ON THE FORMATION OF THYMIDINE-3'-TRIPHOSPHATE BY A MAMMALIAN CELL-FREE SYSTEM

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Although at present deoxyribonucleoside-3'-triphosphates have yet to be isolated from natural sources, their role in nucleic acid metabolism is not excluded. During the course of experimental work dealing with the chemical synthesis of deoxynucleoside-3'-triphosphates and their possible utilization for the biosynthesis of polynucleotides*, the enzymic formation of thymidine-3'-triphosphate (3'-TTP) was investigated. Crude cell-free extracts prepared from regenerating rat liver converted chemically synthesized C^{14}-thymidine-3'-diphosphate (3'-TDP) to C^{14}-3'-TTP in the presence of ATP and an ATP regenerating system.

Materials: 2-C^{14}-3'-TDP, 2-C^{14}-3'-TTP, 3'-TMP, 3'-TDP, 3'-TTP and thymidine-3',5'-diphosphate (pTp) were synthesized by modifications* of known methods (e.g. Tenner 1961, Moffatt 1964).

Partial hepatectomies were performed on male albino Sprague Dawley rats weighing 160-185 gm. A 105,000 x g supernatant of a 36 hour regenerating rat liver homogenate (60% w/v) in 0.025 M potassium phosphate pH 7.4 or 0.1 M tris-HCl pH 7.4, was used as the "enzyme extract" (Table I). The conditions for the enzymatic reaction are described with the tabular data.

Methods of Assay: After deproteinization of the incubation mixture, the C^{14}-thymidine triphosphates (3'- and 5'-) were recovered practically undegraded and well separated from the unconverted C^{14}-3'-TDP and other reactants by chromatography on DEAE columns using the systems successfully applied in the separation of oligonucleotides

* C. Coutsogeorgopoulos: Unpublished experiments and work in progress.

(e.g. Coutsogeorgopoulos and Khorana 1964, Moffatt 1964). The radioactive triphosphate fraction (peak III, Fig. 1) was analyzed by the two methods described below.

Assay A: The material from peak III (Fig. 1) (3,000-30,000 cpm) in 0.1 N HCl was heated in a boiling water bath for 2 hrs. Control experiments with C^{14}-3'-TTP show that during the acid hydrolysis, 90% of 3'-TTP is hydrolyzed to 3'-TMP, 5% is left unhydrolyzed and 5% is converted to thymidine (TdR). After evaporation, the residue was mixed with pTp, 3'-TMP, 5'-TMP, and TdR markers (one to two O.D. units at 260 mμ of each) applied on No. 1 paper and chromatographed in isobutyric acid-conc.

TABLE I

Requirements for 3'-TTP Formation

Conditions	3'-TTP formed 40 min. 37°
Complete system	100 *
Omit ATP	< 1
Omit enzyme extract	< 1
Omit enzyme extract and ATP	< 1

The complete system (1.5 ml) contained: tris-HCl buffer pH 7.4 (50 μmoles), $MgCl_2$ (20 μmoles), ATP (10 μmoles), KCl (40 μmoles), sodium phosphoenolpyruvate (1.6 μmole), pyruvate kinase (60 μgm), 2-C^{14}-3'-TDP (200 mμmoles, 600,000 cpm), enzyme extract (450 μgm of protein). At the end of the incubation period at 37°, the reaction mixture was chilled, diluted tenfold with cold water and deproteinized by passing on a Dowex-2 (carbonate) column (8 mm x 70 mm). The column was washed with water (15 ml) and stripped of all the nucleotidic material with 70-80 ml of 2 M triethylammonium bicarbonate, pH 7.5. The latter eluate was evaporated in vacuo until salt-free, taken up in water (3 ml) and placed on a DEAE column. (see Fig. 1).

* 100 corresponds to 9% conversion of C^{14}-3'-TDP to C^{14}-TTP (3'- and 5'-).

ammonia-water (66 : 1 : 33) pH 3.7 (solvent A). The paper band which contained the 3'-TMP and 5'-TMP markers (they move almost together) was cut, eluted and the eluted material was incubated with snake venom (Crotalus adamanteus) 5'-nucleotidase (Table II). Only 5'-TMP is degraded to TdR. The undegraded C^{14}-3'-TMP represents the C^{14}-3'-TTP fraction of peak III whereas the C^{14}-TdR represents the C^{14}-5'-TTP fraction of peak III, produced by the crude extract. The radioactivity associated with the pTp marker, which moves like 5'-TTP and 3'-TTP in solvent A,

is taken to represent the unhydrolyzed fraction of peak III. The ATP contamination of peak III is converted to adenine which moves ahead on the paper.

