'POLY C' SYNTHESIS AND CDP-DIGLYCERIDE FORMATION BY ISOLATED BRAIN NUCLEI

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Received 30 September 1974
Revised version received 25 October 1974

1. Introduction

Recent reports have claimed that cell nuclei isolated from the mammalian cerebral cortex contain an active poly(C) polymerase enzyme [1–3]. The original evidence for this claim was obtained using a mixed population of nuclei from rat brain, these nuclei were shown to incorporate CTP into an acid-insoluble product from which acid soluble radioactivity could be progressively released by digestion with crude snake venom, while nearest neighbour frequency analysis indicated that 84% of incorporated CMP residues were adjacent to other CMP residues [1]. Two further reports [2,3], have shown that populations of neuronal nuclei from either the guinea-pig cerebral cortex [2], or from whole rat brain [3], are considerably more active in incorporating CTP into an acid-insoluble product than are populations of glial cell nuclei, and consequently poly(C) synthesis has been suggested to be a property of the neuronal nucleus. No biological role is known for poly(C), but in view of recent evidence on the role of poly(A) in eukaryotic systems [4] the localization of poly(C) synthesis within the nucleus of a highly specialized cell such as a neurone could have important implications in understanding neuronal function. Following the development of a rapid method for the separation of two populations of nuclei from the rabbit cerebral cortex [5], I have observed an active incorporation of CTP by neuronal nuclei which has several similar characteristics to the incorporation originally described in rat brain nuclei [1]. However, evidence is represented that this incorporation does not represent poly(C) synthesis but rather the synthesis of the unique liponucleotide CDP-diglyceride.

2. Materials and methods

5[H^3]cytidine-5′-triphosphate was from the Radiochemical Centre, Amersham; pancreatic RNAase, DNAase and *Crotalus atrox* venom were from Sigma, London actinomycin D was from Merck, Sharp and Dohme, U.S.A.; α-amanitin was originally a generous gift of Professor T. Weiland, Heidelberg.

2.1. *Isolation of nuclei and CTP-incorporation assay*

The N′ nuclear population was isolated as previously described [5]. The assay medium contained (final concentrations); 50 mM Tris–HCl pH 7.0, 1 mM cytidine-5[H^3] 5′-triphosphate (10 μCi/μmole), and 20 mM $MgCl_2$. The reaction was started by the addition of approximately 3×10^6 nuclei suspended in 0.1 ml of 0.32 M sucrose, the final vol of the reaction mixture was 0.2 ml. Incubation was at 37°C for 7 min, during which time the incorporation remained linear. Reactions were terminated by the addition of an equal volume of 10% TCA containing 20 mM sodium pyrophosphate. After standing on ice, precipitated reaction mixtures were filtered onto Whatman GF/C discs in a Millipore apparatus and washed 5 times with 3 ml quantities of 5% TCA containing 10 mM sodium pyrophosphate and twice with 3 ml quantities of 5% TCA. The discs were then dried and counted in toluene scintillant (5 g of 2,5-diphenyoxazole and 0.2 g of 1,4-di(2-5-phenyloxazolyl)benzene per litre of toluene)

in a Packard scintillation counter. Blank assay tubes incubated in parallel on ice were included, these always gave less than 10% of the experimental values.

2.2. *Alkaline hydrolysis of the acid-insoluble radioactive product*

Assays were performed as above, except that the volume of the incubation mixture was increased five-fold and the precipitates were washed by centrifugation rather than by filtration. Alkaline hydrolysis was in 0.3 N KOH at 37°C for 18 hr. After neutralization with perchloric acid and centrifugation, the clear supernatant was applied to Whatman 3 mm papers and chromatographed overnight in the solvent systems [6] shown and in the presence of suitable standards. After chromatography spots were cut out, placed in scintillation vials and counted after the addition of 2 ml glass-distilled water and 18 ml of Triton/toluene scintillant [5].

2.3. *Lipid extraction and silica gel chromatography*

The assay and washing of precipitates were performed as described for alkaline hydrolysis. Precipitates were extracted at 55°C for 3 min with chloroform–methanol (1:1) containing 0.1 N HCl and the lipid extracts streaked directly onto silica gel plates (E. Merck, Darmstadt). Synthetic CDP-dipalmitin (International Enzymes, Windsor) was dissolved in acidified chloroform–methanol and run with the experimental samples. Plates were run in chloroform–methanol–acetic acid–water (50:25:7:3). After drying, areas of the plate were scraped into scintillation vials and counted after the addition of 2 ml of 20 mM Tris–HCl pH 7.5 and 18 ml of Triton/toluene scintillant. Synthetic CDP-dipalmitin was detected by spraying with Rhodamine 6G.

