Walter, P., Peatkau, V., and Lardy, H. A. (1966), *J. Biol. Chem. 241*, 2523.
Weber, G., Glazer, R. I., and Ross, R. A. (1970), *Advan. Enzyme Regul. 8*, 13.
Wollenberger, A., Ristau, O., and Schoffa, G. (1960), *Pfluegers Arch. Gesamte Physiol. Menschen Tiere 270*, 399.

Polyamine Accumulation During Lymphocyte Transformation and Its Relation to the Synthesis, Processing, and Accumulation of Ribonucleic Acid†

Robert H. Fillingame‡ and David R. Morris*

ABSTRACT: Large increases in the cellular levels of putrescine, spermidine, and spermine, as well as elevations of cellular RNA and protein, were found during concanavalin A-induced transformation of small lymphocytes isolated from bovine lymph nodes. The magnitude of these changes was: putrescine, 10–15-fold; spermidine, 5–7-fold; spermine, 2–2.5-fold; RNA, 3–5-fold; and protein, 2–3-fold. A careful analysis of the initial sequence of changes showed that the cellular RNA level was elevated within 6 hr of lymphocyte stimulation, whereas putrescine did not increase significantly until 8 hr, and elevations in the spermidine and spermine level were not observed until 10 hr.

Methylglyoxal bis(guanylhydrazone), a potent inhibitor of *S*-adenosyl-L-methionine decarboxylase, completely prevented intracellular accumulation of spermidine and spermine in cultures of transforming lymphocytes. No abnormalities in synthesis, processing, and accumulation of RNA were observed in these cultures. RNA accumulation was unchanged over a 40-hr interval and the absolute rate of RNA synthesis, estimated by measuring the incorporation of [8-^{3}H]adenosine into RNA and the specific activity of the ATP pool, doubled after 20 hr of concanavalin A treatment in the presence or absence of spermidine and spermine accumulation. All classes of RNA were synthesized (in the same proportion as in controls) in the polyamine-deficient cultures and the rate of processing of rRNA precursors was not altered. These results indicate that increases in the spermidine and/or spermine levels are not essential in mediating the large increases in cellular RNA observed during lymphocyte transformation.

The aliphatic polyamines spermidine and spermine, and their precursor putrescine, have been implicated in numerous growth processes (reviewed by Cohen, 1971). In the time since parallel increases in spermidine and RNA were first reported by Raina (1963) in developing chick embryo and subsequently by Dykstra and Herbst (1965) in regenerating rat liver, hypotheses casually relating polyamine levels and RNA metabolism have been frequently advocated. For example, it has been suggested that polyamines may serve a role in the regulation of RNA metabolism in eukaryotes (Caldarera *et al.*, 1965; Russell, 1970; Raina and Jänne, 1970; Russell and Lombardini, 1971), and it has recently been proposed that polyamines are specific regulators of rRNA synthesis (Russell and McVicker, 1973). In contrasting proposals, polyamines were suggested to increase in parallel with RNA in order to neutralize a fraction of the RNA phosphate residues and stabilize the RNA (Raina *et al.*, 1966; Raina and Jänne, 1968). Unfortunately, even in the most thoroughly studied system, regenerating liver, it is not yet clear whether spermidine accumulation precedes or occurs simultaneously with RNA accumulation (Dykstra and Herbst, 1965; Raina *et al.*, 1966; Raina *et al.*, 1970; Russell *et al.*, 1970; Russell and Lombardini, 1971). Obviously, a definite answer to this question is critical in formulating models for polyamine action, since polyamines should increase prior to RNA if they are serving a regulatory role.

