Bot. Bull. Academia Sinica **16**: 25-35 (1975)

DNA SYNTHESIS OF WALKER-256 CARCINOSARCOMA NUCLEI *IN VITRO*:

I. Existence of TTP Phosphatase Activity and Its Inhibition by ATP[1,2]

YAW-HUEI LIN and R. A. SMITH

Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China and Department of Chemistry, University of California, Los Angeles, California 90024, U.S.A.

(Received for publication August 10, 1974)

Abstract

Using radioactive γ-^{32}P-TTP as substrate, a TTP phosphatase activity was found in Walker-256 carcinosarcoma nuclear preparations. ATP exerts its inhibitory effect by competing with TTP. The effects of GTP, CTP, *d*-GTP, and *d*-CTP on the enzymic activity were also studied.

Introduction

Isolated nuclei from Walker-256 carcinosarcoma can be fractionated into nucleoplasmic, acidic protein, and DNA-Histone complex (or Deoxyribonucleoprotein=DNP) fractions according to Wang (1967). Ballal *et al.* (1970) showed that high concentration of ATP (2 mM) inhibits TTP incorporation into DNA by acidic protein fraction when either native DNA or denatured DNA were used as template. However, ATP stimulates TTP incorporation into DNA by using either acidic protein fraction or partially purified DNA polymerase as enzyme source and DNP as template. Other nucleoside triphosphates can not replace ATP for this stimulating effect. They also showed that ^{3}H-TTP can be hydrolyzed to thymidine by crude nuclear extract. ATP as well as other nucleoside triphosphates inhibits this hydrolysis. This suggests that the

(1) This is part of Y.-H. Lin's Ph. D. dissertation done at Department of Chemistry, UCLA, California, U. S. A. during his on leave from Institute of Botany, Academia Sinica, Taipei, Taiwan, R. O. C.

(2) Paper No. 147 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

(3) The following abbreviations are used: ATP, adenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; TDP, thymidine 5′-diphosphate; TMP, thymidine 5′-monophosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; *d*-GTP, deoxy-guanosine 5′-triphosphate; *d*-CTP, deoxy-cytidine 5′-triphosphate.

stimulating effect of ATP for TTP incorporation into DNA may be partially explained in terms of its inhibitory effect to the hydrolysis of TTP by dephosphorylating enzyme(s). So far, few papers concerning the enzymic hydrolysis of TMP, TDP, or TTP in higher animal cells have been published. People do not know whether two or three different enzymes or only one enzyme with broad substrate specificity is involved.

Using radioactive γ-^{32}P-TTP as substrate, a TTP phosphatase activity and its inhibition by ATP were studied in this work.

Materials and Methods

Preparation of γ-^{32}P-ATP

Preparation of γ-^{32}P-ATP was mainly according to the method of Glynn and Chappell (1964) with only a small modification. The following were added, in the order given, to a 20 ml glass-stoppered tube: 5 ml of water; 0.5 ml of 1 M Tris adjusted to pH 8.0 with HCl; 0.06 ml of 1 M $MgCl_2$; 1 ml of 0.1 N NaOH; 20 μmoles of cystein as the free base; 60 μmoles of crystalline ATP as the disodium salt; 10 μmoles of 3-phosphoglycerate as the tricyclohexylammonium salt; 2 mg of muscle glyceraldehyde 3-phosphate dehydrogenase suspended in 0.1 ml of 2.5 M $(NH_4)_2SO_4$ (The source of the glyceraldehyde 3-phosphate dehydrogenase is important, because NAD, which is present in bound form in the enzyme prepared from muscle, is necessary for the reaction.); 0.2 mg of yeast phosphoglycerate kinase suspended in 0.05 ml of a solution of 2.4 M $(NH_4)_2SO_4$ in 0.04 M $Na_4P_2O_7$. About 15 mCi of ^{32}P was added as "carrier-free" inorganic phosphate in 0.01 N HCl. Water was added so that the final volume would be 10 ml.