2.4. *Release of acid-soluble radioactivity by snake venom*

The assay was performed in a final volume of 0.2 ml and filtered onto lass fibre discs as described above. After drying, the glass fibre discs were immersed in 3 ml of 100 mM Tris–HCl buffer, pH 8.5, containing 0.5 mM $MgCl_2$, and either with or without 2 mg/ml crude *Crotalus atrox* venom. After incubation at 37°C for the times shown the discs were removed and the acid-soluble radioactivity determined. Synthetic CDP-dipalmitin was dissolved in the same buffer and similarly incubated with and without snake venom the release of acid-soluble material absorbing at 260 mμ was followed. There was negligible release of either radioactivity or UV-absorbing material in an acid-soluble form from discs incubated in buffer alone.

3. Results and discussion

Table 1 summarises some characteristics of the incorporation of [H^3]CTP into an acid-insoluble product by a population of nuclei from the rabbit cerebral cortex. (Approximately 80% of this nuclear population – the N′ nuclear population [5] consists of nuclei with the morphological characteristics of neuronal nuclei). The incorporation showed a relatively sharp pH optimum at pH 7.0 and was maximal in the presence of 1 mM CTP and with 20 mM $MgCl_2$ or 5 mM $MnCl_2$, exactly analogous results being obtained in the presence of either ion (unpublished results). Table 1 shows that pyrophosphate, but not

Table 1
Characteristics of [H^3]CTP incorporation

	pmoles [H^3]CTP incorporated/nuclear sample
Control	282
+ 1 mM sodium pyrophosphate	55.2
+ 1 mM orthophosphate	286
+ Actinomycin D (20 μg/ml)	280
+ α-amanitin (10 μg/ml)	278
+ $(NH_4)_2SO_4$ (300 mM)	70
+ inositol (1 mM)	58
Control	280
Preincubate with DNAase (50 μg/ml)	278
Control	280
+ Phosphatidic acid (250 μg/ml)	995

Incubations were performed in a final volume of 0.2 ml as described in Materials and methods. Additions to the control incubation were made to the final concentrations shown above. In the case of preincubation with DNAase, both control and experimental samples were preincubated for 3 min at 37°C in 0.32 M sucrose. In the case of phosphatidic acid stimulation, the lipid was suspended in the assay medium without $MgCl_2$ by brief sonication and the $MgCl_2$ was added after the nuclei [11], control incubations being treated in the same way. The values shown are the means of duplicate determinations, all duplicates agreeing to within 5%.

Table 2
Chromatography of alkaline hydrolysate

Solvent system	Total c.p.m. applied	c.p.m. recovered			
		GMP	UMP	CMP	AMP
Ammonia–isobutyric	517	–	–	500	–
			CMP	Cytidine	
Ethanol–ammonium acetate	1650		1602	18	
		5′-CMP		2′,3′ CMP	
Ethanol–ammonium acetate–borate	1130	1060		50	

Alkaline hydrolysis and chromatography were performed as described in Materials and methods, using the solvent systems shown [6]. Values are the means of duplicate determinations agreeing to within 5%.

inorganic phosphate, was markedly inhibitory, suggesting that pyrophosphate was released during incorporation. Actinomycin D and DNAase, at concentrations which virtually abolished RNA synthesis by this nuclear population [5] did not inhibit CTP incorporation, suggesting that a DNA-dependent process was not involved. Ammonium sulphate, at a concentration which markedly stimulates RNA polymerase II type activity in these nuclei [5] produced an inhibition of CTP incorporation, while α-amanitin at over ten times the concentration needed to abolish RNA polymerase II type activity in these nuclei [5] was without effect. The presence of poly(C) at 10 μg/ml, whole cell RNA at 100 μg/ml, or pancreatic RNAase at 50 μg/ml did not affect CTP incorporation.

The results of alkaline hydrolysis of the radioactive product of CTP incorporation by N′ nuclei are shown in table 2. In the solvent systems shown essentially all of the radioactivity present in the alkaline hydrolysate co-chromatographed with 5′-CMP and not with 2′,3′ CMP or with cytidine. In view of the virtual absence of radioactivity in 2′,3′CMP or in cytidine following alkaline hydrolysis, the observed incorporation of CTP cannot represent the insertion of CMP into the interior of a polynucleotide chain or the terminal addition of CMP to a pre-existing polynucleotide chain. Further evidence against the observed incorporation being due to poly(C) synthesis is presented in table 3, in which it is shown that the great majority of the radioactive product is soluble in lipid solvents, providing that the lipid solvents are acidified. This behaviour suggests that the true nature of the product of CTP incorporation by N′ nuclei might be the liponucleotide CDP-diglyceride which is soluble in lipid solvents at acid pH and soluble in aqueous solvents at alkaline pH [7], and which would be expected to yield 5′-CMP on alkaline hydrolysis. Table 1 shows that the incorporation of CTP into an acid insoluble product by N′ nuclei was markedly stimulated by the addition of phosphatidic acid and inhibited by the addition of inositol, as would be expected if the acid-precipitable radioactivity were contained in CDP-diglyceride [9].