Small lymphocytes, stimulated to transform by concanavalin A,[1] present an excellent system in which to define the relationship between polyamine and nucleic acid accumulation. Most small lymphocytes are normally quiescent both *in vivo* and in culture (Nowell, 1960; Robbins, 1964). However, upon stimulation *in vitro* by Con A or phytohemagglutinin (*Phaseolus vulgaris*), lymphocytes are transformed into large blast cells capable of division (Robbins, 1964; Ling, 1968; Powell and Leon, 1970; Novogradsky and Katchalski, 1971). Among the early events in lymphocyte transformation are large increases in the rate of uptake of radioactive precursors and their incorporation into RNA and protein (Kay and Korner, 1966; Kay, 1968; Lucas, 1967; Hausen *et al.*, 1969). This is followed by net RNA and protein accumulation at later times (Hausen *et al.*, 1969; Fisher and Mueller, 1969; Forsdyke, 1967). DNA synthesis begins approximately 24 hr after stimulation (Powell and Leon, 1970; Novogradsky and Katchalski, 1971; Loeb and Agarwal, 1971) and the cells begin dividing on the second and third days of culture (Bender and Prescott, 1962).

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received May 21, 1973*. This work was supported by a research grant from the National Institute of General Medical Sciences (GM 13957), the University of Washington institutional grant from the American Cancer Society, and the University of Washington Graduate School Initiative 171 Funds.

‡ Supported by Training Grant GM 00052 from the National Institutes of Health.

[1] Abbreviations used are: ConA, concanavalin A; MGBG, methylglyoxal bis(guanylhydrazone).

We have utilized this cellular system to examine first the temporal relationship between polyamine accumulation and changes in RNA metabolism, and second the effect of an inhibitor of spermidine and spermine synthesis, methylglyoxal bis(guanylhydrazone) (Williams-Ashman and Schenone, 1972), on the accumulation, synthesis, and processing of RNA. Portions of this work have been presented in preliminary form (Fillingame and Morris, 1973a,b).

Experimental Section

Materials. The culture medium used in these experiments was 90% RPMI 1640 (Grand Island Biological Co.) and 10% newborn calf serum (Microbiological Associates, Inc., or Flow Laboratories, Inc.). The following antibiotics were added to the medium: penicillin (100 units/ml), streptomycin sulfate (50 μg/ml), and amphotericin B (3.2 μg/ml). Con A that was crystallized three times was obtained from Research Division of Miles Laboratories, Inc., as a lyophilized powder, and was dissolved in 0.137 M NaCl and buffered with 0.22 mM phosphate at pH 7.0. The medium and all solutions added to cultures were sterilized by filtration through a 0.45 μ MF-Millipore filter. Methylglyoxal bis(guanylhydrazone) dihydrochloride monohydrate was obtained from Aldrich Chemical Co. Iproniazid phosphate was purchased from Sigma Chemical Co. These compounds were dissolved in phosphate-buffered saline, neutralized with NaOH, and sterilized by filtration. The phosphate-buffered saline used in these experiments was Puck's saline G (Puck *et al.*, 1958). Other materials and chemicals were used directly as obtained from commercial sources.

Cell Culture. Two suprapharyngeal lymph nodes were removed from the head of a freshly slaughtered cow, freed of surrounding tissue and fat, and placed in ice-cooled culture medium during transport to the laboratory. The small lymphocytes were purified essentially as described by Hausen *et al.* (1969) using standard asceptic procedures. The final yield was 1–2 $\times 10^{10}$ small lymphocytes from two lymph nodes. Prescription bottles (32 oz), containing 100 ml of medium which had been equilibrated overnight with 5% CO_2–95% air at 37°, were inoculated with 3×10^8 purified lymphocytes. The bottles were placed on their broad side in a tissue-culture incubator and agitated as little as possible throughout the course of the experiment. A water-saturated atmosphere of 5% CO_2–95% air was maintained to keep the medium at pH 7.2. The depth of the medium was 0.7 cm; at greater depths the transformation-associated increases in DNA and RNA were reduced. The cultures were preincubated for 24 hr prior to addition of ConA at a final concentration of 12 μg/ml. ConA concentrations of 10–15 μg/ml were shown to result in optimal accumulation of RNA and DNA and in maximal incorporation of nucleic acid precursors. The optimal dose of ConA was independent of the lymphocyte concentration.

