Cytidylate cyclase: development of assay and determination of kinetic properties of a cytidine 3',5'-cyclic monophosphate-synthesizing enzyme

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A method is described for the separation of cytidine 3',5'-cyclic monophosphate (cyclic CMP) from cytidine tri-, di- and mono-phosphates and from cytidine 3',5'-cyclic pyrophosphate, cytidine 2'-monophosphate-3',5'-cyclic monophosphate, cytidine 2'-*O*-aspartyl-3',5'-cyclic monophosphate and cytidine 2'-*O*-glutamyl-3',5'-cyclic monophosphate, compounds previously shown to be the result of putative cytidylate cyclase activity. This separation, involving elution of a novel bilayer column of QAE-Sephadex and alumina with 0.03 M-HCl, has been incorporated into an assay protocol to determine the enzyme-catalysed conversion of radiolabelled CTP to cyclic CMP. By this assay, cytidylate cyclase activity has been shown to be present in rat lung, spleen, ovary, testes, brain, stomach, liver, heart and kidney preparations; the activity was of a similar order in each tissue and had a sharp pH optimum of 7.0–7.5. The liver preparation had a $V_{\max}$ of 1.2 nmol of cyclic CMP formed/min per mg, and a K_m of 220 μ M-CTP, and although active in the absence of added cations, it was stimulated by Fe^{2+} and Mn^{2+} ions. In several of the tissues examined, the cytidylate cyclase activity was inversely proportional to age of the animals.

INTRODUCTION

Cyclic AMP and, to a lesser extent, cyclic GMP are established as crucial factors in metabolic regulation, but the natural occurrence of a third cyclic nucleotide, cytidine 3',5'-cyclic monophosphate (cyclic CMP), has been a topic of debate. Initial evidence that cyclic CMP was involved in regulation of cell proliferation [1,2] was criticized on the basis of ambiguous identification of the putative cyclic CMP; further evidence involving estimation of cyclic CMP by radioimmunoassay [3–7] was subjected to the same criticism [8,9]. However, the occurrence of cyclic CMP in mammalian tissues has now been demonstrated unambiguously by means of large-scale tissue extraction followed by a sequential chromatographic procedure, yielding a product confirmed as 3',5'-cyclic CMP by i.r. and n.m.r. spectroscopy and fast-atom-bombardment (f.a.b.) m.s. with mass-analysed ion kinetic energy spectrum (m.i.k.e.s.) scanning [10].

This demonstration of the natural occurrence of cyclic CMP has again posed the question of the biosynthetic origin of this third cyclic nucleotide. The presence of a cyclic CMP-generating system was initially reported in mouse myeloid leukaemic tumours and normal mouse liver and spleen [11–15]. The enzyme was characterized in mouse liver by monitoring the formation of cyclic [^{32}P]CMP from [α - ^{32}P]CTP in tissue extracts [11–15], with unconverted [α - ^{32}P]CTP being separated chromatographically from cyclic [^{32}P]CMP by passage through a neutral alumina column. The identity of the principal product of this enzyme-catalysed reaction was verified as being cyclic CMP by comparison with authentic cyclic CMP in a number of systems, including column chromatography on Dowex-1-formate and PE1 cellulose, t.l.c. on PE1 cellulose, crystallization to specific

activity, resistance to degradation by bovine heart phosphodiesterase and radioimmunoassay [11–15]. The properties of the putative cytidylate cyclase enzyme were found to differ from those of the adenylate and guanylate cyclases in a number of respects, including substrate specificity, optimal stimulation of enzyme activity with low rather than high concentrations of Mn^{2+} , stimulation by Fe^{2+} and lack of effect of ATP, GTP and adrenaline. On this evidence it was deduced that cytidylate cyclase was a distinct enzyme from the adenylate and guanylate cyclases.