After incubation at room temperature for 60 to 90 min, the contents of the tube were poured into a 500 ml round-bottomed flask containing 50 ml of 95% ethanol. Then 10 ml of 17% (v/v) ethanol was used to wash the tube and the washings were added to the bulk mixture. The ethanol-treated mixture, which at this stage was cloudy, was allowed to stand for a few min and then clarified by the addition of about 15 ml of water. A small sample (0.1 ml) was withdrawn so that the total radioactivity present could be determined. The volume of the bulk mixture was reduced to 5-8 ml on a rotary evaporator at 40°C.

(1) *Preparation of column*

About 1 gm of Dowex AG 1-X4 (200-400 mesh) was used. The column was 7 mm in diameter and about 35 to 38 mm long. The resin was first washed with 2 N HCl, water, 2 N NaOH, and then water again, and this process

was repeated a few times. The column then was washed with 2 N HCl and with water wash to remove the excess HCl (tested with $AgNO_3$). Finally, the resin was washed with 0.2 M Tris-HCl pH 8.0 (5–6 ml) followed by water again.

(2) *Separation of γ-^{32}P-ATP*

After concentration, the reaction mixture was filtered through Whatman no. 1 paper directly onto the Dowex 1 column. The filter paper was washed with 10 ml of water and the washings were added to the column. To remove both unlabeled and radioactive Pi and ADP, the column was eluted with 200 ml of a solution containing 0.02 N NaCl and 0.01 N HCl. To remove Na^+, the column was eluted with 10 to 20 ml of 0.01 N HCl. Finally, to obtain the γ-^{32}P-ATP, the column was eluted with 0.25 N HCl. The eluate was collected in three 5 ml portions in test tubes immersed in ice, and 1 M Tris base was added in small measured portions as the eluate was collected to give a final pH about 7–8. The first tube had the material with the highest specific activity.

(3) *Assay of γ-^{32}P-ATP*

The fractions were analyzed for specific activity by taking optical density readings at 259 nm for ATP on suitable dilutions. The molar extinction coefficient was taken to be 15.4×10^3 at pH 7. Aliquots of radioactive ATP solution of known volume were applied to Whatman 3 MM filter paper discs. The filters, dried under a lamp, were then counted in 10 ml PPO/POPOP toluene in a Beckman liquid scintillation counter (Model LS-100). γ-^{32}P-ATP preparations with an average specific activity about 10^{10} cpm/μmole were obtained.

(4) *Precautions against radiation*

Lead screens were necessary to decrease radiation.

Preparation of γ-^{32}P-TTP

(1) *Preparation of **E. coli** kinase fraction*

The procedure described by Hurlbert and Hurlong (1967) was followed.

(2) *Procedure for making γ-^{32}P-TTP*

The followings were mixed in order: 1) 0.50 ml of 1.0 M Tris-HCl pH 8.1; 2) 0.05 ml of 1.0 M $MgCl_2$; 3) 0.0186 gm solid KCl; 4) 0.01 ml of 1 M 2-mercaptoethanol; 5) 0.10 ml of 0.05 M sodium EDTA (pH 8.3); 6) 5 μmole TDP; 7) 0.90 ml of *E. coli* kinase fraction; 8) 0.84 μmole of γ-^{32}P-ATP (3.5 ml of ATP of specific activity 4.5×10^9 cpm/μmole); and 9) 10 μmoles of cold ATP (0.05 ml