Polyamine Analysis. Lymphocytes were collected from culture by centrifugation at 300*g* for 10 min. The cellular pellet was extracted twice with 10 ml of ice-cold 5% trichloroacetic acid. The first extraction contained more than 95% of the cellular polyamines. The trichloroacetic acid supernatant solution containing the polyamines and the precipitate, under absolute ethanol, was stored at −20° for periods up to a month.

For polyamine analysis, the trichloroacetic acid supernatant solution was extracted three times with two volumes of anhydrous ether and the aqueous solution lyophilized. The dried powder was redissolved and analyzed for polyamines (Morris *et al.*, 1969).

In order to check for leakage of polyamines during harvesting of the cells, cultures were made 5 mM in iproniazid phosphate (neutralized with NaOH) 15 min prior to harvest. The preincubation with iproniazid was required to inhibit a potent spermine (spermidine) oxidase present in the calf serum of the medium (Tabor *et al.*, 1954). Oxidation of spermine and spermidine was inhibited by 85% and greater than 95%, respectively. The medium (100 ml) was cooled on ice and filtered through a 3.0 μ MF-Millipore filter. The serum proteins in the medium were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The supernatant solution was extracted three times with two volumes of anhydrous ether and evaporated to dryness. The dried medium was taken up in 5 ml of H_2O and adjusted to pH 13 with 50% NaOH and the polyamines were extracted into 5 ml of 1-butanol (Pohjanpelto and Raina, 1972). The butanol phase was evaporated to dryness after adding 0.4 ml of 5 N HCl, taken up in several milliliters of water and applied to a 7-ml Bio-Rad AG-50 column. The column was washed with 70 ml of 1 N HCl after which the polyamines were eluted with 50 ml of 5 N HCl. The 5 N HCl fraction was evaporated to dryness, resuspended in 0.23 M pyridine acetate buffer (pH 5.7), and analyzed for polyamines as previously described (Morris *et al.*, 1969). When polyamines were added to culture medium at 4° and the solution was acidified immediately by the addition of trichloroacetic acid, the recovery was 75% for putrescine and spermidine and 60% for spermine.

Nucleic Acid and Protein Analyses. The trichloroacetic acid precipitate (obtained after polyamine extraction of the cells) was subjected to base hydrolysis and the acid-soluble material was assayed for RNA using the orcinol method (Schneider, 1957). Adenosine was used as a standard and the values were converted to micrograms of RNA using the factor 0.57 μg of RNA/nmol of adenosine. For DNA analysis, the acid-insoluble material remaining after base hydrolysis was heated in 5% Cl_3CCOOH at 90° for 20 min and, after centrifugation, the supernatant solution was assayed for DNA by the diphenylamine reaction (Burton, 1956). Calf thymus DNA (Calbiochem, A grade) was used as a standard.

When the rate of thymidine incorporation into DNA was determined, a 2-ml sample from a 100-ml culture was labeled with 5.0 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) for 2 hr. The radioactive cellular pellet was stored under ethanol and later treated as described above for nucleic acid analysis. A portion of the DNA hydrolysate was counted in a dioxane scintillation fluid containing 100 g of naphthalene and 5 g of 2,5-diphenyloxazole/l. The DNA content of the solutions was determined as described above.

Protein analyses were done on samples that were removed from the cultures prior to harvesting.[2] The cells were washed once with saline G, resuspended in 5% trichloroacetic acid, and stored at −20°. After thawing, the suspension was heated at 90° for 20 min and centrifuged and the pellet was washed once with absolute ethanol and twice with anhydrous ether and dissolved in 0.5 N NaOH. Protein was determined (Bailey, 1967) using crystalline bovine serum albumin (Miles) as a standard.