However, a conflicting view was expressed by Gaion & Krishna [16,17], who repeated the assay procedure of Ignarro's group described above, obtaining a ^{32}P -labelled product in similar quantities to those previously reported. This labelled product co-chromatographed with authentic cyclic CMP on alumina, but when subjected to further chromatography it did not behave identically with authentic cyclic CMP. Rechromatography on Dowex 50- H^+ resulted in the ^{32}P -labelled product separating into three major peaks, two of which coincided with 5'-CDP and 5'-CMP on PE1 cellulose, and none of which co-migrated with cyclic CMP. It was concluded by Gaion & Krishna that the product formed was not cyclic CMP [16,17]; later, a third group also reported a plurality of cyclic CMP-immunoreactive products from the cytidylate cyclase incubation system [18]. More recently, we have shown by a combination of selective hydrolysis, chromatography, dual labelling with ^{32}P and ^{14}C and f.a.b./m.i.k.e.s. scanning that incubation of the putative cytidylate cyclase preparation with CTP yielded seven identified products [19]. In addition to unchanged CTP plus its hydrolytic products CDP and CMP, cyclic CMP was unequivocally identified as a product, together with four novel cyclic CMP analogues, cytidine 3',5'-cyclic pyrophosphate, cytidine 2'-monophosphate-3',5'-cyclic

monophosphate, cytidine 2'-*O*-glutamyl-3',5'-cyclic monophosphate and cytidine 2'-*O*-aspartyl-3',5'-cyclic monophosphate. The existence of the last four novel compounds constitutes a plausible explanation of the controversy concerning cytidylate cyclase activity. In order to study this enzyme further, having established the identity of one product as cyclic CMP, it is necessary to devise a method which resolves cyclic CMP from the other labelled components present at the completion of the reaction, and to incorporate this procedure into an unambiguous assay for cytidylate cyclase. Here we describe the development of such an assay and report some of the properties of cytidylate cyclase determined by its application.

EXPERIMENTAL AND RESULTS

Materials

Radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.). Nucleotides were obtained either from the Boehringer Corp. (London, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.). All other biochemicals were from either Sigma Chemical Co. or BDH Chemicals (Poole, Dorset, U.K.), and all other chemicals were from either BDH Chemicals or the Aldrich Chemical Co. (Gillingham, Kent, U.K.) unless specified otherwise. All items were of the highest purity commercially available.

Chromatographic separation of products of putative cytidylate cyclase

A variety of column systems were examined for their ability to separate cyclic CMP from CTP and the six other cytidine-containing compounds present at the end of the cytidylate cyclase incubation [19]. Neutral alumina, PEI cellulose, QAE Sephadex, Dowex-50, Dowex-1 and phenyl boronate (Affigel 601, Bio-Rad) were tested in plastic columns (11 cm $\times$ 0.8 cm; Amicon) with column lengths of 0.5–8 cm using different eluting solvents. Elution profiles were obtained by adding $(2-40) \times 10^5$ d.p.m. of each ^3H -, ^{14}C - or ^{32}P -labelled component, both singly and in mixtures, within a total application of at least 5 nmol of material. Fractions of 0.5 ml were collected and their radioactivity was determined as described previously [20]. No single column system on a small scale was capable of satisfactorily resolving cyclic CMP while still providing reasonable recovery. For example, with a neutral alumina column of 3 cm length eluted with distilled water, only 68 % of the applied cyclic CMP was recovered in the first 8 ml of eluent, which also contained 12 % of the applied CTP. Use of 50 mM-phosphate buffer, pH 7.4, or imidazole/HCl buffer, pH 7.3, only reduced contamination by CTP to 9 % and improved recovery of cyclic CMP by 5 %; elution with 50 mM-Hepes/NaCl, pH 7.4, however, provided a recovery of more than 90 % of the applied cyclic CMP in the first 8 ml, but still contained significant quantities of CTP and the other cytidine-containing components in this same volume. Separation of cyclic CMP on PEI cellulose (3 cm $\times$ 0.5 cm) eluted with 50 mM-LiCl, based on the method in [21], gave a recovery of more than 60 % of cyclic CMP in a 3 ml volume, but despite the advantage of a much smaller eluting volume, the retained fraction still contained up to 30 % of each of the other cytidine-containing compounds. Phenyl boronate proved inconsistent. Dowex-1 and Dowex-50 failed to totally

resolve cyclic CMP, and although QAE-Sephadex did so more successfully, this was only with column lengths giving unsatisfactory recovery and long running times. More successful separation was achieved by using sequential chromatography involving two columns, first on neutral alumina eluted with 8 ml of distilled water and then on boronate eluted with 4 ml of 50 mM-Hepes/NaCl, pH 8.4, which resolved cyclic CMP from the other components with a recovery of 70 %. The use of two sequential columns, however, constituted a tedious process with a tendency to a loss of reproducibility, and it was thus resolved to examine the feasibility of developing a single column containing tiers of different matrices eluted with a common solvent.

