# MURINE TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE: CELLULAR DISTRIBUTION AND RESPONSE TO CORTISONE* ‡

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Terminal deoxynucleotidyl transferase (TdT)[1] is an enzyme which has the unique property of polymerizing deoxyribonucleotides onto a primer in the absence of a template (1–4). Chang (4) first reported that TdT is present only in the thymus among various avian and mammalian tissues examined. Her finding was extended to humans by McCaffrey et al. (5), who found that the enzyme was restricted to the thymus in normal tissues but was found in peripheral blood lymphocytes from a patient with acute lymphoblastic leukemia. The specific association of TdT with the thymus has led to the suggestion that TdT might act as a somatic mutator and could be implicated in the generation of immunological diversity (6).

Since TdT was thought to be restricted to the thymus, its distribution among thymocytes has been a critical problem. At least two major thymocyte subpopulations have been identified in mice and other animals (7–13). The major subpopulation is immunoincompetent, cortisone-sensitive, rich in Thy, TL, $G_{1X}$, and Ly antigens, but bears little or no *H-2* antigen. The minor subpopulation, on the other hand, is immunocompetent, cortisone-resistant, rich in *H-2* but low in Thy and Ly antigens, and carries no detectable TL or $G_{1X}$. Whether the minor subpopulation is derived from the major one is still debatable (12).

In this communication we present evidence for two forms of TdT in murine thymocytes and for their distribution between two thymocyte subpopulations. Also, the occurrence of TdT in murine bone marrow cells is reported.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DTT, dithiothreitol; HBSS, Hanks' balanced salt solution; TdT, terminal deoxynucleotidyl transferase.

## Materials and Methods

*Cell Preparation.* Thymuses removed from 5- to 8-wk old mice (C57BL/6J; Jackson Laboratories, Bar Harbor, Maine) were gently pushed through a stainless steel screen (100 mesh, Cistron Corp., Elmsford, N.Y.) in Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N.Y.). The cell suspension was then filtered through absorbent cotton (Johnson and Johnson, New Brunswick, N.J.). Nucleated cells were counted in 3% acetic acid with a hemocytometer. Cell viability was determined by the exclusion of 0.05% trypan blue.

*Extraction of DNA Polymerases from Tissues and Cells.* Whole thymuses or other tissues were homogenized in a Waring blender at high setting for 2 min in the presence of TEM (0.05 M Tris-HCl, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol) at 4°C. KCl was immediately added to a final concentration of 1.5 M, and Triton X-100 to 0.5%. The homogenate was mixed thoroughly, stirred at 4°C for 2 h, and dialyzed overnight against 100 vol of TEM-50 mM KCl. The dialysate was centrifuged at 10,000 *g* for 10 min followed by another 100,000 *g* centrifugation for 60 min. The final supernate (S-100) was saved for phosphocellulose chromatography.

Where cells were the starting material they were washed repeatedly in HBSS and resuspended in TEM. The resuspended cells were subjected to three cycles of rapid freezing and thawing and thereafter processed in a manner identical to the crude tissue homogenate described above.

*Phosphocellulose Chromatography.* Phosphocellulose (Whatman P-11) was prepared by the method of Burgess (14). The resin was equilibrated with 50 mM KCl in TEMG (TEM-20% glycerol) and a 0.5 x 12 cm column was prepared. In a typical purification, the S-100 supernate (10 ml) from 1–3 $\times$ $10^8$ cells was applied to the column at a flow rate of 0.2 ml/min. After a 10 ml wash with 50 mM KCl in TEMG, the column was eluted with a 60 ml linear gradient of 0.05-1 M KCl in TEMG buffer. Fractions of 0.9 to 1 ml were collected.

