Selective Stimulation by Tri-iodothyronine of Myocardial Deoxyribonucleic Acid Polymerase- α in Neonatal Rats

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Three forms of DNA polymerase (α , β and γ) were separated from isolated rat myocardial cells on the basis of template, pH and ionic requirements, sensitivity to *N*-ethylmaleimide and position on sucrose gradients. Tri-iodothyronine administration ($20\mu\text{g}/100\text{g}$ intraperitoneally) to 3-week-old rats resulted in selective stimulation of DNA polymerase- α (198 ± 7.1 versus 102 ± 5.8 pmol of [^3H]dTMP/30 min per mg of protein in untreated controls, $P < 0.01$), with no change in polymerases- β and - γ . [^3H]Thymidine incorporation into myocardial DNA was also enhanced in tri-iodothyronine-treated neonatal rats (132 ± 11.2 versus 53 ± 4.1 c.p.m./ μg of DNA in controls, $P < 0.001$). Increased incorporation was associated with an expansion of deoxyribonucleoside 5'-triphosphate pools, especially that of dTTP (24 ± 1.6 versus 10 ± 1.1 pmol/mg of DNA, $P < 0.01$). Neither DNA polymerase activities nor [^3H]thymidine incorporation were changed in 6-month-old rats in response to tri-iodothyronine. Unstimulated adult myocardial cells had DNA polymerase activities comparable with those in 3-week-old animals, but significantly lower [^3H]thymidine incorporation and deoxyribonucleoside triphosphate concentrations. Enhancement of both DNA polymerase- α activity and [^3H]thymidine incorporation in tri-iodothyronine-treated young rats was prevented by concomitant administration of either vinblastine ($1\mu\text{g}/\text{g}$) or daunomycin ($2\mu\text{g}/\text{g}$); actinomycin D ($0.1\mu\text{g}/\text{g}$) or cycloheximide ($8\mu\text{g}/\text{g}$), on the other hand, prevented the increase in [^3H]thymidine incorporation, but not DNA polymerase- α activation. These results demonstrate an age-dependent stimulation of myocardial DNA replication by tri-iodothyronine and suggest an inter-relationship between DNA synthesis and subsequent entry into mitosis.

Both proliferative activity (percentage of cells engaged in mitosis) and proliferative capacity (ability to undergo mitosis in response to appropriate stimuli) of myocardial cells decline rapidly during the early post-natal period (Grohmann, 1961; Rumyantsev, 1963; Klinge & Stocker, 1968). Proliferative activity probably ceases when the heart attains its (genetically determined) full complement of cells. Restriction of proliferative capacity, on the other hand, reflects a progressive inability of myocardial cells to respond to injury or increased functional demands with hyperplasia (increased cell number), relying instead on hypertrophy (increased cell mass). The mechanisms of the progressive decline in myocardial mitotic potential have not been adequately studied.

Since transition from quiescence to mitosis in response to proliferative stimuli requires prior DNA synthesis, the possibility has been examined that low activities of the DNA-synthetic enzymes in adult myocardium might be the limiting factor. A number of studies have, indeed, reported a dramatic

fall in DNA polymerase and thymidine kinase activities during the first few weeks of post-natal development (Doyle *et al.*, 1974; Claycomb, 1975; Hübscher *et al.*, 1977; Claycomb, 1977). In the rat, for example, DNA polymerase activity falls to negligible values within 17 days after birth (Claycomb, 1975) and does not change thereafter. The implication (Claycomb, 1976), however, that this decline is responsible for the restriction in proliferative capacity of myocardial cells is complicated by two considerations. (a) None of the reported studies (Doyle *et al.*, 1974; Gillette & Claycomb, 1974; Claycomb, 1975, 1976; Hübscher *et al.*, 1977; Claycomb, 1977) has taken into consideration that myocardial cell size increases considerably during early post-natal growth (Sasaki *et al.*, 1968; Katzberg *et al.*, 1977) and that expression of enzymic activity per mg of protein may grossly overestimate the magnitude of DNA polymerase decline. The cellular heterogeneity of the heart has also been ignored, despite the fact that non-muscle (interstitial and endothelial) cells retain their proliferative capacity