of a 50 mM ATP). The total volume was 5 ml. The reaction mixture was incubated for 2 hr at 37°C, then heated to 95°C for 3 min to stop the reaction, and centrifuged at low speed. The supernatant fluids containing γ-^{32}P-TTP was loaded onto a 1.0×17 cm column of Dowex 1 (formate) and eluted with a linear gradient produced by 50 ml of water in the mixing flask and 50 ml of 4 M formic acid–1 M ammonium formate in the reservoir. ATP was eluted near the end of this gradient and recognized by the high absorbance and low radioactivity. The column was washed with water then with 100 ml of 0.2 M Na_2CO_3 to change to the carbonate form and again washed with water to remove excess salt. Then 50–75 ml of 0.75 M ammonium bicarbonate was used to elute TTP. The fractions containing the γ-^{32}P-TTP were evaporated and desalted *in vacuo* in a rotary evaporator at 40°C, and taken up in 3–5 ml of water. The nucleotide was assayed and stored frozen. γ-^{32}P-TTP of specific activity around 10^8 cpm/μmole was prepared by this method.

Animals and Preparation of Tissues

Female Sprague-Dawley rats were used. The techniques of transplantation of the tumors, anesthesia, exsanguination, and initial treatment of the tissues were according to those described by Busch *et al.* (1958).

Isolation of Walker-256 Carcinosarcoma Cell Nuclei

All operations were done at 0–4°C. Ice-cold fresh Walker tumor tissues were minced with scissors in 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$. Minced tissues were then homogenized in a Potter-Elvehjem glass homogenizer with a tightly fitting Teflon pestle (clearance: 3×10^{-4} inches; drill speed: 1150 rpm) with 10 volumes of the same sucrose solution (v/w) for 10 strokes. The homogenized solution was filtered through one-layer coarse, one-layer fine, and 8-layer fine cheese cloth, successively. The filtrate was centrifuged at 3,000 rpm using a Servall SS-34 rotor (1,085 g) for 15 min. Crude nuclei were collected from the bottom of the centrifuge tubes and then were suspended in 10 volumes of 2.2 M sucrose containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$. Suspended nuclei were transferred to a Parr pressure bomb (Parr instrument Co., Moline, Ill., U. S. A.) and then were subjected to 400 pound/square inch pressure for 20 min. At this stage fine intact nuclei were observed when examined under phase-contrast microscope. Pure nuclei were obtained after the pressed suspension being centrifuged at 105,000 g using a Beckman SW-41 rotor for 60 min.

Preparation of Crude Nuclear Extract

All operations were done at 4°C. Isolated Walker-256 tumor nuclei were

suspended in 10 volumes (v/w) of 0.14 M NaCl–0.05 M phosphate buffer (pH 7.2) —1 mM $MgCl_2$ and sonicated for 1 min. The sonicated solution was centrifuged at 12,000 rpm with a Servall SS-34 rotor for 30 min. The supernatant fluid was saved and used directly for TTP phosphatase activity assays.

(1) *Preparation of different nuclear fractions*

All operations were done at 4°C (Wang and Johns, 1968; Kostraba and Wang, 1971). Nuclei were suspended in 20% glycerol containing 1 mM 2-mercaptoethanol, 0.05 M imidazole buffer (pH 7.4), and 0.14 M NaCl. The suspension was sonicated in a Waring Blender for a total of 4 min keeping the temperature under 4°C at all times. The sonicated suspension was centrifuged at 12,000 g for 20 min. The supernatant fluid was designated as NaCl-extract I. The pellet was resuspended in the same solution, sonicated and centrifuged in the same way. The supernatant fluid was designated as NaCl-extract II. The nuclear pellet after second 0.14 M NaCl extraction was stirred in 20 volumes of a solution containing 1 mM 2-mercaptoethanol, 0.05 M imidazole buffer (pH 7.4), and 1.0 M NaCl for at least 3 hr. The stirred suspension was centrifuged at 12,000 g for 30 min to get supernatatant fluid. The pellet was saved for the same treatment. The supernatant fluid was dialyzed overnight against exactly 6 volumes of 0.05 M imidazole buffer (pH 7.4) with 1 mM 2-mercaptoethanol. Precipitates formed during dialysis were separated from the supernatant fluid by centrifuging at 12,000 g for 30 min. The supernatant fluid contained acidic proteins (or non-histone proteins) was designated as AP-I, while the precipitate fraction was designated as DNP-I. The pellet from 1.0 M NaCl solution was treated again by 1.0 M NaCl extraction twice to get AP-II, DNP-II; AP-III, and DNP-III.