Rate of RNA Synthesis. The rate of RNA synthesis was

[2] This was necessary since washing the cellular pellet (used for polyamine determinations) with phosphate-buffered saline resulted in some leakage of the polyamines into the saline. A saline wash of the cells for protein analysis is necessary to remove serum proteins.

quantitatively determined by measuring incorporation of [8-^{3}H]adenosine (18.3 Ci/mmol; Schwartz) into RNA and the specific radioactivity of the ATP pool. The procedure of Emerson and Humphreys (1971) was modified as follows: 10-ml cultures containing 3×10^7 cells were labeled with 20 μCi of [8-^{3}H]adenosine for various periods (15–180 min) at 37°. The cultures were centrifuged at 200*g* during the last 5 min of the labeling period (also at 37°). The medium was removed and the cells were immediately suspended and homogenized in 2 ml of ice-cold 0.5 N $HClO_4$. After centrifugation of the acid precipitate, nucleotides in the acid-soluble fraction were adsorbed to Norit A, washed, and eluted (Emerson and Humphreys, 1971). After evaporation, the residue was taken up in a small volume of water and applied to cellulose-PEI (poly(ethylenimine)) thin-layer plates, Baker-flex, from Baker Chemical Co. The plates were developed once with water and then with 1 M LiCl in 1 M acetic acid. ATP ran with an R_F of 0.19 as compared with values of 0.07 (GTP), 0.32 (GDP), and 0.58 (ADP). The ATP region was cut out and eluted by incubation in 4 M NH_4OH for 30 min. The eluate was evaporated to dryness and dissolved in water. One portion of this fraction was counted in toluene–Bio-Solv (Beckman) (9:1) containing 5 g of 2,5-diphenyloxazole/l. ATP was quantitatively determined with luciferase (Sigma FLE-250) as described by Emerson and Humphreys (1971). Three samples of varying size (containing 1–10 pmol) were assayed, usually with an average deviation of 5% or less.

The acid-soluble material (*vide supra*) was washed five times with 0.5 N $HClO_4$ and then hydrolyzed in 0.3 N KOH at 37° for 1 hr. A sample of the hydrolysate was mixed with an equal volume of 1.3 N $HClO_4$ and centrifuged. The supernatant solution was counted in dioxane scintillation fluid containing 100 g of naphthalene and 5 g of 2,5-diphenyloxazole/l. The DNA content of the acid-soluble pellet was determined as described above.

The label from [8-^{3}H]adenosine was incorporated into RNA as both AMP and GMP. The proportion incorporated as AMP was determined after complete alkaline hydrolysis (16 hr in 0.3 N KOH at 37°) and separation of the nucleoside monophosphates on PEI-cellulose using the same developing procedure as for ATP. The R_F values were 0.74 (AMP) and 0.63 (GMP). The AMP and GMP areas were cut out and eluted with 0.4 ml of 2 M NH_4OH for 20 min in scintillation vials. Toluene–Bio-Solv scintillation fluid (15 ml) was added and the vials were counted. Tritium in the 8 position of AMP or GMP exchanges at different rates in dilute base (Brandhorst and Humphreys, 1971). The rate of exchange was determined by hydrolyzing the RNA for 16, 28, or 40 hr (in 0.3 N KOH at 37°) and then determining the radioactivity remaining in AMP or GMP. This exchange followed first-order kinetics and showed that the radioactivity remaining after 16 hr of hydrolysis was 37% (AMP) and 69% (GMP) of the original. These exchange rates agree with the results of Brandhorst and Humphreys (1971). The data from total incorporation were corrected for both exchange and the incorporation of GMP and are presented as dpm of AMP incorporated/μg of DNA.