to adulthood (Morkin & Ashford, 1968; Grove *et al.*, 1969). We have re-examined these issues (Limas & Limas, 1978) by separately determining the activity of three DNA polymerases (α , β and γ) from muscle and non-muscle cells of the developing myocardium. Decline in muscle cell polymerases was much less (30–40% compared with day 1) than previous studies (Doyle *et al.*, 1974; Gillette & Claycomb, 1974; Claycomb, 1975; Hübscher *et al.*, 1977; Claycomb, 1977) have indicated and occurred entirely within the first 2 weeks of post-natal life; non-muscle-cell polymerases were essentially unchanged when expressed as [^3H]dTMP/ 10^6 cells. (b) DNA polymerase activities in quiescent cells cannot predict the ability of the cells to proliferate in response to stimuli. Neffgen & Korecky (1972), for example, have shown that myocardial-cell proliferation occurs in 3–4-week-old rats after induction of nutritional anaemia; the same stimulus was ineffective in inducing muscle-cell mitosis in adult myocardium. Similar results have been obtained by Dowell & McManus (1978) with aortic constriction in rats and by Bishop (1971) in young puppies. Since no interim change in DNA polymerase activity occurs between 3–4 weeks of age and adulthood, this enzyme cannot be the sole determinant of proliferative ability.

The present study attempts to obtain more detailed information on the control of myocardial DNA synthesis in response to tri-iodothyronine. This model was selected because thyroid hormones induce cardiomegaly affecting only myocytes and without deleterious effects on cardiac performance (Bartosova *et al.*, 1969). Both young (2 weeks old) and adult (6 months old) rats were utilized. The results indicate an age-dependent stimulation of DNA synthesis and DNA polymerase- α .

Materials and Methods

Experiments were carried out on male Sprague-Dawley rats, 3 weeks or 6 months of age. Tri-iodothyronine (20 μg /100 g body wt.) or 0.9% NaCl (controls) was injected intraperitoneally daily for 3 days. The amounts of inhibitors, when given, were (per 100 g): actinomycin D, 10 μg ; cycloheximide, 0.8 mg; daunomycin, 200 μg ; and vinblastine, 100 μg .

Myocardial-cell isolation

Myocardial cells were isolated from rat hearts by a modification of the method described by Glick *et al.* (1974). Cardiac ventricles from either young or adult rats were diced into 3 mm slices and washed twice with Ca^{2+} -free phosphate buffer (composition in g/litre: NaCl, 6.8; KCl, 0.4; glucose, 0.9; Na_2HPO_4 , 0.21; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.06; pH 7.4). The tissue was then placed into 50 ml Erlenmeyer flasks containing 5 ml of the same buffer and enzymes (1 mg of col-

lagenase and 1 mg of hyaluronidase/ml). The flasks were shaken at 100 strokes/min at 37°C. Cells were decanted from the tissue pieces at 20-min intervals (harvests 1–4) and the tissue was resuspended in fresh phosphate buffer. Harvests 1 and 2 were mostly erythrocytes and fragmented cells and were discarded. Cells from harvests 3 and 4 were washed twice with Ca^{2+} -free phosphate buffer, pooled, and centrifuged through a 3% (v/v) Ficoll solution containing 0.1% albumin at 60 g for 5 min. The myocytes thus isolated were washed twice with phosphate buffer and were immediately used for the enzyme isolation. Approx. 93–95% of the cells were myocytes and 85% of the latter excluded Trypan Blue.