For enzyme activity assays of NaCl-extract I, NaCl-extract II, or acidic proteins, each fraction was brought to 0.7 ammonium sulfate saturation, allowed to stand for one hr, and then centrifuged at 12,000 g for one hr to get protein pellet. Protein pellet was dissolved in a suitable volume of 20% glycerol, 1 mM 2-mercaptoethanol, and 0.05 M imidazole buffer (pH 7.4), and then dialyzed for at least 4 hr against 100 volumes of the same solution twice before use.

For enzyme activity assays of DNP, salt was removed by washing with 20% glycerol containing 1 mM 2-mercaptoethanol, and 0.05 M imidazole buffer (pH 7.4) several times before use.

Assay of TTP Phosphatase Activity

The reaction of the enzyme is:

$$\text{Thymidine} - Ⓟ - Ⓟ - {}^{32}Ⓟ + H_2O \longrightarrow \text{Thymidine} - Ⓟ - Ⓟ + {}^{32}Pi$$

The hydrolized ^{32}P was precipitated togother with carrier orthophosphate by adding Sugino-Miyoshi mixture (1 N $HClO_4$: 10% ammonium molybdate: 0.2 M triethylamine-hydrochloride = 4:2:1 by volume). The precipitate was dissolved in ammonia/acetone (1:1) and an aliquot was applied on Whatman 3 MM filter paper discs, dried under a heating lamp, and counted by a Beckman liquid scintillation counter.

Results

Effect of ATP, GTP, CTP, d-GTP, and d-CTP on TTP phosphatase activity in crude Walker-256 nuclear extract

The conditions used for these experiments were the same. But for clarity, the results are ploted on different charts, namely, Fig. 1 to Fig. 4. Using γ-^{32}P-TTP as substrate, the enzyme activities are expressed as picomole inorganic phosphate liberated as shown on ordinate.

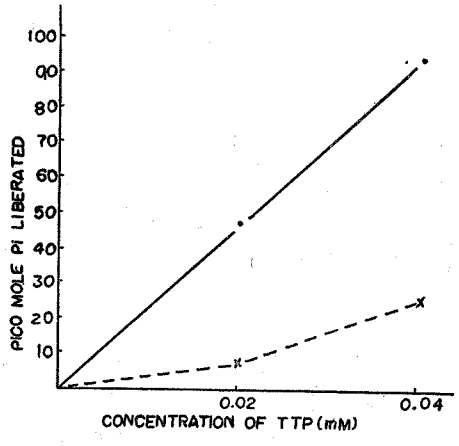

Fig. 1. Effect of 2 mM ATP on TTPase activity at two concentrations of TTP. Enzyme source: Walker-256 tumor crude nuclear extract. Each tube contained in 0.25 ml 0.1 M imidazole buffer, pH 7.4; 0.01M $MgCl_2$; 2mM ATP; 0.02 mM or 0.04 mM cold TTP; about 253,000 cpm γ-^{32}P-TTP; 0.015 mg protein. The reaction lasted for 15 min and was stopped by boiling for 3 min.
— • — • —: without ATP;
--- × --- × ---: with 2 mM ATP.