The most reproducible of the single-column systems examined had been neutral alumina eluted with distilled water and QAE-Sephadex eluted with dilute HCl. As dilute acid was found to alter the retention properties of alumina to only a small extent, the elution of cyclic CMP, CTP and the other cytidine components present at the completion of the cytidylate cyclase incubation was examined using a series of columns composed of 0.5–3.5 cm of QAE-Sephadex on top of 0.3–0.8 cm of alumina and eluted with 0.01–0.06 M-HCl as the single eluting agent. A bilayer column of 0.5 cm of alumina and 2.0 cm of QAE-Sephadex when eluted with 0.03 M-HCl successfully resolved cyclic CMP from the other components (Fig. 1a) with a recovery of 87 %, whereas the major component of the assay, CTP, was only eluted by increasing the concentration of the eluting solvent to 0.09 M-HCl (Fig. 1b). Collection and examination by the method as described for f.a.b./m.i.k.e.s. scanning [22,23] of the first 2.5 ml eluted from the bilayer column after individual application of 50 nmol of each of the cytidine nucleotides confirmed that only cyclic CMP was eluted in this fraction.

Before the application of this separation procedure to an assay of cytidylate cyclase activity, it was necessary to determine whether the presence of buffer components, protein fragments, protein precipitants or cations in an enzymic incubation had any significant effect upon the performance of the bilayer column. Tissue homogenates were prepared as follows. Lister hooded rats were killed as described previously [1] and tissues were rapidly removed and homogenized in 50 mM-Tris/HCl, pH 7.4, at 4 °C (1:9, w/v) using a Braun-Melsinjen homogenizer. The tissue preparations were centrifuged at 500 *g* for 5 min at 4 °C, and the resulting supernatants were dialysed for 4 h against 12 vol. of the same buffer with three changes, and then used immediately in the enzyme incubation system. The protein content was determined by the method of Bradford [24].

The resultant protein preparations were included in cytidylate cyclase incubations in a total volume of 500 μl of 50 mM-Tris/HCl buffer, pH 7.4, containing 50–100 μl of protein preparation, 5–500 nmol of CTP, $(2-10) \times 10^5$ d.p.m. of $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ or $[\text{G-}^{14}\text{C}]\text{CTP}$ and various quantities of divalent metal ions, and were incubated for 10–12 min at 37 °C. The reaction was stopped either by addition of 55 % (v/v) trichloroacetic acid or by heating at 90 °C for 1.5 min; the precipitated protein was removed by centrifugation and the supernatant was rapidly applied to the bilayer column system. Another series of incubations was set up as above but without radiolabelled CTP present. A no-enzyme control and a boiled enzyme control were each included, together with a second set of

Fig. 1. Recovery of radiolabelled nucleotides from bilayer column

(a) Elution of 50 nmol (2×10^5 d.p.m.) of radiolabelled cyclic CMP (●), CMP (■), CDP and cyclic CMP-*P* (◆), CTP and amino acyl cyclic CMP (▲) and cyclic CDP (△). Fractions of 0.5 ml were collected. (b) Elution of 50 nmol (2×10^5 d.p.m.) of cyclic [^3H]CMP (●) and 50 nmol (2.5×10^5 d.p.m.) of [^{14}C]CTP (■) by 4 ml of 0.03 M-HCl followed by 4 ml of 0.09 M-HCl. Fractions of 0.5 ml were collected. Each point is the mean of six replicates; S.E.M. values were within $\pm 5\%$. For further details, see the text.

controls in which $(1-1.5) \times 10^5$ d.p.m. of cyclic [^3H]CMP and 50 nmol of unlabelled cyclic CMP, replacing the CTP components, were included in no-enzyme and boiled enzyme controls.