Isolation of DNA polymerases

Myocardial cell suspension from three to five rat hearts was homogenized for 30 s in a solution containing 10 mM-phosphate buffer (pH 7.5), 10 mM-KCl, 1.5 mM- MgCl_2 and 0.5 mM-dithiothreitol. The homogenate was centrifuged at 800 g for 15 min and the supernatant was dialysed overnight against 50 mM-phosphate buffer (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA. Portions of the diffusate were either assayed for DNA polymerase activity or layered on top of a 5–20% (w/v) sucrose gradient in 0.1 M- KH_2PO_4 (pH 7.2)/0.1 M-KCl/1 mM-dithiothreitol. After centrifugation at 100 000 g for 16 h, fractions were collected and assayed for enzymic activity. The assay medium for DNA polymerase- α contained 20 mM- KH_2PO_4 (pH 7.2), 0.1 mM-EDTA, 0.5 mM-dithiothreitol, 10 mM- MgCl_2 , activated (Aposhian & Kornberg, 1962) calf thymus DNA (200 $\mu\text{g}/\text{ml}$), dATP, dCTP and dGTP (each 80 μM) and 16 μM -[^3H]dTTP (0.5 Ci/mmol; Amersham/Searle, Arlington Heights, IL, U.S.A.). The assay for polymerase- β was carried out in 50 mM-Tris/HCl (pH 8.5) containing 0.1 M-KCl, 10 mM- MgCl_2 , activated calf thymus DNA (200 $\mu\text{g}/\text{ml}$), 0.5 mM-dithiothreitol, dATP, dCTP and dGTP (each 50 μM) and 16 μM -[^3H]dTTP. For comparisons of polymerase activities in the diffusate before sucrose gradient centrifugation, dC₅₀₀·dG_{5–12} replaced DNA as the template for polymerase- β assay, which was carried out with 10 mM-MnCl₂ and [^3H]dGTP (instead of [^3H]dTTP); under these conditions, the reaction is specific for DNA polymerase- β and only one radioactive nucleotide is being polymerized. When activated DNA at pH 7.2 is used as template, both polymerases- α and - β respond, although the pH of the reaction favours the α form. Finally, the assay medium for polymerase- γ contained 50 mM-Tris/HCl (pH 8.5), 50 mM- KH_2PO_4 (pH 8.5), 0.13 M-KCl, 0.5 mM-MnCl₂, 1 mM-dithiothreitol, poly(rA)·dT_{25–30} (50 $\mu\text{g}/\text{ml}$) and 16 μM -[^3H]dTTP. Under these conditions, the assay was specific for polymerase- γ , since polymerase- β activity on poly(rA)·dT_{25–30} is inhibited by phosphate

(Knopf *et al.*, 1976) and polymerase- α cannot utilize this polynucleotide (Chappeville & Brun, 1977). The DNA polymerase reaction was routinely carried out at 37°C for various lengths of time and was stopped with 0.5 ml of 1 M-NaOH containing 100 μ g of heat-denatured calf thymus DNA and 1 mg of bovine serum albumin. The incorporation of [3 H]dTTP into DNA was measured as described by Lynch *et al.* (1970).

[3 H]Thymidine incorporation

Control or tri-iodothyronine-treated rats were given 2 μ Ci of [methyl- 3 H]thymidine (Amersham/Searle, specific radioactivity 60 Ci/mmol) per g body wt. subcutaneously and were killed 1 h later. The ventricles were washed and weighed: cardiac myocytes were isolated as described above and homogenized in ice-cold 0.15 M-NaCl. To the homogenate was added an equal volume of 10% (v/v) HClO₄, and the acid-insoluble material, collected by centrifugation at 2000g for 10 min, was washed twice with ice-cold 5% HClO₄ and twice with ethanol/ether (3:1, v/v). It was then resuspended in 5% HClO₄ and heated at 70°C for 30 min. The hydrolysed DNA in the acid-soluble fraction was measured by Burton's (1956) modification of the diphenylamine reaction. Portions of the acid-soluble fraction were also dissolved in NCS solubilizer and radioactivity was counted in 10 ml of a toluene-based scintillation fluid (Econofluor; New England Nuclear Corp.).

Uptake and catabolism of [3 H]thymidine

Rats were injected with 2 μ Ci of [3 H]thymidine/g. Cardiac myocyte homogenates were prepared and cold 10% HClO₄ was added as described above. After centrifugation, samples of the acid-soluble material were dissolved in NCS solubilizer and counted for radioactivity. Thymidine and pyrimidine derivatives in samples of the acid-soluble fraction were separated from non-aromatic degradation products by charcoal absorption (Claycomb, 1975). A 2.0 ml portion of the HClO₄-soluble fraction was added to 50 mg of activated charcoal; mixing was achieved by frequent (every 5 min) vortexing for 30 min. After centrifugation at 1000g for 10 min, a portion of the supernatant was dissolved in NCS solubilizer and counted for radioactivity to measure unadsorbed tritiated derivatives. Radioactivity adsorbed on charcoal represents labelled thymidine and aromatic pyrimidine derivatives and gives an estimate of the [3 H]thymidine taken up and not catabolized into non-aromatic compounds.