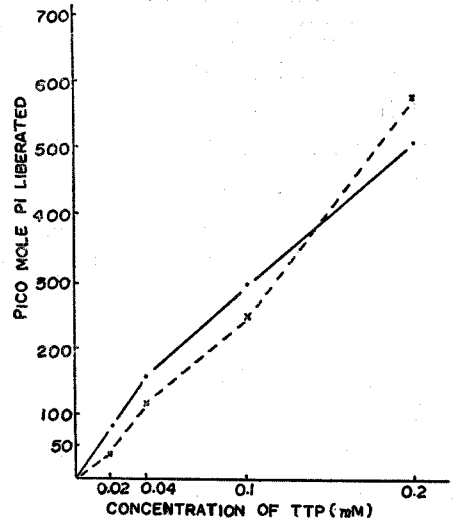

Fig. 2. Effect of 0.4 mM ATP on TTPase activity at various concentrations of TTP. Reaction conditions were the same as those in Fig. 1.
— • — • —: without ATP;
--- × --- × ---: with 0.4 mM ATP.

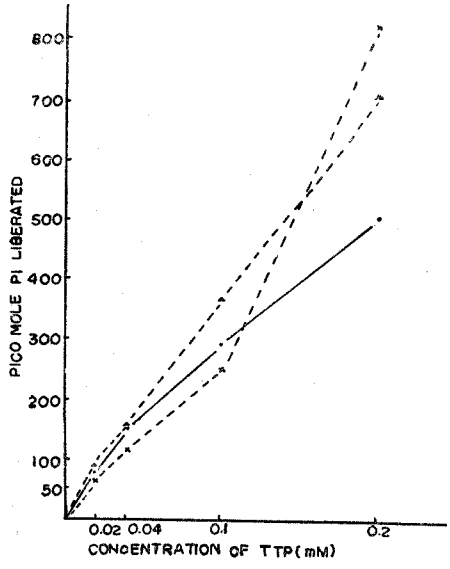

Fig. 3. Effect of 0.1 mM GTP or 0.1 mM CTP on TTPase activity at various concentrations of TTP. Reaction conditions were the same as those in Fig. 1 except that GTP or CTP replaced ATP.
—•—•—: without either GTP or CTP;
---×---×---: with 0.1 mM GTP;
---▲---▲---: with 0.1 mM CTP.

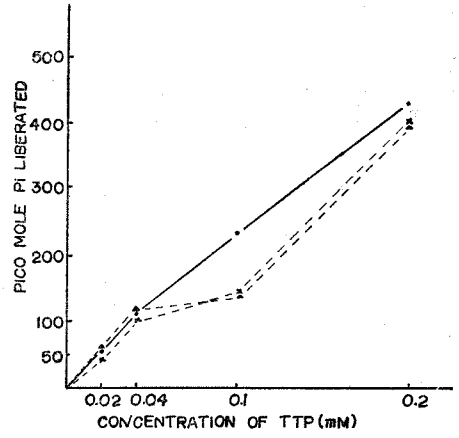

Fig. 4. Effect of 0.1 mM *d*-GTP or *d*-CTP on TTPase activity at various concentrations of TTP. Reaction conditions were the same as those in Fig. 1 except that *d*-GTP or *d*-CTP replaced ATP.
—•—•—: without either *d*-GTP or *d*-CTP;
---×---×---: with 0.1 mM *d*-GTP;
---▲---▲---: with 0.1 mM *d*-CTP.

Both ATP and GTP showed similar effect on TTP phosphatase activity. They had some inhibitory effect when substrate (TTP) concentrations were low, but stimulatory effect when TTP concentrations were high. CTP had stimulatory effect when TTP concentrations were high.

Both *d*-GTP and *d*-CTP at 0.1 mM showed 39% inhibition when TTP was at 0.1 mM.

The results on Fig. 1 and 2 are summaried in Table 1.

Table 1. *Per cent inhibition of TTP phosphatase activity by ATP*
Reaction conditions are described in Fig. 1 and 2. Minus sign stands for stimulation.