Minor modifications of sample application were examined and the most effective system was found to be as follows. Immediately before use, the bilayer column was washed with 2 ml of 0.03 M-HCl and then 550 μl of supernatant was added, followed immediately by 2.5 ml of 0.03 M-HCl. The first 2.5 ml eluted, after discarding the 2 ml pre-sample washings, was collected and the radioactivity was counted. Using such a procedure, only 0.3-4% of the applied [^{14}C]CTP or [^{32}P]CTP was recovered in this fraction from the boiled enzyme and no-enzyme controls, indicating that the inclusion of the enzyme incubation components had not seriously affected the bilayer column's ability to retain CTP. In the controls containing cyclic [^3H]CMP, 78-86% of the added cyclic CMP was recovered in the first 2.5 ml, confirming that the incubation media components had not affected the separation system. In the incubates containing active proteins, the cyclase activities observed in replicate assays

showed a variation of $\pm 8\%$, which was considered an acceptable level of reproducibility. Finally, the initial 2.5 ml eluents obtained from incubations containing active protein but non-radioactive substrate were examined by f.a.b./m.i.k.e.s., by the methods described [22,23] after concentration to dryness and addition of 50 μl of glycerol/water (1:1, v/v), and were found to contain only cyclic CMP with no evidence of the characteristic ions of CTP, CDP, CMP, cyclic CDP, cyclic CMP-*P*, glutamyl or aspartyl cyclic CMP present.

As a final confirmation that the generation of cyclic CMP was enzymically and not artefactually catalysed in such a system, product yields against enzyme concentration and time were determined (Fig. 2). These plots obtained with liver preparations provide a near-linear response of activity against enzyme concentration (Fig. 2a) and product against time for up to 10-12 min (Fig. 2b). Essentially similar plots were obtained with the other preparations from heart, brain, testis and kidney. It could thus be concluded that the cyclic CMP was biosynthesized enzymically.

This protocol was thus employed as the standard

Fig. 2. Validation of cytidylate cyclase assay protocol

(a) Plot of activity against enzyme concn.; liver homogenates containing 1-10 mg of protein were incorporated into the standard assay containing 50 nmol of CTP and 50 nmol of $\text{Mn}(\text{CH}_2\text{COO})_2$, and the cytidylate cyclase activity was determined. (b) Plot of activity against time; 200 μl of liver homogenate was included in the standard incubation containing 50 nmol of CTP and 50 nmol of $\text{Mn}(\text{CH}_3\text{COO})_2$ for 1.5-30 min, and then product formed was determined. Each point is the mean of six replicates, and S.E.M. values were within $\pm 6\%$. For further details, see the text.

Fig. 3. Variation of liver cytidylate cyclase activity with pH

Cytidylate cyclase activity of 50 μ l of dialysed liver homogenate was measured between pH 5.5 and pH 9.0 by the standard assay containing 50 nmol of CTP and 50 nmol of $\text{Mn}(\text{CH}_3\text{COO})_2$. Buffers utilized were: pH 5.5–6.5, 50 mM-acetate/sodium acetate, and pH 6.0–9.0, 50 mM-Tris/HCl. Each point is the mean of four replicates with S.E.M. within $\pm 8\%$. For further details, see the text.

cytidylate cyclase assay and used in studying the tissue distribution, kinetics and properties of this enzyme. Unless specified otherwise, the reaction was carried out at pH 7.4 at 37 °C for 10 min and then stopped by heating at 90 °C for 1.5 min, and included a boiled enzyme control and a no-enzyme control for each data point (although in most experiments little variation was observed between controls). In a typical set of assays using this system, inclusion of $(1-2) \times 10^5$ d.p.m. of [^3H]-, [^{14}C]- or [^{32}P]CTP substrate yielded no-enzyme and boiled enzyme control values of $(6-80) \times 10^2$ d.p.m. and experimental control values of $(2-16) \times 10^3$ d.p.m., with routine reproducibility of replicates being within $\pm 8\%$. Consequently, all replicates diverging by more than $\pm 10\%$ were discarded. With such a system, it has been possible to estimate cytidylate cyclase activity to a minimum level of 1–2 pmol/min while still discarding experimental values less than double the controls.