Deoxyribonucleoside triphosphate pools

Myocardial deoxyribonucleotide concentrations were determined either in whole-heart homogenates or in myocytes isolated as described above and sedi-

mented by centrifugation at 900g for 15 min. Tissues were extracted by the addition of 5 vol. of ice-cold 0.6 M-HClO₄. After centrifugation at 2000g for 15 min, the supernatant was neutralized with 2 M-KOH. The precipitate was removed by centrifugation (2000g for 15 min) at 4°C and the clear supernatant was used for determination of the deoxyribonucleoside triphosphates. dATP and dTTP were determined essentially as described by Lindberg & Skoog (1970). Standard curves were obtained with the following reaction mixture: 20 μ mol of potassium phosphate buffer (pH 7.4), 2 μ mol of MgCl₂, 0.4 μ mol of dithiothreitol, 10 nmol of poly[d(A-T)], 80 pmol of 3 H-labelled deoxyribonucleoside triphosphate (dTTP and dATP respectively) and 4.5 units of large-fragment DNA polymerase from *Escherichia coli* in a final volume of 0.3 ml. DNA polymerase was added immediately before incubating the tubes at 37°C. The incorporation of radioactivity into an acid-insoluble product was followed by applying 50 μ l samples on Whatman GF/C filters; the filters were washed and counted for radioactivity as described by Klenow & Henningsen (1970). The amounts of dATP or dTTP in the tissue homogenates were measured by replacing the limiting deoxyribonucleoside triphosphate (dATP or dTTP) with an adequate volume (adjusted so that at least 2 pmol of dATP or dTTP was present) of tissue extract. The incorporated radioactivity corresponds to the amounts of dATP or dTTP in the extract (after correction for the presence of unlabelled deoxyribonucleoside triphosphate in the tissues) as described by Munch-Petersen *et al.* (1973). dCTP and dGTP were similarly determined (Tyrsted, 1975), except that the limiting deoxyribonucleoside triphosphates were different (dGTP and dCTP respectively) and 0.8 nmol of poly[d(I-C)] was used instead of poly[d(A-T)].

Materials

The oligonucleotides dT₁₂₋₁₈, dG₅₋₁₂, rA₁₂₋₁₈ and dC₁₂₋₁₈ were obtained from Collaborative Research, Waltham, MA, U.S.A. Poly(A), poly(C) and poly(dA) were obtained from Miles Laboratories, Kankakee, IL, U.S.A. Oligodeoxyribonucleotides were annealed to homopolymers at a proportion of 3 parts of oligonucleotide to 7 parts of homopolymer, by weight. Collagenase (type II, from *Clostridium histolyticum*) and hyaluronidase (type I, from bovine testes) were purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.) and Sigma (St. Louis, MO, U.S.A.) respectively.

Results

The DNA polymerase reaction in the tissue extracts after dialysis was linear with time for at least 1 h and has proceeded linearly for more than 4 h

Fig. 1. Sucrose-density-gradient centrifugation of myocardial tissue extracts from adult (a) and neonatal (b) rats (a) DNA polymerase activities from control (●, ■, ▲) or tri-iodothyronine-treated (○, □, △) adult rats were separated from tissue extracts containing 49.5 and 44.8 mg of DNA respectively. Portions (0.25 ml) of the extracts were loaded on linear 5–20% (w/v) sucrose gradients in 0.1 M-KH₂PO₄ (pH 7.2)/0.1 M-KCl/1 mM-dithiothreitol. After centrifugation at 100000g for 16 h at 2°C, the bottom of the centrifuge tube was punctured and 2.0 ml fractions were collected. Each fraction was tested for DNA polymerase activities (α, β, γ), as described in the text. (b) DNA polymerase activities from control (●, ■, ▲) or tri-iodothyronine-treated (○, □, △) neonatal rats from tissue extracts containing 49.3 and 51.6 mg of DNA respectively. Conditions of sucrose-gradient centrifugations are as described in (a).

Table 1. Recoveries of DNA polymerases in crude extracts from sucrose gradients

Reactions were carried out as described in the text, by using activated calf thymus DNA for polymerase-α (with MgCl₂), dC₅₀₀·dG_{5–12} for polymerase-β (with MnCl₂), and poly(rA)·dT_{25–30} for polymerase-γ (with MnCl₂). Activities are expressed as pmol of nucleotide incorporated/30 min per mg of protein and represent means ± S.E.M. for six experiments in each group.

