 nm was measured. Cyclic AMP phosphodiesterase activity (–●–●–●–) was measured by assay procedure III.

Table 1. Inhibition of rat liver cyclic 3',5'-nucleotide phosphodiesterase by substituted xanthines*

Xanthines	% Inhibition Inhibitor concn 2 mM	3 mM	30 μM	80 μM
	1 mM Cyclic AMP		1 μM Cyclic AMP	
1-Ethyl-3-propyl-	71	78	37	54
1-Methyl-3-isobutyl-	69	77	44	61
1-Ethyl-3-butyl-	69	76	32	56
1-Methyl-3-butyl-	57	67	25	42
1,3-Diethyl-	52	60	13	32
1,3-Dimethyl-7-acetic acid-			11	37
1,3-Dimethyl- (theophylline)	33	42	5	16
3,7-Dimethyl- (theobromine)	20	24	0	11
1,3,7-Trimethyl- (caffeine)	11	22	2	8
Xanthine	0	0	0	0

* Cyclic AMP hydrolysis was measured by assay procedures I and II at 1 mM and 1 μM substrate concentrations respectively.

was followed at both high and low concentrations of cyclic AMP. Thus, increased size of the substituted alkyl groups at positions N_1 and N_3 leads to increased inhibitory activity toward the rat liver phosphodiesterase as well.

Since the cyclic AMP phosphodiesterase activity has been reported to be enhanced by cyclic GMP using less purified fractions from rat liver [11, 15, 16], a plot of fractional inhibition (i) against inhibitor concentration [I] over a range of 1–150 μM was carried out (Fig. 2). Activity was not enhanced in the presence of cyclic GMP, cyclic IMP, cyclic 3',5'-tubercidin monophosphate (cTuMP) or the 5'-methylene phosphonate analog of cyclic AMP (5'-MetP-cAMP). The nucleotides inhibited the hydrolysis of cyclic AMP in a hyperbolic fashion. The failure to detect stimulation of cyclic AMP hydrolysis even in the presence of cyclic GMP ranging in concentrations as low as 0·3 to 1·5 μM is in contrast to previous reports [11, 15, 16] and remains unexplained. However, loss of both substrate cooperativity and cyclic GMP activation has been observed for the liver enzyme [16]. The analogs, N^6, $O^{2'}$-dibutyryl cyclic AMP and 5-amino-imidazole-4-carboxamide-1-riboside-3',5'-cyclic monophosphate, were not inhibitory. The apparent competitive inhibitor constants derived from the slopes of the double reciprocal plots [17] were 214, 78, 21 and 11 μM for cTuMP, cIMP, cGMP and 5'-MetP-cAMP respectively. In addition to inhibition by cyclic nucleotides and substituted xanthines, the liver enzyme was potently inhibited in a competitive manner by both papaverine and thyroxine with inhibition constants of 6 and 27 μM respectively.

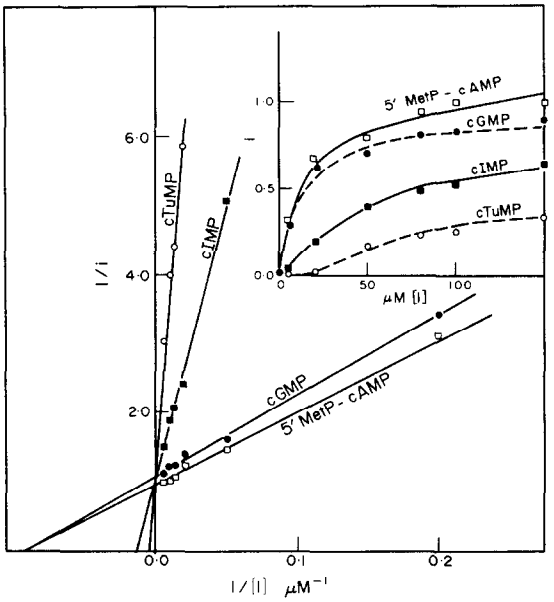

Fig. 2. Fractional inhibition of cyclic AMP phosphodiesterase as a function of inhibitor concentration. Fractional inhibition is defined as $1-(v_i/v_c)$, where v_i is the initial inhibited velocity and v_c is the initial control velocity in the absence of inhibitor. Enzyme activity was measured using [8-^{3}H]cyclic AMP at a concentration of 1 μM. Unlabeled inhibitors were included in the reaction mixture at concentrations ranging from 1 to 150 μM with 7·7 μg protein.

The present study shows that a high molecular weight phosphodiesterase of rat liver observes a similar order of inhibitory potency by substituted xanthines as previously reported for enzymes from rat adipose tissue [2] and beef heart [3]. The 5'-methylene phosphonate analog of cyclic AMP, and cyclic GMP were substantially more effective as inhibitors of cyclic AMP hydrolysis than either cyclic IMP or the 7-deaza analog of cyclic AMP.

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