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Incorporation of ^{3}H-Labeled Nucleosides and ^{3}H-Labeled Deoxynucleosides into Detergent Soluble DNA (42127)

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Abstract. Detergent soluble DNA from splenocytes of immunologically stimulated mice has been shown to incorporate [^{3}H]dThd more rapidly than detergent insoluble DNA. In this report we compare the incorporation of other ^{3}H-labeled nucleosides and ^{3}H-labeled deoxynucleosides and the distribution of ^{3}H in the different size classes of detergent soluble DNA. The order of incorporation into DS DNA is [^{3}H]dThd > [^{3}H]dCyd > [^{3}H]Ado > [^{3}H]dGuo ≈ [^{3}H]Cyd > [^{3}H]dAdo > [^{3}H]Guo. We also show that the previously reported slight enrichment in Gua + Cyt content is not due to preferential incorporation of dGuo or of dCyd into any one size class.

Detergent soluble (DS) DNA from splenocytes of immunologically activated mice is metabolically active in the sense that it incorporates [^{3}H]dThd[2] more rapidly than the DNA of bulk chromatin (1–3). It is nonmitochondrial in origin as shown by Southern blots using mouse mitochondrial DNA as the probe. Accordingly, the metabolic activity is inhibited by aphidicolin and hydroxyurea, two inhibitors of nuclear replication, but not by ethidium bromide which inhibits mitochondrial DNA synthesis (1, 2).

DS DNA, which is slightly enriched in its Gua + Cyt content in comparison with detergent insoluble (DI) DNA, is size heterogeneous with discrete classes between 200 and 5000 base pairs in length. [^{3}H]dThd first appears in oligonucleotides 20–100 base pairs long. As the [^{3}H]dThd in the oligonucleotides diminishes, radiolabel appears in the larger size classes. All size classes ≥ 200 base pairs appear to label simultaneously so that no progression is seen from smaller to larger fragments. At the same time, degradation from larger to smaller fragments is also not seen.

Three reports of pulse labeled low-molecular-weight DNA fragments in eucaryotic cells have appeared recently. In the first study, lymphocytes of patients with systemic lupus erythematosis were shown to contain low-molecular-weight DNA fragments enriched in Gua + Cyt (4). In the second report, HeLa cells accumulated replication-associated low-molecular-weight DNA after treatment with interferon. Accumulation was associated with enhanced [^{3}H]dThd but not [^{3}H]dGuo incorporation (5). In the third report, Chinese hamster cells were shown to incorporate both [^{3}H]dThd and [5-^{3}H]dCyd into oligonucleotide fragments of various sizes (6).

In this study we examined the efficiency of incorporation of various ribo- and deoxyribonucleosides into DS and DI DNA. As we were aware that the specific activity of [^{3}H]dThd was not constant in the different size classes of DS DNA, we were also interested in whether some size classes might incorporate certain nucleosides preferentially.

Methods. *Preparation of cells.* Nonadherent spleen cells from concanavalin A (Con A)-stimulated, male outbred Swiss mice (Sendai virus free, CD-1, 4–8 weeks old, from Charles River Breeding Farms, Wilmington, Mass.) were prepared as described previously (1, 2, 7). Stimulated mice had been injected intravenously with 250 μg Con A 24–48 hr prior to sacrifice. Under these conditions the spleens became enlarged, numerous mitotic figures were observed (8), and dThd transport was pronounced (7, 9).

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[2] Abbreviations used: Adenine, Ade; adenosine, Ado; deoxyadenosine, dAdo; cytosine, Cyt; cytidine, Cyd; deoxycytidine, dCyd; disodium ethylene diaminetetraacetic acid, Na_2 EDTA; guanine, Gua; guanosine, Guo; deoxyguanosine, dGuo; hypoxanthine, HX; inosine, Ino; Nonidet-P40, NP-40; thymine, Thy; thymidine, dThd.

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Chemicals were obtained from the sources previously listed (1, 2). Radiolabeled compounds were obtained from Amersham Corp., (Arlington Heights, Ill.), or New England Nuclear Corp., (Boston, Mass.). The specific activities of the individual ribo- and deoxyribonucleosides were as follows: [2,8-^{3}H]Ado, 33 Ci/mmole; [1′,2′,2,8-^{3}H]dAdo, 76 Ci/mmole; [5′-^{3}H]Guo, 25 Ci/mmole; [1′,2′-^{3}H]dGuo, 30 Ci/mmole; [5-^{3}H]Cyd, 20 Ci/mmole; [1′,2′,5-^{3}H]dCyd, 62 Ci/mmole; [*methyl*-^{3}H]dThd, 88 Ci/mmole. Deoxycoformycin was the kind gift of Dr. Linda Thompson.