Sucrose Density Gradient Centrifugation of RNA. Cultures (50 ml) containing 1.5×10^8 cells were labeled with [5-^{3}H]-uridine or [2-^{14}C]uridine (New England Nuclear). The [5-^{3}H]-uridine was diluted with unlabeled uridine to a specific activity of 540 Ci/mol, which was 10 times the specific activity of the commercial [2-^{14}C]uridine. For 1-hr labeling periods, 18 μCi of [2-^{14}C]uridine or 180 μCi of [5-^{3}H]uridine were used. During 3-hr labeling periods 10 or 100 μCi was used. In chase experiments 250 μg of actinomycin D (Calbiochem) and a 100-fold excess of unlabeled uridine were added to each 50-ml culture.

RNA was extracted and displayed on 5–20% linear sucrose gradients prepared as described by Torelli *et al.* (1968). Effluent from the gradients was continuously monitored at 260 nm and fractions were collected. These fractions were either counted directly in Triton X-100–toluene (3:7) scintillation fluid containing 3.5 g of 2,5-diphenyloxazole/l., or were precipitated with trichloroacetic acid (with 1.5 mg of carrier serum albumin). These precipitates were hydrolyzed in 0.3 N KOH and acidified with an equal volume of 1.3 N $HClO_4$, and the acid-soluble supernatant solution was counted in the dioxane scintillation fluid described above. Either method gave equivalent results.

Results

Response of Bovine Lymphocytes to ConA. After purification of lymphocytes as described in the Experimental Section, 10–15% of the components in the suspension appeared to be nuclei from damaged cells on microscopic examination. After 24 hr of incubation in culture in the absence of ConA, most of this material had disappeared, leaving greater than 98% of the components as normal cells (as judged by microscopic examination and the absence of uptake of Trypan Blue). More than 95% of these cells were small or medium lymphocytes (usually 2–3% were large lymphocytes and 1–2% erythrocytes). After the preincubation period, the decline in cell number was less than 10% per day in the absence of mitogen. Approximately 40% of the cells transformed to large lymphoblasts and approximately 50% to transitional lymphocytes within 36 hr after the addition of ConA to cultures. By 66 hr more than 60% of the cells were lymphoblasts, 20–30% transitional cells, and 10% remained as small lymphocytes.

Cell Composition During Transformation. There is generally a 15% decline in the content of DNA in an average culture during the 24-hr preincubation which correlates with the disappearance of material from damaged cells (*vide supra*). In unstimulated cultures or stimulated cultures prior to the initiation of DNA synthesis, the DNA content was a convenient index of the number of cells per culture. After preincubation, there was a loss of approximately 7% of the cells per day during 3 days of culture in the absence of ConA (Figure 1). During the first 30 hr of culture in the presence of ConA (12 μg/ml), the DNA values paralleled the untreated controls; thus ConA did not decrease viability. Increases in DNA normally became apparent after 36–42 hr of culture in the presence of ConA. In the experiment shown in Figure 1, the amount of DNA per culture increased continuously from 37 until 72 hr; the net increment was 1.5-fold. In other experiments, increases in DNA have continued beyond 84 hr. Consistent with the net accumulation of DNA, the rate of incorporation of [^{3}H]-thymidine during 2-hr pulses increased rapidly between 24 and 48 hr after the addition of ConA (Figure 1). Negligible incorporation (<1% of the optimal incorporation rate) was seen in cultures lacking ConA or in ConA-stimulated cultures prior to 20 hr.

Since changes in the DNA contents of the cultures were rather small prior to 48 hr, we have chosen to normalize RNA, protein, and polyamine data to DNA. This normalization allowed a more precise analysis of the initial sequence of events since it corrected for differences in the number of cells per culture.