With rat liver, heart and kidney preparations, the enzyme was found to be active at pH values between 5.5 and 9, but had a sharp optimum occurring between pH 7.0 and 7.5, as can be seen from the representative data shown for cytidylate cyclase activity of a liver preparation in Fig. 3, in which the pH optimum is 7.5.

Incubation of preparations from liver, brain, heart and kidney in the standard assay each showed linear

Table 1. Effect of addition of unlabelled product on cytidylate cyclase activity

The cytidylate cyclase activity of 100 μ l of dialysed kidney homogenate was determined in the presence of 0–100 μ mol of unlabelled cyclic CMP by the standard assay containing 50 nmol of CTP and 50 nmol of $\text{Mn}(\text{CH}_3\text{COO})_2$. Results are the means $\pm$ S.E.M. for six replicates. For further details, see the text.

Unlabelled cyclic CMP added (nmol)	Cytidylate cyclase activity (% of control)
0	100.0 $\pm$ 4.7
1	97.2 $\pm$ 6.2
10	98.6 $\pm$ 7.2
1×10^2	93.5 $\pm$ 3.8
1×10^3	105.0 $\pm$ 8.2
1×10^4	83.2 $\pm$ 6.3
1×10^5	74.8 $\pm$ 5.4

kinetics for up to 12 min, but after this the amount of product did not increase. This effect could have been due to consumption of the CTP substrate for other purposes, product inhibition of the enzyme, slow hydrolysis of the cyclic CMP product by phosphodiesterase (perhaps only activated at a certain minimal level of substrate), denaturation of the cyclase, or any combination of these. The possibility that the non-linear kinetics after 12 min were due to either product inhibition or phosphodiesterase activity was tested by adding between 1 nmol and 100 μ mol of cyclic CMP to the standard assay. As shown in Table 1, the addition of unlabelled cyclic CMP did not significantly increase or decrease the cytidylate cyclase activity as would be anticipated if the preparation was subject either to phosphodiesterase hydrolysis of product or to product inhibition. It was therefore concluded that neither of these two factors was responsible for the decay of activity. At the highest concentrations of cyclic CMP, a decrease in activity was observed which could indicate sensitivity to product inhibition, but this appeared at a cyclic CMP concentration vastly in excess of that produced by the cytidylate cyclase preparation.

The potential loss of substrate during the incubation other than by conversion to cyclic CMP was tested by the inclusion of a CTP-regenerating system shown in preliminary studies to be capable of converting CMP and CDP to CTP (A. Carey & R. P. Newton, unpublished work). As shown in Table 2, the regenerating system did not significantly increase the apparent cytidylate cyclase

Table 2. Effect of CTP-regenerating enzyme on cytidylate cyclase activity

The cytidylate cyclase activities of 50 μ l portions of heart, brain, liver and testis dialysed homogenates were determined in the presence (+) and absence (–) of 80 units of phosphocreatine kinase and 10 mM-phosphocreatine by the standard assay containing 50 nmol of CTP and 50 nmol of $\text{Mn}(\text{CH}_3\text{COO})_2$. Results are the means $\pm$ S.E.M. for six replicates. For further details, see the text.

CTP regenerating system	Tissue . . .	Cytidylate cyclase activity (nmol/min)			
		Heart	Brain	Liver	Testis
–		0.18 $\pm$ 0.009	0.22 $\pm$ 0.015	0.36 $\pm$ 0.005	0.48 $\pm$ 0.009
+		0.20 $\pm$ 0.018	0.21 $\pm$ 0.013	0.33 $\pm$ 0.011	0.48 $\pm$ 0.012

Table 3. Effect of cations on cytidylate cyclase activity

The cytidylate cyclase activities in 50 μ l portions of liver, kidney and heart dialysed homogenates were determined in the presence of different cations and 50 nmol of CTP in the standard assay protocol. Results are the means $\pm$ S.E.M. for six replicates. For further details, see the text.