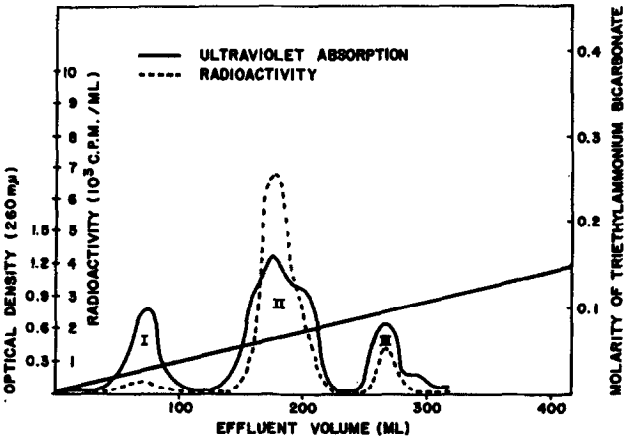

Fig. 1. Fractionation of the deproteinized incubation mixture on a DEAE (carbonate) column (10 mm x 200 mm). A linear gradient of triethylammonium bicarbonate pH 7.5, from 0.005 M (500 ml) to 0.35 M (500 ml) was used for elution and 5 ml fractions per 10 min. were collected. The distribution of radioactivity was followed and is shown in the diagram along with the positions for 3'-TMP (peak I), 3'-TDP (peak II) and 3'-TTP (peak III) determined in parallel runs with known markers. The 3'-TTP peak coincides with the beginning of the ATP peak, but this ATP contamination does not interfere with either of the Assays A or B. The radioactive peaks (recovered radioactivity 90% of the input) were pooled and evaporated in vacuo, until salt-free. A very similar distribution of the radioactivity was obtained on a Dowex-1 formate column using the pH 4.2 ammonium formate system (Canellakis and Mantsavinos 1958) but the problems associated with the recovery of the radioactive material in the peaks made the DEAE system a preferable one.

Assay B: The material from peak III (Fig. 1) (3,000 - 30,000 cpm) was mixed with nonradioactive 5'-TTP, 3'-TTP and 3'-TDP markers (one to two O.D. units at 260 mμ of each) and treated with snake venom phosphodiesterase (Table III). Under these conditions all of the ATP contaminant was converted to AMP, and all of the 5'-TTP was converted to 5'-TMP. The 3'-TDP and 3'-TTP markers were not degraded by the diesterase and, after paper chromatography in solvent A, were very well separated from each other and from 5'-TMP and AMP. The 5'-TMP, 3'-TDP and 3'-TTP paper bands were cut and counted in a Tricarb scintillation counter. Alternatively the 3'-TTP paper band was eluted and further analyzed. Ninety percent of the phosphodiesterase resistant radioactive material in the 3'-TTP band represents

the original C^{14}-3'-TTP fraction of peak III, whereas the radioactivity found in the 5'-TMP band represents the C^{14}-5'-TTP fraction of peak III. The results of assays A and B agree well within the experimental error.

TABLE II

5'-Nucleotidase Treatment (Assay A)

	Resistant thymidine monophosphate %	Thymidine %
TMP* (3'- and 5'-) from peak III	25	75
C^{14}-3'-TMP (synthetic)	98	2
C^{14}-5'-TMP (commercial)	1	99

The incubation mixture (0.2 ml) contained: Glycine buffer pH 8.6 (100 μmoles), $MgCl_2$ (10 μmoles), thymidine monophosphate, (2 - 3 O.D. units at 260 mμ, 3,000 - 4,000 cpm), 5'-nucleotidase (45 μgm) purified from Crotalus adamanteus crude venom. After 60 min. at 37°, 5'-TMP or 3'-TMP and TdR markers were added and the mixture was applied on No. 1 paper and chromatographed in n-propanol-conc. ammonia-water (55 : 10 : 35). The 3'-TMP or 5'-TMP and the TdR bands were cut, and counted in a Tricarb scintillation counter.

*Acid hydrolyzable portion (85%) of peak III.