*Assay of DNA Polymerases.* All reactions were performed at 37°C for 15–30 min in 0.1 ml with 0.05 M Tris-HCl, pH 8.3. Reaction mixtures contained 30-$\mu$l portions of the phosphocellulose fractions. Templates, primers, and deoxynucleotide triphosphates were obtained from commercial sources (15). Standard mixtures contained one of the following sets of reagents (5): (*a*) For terminal deoxynucleotidyl transferase: oligo(dA), 2 $\mu$g; $MnCl_2$, 0.6 mM; dithiothreitol (DTT), 2 mM; [$^3$H]dGTP, 450 pmoles, 2600 cpm/pmol. (*b*) For DNA polymerase-$\alpha$: poly(dC), 1 $\mu$g; poly(dI), 0.5 $\mu$g; Mg acetate, 4 mM; [$^3$H]dGTP, 450 pmol, 2,600 cpm/pmol. (*c*) For DNA polymerase-$\beta$: poly(dA-dT), 2 $\mu$g; Mg acetate, 6 mM; DTT, 6 mM; [$^3$H]dTTP, 400 pmol, 2,300 cpm/pmol; dATP 20 nmol.

Reactions were terminated and processed as previously described (5). One unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 nmol of deoxynucleotide monophosphate into acid-insoluble material per hour. Enzyme units in a given sample were calculated by integrating the activity in all fractions containing enzyme activity which eluted from phosphocellulose. Specific activity (enzyme units/$10^8$ cells) was computed on the basis of nucleated cells. When enzyme activity was less than four times higher than the background, these fractions were reassayed by increasing the reaction volume or using higher specific activity of [$^3$H]dGTP.

*Discontinuous Bovine Serum Albumin Density Gradients.* Fractionation of mouse thymocytes was carried out by centrifugation on discontinuous bovine serum albumin (BSA) gradients according to Dicke et al. (16). The gradient was prepared by sequentially layering 1-ml portions of BSA solutions, in 2% decreasing concentration from 35 to 19% into a 16 x 125 mm plastic tube (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The solutions of varying concentrations of BSA were prepared by diluting a stock solution of 35% BSA (Fraction V, Sigma Chemical Co., St. Louis, Mo.) in 0.15 M Tris buffer (pH 7.2) with sodium phosphate buffer (0.15 M NaCl, 0.01 M Na phosphate, pH 7.2). The osmolarities of the final solutions were controlled in this way at 300 mOsm/liter. Washed thymocytes (1–4 $\times$ $10^8$ cells) were suspended in 17% BSA before layering this solution on top of the gradient. The gradient was centrifuged at 1,500 *g* for 30 min at 6°C in an International Model PR-J centrifuge using the no. 269 rotor (International Equipment Corp., Needham Hts., Mass.). Cell fractions formed at the interfaces of neighboring BSA solutions were carefully removed with a Pasteur pipet and washed in HBSS before cell count and storage at −70°C. Cell fraction 1 was between 17–19% layers, fraction 2 between 19–21% layers etc.

*Administration of Cortisone.* C57BL/6J mice (4–8 wk old) were injected intraperitonially with cortisone acetate (150 mg/kg body weight, Upjohn Co., Kalamazoo, Mich.). After the period indicated as the time of sampling, the mice were chloroformed, exsanguinated by cardiac puncture, and the thymus removed and suspended in ice-cold HBSS. Contaminating blood vessels and

parathymic lymph nodes were cleaned away from the thymuses. The organs were then processed for the determination of cell number and the assay of DNA polymerases. Control samples were prepared using a sterile standard vehicle (Upjohn).

## Results

*DNA Polymerases of the Thymus.* To characterize the DNA polymerases from thymus, crude thymic extracts in the high salt buffer were prepared and the soluble fractions were chromatographed on a phosphocellulose column. The fractions from the phosphocellulose column were assayed with different template·primers to identify the various DNA polymerases (Fig. 1). DNA polymerase-$\alpha$,