Preparation and analysis of DS DNA. Cells were incubated for 2 hr at 37°C with ^{3}H-labeled nucleoside (6 μCi/ml) and washed as described earlier (1). For [^{3}H]Ado and [^{3}H]dAdo and some [^{3}H]Guo and [^{3}H]dGuo experiments, deoxycoformycin, a tight binding inhibitor of adenosine deaminase (10, 11), was added to a final concentration of 10 μM $\frac{1}{2}$ hr before the addition of isotope. Cells were lysed with chilled 0.5% Nonidet-P40 (NP-40) containing 25 mM Na_2 EDTA followed immediately by rapid separation into DS and DI fractions by microfuge centrifugation at 4°C. DNA was purified from the DS fraction as described earlier (1, 2) and analyzed by means of agarose gel electrophoresis under either native or denaturing (30 mM NaOH) conditions. DNA in the DI fraction was solubilized in 1% sodium dodecyl sulfate in 0.1 mM Tris · HCl (pH 8) containing 0.1 mM EDTA (pH 8) and 8.0 M urea. The distribution of tritium in agarose gels was determined by cutting the lane into 2-mm-wide slices. Each slice was dissolved as described earlier (1, 2) and counted by scintillation spectrometry.

Metabolism of ^{3}H-labeled nucleosides. Aliquots of the NP-40 soluble fractions were spotted onto Whatman 3MM paper along with appropriate standards including Ade, Ado, ATP, dAdo, dATP, Ino, IMP, and HX; dThd, TTP, and Thy; Cyt, Cyd, CTP, dCyd, and dCMP; Gua, Guo, GTP, dGuo, and dGTP. Ascending chromatography was performed using one of several systems described by Fink *et al.* (12). The most useful system consisted of *t*-butyl alcohol, methyl ethyl ketone, water, ammonium hydroxide (40:30:20:10), which was able to separate ribo- from deoxyribonucleotides. Before the NP-40 supernate was spotted on the paper, it was treated with 1 U/ml bacterial alkaline phosphatase (BAP) in 0.05 M Tris · HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine-hydrochloride for $1\frac{1}{2}$–4 hr. BAP removed 5′-phosphates so that nucleotides were converted to nucleosides. Longer exposure or higher concentrations of enzyme did not alter the results except to increase the percentage of [^{3}H]HX recovered from [^{3}H]Ado and [^{3}H]dAdo. Even though the cells were pretreated for 30 min with 10 μM deoxycoformycin, some 10% of [^{3}H]Ado and [^{3}H]dAdo was obtained as HX or Ino. These metabolites may have reflected the activity of adenylate deaminase or that a longer preincubation with deoxycoformycin was required to completely inhibit adenosine deaminase.

Results. The accumulation of the various ^{3}H-labeled ribonucleosides and ^{3}H-labeled deoxynucleosides into the DS and DI fractions is shown in Table I. Accumulation in the DS fraction reflects the net balance among a variety of processes including transport across the plasma membrane (efflux as well as influx), metabolism, and incorporation into macromolecules. Accumulation in the pellet reflects incorporation into both RNA and DNA. [^{3}H]Ado was accumulated in the detergent lysate to a greater extent than other ^{3}H-labeled nucleosides and ^{3}H-labeled deoxynucleosides. [^{3}H]dThd, [^{3}H]dCyd, and [^{3}H]Ado were the most efficient precursors for DS DNA as shown by the amount of radiolabeled precursor incorporated into purified DS DNA. We presumed that incorporation of ^{3}H-labeled ribonucleosides reflected conversion to ^{3}H-labeled deoxyribonucleosides since about 2% of each ^{3}H-labeled ribonucleoside was recovered as the ^{3}H-labeled deoxyribonucleoside. Also the preparation of DS DNA included RNase treatment and there is no evidence for the presence of extensive ribonucleotide linkage in DS DNA (1). A greater percentage of each ^{3}H-labeled deoxyribonucleoside (20–47%) as opposed to each ^{3}H-labeled ribonucleoside (4–7%) was found in DS DNA in comparison to the DI fraction. In two other experiments in this series, the percentage of [^{3}H]dThd incorporated into DS DNA was 35 and 27%.