Addition (2.5 mM)	Tissue...	Cytidylate cyclase activity (nmol/min)		
		Liver	Kidney	Heart
None		1.8 $\pm$ 0.11	1.1 $\pm$ 0.08	2.2 $\pm$ 0.15
FeCl ₂		3.1 $\pm$ 0.23	2.3 $\pm$ 0.10	2.5 $\pm$ 0.15
FeCl ₃		3.0 $\pm$ 0.20	2.7 $\pm$ 0.13	2.7 $\pm$ 0.13
KCl		1.6 $\pm$ 0.06	2.0 $\pm$ 0.21	3.1 $\pm$ 0.12
CaCl ₂		2.0 $\pm$ 0.13	3.3 $\pm$ 0.17	4.2 $\pm$ 0.18
Mg(CH ₃ COO) ₂		2.5 $\pm$ 0.22	2.0 $\pm$ 0.01	3.8 $\pm$ 0.21
Mn(CH ₃ COO) ₂		3.2 $\pm$ 0.12	3.8 $\pm$ 0.13	2.2 $\pm$ 0.09

Fig. 4. Effect of substrate concentration on cytidylate cyclase activity

Cytidylate cyclase activity of 50 μ l of liver dialysed homogenate was determined by the standard assay protocol containing a series of substrate concentrations between 0.025 mM- and 5.0 mM-CTP, and 2.5 mM-Mn(CH₃COO)₂, to provide data for the Lineweaver-Burk plot. Each point is the mean of six replicates with the S.E.M. value within $\pm$ 7%. $V_{\max}$ = 1.2 nmol of cyclic CMP formed/min per mg of protein; K_m = 0.22 mM. For further details, see the text.

activity, thereby indicating that loss of the CTP substrate due to phosphatase activity was not the cause of the rapid decay of the cyclase activity. Preincubation of the cytidylate cyclase preparations from liver, kidney and heart at 37 $^{\circ}$ C for 20 min, in both the presence and absence of 50 nmol of Mg(CH₃COO)₂, led to loss of 80–90 % of enzyme activity, suggesting that the decay of cyclase activity observed during the time course was due to either heat denaturation and/or proteolytic modification.

Previous reports [11–15] suggested that cytidylate cyclase preparations, at least in crude and partially purified states, were active in the absence of additions of cations, but were stimulated by their presence. The data obtained here (Table 3) show that whereas the enzyme from liver, heart and kidney was active in the absence of additional cations, the presence of cations was generally stimulatory. With the liver preparation, Fe³⁺ and Mn²⁺ ions had the greatest stimulatory effect, whereas K⁺ had no effect. In the kidney, Mn²⁺ and Ca²⁺ had the greatest positive effect, and Ca²⁺ provided the greatest activation in the heart preparation. The absence of any observed retardation of the decay of cyclase activity by the addition of cations suggests that their effects are not merely a result of enzyme stabilization against either heat denaturation or proteolysis.

The effect of substrate concentration upon the activity of cytidylate cyclase was determined with liver, heart, kidney and lung preparations. The v against $[S]$ response observed with the liver preparation between 0.1 and 5 mM-CTP was hyperbolic and was closely similar to those observed with the three other tissues. In a Lineweaver-Burk plot, the data obtained (Fig. 4) gave a straightforward linear relationship with no evidence of allosteric kinetics, with a K_m of 0.22 mM and a $V_{\max}$ of 1.2 nmol/min per mg.

The tissue variation in cytidylate cyclase activity was determined in three age groups of rats and showed activity of similar levels in each of the tissues examined (Table 4), with the highest activities being present in the lung, ovary, testes and liver preparations of various ages. Rather than significant differences between tissue activities, a more interesting observation is the variation in each tissue with age. In the heart, liver, testes and brain preparations, cytidylate cyclase activity was inversely proportional to age, with the activity in the young testes being over double that of the adult tissue which, in turn, was 80 % greater than that of the testes of the aged rat. In the stomach, the activity was independent of age; in the ovary and lung the highest activity was in the older

Table 4. Cytidylate cyclase activity in tissues from rats of different ages

Cytidylate cyclase activities of 100 μ l portions of dialysed tissue homogenates from animals aged 2–6 weeks (young), 16–24 weeks (adult) and 38 weeks (aged) were determined at a substrate concentration of 2.5 mM-CTP in the presence of 2.5 mM-Mn(CH₃COO)₂. Three animals were taken from each group for each tissue determination and three replicates were taken for each individual tissue assayed. Results are means $\pm$ S.E.M. For further details, see the text.