The transformation-associated changes in RNA and protein

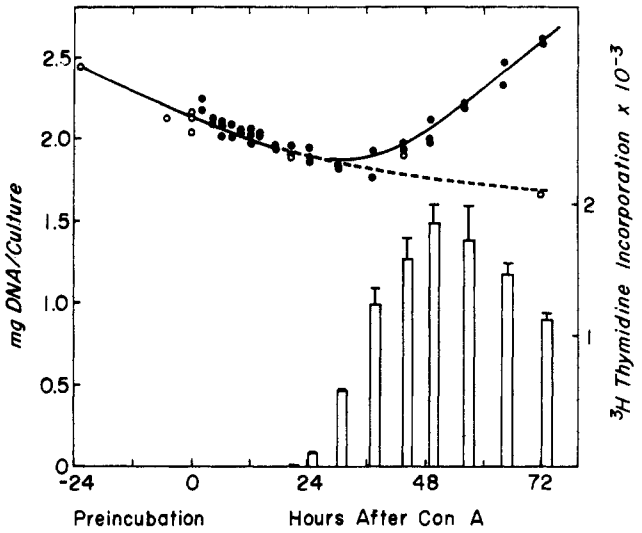

FIGURE 1: DNA accumulation and synthesis in lymphocyte cultures. Cultures (100 ml) were inoculated at −24 hr and harvested at the indicated times. ConA was added to a portion of the cultures at the end of the 24-hr preincubation period (●). Other cultures were not stimulated by ConA (○). Each point represents the average of duplicate DNA determinations, the difference in duplicates not exceeding 5%. The kinetics of incorporation of [*methyl*-^{3}H]thymidine into DNA during a 2-hr pulse with aliquots from the mother culture are shown in the bar graph (cpm/2 hr/μg of DNA). The incorporation was normalized to the DNA content of the aliquots.

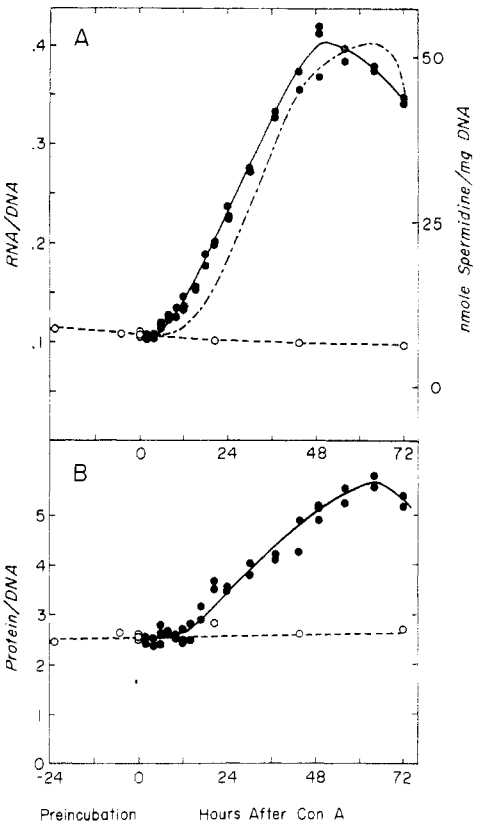

FIGURE 2: Changes in cellular RNA (A) and protein (B) content during ConA-induced lymphocyte transformation. Each point represents the average of duplicate determinations from the same cultures cited in Figure 1. The maximal difference in duplicates for RNA was 5% and for protein 10%. The average RNA and protein contents (expressed in mg) were normalized to the average DNA content (also in mg) of each culture. ConA was added to most of the cultures at 0 hr (●); control cultures were not stimulated by ConA (○). In (A), the normalized spermidine content of cultures (from Figure 3) is indicated by the line (· · · ·).

are shown in Figure 2 and the changes in polyamines in Figure 3. The magnitude of the changes is about the same whether they are based on the amount per culture or normalized to DNA. After a short lag, RNA increased fourfold over a 48-hr period (Figure 2A). After a more pronounced lag, protein increased a little more than twofold between 12 and 60 hr (Figure 2B). Of the polyamines, putrescine increased the most, 15-fold by 48 hr. After remaining at their basal levels for approximately 12 hr, spermidine and spermine increased markedly, the respective increments being six- to sevenfold and approximately twofold by 60 hr. In general, the decline in the curves (Figures 2 and 3) between 48 and 72 hr was due to increases in DNA. Spermine and protein per culture continue increasing until 72 hr. RNA per culture was constant from 56 to 72 hr. However, spermidine and putrescine did decline somewhat (5–10% per culture) by 72 hr.