	DNA polymerase					
	α		β		γ	
	Extract	Sucrose gradient	Extract	Sucrose gradient	Extract	Sucrose gradient
Young, control	14.8 ± 0.6	105 ± 5.8	11.8 ± 0.9	83 ± 2.4	8.2 ± 0.7	67 ± 5.3
Young, tri-iodothyronine-treated	27.2 ± 0.8	198 ± 7.1	11.6 ± 0.3	84 ± 2.2	8.5 ± 0.6	69 ± 4.7
Adult, control	13.8 ± 0.6	101 ± 4.7	12.1 ± 0.5	86 ± 2.6	8.0 ± 0.4	64 ± 4.3
Adult, tri-iodothyronine-treated	14.1 ± 0.5	106 ± 3.8	11.9 ± 0.6	82 ± 2.4	8.4 ± 5.3	69 ± 5.1

(results not shown). All three unlabelled deoxyribonucleoside triphosphates are needed for optimal activity; deoxyribonuclease and actinomycin D strongly inhibit the reaction, whereas ribonuclease is ineffective. Sucrose-density-gradient centrifugation separated two peaks of DNA polymerase activity. The first corresponded to polymerases-α and -γ (distinguishable on the basis of template, pH and ionic requirements; see below), whereas the second

peak represented polymerase-β activity (Fig. 1). Comparison of Figs. 1(a) and 1(b) demonstrates that, although no significant difference was noted in baseline DNA polymerases between the two groups of animals, stimulation of enzymic activity occurred only in the 3-week-old rats and involved exclusively polymerase-α. The possibility that differences in recoveries of the enzymes from the tissue extracts accounted for these results was excluded by the data

Table 2. Effects of *N*-ethylmaleimide (NEM; 0.8mM) on DNA polymerases from myocardial cells of control and tri-iodothyronine-treated rats

The reaction was carried out with activated calf thymus DNA (200mg/ml) and 0.1 ml of tissue extract (0.5mg of protein) at 37°C for 30min. Results are expressed as pmol of nucleotide incorporated/30min per mg of protein and represent means $\pm$ S.E.M. for six experiments.

	DNA polymerase activity					
	α		β		γ	
	-NEM	+NEM	-NEM	+NEM	-NEM	+NEM
Young	101 $\pm$ 6.7	23 $\pm$ 1.8	82 $\pm$ 2.4	84 $\pm$ 3.1	68 $\pm$ 5.1	34 $\pm$ 2.1
+Tri-iodothyronine	196 $\pm$ 7.2	34 $\pm$ 1.9	86 $\pm$ 3.2	82 $\pm$ 1.8	71 $\pm$ 4.7	32 $\pm$ 2.6
Adult	98 $\pm$ 6.2	22 $\pm$ 1.3	77 $\pm$ 2.2	73 $\pm$ 2.9	65 $\pm$ 4.7	31 $\pm$ 2.3
+Tri-iodothyronine	100 $\pm$ 6.3	24 $\pm$ 1.6	80 $\pm$ 2.4	78 $\pm$ 3.1	66 $\pm$ 3.2	33 $\pm$ 1.8

Fig. 2. Effects of KCl on myocardial DNA polymerases. Reactions were carried out at 37°C for 30min by using myocardial tissue extract containing 61.8mg of DNA in the medium appropriate for DNA polymerase- α (○), - β (●) or - γ (Δ), as described in the text.

Fig. 4. [3 H]Thymidine incorporation into myocardial DNA of control (solid column) and tri-iodothyronine-treated (hatched column) rats

Results represent means $\pm$ S.E.M. for eight experiments in each group. *No statistical difference from untreated animals ($P>0.2$); ** $P<0.01$ compared with untreated controls.

Fig. 3. Effects of pH on myocardial DNA polymerases. Reactions were carried out at 37°C for 30min with myocardial tissue extract (65.9mg of DNA) in incubation medium appropriate for DNA polymerase- α (○), - β (●), or - γ (Δ), as described in the text.

in Table 1. Recovery of DNA polymerase- α (70%), - β (72%) and - γ (80%) did not differ significantly among the experimental groups. The pellet obtained from the initial low-speed (800g) centrifugation of the homogenate contained 3–5% of the total DNA polymerase activity and was therefore disregarded.