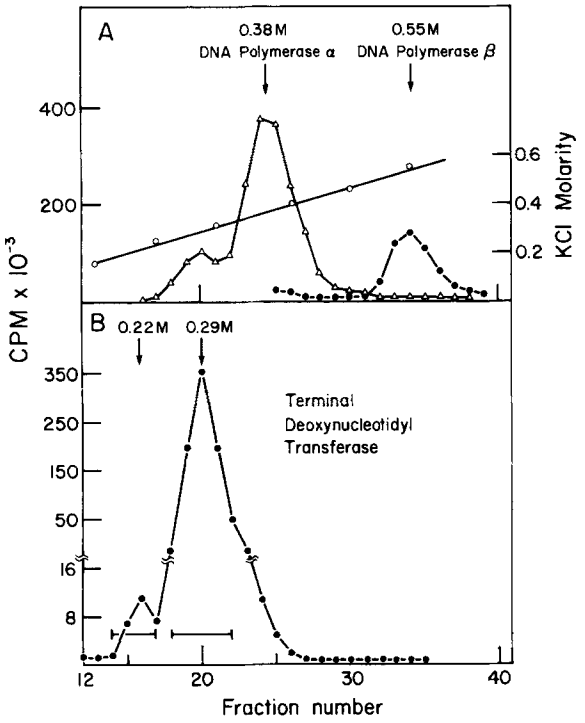


FIG. 1. Phosphocellulose chromatography of DNA polymerases and terminal transferase from 1.5 × $10^9$ thymocytes. (A) Assays of DNA polymerases. Δ, polymerase-$\alpha$ assay; ●, polymerase-$\beta$ assay; O, KCl molarity. (B) Assay of terminal transferase.

which eluted at approximately 0.35 M KCl, was localized using poly(dC)·poly(dI). DNA polymerase-$\beta$ (the nuclear enzyme) was localized using poly (dA-dT) as a template·primer and was found to elute at approximately 0.55 M KCl (Fig. 1 A).[2]

TdT eluted from the column before either of the two replicative DNA polymerases. Two peaks of TdT activity could be detected using oligo(dA) as a primer and dGTP as a substrate (Fig. 1 B). The minor peak, called peak I, eluted

[2] The nomenclature for DNA polymerases used here was agreed to by a number of workers in the field during a recent meeting. Publication of its rationale is planned. DNA polymerase-$\alpha$ was previously called "C" by us while DNA polymerase-$\beta$ was previously called "N" (5).

at 0.22 M KCl while the major peak II eluted at 0.29 M KCl. In various preparations, the elution positions of these two peaks have varied by about 0.02 M KCl but their separation has been constant. When each of the peaks was separately pooled and rechromatographed on a phosphocellulose column, each of them eluted at approximately the same salt concentration as it had originally (Fig. 2). Thus there appeared to be two forms of terminal transferase in murine thymic extracts. Two forms of human TdT have been observed by McCaffrey et al. (17) using similar methodology. In the thymus of both species the later eluting peak is the predominant one with about 3–10% of the total activity appearing in peak I.

To determine how best to assay murine TdT, all four possible oligonucleotide primers and all four possible substrates were varied systematically. The results in

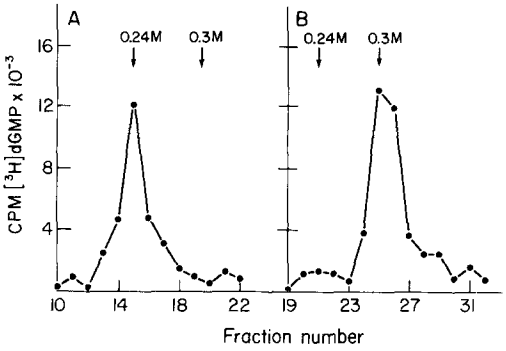


FIG. 2. Rechromatography on phosphocellulose of peak I and peak II terminal transferase. Fractions from the column described in Fig. 1 were pooled, dialyzed against 50 mM KCl-TEMG, and rechromatographed. (A) 3 ml of pooled fractions from 0.22 M peak in Fig. 1 B. (B) 1 ml of pooled fractions from 0.29 M peak in Fig. 1 B.