TABLE I. ACCUMULATION OF ^{3}H-LABELED DEOXY- AND RIBONUCLEOSIDES IN THE NP40 SUPERNATE AND THEIR INCORPORATION INTO PURIFIED DS DNA

Nucleoside	A Amount accumulated in supernate fraction (dpm × 10^{-6}/ 5×10^7 cells)	B Amount incorporated into DS DNA (dpm × 10^{-4}/ 5×10^7 cells)	C Amount in pellet fraction (dpm × 10^{-4}/ 5×10^7 cells)	D Specific activity of starting compound (Ci/mmole)
Pyrimidines				
Thymidine	1.9	66 (44)[a]	73.4	88
Cytidine	5.4	3.8 (4)	77.9	30
Deoxycytidine	2.2	20 (20)	79.3	62
Purines				
Adenosine	46.9	13 (7)	167.4	33
Deoxyadenosine	2.5	1.4 (20)	5.6	76
Guanosine	5.3	0.6 (4)	12.3	25
Deoxyguanosine	2.6	3.9 (40)	5.7	30

Note. Cells were incubated with the indicated ^{3}H-labeled nucleoside as described in the legend to Fig. 1, washed, and lysed with NP-40. The dpm accumulated in the supernate and pellet fractions are shown in Columns A and C, respectively. The amount of ^{3}H-labeled nucleoside incorporated into purified DS DNA from the supernate fraction is shown in Column B. The specific activity of the starting compound is shown in Column D. The percentages in parentheses in Column B refer to the relative amounts of precursor incorporated into DS DNA in comparison with the total incorporation into DS DNA as well as that contained in the pellet.

The distribution of ^{3}H-labeled deoxy- and ribonucleosides incorporated into the different size classes of DS DNA is shown in Figs. 1–3. The incorporation patterns were similar to each other and to those published previously (1–3). The incorporation patterns for [^{3}H]Guo and [^{3}H]dGuo data (not shown) were also similar. Therefore, no size class incorporated any nucleoside to the exclusion of the others. In two cases, a size class appeared enriched in a particular deoxynucleoside: [^{3}H]dAdo was preferentially incorporated into very large fragments (6 KB or longer), while [^{3}H]Ado was enriched in the oligonucleotide region (1). Therefore, on the basis of these and other data, enrichment of dGuo or dCyd was not a characteristic of some size classes over others.

Discussion. The metabolism of ribonucleosides and deoxynucleosides in peripheral and stimulated mammalian lymphocytes has been the topic of considerable interest. The metabolic pathways are intimately interrelated either directly or through indirect regulatory mechanisms at the levels of biosynthesis and salvage (13, 14). They are also interrelated at the level of macromolecular biosynthesis. For instance, in calf thymus, cultured human lymphoblasts (MOLT-4F), and Novikoff rat hepatoma cells an excess of dATP inhibits reduction of CDP, UDP, ADP, and GDP by ribonucleotide reductase (EC 1.17.4.1) so that DNA synthesis is ultimately inhibited (13). The inhibitory effects of dGTP are more complex, depending on whether ADP is present. An excess of dTTP results in inhibition of CDP and UDP reduction and stimulation of GDP reduction. Moreover, the entire balance depends on adequate levels of intracellular glutamine which can control cell proliferation by acting as a precursor to *de novo* synthesis of nucleosides (15, 16). In recent years the role of an imbalance in Ado metabolites in lymphocytes from patients with severe combined immunodeficiency disease has served to focus the discussion away from the broader view.

In this study we examined the ability of splenocytes from activated mice to incorporate various ^{3}H-labeled ribo- and deoxyribonucleosides into DS DNA. It should be stressed at the outset, however, that isolation of DS DNA does *not* require incorporation of a tritiated precursor. Indeed, large amounts of DS DNA are routinely prepared in bulk directly from unlabeled cells. [^{3}H]dThd was incorporated to a greater extent than other substrates tested. Usually, the efficient incor-