Results and Discussion. The ATP requirement for the conversion of C^{14}-3'-TDP to C^{14}-3'-TTP are shown in Table I. Phosphoenolpyruvate and pyruvate kinase, with or without ATP, do not phosphorylate 3'-TDP (Table I); similarly, conversion less than 1%, of that obtained with the complete system, was found when the creatine phosphate-creatine kinase system was used. The overall conversion to C^{14}-TTP (3'- and 5'-) increased linearly for 90 min. at 37°, decreasing after longer incubation periods. After incubation for 90 min. approximately 20% of the C^{14}-3'-TDP was converted to a mixture (peak III, Fig. 1) of C^{14}-3'-TTP (about 22% of peak III) and C^{14}-5'-TTP (about 63% of peak III). About 10% of peak III is neither C^{14}-3'-TTP nor C^{14}-5'-TTP, since, it is not converted to C^{14}-TMP (3'- or 5'-) on acid hydrolysis. Roughly the same ratio of 3'-TTP to 5'-TTP in peak III was found, under both assays A and B, in all samples analyzed after different periods of incubation.

TABLE III

Snake Venom Phosphodiesterase Treatment (Assay B)

	Nucleoside monophosphate produced %	Resistant nucleoside triphosphate %
Radioactive material from peak III, mixed with nonradioactive 5'-TTP, 3'-TDP and 3'-TTP markers	67	33
ATP (18 O.D. at 260 mμ)	99	1
5'-TTP (10 O.D. at 260 mμ)	98	2
C^{14}-3'-TTP (synthetic) (3 O.D. units at 260 mμ, 4,000 cpm)	1	99

The incubation mixture (0.1 ml) contained: Tris-HCl buffer pH 8.0 (100 μmoles), nucleoside triphosphate (see above) and Worthington snake venom phosphodiesterase (50 - 100 μg). After 4 - 5 hrs. at 37°, the mixture was applied on No. 1 paper and chromatographed in solvent A for 24 - 30 hrs. The nucleoside mono- and tri- phosphate bands were cut and counted in a Tricarb scintillation counter; when nonradioactive, these bands were eluted and the nucleotidic material was estimated by ultraviolet absorption.

The behaviour of the radioactive material from peak III during Assay A suggest that the fraction behaving like 3'-TTP or 5'-TTP is not a thymidine product phosphorylated at both the 5'- and 3'- hydroxyl groups, as for example pTp, ppTp or pTpp. Any such contaminant of peak III would give pTp by the acid hydrolysis and not TMP (5'- or 3'-).

On the other hand, the behaviour of the same material during Assay B, excludes it from being a column artifact or a contamination from the C^{14}-3'-TDP peak (peak II of Fig. 1), since, after paper chromatography in solvent A, the material resistant to snake venom phosphodiesterase does not travel with the 3'-TDP marker but exclusively with the 3'-TTP marker. A control experiment showed that, by the same treatment (Assay B) the material from peak II (Fig. 1) gave 93% of the radioactivity in the 3'-TDP band, 5% in the 5'-TMP band, and only 2% in the 3'-TTP band. Thus, the phosphodiesterase would convert, during Assay B, only 2% of any contamination from peak II into radioactive material moving with the 3'-TTP band. Furthermore, 90% of the radioactivity associated with the 3'-TTP marker on the paper chromatogram, after phosphodiesterase treatment of peak III, is shown to consist of C^{14}-3'-

TTP by two additional criteria: a) Acid hydrolysis and subsequent 5'-nucleotidase treatment (conditions like those of Assay A) gives only 3'-TMP, and TdR no more than an authentic C^{14}-3'-TMP sample would give. b) Elution and retreatment with snake venom phosphodiesterase (conditions similar to those of Assay B) leaves it unchanged giving no more thymidine monophosphate than a control with authentic C^{14}-3'-TTP.

Thus, the radioactive material produced, by the regenerating rat liver extract, from synthetic C^{14}-3'-TDP, after the above treatments (Assays A and B) behaves identically with synthetic 3'-TTP and not with the original 3'-TDP, in two different ion exchange columns and one paper chromatographic system. Moreover, the same treatments exclude the possibility that the product could be 5'-TTP.

While these studies were in progress, Canellakis et al. (1965) reported on the formation of 3'-TTP from 3'-TDP by a cell-free system from B. subtilis.

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