Table I indicate that for peak II the most active substrate for polymerization under our assay conditions was [³H]dGTP and the most active primer was oligo(dA). Polymerization of dGMP onto any of the four oligomers occurred at a higher rate than the polymerization of any other nucleotide. Similarly, polymerization onto oligo(dA) occurred at a faster rate for all substrates than polymerization onto any other primer. No significant unprimed activity was demonstrable. Results with peak I have been identical. These specificities are similar to those of human TdT (17). The specificities differ markedly from those reported for calf TdT, but the assays in those studies were done under conditions very different from ours (18).

*Tissue Distribution of TdT in Mice.* TdT was originally reported to be restricted to the thymus (4). Recently low activities of TdT have been detected in bone marrow of healthy human subjects (17, 20). Although in mice the thymus has the highest level of TdT of any tissue we have assayed, a low specific activity has also been found in the bone marrow (Table II). Other tissues have no demonstrable activity. The TdT activity in bone marrow elutes from phosphocellulose at the same salt concentration as peak II.

*TdT in Separated Thymocytes.* Suspensions of thymocytes were prepared by

TABLE I

*Substrate and Primer Preferences of Mouse Terminal Transferase Peak II*

| Substrate | Primer | | | | |
|---|---|---|---|---|---|
| | $(dA)_{15}$ | $(dC)_{15}$ | $(dG)_{15}$ | $(dT)_{15}$ | Unprimed |
| dATP | 1.8 | 0 | 1.3 | 0.2 | 0 |
| dCTP | 15 | 0 | 1.7 | 2 | 0 |
| dGTP | 100 | 1.6 | 21.4 | 15 | 0.2 |
| dTTP | 7.5 | 0.4 | 4 | 2 | 0.1 |

Peak fractions from the phosphocellulose column shown in Fig. 2 B were pooled and assayed with concentrations of primer and substrate corresponding to those described for oligo(dA) and dGTP in Materials and Methods. The amount of substrate incorporated in each case was normalized to the value observed for oligo(dA) and dGTP, which is taken as 100%.

TABLE II

*Tissue Distribution of Mouse Terminal Transferase*

| | Enzyme unit/$10^8$ cells |
|---|---|
| Thymus | 1.5 |
| Marrow | 0.05–0.1 |
| Spleen | 0 |
| Lymph nodes | 0 |
| Liver | 0 |
| Brain | 0 |
| Heart | 0 |

Thymocytes ($2 \times 10^8$ cells), bone marrow ($10 \times 10^8$ cells), spleen ($2.2 \times 10^9$ cells), lymph nodes (0.59 g), brain (1.7 g), liver (6 g), and heart (2.5 g) were assayed in these experiments. All tissues were collected from 5- to 7-wk old mice. The limit of the enzyme assay was about 0.02 unit.

gently pushing whole thymuses through a stainless steel screen. More than 98% of the cells were viable after this treatment as judged by their ability to exclude trypan blue and most of the TdT activity was recovered in the cell suspension. The thymocytes were fractionated on a 17–35% discontinuous BSA gradient and nine cell fractions plus a pellet were obtained. The distribution of cells in the various fractions is shown in Fig. 3.

To determine the activity of TdT in each of the cell fractions, extracts were chromatographed on phosphocellulose columns. Fig. 4 A shows the activities of peak I and peak II in each of the thymocyte fractions. Fraction 5, which contained 35% of the total thymocytes, had the highest specific activity of peak II TdT (2.1 U/$10^8$ cells). Both the lighter and heavier fractions had lower activities with minimal activities in fractions, 1, 2, 3, 8, 9, and the pellet.

The distribution of peak I TdT through the BSA gradient was different from that of peak II (Fig. 4 A). The highest specific activities of peak I were found in the upper fractions (fractions 1, 2, 3). Detectable levels of TdT peak I were