Chromatography on silica gel as described in Materials and methods showed that approximately

Table 3
Effect of washing discs with lipid solvents

Discs washed	cpm recorded
Control	900
3 × with 99% ethanol	400
3 × with 99% ethanol/0.1N HCl	140
3 × with chloroform/methanol (1:1)	410
3 × with chloroform/methanol (1:1) + 0.1N HCl	95

Assays were performed as for table 1. After washing with 5% TCA, the glass fibre discs were washed 3 × with 3 ml quantities of the solvents shown above, dried, and counted. Values are the means of duplicate determinations agreeing to within 5%.

85% of the radioactive product synthesized either in the presence or absence of phosphatidic acid migrated with an RF of 0.62, co-chromatographing with synthetic CDP-dipalmitin. The remainder of the product chromatographed with an RF of 0.33 and was not identified.

It is therefore suggested that the great majority of the incorporation of CTP performed by the present nuclear population can be accounted for by CDP-diglyceride synthesis. However, the incorporation described here closely resembles the incorporation described as poly(C) synthesis in rat brain nuclei [1] in having a similar pH optimum, and response to raised salt concentrations. It is proposed that the evidence presented for the occurence of poly(C) synthesis in brain nuclei [1] can be explained further in terms of CDP-diglyceride synthesis for, as shown in fig. 1, radioactivity from the acid-insoluble product synthesised by N′ nuclei both in the presence and absence of phosphatidic acid, and UV-absorbing material from synthetic CDP-dipalmitin itself was rendered progressively acid soluble by digestion with crude snake venom under

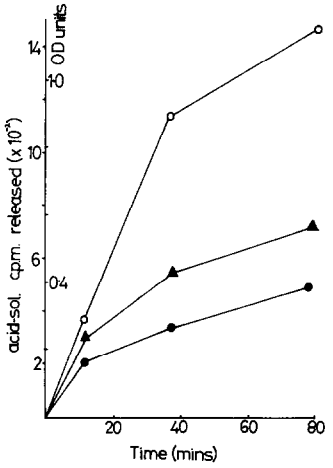

Fig. 1. Effects of incubation with snake venom. The release of acid-soluble radioactivity from the glass fibre discs on incubation with crude *Crotalus atrox* venom was followed as described in Materials and methods. (○–○–○), radioactivity released from the product synthesized in the presence of phosphatidic acid (250 μg/ml); (●–●–●), radioactivity released from the product synthesized in the absence of phosphatidic acid; (▲–▲–▲), UV-absorbing material released from synthetic CDP-dipalmitin incubated with snake venom. Values are the means of duplicate determinations agreeing to within 5%.

similar conditions to those used in the original description of 'poly(C)' synthesis [1]. It is not at present known whether phosphodiesterase activity contained in the crude snake venom was actually responsible for this effect. Finally, in nearest neighbour frequency analysis with CT[α-P^{32}]P, the product of alkaline hydrolysis would be radioactive 5′-CMP in the case of CDP-diglyceride synthesis and mainly radioactive 2′,3′ CMP in the case of poly(C) synthesis. Since high voltage paper electrophoresis at acid pH does not widely separate 5′CMP from 2′,3′ CMP (10) the appearance of radioactivity in 5′ CMP from CDP-diglyceride hydrolysis (radioactivity which has in fact never left the 5′ position of the original CT[α-P^{32}]P) could be confused with radioactivity which has been transferred to the 2′,3′ position of an adjacent cytidine residue. CDP-diglyceride synthesis can, therefore, superficially resemble poly(C) synthesis in that CDP-diglyceride is adic-precipitable [7], is only partially soluble in lipid solvents unless these solvents are acidified (table 2), is digested by crude snake venom (fig. 1), and yields a cytidine monophosphate upon alkaline hydrolysis [8]. Previous reports on the subcellular distribution of CDP-diglyceride synthetase in nervous tissue [12,13] have proposed a mitochondrial location. Purified brain nuclei, however, have not previously been examined for CDP-diglyceride synthesis.

Acknowledgements

Professor C.N. Hales is thanked for his support and encouragement. D. M. Mackay is thanked for expert technical assistance.

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