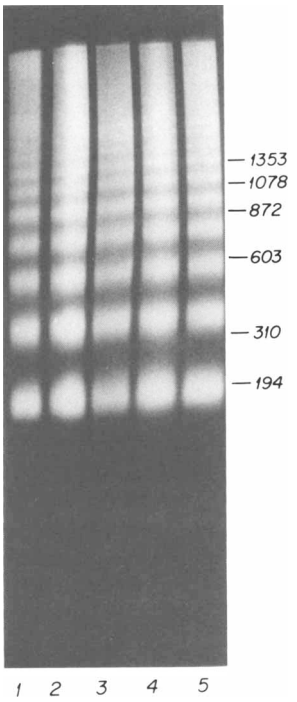

FIG. 1. Gel electrophoretogram of DS DNA. Splenocytes (5×10^6/ml, total 10 ml) were incubated for 2 hr with the indicated ^{3}H-labeled nucleoside at 6 μCi/ml, washed, and lysed with NP-40. DNA, isolated from the DS fraction, was dissolved in 25 μl Tris–HCl (pH 8.0) and analyzed by electrophoresis under native conditions using 1.2% agarose. After staining the gel with ethidium bromide, the individual lanes were cut and distribution of incorporated radioactivity determined by scintillation spectrometry. This figure represents the ethidium bromide staining patterns for Figs. 2 and 3. Lane 1, [^{3}H]thymidine; lane 2, [^{3}H]cytidine; lane 3, [^{3}H]deoxycytidine; lane 4, [^{3}H]adenosine in the presence of deoxycoformycin; lane 5, [^{3}H]deoxyadenosine in the presence of deoxycoformycin. The amount and distribution of DNA in the different size classes are unaltered.

poration of [^{3}H]dThd is ascribed to low intracellular concentrations of dTTP in comparison to other deoxynucleotides. Calculating the actual amount of precursor incorporated into DS DNA requires thorough knowledge of the concentration of individual metabolites and the flux rate through each pathway as well as the initial exogenous specific activities. While we were able to calculate the percentage distribution of various ^{3}H-labeled metabolites, the intracellular amounts were not measured in this study. In other studies, however, the intracellular levels

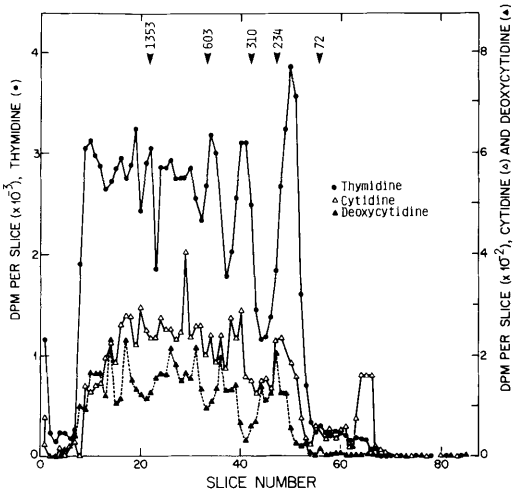

FIG. 2. Incorporation of [^{3}H]dThd, [^{3}H]Cyd, and [^{3}H]dCyd into detergent soluble DNA. Splenocytes were incubated in the presence of [^{3}H]dThd, [^{3}H]Cyd, or [^{3}H]dCyd and DS DNA was prepared and analyzed as described in the legend to Fig. 1. After being photographed (see Fig. 1), the gel was sliced and the distribution of incorporated ^{3}H was determined by scintillation spectrometry.

of dATP and dTTP were shown to be similar under a variety of conditions (17). In peripheral blood lymphocytes, on the other hand, the intracellular levels of dGTP, dCTP, and dTTP were similar and about one-eighth that of dATP, while in thymocytes dATP and

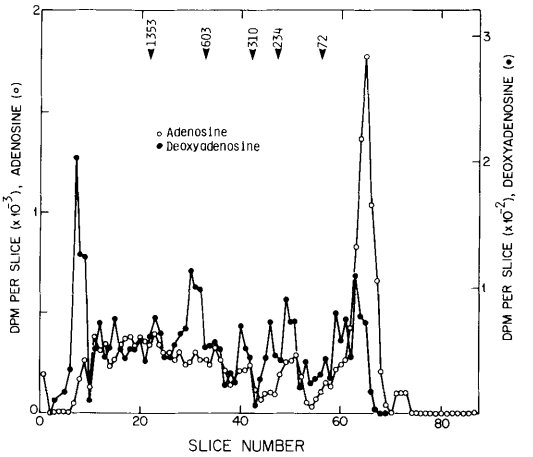

FIG. 3. Incorporation of [^{3}H]Ado and [^{3}H]dAdo. Splenocytes were incubated in the presence of deoxycoformycin with [^{3}H]Ado or [^{3}H]dAdo and DS DNA was prepared and analyzed as described in the legend to Fig. 2. See Fig. 1 for ethidium bromide staining patterns.

dGTP levels were well below those of dTTP (18). Thus, the assumption regarding intracellular pools does not necessarily hold.

All the incorporated precursors became distributed within all the different size classes of DS DNA. In only two cases did a ^{3}H precursor appear enriched in any size class. In particular, [^{3}H]dAdo was preferentially incorporated into the largest size class, and [^{3}H]Ado was enriched in the oligonucleotide region (1). Therefore, the slight enrichment of Gua + Cyt in DS DNA from murine splenocytes is not due to selective incorporation into any one size class.

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