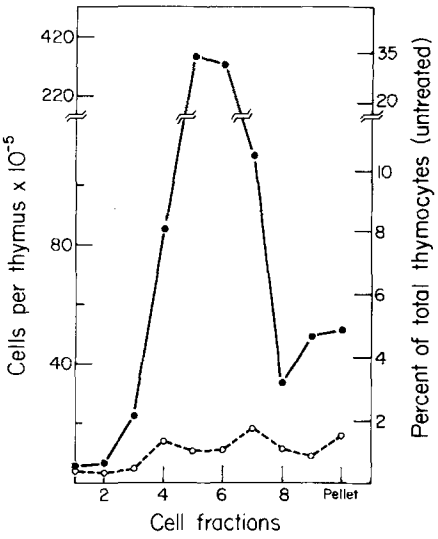


FIG. 3. Fractionation of normal thymocytes and cortisone-resistant thymocytes on BSA gradients. Data are given per thymus, but for normal cells, thymuses from four mice were pooled and for the cortisone-resistant thymocytes, six mice treated for 24 h with cortisone were used. ●, Normal; O, cortisone-treated.

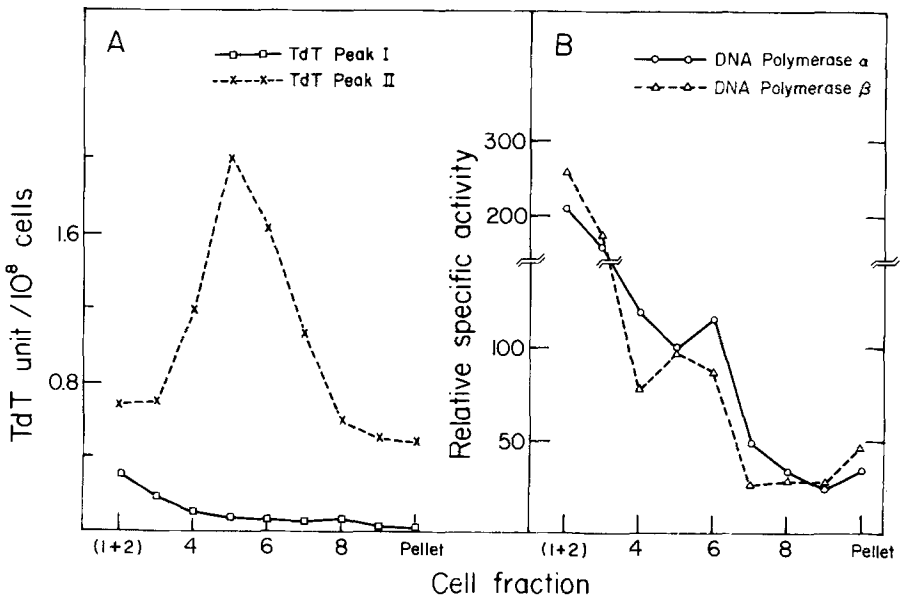


FIG. 4. Specific activities of terminal transferase and DNA polymerases in thymocytes fractionated in a BSA gradient. Values are averages of two to three determinations. For each assay, 0.5–2 × $10^8$ cells were pooled from the same fraction of multiple gradients and assayed. For purposes of comparison, the DNA polymerase values were normalized to fraction 5.

present throughout the rest of the gradient. Thus the lowest density fractions had the highest ratio of peak I to peak II TdT activity.

The amounts of DNA polymerases-$\alpha$ and $\beta$ were also determined in the various fractions from the BSA gradient (Fig. 4 B). Fractions 1, 2, and 3 had the highest specific activities of both DNA polymerases.

*Effect of Cortisone.* To determine whether the cells containing TdT were sensitive or resistant to cortisone, mice were treated with cortisone and their thymuses removed at various times thereafter. Fig. 5 shows that the number of

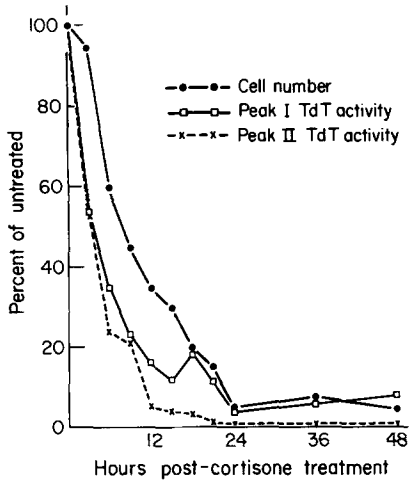


FIG. 5. Effect of cortisone on thymocyte numbers and terminal transferase activities. Each enzyme determination represents assays of 3–6 $\times$ $10^8$ thymocytes pooled from as many animals as necessary. Each point represents the total enzyme activity per animal and is an average of two determinations.