Several properties of the enzymic forms of DNA polymerase were studied and were found to be independent of animal age. Activated DNA is the preferred template over either heat-denatured or native DNA, both of which are poorly utilized by all three forms of the enzyme.

Sensitivity of the polymerases to *N*-ethylmaleimide is shown in Table 2: polymerase- β is resistant, whereas with activated DNA or poly(rA)·dT_{25–50} as template/primer, respectively, polymerases- α and

Table 3. *Variations in myocardial deoxyribonucleoside 5'-triphosphate concentrations in control and tri-iodothyronine-treated animals*

Myocardial cells isolated as described in the text were sedimented by centrifugation at 900g for 15 min and were then extracted by the addition of 5 vol. of ice-cold 0.6M-HClO₄. After centrifugation at 2000g for 15 min the supernatant was neutralized with 2M-KOH. The precipitate was removed by centrifugation at 4°C and the clear supernatant was used for determination of deoxyribonucleoside triphosphates as described in the text. Results are expressed as pmol/μg of DNA, and are means ± S.E.M. for six experiments in each group.

Deoxyribonucleoside 5'-triphosphate	Experimental group			
	3-week-old		6-month-old	
	Control	Tri-iodothyronine-treated	Control	Tri-iodothyronine-treated
dATP	20 ± 1.6	28 ± 1.3	13 ± 0.9	14 ± 1.1
dTTP	10 ± 1.1	24 ± 1.6	10 ± 1.0	11 ± 1.2
dCTP	23 ± 1.8	32 ± 1.7	14 ± 1.1	13 ± 1.4
dGTP	22 ± 1.4	29 ± 1.3	12 ± 0.8	12 ± 0.7

Table 4. *Effects of inhibitors on thymidine incorporation and tri-iodothyronine-induced stimulation of DNA polymerase-α activity in 3-week-old rats*

Control animals received 20 μg of tri-iodothyronine/100 g body wt. intraperitoneally daily for 3 days; experimental rats received, in addition to tri-iodothyronine, the indicated (per 100g) amount of the inhibitor. Results represent means ± S.E.M. for seven experiments in each group.

Inhibitor	[³ H]Thymidine incorporation (c.p.m./μg of DNA)	DNA polymerase-α (pmol of [³ H]dTTP/30 min per mg of protein)
None (control)	132 ± 9.2	198 ± 8.9
Actinomycin D (10 μg)	101 ± 8.3	192 ± 9.6
Cycloheximide (0.8 mg)	96 ± 3.9	202 ± 8.7
Vinblastine (100 μg)	78 ± 2.2	83 ± 7.1
Daunomycin (200 μg)	82 ± 2.3	89 ± 6.5

-γ were inhibited to a significant extent. The effects of KCl on the activities of different DNA polymerases are shown in Fig. 2: polymerase-α is rapidly inactivated at KCl concentrations higher than 50 mM, whereas polymerase-β activity shows a slow rise to a maximum between 100 and 150 mM-KCl. A similar dependency was found for polymerase-γ in the presence of 50 mM-potassium phosphate.

The pH-dependency of the polymerases is shown in Fig. 3: polymerase-α has an optimum between pH 7.0 and 7.4, whereas polymerases-β and -γ have optima at more alkaline pH (8.0–8.5). The incorporation of [³H]thymidine into nuclear DNA was higher in young rats (Fig. 4) and was significantly increased by tri-iodothyronine, which, in contrast, was ineffective in 6-month-old animals. The possibility that decreased [³H]thymidine incorporation in 6-month-old rats was due to differences in uptake and catabolism of injected [³H]thymidine was excluded by similar radioactivity recovery in the acid-soluble fraction (1400 ± 172 d.p.m./mg wet wt. of isolated myocytes in 3-week-old versus 1298 ± 161 d.p.m./mg in the adult rats). Similarly, the percentage of radioactivity retained by charcoal 1 h after [³H]-

thymidine administration was 23 ± 1.1% (n = 8) in the 3-week-old rats compared with 20 ± 1.4% (n = 8) in 6-month-old animals.