Age of rat (weeks)	Tissue...	Cytidylate cyclase activity (nmol/min per mg)								
		Lung	Kidney	Heart	Brain	Stomach	Liver	Spleen	Ovary	Testis
2–6		0.75 $\pm$ 0.23	0.49 $\pm$ 0.08	0.86 $\pm$ 0.06	0.94 $\pm$ 0.11	0.62 $\pm$ 0.08	1.33 $\pm$ 0.14	0.46 $\pm$ 0.08	0.93 $\pm$ 0.15	1.38 $\pm$ 0.13
16–24		0.78 $\pm$ 0.09	1.02 $\pm$ 0.09	0.61 $\pm$ 0.04	0.63 $\pm$ 0.13	0.59 $\pm$ 0.09	0.75 $\pm$ 0.09	0.51 $\pm$ 0.12	0.90 $\pm$ 0.09	0.68 $\pm$ 0.08
38+		1.34 $\pm$ 0.11	0.79 $\pm$ 0.07	0.46 $\pm$ 0.05	0.47 $\pm$ 0.08	0.63 $\pm$ 0.21	0.47 $\pm$ 0.08	0.21 $\pm$ 0.09	1.33 $\pm$ 0.19	0.46 $\pm$ 0.03

tissue; whereas in the kidney and spleen, the greatest activity was found in the adult tissue.

DISCUSSION

The identification of cytidine 3',5'-cyclic pyrophosphate, cytidine 2'-monophosphate-3',5'-cyclic monophosphate, cytidine 2'-*O*-aspartyl-3',5'-cyclic monophosphate and cytidine 2'-*O*-glutamyl-3',5'-cyclic monophosphate as side-products of or intermediates in the reaction mechanism of cytidylate cyclase activity [19] has provided an explanation for the previous controversy regarding the natural occurrence of cyclic CMP and cytidylate cyclase. The novel chromatographic separation involving a bilayer column developed here has been demonstrated to be capable of resolving cyclic CMP from the above four compounds and from CTP, CDP and CMP; this technique in combination with the incubation protocol described in the Experimental and results section constitutes the first unambiguous means of estimating cytidylate cyclase activity. The assay protocol developed provides a rapid, reproducible, sensitive and specific means of estimation, with the only disadvantage to its use being the requirement to remove the QAE-Sephadex and alumina layers from the columns and to separate them before regeneration. (An analogous assay is of potential value in estimations of adenylate and guanylate cyclase activities.)

Cytidylate cyclase activity has been found in each of the tissues examined and the activity in each has been of a similar order, suggesting that any biochemical function of cyclic CMP is general rather than tissue-specific. No evidence is provided concerning the specificity of this cyclic CMP-biosynthesizing capacity, and as the observed activity is not greater than the adenylate or guanylate cyclase activities in the same tissues [25], then non-specific activity of the latter two enzymes is not on this evidence precluded as the source of the observed cytidylate cyclase activity. The data shown here, with rat liver cytidylate cyclase having a K_m of 220 μM -CTP and a V_{max} of 1.2 nmol of cyclic CMP formed/min per mg, are in broad agreement with those of Ignarro's group [11–15], with reports of cytidylate cyclase with a K_m of 190 μM and a V_{max} of 0.98 nmol/min/mg.

The non-obligatory requirement for cations, but stimulation by Fe^{2+} , Mg^{2+} and Mn^{2+} , was also in broad agreement with these authors' data. This K_m value, in view of the apparent liver concentration of CTP of approx. 5.3 $\mu\text{mol/g}$ [26], suggests that, in the absence of any compartmentation effects or stimulation, cytidylate cyclase operates well below the potential maximum activity in the liver. The variation observed in several tissues suggested that cytidylate cyclase activity decreases with age; it is interesting to note that the reverse is true of cyclic CMP phosphodiesterase activity, with an observed increase with age [27], and these observations are consistent with previously proposed theories of cyclic CMP possessing a role in the regulation of cell growth and proliferation [28]. Further systematic studies of the specificity, effector sensitivity and subcellular distribution

of cytidylate cyclase will assist in the elucidation of any role of cyclic CMP in this process or in the recently proposed models for luteal cell structure development [29] and hatching and attachment of the mouse blastocyst [30].

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