cells per thymus decreased rapidly after cortisone treatment and the levels of peak II TdT activity decreased even more dramatically. Peak I TdT activity also fell, initially at a very rapid rate, but after about 15 h a residual level of peak I activity remained while peak II activity declined to almost immeasurable levels. The elution profiles from phosphocellulose of the TdT activity remaining after 24 and 48 h of cortisone treatment are shown in Fig. 6 A & B. Virtually the only activity demonstrable by 48 h was peak I.

The data on the effect of cortisone on various DNA polymerase activities is plotted in Fig. 7 as activity per $10^8$ cells remaining in the thymus at various times after cortisone treatment. It is evident that the remaining cell populations change their characteristics. First a population deficient in both TdT activities and DNA polymerase-$\alpha$ is selected for but after 12 h of cortisone treatment the cells remaining have increasing levels of peak I TdT and after 36 h they have increasing levels of DNA polymerases-$\alpha$ and $\beta$. Peak II TdT per cell shows a monotonic decline over 48 h. The cell population remaining 48 h after treatment is similar to the cells in fractions 1 and 2 from the BSA gradient except that more TdT peak II is present per cell in the least dense normal cells.

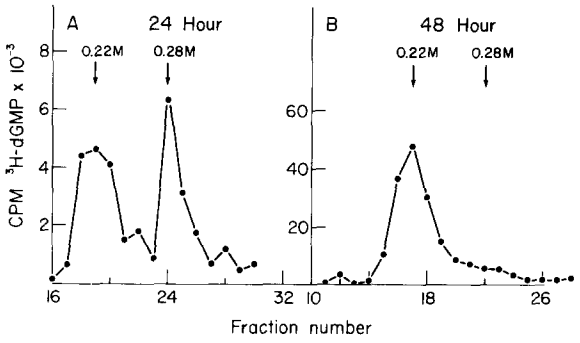


FIG. 6. Phosphocellulose chromatography of terminal transferase remaining in thymocytes after 24 or 48 h of cortisone treatment. For each time point $3 \times 10^8$ cells were analyzed.

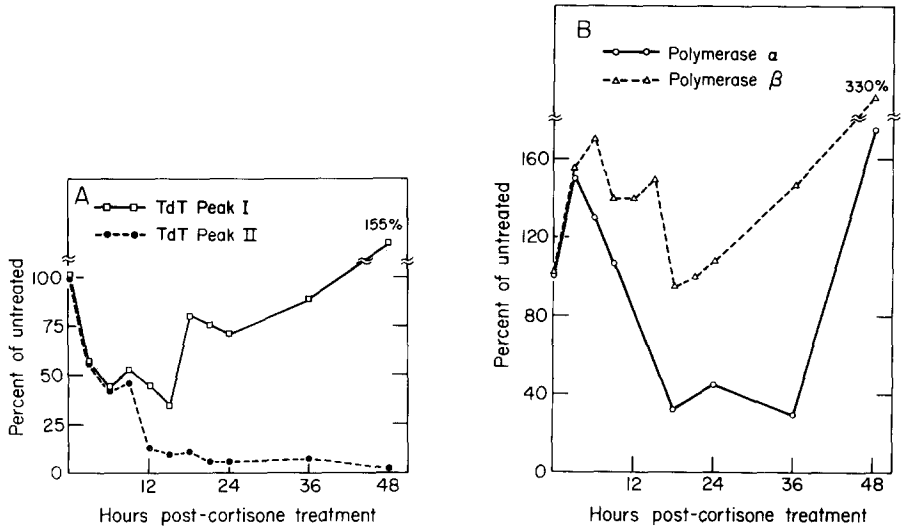


FIG. 7. Terminal transferase and DNA polymerase activities after cortisone treatment expressed as specific activity. Data are recalculated from Fig. 5 as the specific enzyme activity per $10^8$ remaining nucleated cells after various times of cortisone treatment. The values are given as percent of untreated thymocytes.