In young rats, increased thymidine incorporation in response to tri-iodothyronine was accompanied by an expansion of the deoxyribonucleoside triphosphate pools, particularly that of thymidine; pool sizes were lower in adult animals and did not increase in response to tri-iodothyronine (Table 3).

The effects of various inhibitors on [³H]thymidine incorporation and DNA polymerases of 3-week-old rats treated with tri-iodothyronine are shown in Table 4. Daunomycin and vinblastine inhibited the enhancement by tri-iodothyronine of both [³H]thymidine incorporation and DNA polymerase-α activity, whereas actinomycin D and cycloheximide prevented the increase in [³H]thymidine incorporation in response to tri-iodothyronine administration, but left DNA polymerase-α unaffected.

Discussion

Multiple DNA polymerases were isolated from rat myocardial cells, having different template, pH, and ionic requirements and variable sensitivity to

N-ethylmaleimide (Table 2, Figs. 2 and 3). On the basis of these properties and their fractionation patterns on sucrose density gradients, we distinguish three DNA polymerases (α , β and γ), in agreement with findings in other cell systems. The significance of this enzymic multiplicity for the regulation of DNA synthesis and cell proliferation is still unclear. On the basis of the preferential increase of DNA polymerase- α in response to proliferative stimuli (Chang & Bollum, 1972; Chang *et al.*, 1973; Baril *et al.*, 1973; Mayer *et al.*, 1975; Roodman *et al.*, 1975), it has been suggested that this is the replicative enzyme, whereas polymerase- β may be concerned with DNA repair. For example, when L cells are stimulated to divide, polymerase- α increases, with little change in the activity of polymerase- β (Chang *et al.*, 1973). Similar results have been obtained with induced erythropoiesis in the mouse spleen (Roodman *et al.*, 1975). In addition, investigations with synchronized cell cultures (Spadari & Weissbach, 1974) have shown a positive correlation between the rate of DNA synthesis and the activity of polymerase- α . These findings are in agreement with the preferential increase of myocardial DNA polymerase- α in response to tri-iodothyronine. On the other hand, instances of concordant increase in all three polymerases have also been reported, e.g. in phytohaemagglutinin-stimulated lymphocytes (Mayer *et al.*, 1975). This pattern may indicate synthesis *de novo* of DNA-synthetic enzymes rather than selective stimulation of polymerase- α activity. Whether this is a difference dependent on the tissue rather than the nature or intensity of the stimulus to proliferation is not known.

The tri-iodothyronine-induced enhancement of myocardial DNA polymerase- α in neonatal rats was accompanied by a significant increase in [³H]-thymidine incorporation into DNA. The size of the deoxyribonucleoside triphosphate pool was also increased, with dTTP showing the largest proportional expansion. An expansion in deoxyribonucleoside triphosphate pools is regularly observed after proliferative stimuli (Adams *et al.*, 1971; Bray & Brent, 1972; Skoog *et al.*, 1973). Bray & Brent (1972) found in synchronized HeLa cells that a sharp increase occurred in the pyrimidine deoxynucleotide pools with the onset of or just before DNA replication, whereas the pools of the purine deoxynucleotides began to increase more slowly at this time; a similar pattern was observed in L929 cells (Adams *et al.*, 1971). In synchronized Chinese-hamster cells, Skoog *et al.* (1973) found that the pool sizes of dATP and dGTP increased at the same time as DNA synthesis, whereas dCTP and dTTP pools increased slightly after the onset of DNA synthesis. However, Walters *et al.* (1973) observed, in the same cell system, that the initial rise in pool size preceded DNA synthesis by 0.3–0.6 h for all deoxyribonucleo-

tides. Similar results have been reported in lymphocytes by Tyrsted & Munch-Petersen (1977). The induction of deoxyribonucleoside triphosphate pools may indicate synthesis *de novo* of ribonucleoside diphosphate reductase(s) or its activation by nucleotide effectors when these have reached appropriate concentrations (Riechard, 1978).