$\frac{m\mu mole\ ATP}{m\mu mole\ TTP}$ in 0.25 ml reaction mixture	$\frac{100}{50}=2$	$\frac{100}{25}=4$	$\frac{100}{10}=10$	$\frac{100}{5}=20$	$\frac{500}{10}=50$	$\frac{500}{5}=100$
% inhibition	−13.7%	16%	25%	54%	72%	83%

Table 2. *ATP effect on TTPase activity and TTP effect on ATPase activity of Walker-256 nuclear fractions*

The reaction mixture for studying ATP effect contained in a volume of 0.25 ml 0.1 M imidazole buffer (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP (if included), 0.8 mM TTP, ca. 250,000 cpm γ-^{32}p-TTP, and enzyme solution as shown in table.

The reaction mixture for studying TTP effect contained in a volume of 0.25 ml 0.1 M imidazole buffer (pH 7.4), 2 mM $MgCl_2$, 0.8 mM TTP (if included), 2 mM ATP, 75,990 cpm γ-^{32}P-ATP, and enzyme solution as indicated in table.

Fraction	Protein Concentration mg/ml	Amount of protein added 50 μl	γ-^{32}-P-TTP as substrate				γ-^{32}-P-ATP as substrate			
			mμmole Pi liberated in 0.25 ml reaction mixture		Specific activity mμmole Pi/mg protein	% inhibition	mμmole Pi liberated in 0.25 ml reaction mixture		Specific activity mμmole Pi/mg protein	% inhibition
1) 0.25 M sucross	16.490	mg 0.835	+ ATP — ATP	33.90 59.10	41.0 71.6	% 43	+ TTP — TTP	31.5 68.7	38.2 83.1	% 54
2) 2.2 M sucrose	3.290	0.165	+ ATP — ATP	7.56 32.90	45.8 200.0	77	+ TTP — TTP	51.7 71.5	313.0 433.0	28
3) NaCl extract I frozen	0.920	0.046	+ ATP — ATP	3.08 7.52	67.0 163.0	59	+ TTP — TTP	— —	— —	—
4) NaCl extract I refrigerated	0.920	0.046	+ ATP — ATP	3.08 7.45	67.0 162.0	59	+ TTP — TTP	13.8 17.3	299.0 376.0	21
5) NaCl extract II frozen	0.437	0.022	+ ATP — ATP	0.94 8.24	42.9 376.0	89	+ TTP — TTP	— —	— —	—
6) NaCl extract II refrigerated	0.437	0.022	+ ATP — ATP	3.45 14.00	150.0 638.0	76	+ TTP — TTP	0 3.1	0.0 142.0	—
7) Acidic protein	0.263	0.013	+ ATP — ATP	0.36 5.25	25.7 398.0	94	+ TTP — TTP	6.2 24.3	466.0 1,840.0	75
8) DNA-Histone Complex	12.260	0.613	+ ATP — ATP	2.79 23.00	4.6 37.5	88	+ TTP — TTP	17.1 55.2	27.9 90.0	69

(1) *Substrate specificity*

Table 2 shows that in various Walker-256 nuclear fractions ATP inhibited TTPase activity while TTP inhibited ATPase activity. This suggests that there is such an phosphatase with broad substrate specificity which can utilize both TTP and ATP as substrates.

(2) *Alteration of ATP effect by AMP*

Table 3 shows that including AMP in the reaction mixture decreased inhibitory ATP effect on TTPase in most fractions except 40–50% saturation fraction in which AMP enhanced inhibitory ATP effect. This finding is interesting since it suggests that TTPase under study might response to energy charge.

Table 3. *TTP phosphatase activity of various ammonium sulfate precipitation fractions from Walker-256 crude nuclear extract*

The 0.25 ml reaction mixture contained 0.1 M imidazole buffer (pH 7.4), 20 mM $MgCl_2$, 2 mM ATP (if included), 2 mM AMP (if included), 0.8 mM TTP, 250,000 cpm γ-^{32}P-TTP, and enzyme solution.