## Discussion

It is evident that the mouse thymus contains two forms of TdT which are distinguishable both by the salt concentration necessary to elute them from a phosphocellulose column and by their physiology. Peak I TdT shows a higher specific activity in low density thymocytes than in the bulk of the thymocyte population and this form of the enzyme is also more resistant to cortisone treatment than is the major peak II activity (Figs. 4 A, 7 A). This behavior is consistent because cortisone causes a less severe depression to the low density thymocytes than it does to the bulk of the thymic cell population (Fig. 3). Although we have not yet been able to find any other biochemical or physical difference between the two forms of TdT further studies may reveal such distinctions.

In humans (17), rats (19), and mice (present data) the majority of TdT activity is found in the major population of thymocytes which occurs at the center of a BSA gradient. Most of this TdT activity is peak II in both humans and mice. Cells containing the highest density of thymus-specific antigens, such as Thy-1, TL, and Ly, are also localized in the central region of the density gradient (10). Whether these cells are precursor to mature T cells or whether they are dead end cells is unclear (12).

The fraction of cells which contains the highest specific activity of peak I TdT is the same fraction which is cortisone-resistant. Cells with these properties have previously been shown to be immunocompetent (7, 8, 10). However, the light fraction of thymocytes appears to include two cell populations (12). The high level of DNA polymerase-$\alpha$ in these cells along with their higher DNA synthetic activity (9) suggests that cells which are rapidly multiplying are found in this region of the gradient. From the relatively small effect of cortisone on DNA polymerase-$\alpha$ activity (Fig. 7 B), it would appear that these dividing cells are resistant to steroid treatment. The light fraction of cells also includes cells which are immunocompetent, have low amounts of certain thymus-specific antigens, and are cortisone-resistant (10). Whether one or both of the two cell populations in the light region of the BSA gradients contain TdT can only be guessed at present. An attractive hypothesis is that TdT peak I is the initially synthesized form of the enzyme and is active in the cortical, dividing cells while peak II is a derivative form of the enzyme found mainly in the bulk population of denser, cortisone-sensitive and $TL^+$ thymocytes which may be destined for death. Alternatively, TdT peak I could be restricted to the immunocompetent thymocytes rather than to the dividing cells but, in that case, the enzyme must be lost when the immunocompetent cells leave the thymus.

The observation of cells bearing TdT in the mouse bone marrow is in agreement with recent human studies (17, 20). Are these stem cells destined for the thymus, or might they instead be B-cell precursors? Further investigation of this cell population will be necessary to answer this question.

From the foregoing discussion it is evident that TdT may be valuable as a biochemical marker for studying the differentiation sequence of T cells. The role of TdT in the physiology of the thymus and bone marrow may become more evident when the particular cells containing the various forms of the enzyme are better understood.

## Summary

The mouse thymus contains two forms of terminal deoxynucleotidyl transferase (TdT) which are distinguishable by the salt concentration necessary to elute them from a phosphocellulose column, by their distribution among the thymocyte subpopulations, and by their sensitivity to cortisone treatment. In the whole thymus the later eluting peak (peak II) is the predominant one with about 3–10% of the total activity appearing in peak I. Both peak I and peak II activities are most sensitively assayed by the polymerization of dGMP onto an oligo(dA) primer. The minor population of thymocytes which is less dense and cortisone-resistant contains a higher specific activity of peak I TdT. The majority of TdT activity is, however, found in the major population of thymocytes which occurs in the center region of a bovine serum albumin gradient and is cortisone-sensitive. A very low level of an activity indistinguishable from peak II TdT activity is also detected in the mouse bone marrow. Other tissues, such as spleen, liver, heart, and brain lack detectable amounts of TdT activity.

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