Neither [³H]thymidine incorporation nor DNA polymerase activity changed in adult myocardial cells in response to tri-iodothyronine. This difference between 3-week- and 6-month-old rats could not be accounted for by differences in the baseline (unstimulated) DNA polymerase activity. In fact, DNA polymerase- α enhancement in young rats was probably not due to synthesis of the enzyme *de novo*, since it was not affected by either actinomycin D or cycloheximide, both of which markedly inhibited DNA synthesis. A similar lack of dependency on RNA and protein synthesis has been found (Limas, 1979) for thymidine kinase, another enzyme involved in DNA synthesis. Such dissociation between the effects of protein-synthesis inhibitors on DNA synthesis and activities of DNA polymerase or thymidine kinase has been previously noted in other cell systems (Taylor, 1965; Kim *et al.*, 1968; Brown *et al.*, 1970; Searle & Simpson, 1975; Weintraub, 1976; Stimac *et al.*, 1977). Although prior RNA and protein synthesis are required for orderly transition to mitosis, this requirement seems to be related not to the DNA-synthetic enzymes but to specific RNA and protein species. In addition to inhibiting DNA synthesis (by decreasing the rate of DNA-replication fork movement and the frequency of new replicon initiation; Stimac *et al.*, 1977), cycloheximide and other protein-synthesis inhibitors affect the structure of chromatin so that it has decreased nucleosome density and is more sensitive to nuclease attack (Searle & Simpson, 1975; Weintraub, 1976). These effects are a reflection of changes in chromatin-associated protein which affect the functional integrity of the chromatin during the transition to mitosis (Tobey *et al.*, 1975).

Although there was a small difference in myocardial deoxyribonucleoside triphosphate pools between neonatal and adult rats, it is not likely that this alone accounts for the differences in proliferative capacity, since there is little correlation between the size of these pools in quiescent cells and subsequent response to proliferative stimuli (Adams *et al.*, 1966). The failure of these pools to expand in adult cells after tri-iodothyronine, however, would impair their capacity for DNA synthesis.

How are these age-dependent differences in DNA synthesis related to the loss of proliferative capacity? The inhibition by daunomycin and vinblastine of the enhancement by tri-iodothyronine of DNA

synthesis and DNA polymerase- α is relevant in this respect. It suggests that DNA synthesis in response to proliferative stimuli is somehow regulated by the subsequent entry of the cell into mitosis and is prevented by mitotic inhibitors. Similar findings have been previously reported for the erythropoietin-induced stimulation of spleen DNA polymerases (Roodman *et al.*, 1975). The mechanism by which the DNA-synthetic apparatus senses the need for mitosis is not known, but is probably related to changes that have already occurred during the pre-replicative phase when the cell was committed to mitosis (Tobey *et al.*, 1975). In view of the rather low concentrations of inhibitors used in the study, it is unlikely that their effect on DNA synthesis represents non-specific toxicity; this conclusion is reinforced by the preservation of high DNA polymerase- α activity after cycloheximide and actinomycin D. In addition, since tri-iodothyronine-induced cell proliferation is in excess of normal growth requirements, it is improbable that its prevention by the inhibitors would have generalized cellular effects.

The implication that the increase in myocardial DNA-synthetic enzymes in response to tri-iodothyronine should be viewed within the context of a more generalized metabolic adaptation of a cell preparing to undergo mitosis has two corollaries: (a) the inability of the adult myocardial cells to increase the sizes of deoxyribonucleoside triphosphate pools, [^3H]thymidine incorporation and DNA polymerase activity in response to tri-iodothyronine may be a consequence of, rather than a reason for, the age-dependent restriction in proliferative capacity; (b) similarly, it may be argued that the decline in DNA polymerase (and other related enzymes) with age may be secondary to the loss of myocardial-cell mitotic ability rather than an irreversible loss of enzymic activity. Our results suggest that a study of these enzymes in respect to control of myocardial cell proliferation may not be productive. A more fruitful approach might be to examine changes in the structure and function of myocardial chromatin in response to proliferative stimuli. This is reinforced by our finding (Limas & Chan-Stier, 1978) of significant chromatin changes during post-natal myocardial development. A progressive decrease in template activity was associated with decreased susceptibility to deoxyribonuclease digestion and lower polylysine binding. Derivative 'melting' profiles showed diminished 'melting' of DNA regions bound by non-histone proteins, with concomitant increase in histone-bound regions as age advanced. These findings are indicative of a decline in accessibility of DNA for gene transcription which accompanies and perhaps determines the loss of mitotic potential by the myocardial cells.

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