Activity (cpm) / Additions / Fraction % Ammonium sulfate saturation	−ATP, −AMP	+ATP, +AMP	+ATP
0-20	2,088	139	89
20-40	2,878	717	405
40-50	1,831	87	622
50-60	1,730	341	131
60-80	287	—	—

Discussion

Although within the TTP concentrations used, both *d*-GTP and *d*-CTP did not show stimulating effect, they probably would if TTP concentration was increased. But whether TTP concentration higher than 0.2 mM is of physiological significance or not is not clear now.

The TTP phosphatase activity under study seems to be a non-specific type. It can utilize both TTP and ATP as substrates. Thus ATP exerts its inhibitory effect by competing with TTP for the catalytic site. However the possibility that ATP can also bind to regulatory site of the enzyme could not be excluded since AMP can alter the effect of ATP on the enzyme.

The fact that substantial TTP phosphatase activity was detected in several ammonium sulfate precipitation fractions might be explained by the existence of multiple forms of TTP phosphatase with different molecular weights in

Walker-256 nuclei. But this could also be due to aggregation between TTP phosphatase and other macromolecules during the preparation process.

The TTP phosphatase under study seems to be a different enzyme from TDP phosphatase. The detail will be described in the next report of this series.

Acknowledgements

This investigation was supported in part by a U. S. Public Health Services grant (CA 13196-02). Y.-H. Lin is grateful to National Science Council, Republic of China for a financial support from 1970 to 1972 when he was on leave from Institute of Botany, Academia Sinica, Taipei, Taiwan, R. O. C. He also wants to acknowledge the receipt of a non-resident tuition grant from Winstein Memorial Fund, Chemistry Department, University of California at Los Angeles, U. S. A.

Literature Cited

BALLAL, N. R., M. S. COLLINS, R. M. HALPERN, and R. A. SMITH. 1970. DNA polymerase activities in fractionated Walker-256 tumor cell nuclei. Biochem. Biophys. Res. Commun. **40**: 1201-1208.

BUSCH, H., J. R. DAVIS, and D. C. ANDERSON. 1958. Labeling of histones and other nuclear proteins with L-lysine-U-C^{14} in tissues of tumor-bearing rats. Cancer Research. **18**: 916-926.

GLYNN, I. M. and J. B. CHAPPELL. 1964. A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. Biochem. J. **90**: 147-149.

HURLBERT, R. B. and N. B. FURLONG. Biosynthetic preparation of ^{32}P-labelled nucloside 5′-phosphates and derivatives. 1967. *In:* Methods in Enzymology, Vol. XII, Part A, (L. Grossman and K. Moldave, eds), pp. 193-202. Academic Press, New York.

KOSTRABA, N. C. and T. Y. WANG. 1972. Differential activation of transcription of chromatin by non-histone fractions. Biochem. Biophys. Acta, **262**: 169-180.

WANG, T. Y. 1967. The isolation, properties, and possible functions of chromatin acidic proteins. J. Biol. Chem. **242**: 1220-1226.

WANG, T. Y. and E. W. JOHNS. 1968. Study of the chromatin acidic proteins of rat liver: heterogeneity and complex formation with histones. Arch. Biochem. Biophys. **113**: 176-183.

Walker-256 惡性瘤細胞核之去氧核酸的合成：

I. 胸腺核苷5′-三磷酸水解酵素活性的存在及腺嘌呤核苷5′-三磷酸對此酵素活性的抑制

林 耀 輝

中央研究院植物研究所

R. A. SMITH

美國加州大學化學系

利用放射性 γ-^{32}P-TTP 爲基質可以檢驗出 Walker-256 惡性瘤細胞核的抽出液中有胸腺核苷5′-三磷酸水解酵素的活性。ATP 也可以被這個酵素所分解，因此所觀察到的抑制作用乃因爲它和 TTP 競爭摧化部位。其他核苷酸，如 GTP, CTP, *d*-GTP, 和 *d*-CTP 對此酵素活性的影響也一起報告。