The order of the initial changes in these levels is of considerable relevance to regulatory hypotheses. The results in Figures 2 and 3 illustrate the sequence of these changes and have been confirmed in five independent experiments. Increases in RNA content were always observed before increases in polyamine levels. The 10% increase in RNA observed by 6 hr was statistically significant whether normalized per DNA ($P < 0.001$)[3] or per culture ($P < 0.05$).[3] In addition, changes in the species of RNA synthesized were readily detectable by 6 hr (Figure 4). In unstimulated cultures, there was proportionally more high molecular weight heterogeneous RNA synthesized during a 1-hr labeling period. After 6 hr of ConA, there was relatively less synthesis of high molecular weight heterogeneous RNA and a distinct increase in synthesis of 28S and 18S rRNA. In single-label experiments, stimulation of ribosomal and ribosomal precursor RNA synthesis has been observed as early as 3 hr after stimulation.

Changes in putrescine levels were observed after these changes in RNA metabolism. The increase was significant by 8 hr when either normalized to DNA ($P < 0.02$)[3] or per culture ($P < 0.02$).[3] The spermidine/DNA level did not increase significantly in the experiment shown in Figure 3 until 12 hr ($P < 0.05$). However, when data from six experiments were combined, the increase normalized to DNA was significant by 10 hr ($P < 0.05$), and by 12 hr ($P < 0.001$) on a per culture basis. A graphic illustration of the relationship between RNA and spermidine accumulation is shown in Figure 2A. Although spermidine and RNA accumulated over the same period of time, it is clear that the initial increase in RNA preceded the initial increase in spermidine. The spermine/DNA level did not increase significantly in the experiment shown in Figure 3 until 17 hr ($P < 0.05$). However, the cumulative data from six experiments indicate significant increases by 10 hr ($P < 0.02$) normalized to DNA and by 12 hr per culture ($P < 0.05$).

In order to unequivocally verify that polyamine accumulation lags behind RNA accumulation, it was necessary to determine whether polyamines leaked from the cells during harvest of cultures. Cultures were preincubated with iproniazid for 15 min prior to harvest (see Experimental Section) in order to inhibit the amine oxidase which is present in the serum (Tabor

[3] The statistical values given were calculated by Student's t test. The values given for RNA and putrescine are from the combined data of six experiments. However, these increases were also significant ($P < 0.05$) at the time indicated for the single experiment shown in Figures 2 and 3.

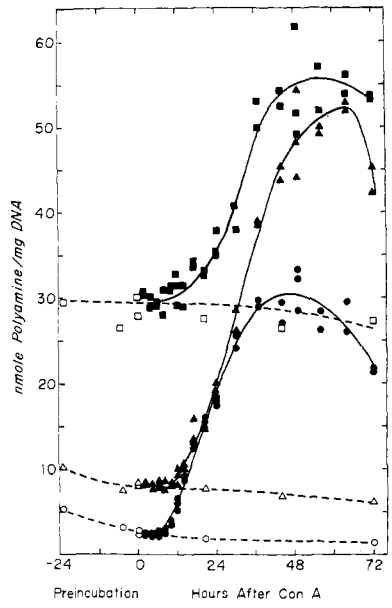

FIGURE 3: Changes in polyamine levels during ConA-induced lymphocyte transformation. The polyamine levels were determined in the same cultures as used in Figures 1 and 2 and these values were normalized to the DNA content. Putrescine (●), spermidine (▲), and spermine (■) were determined in control cultures not stimulated by ConA (open symbols) and in stimulated cultures (closed symbols).

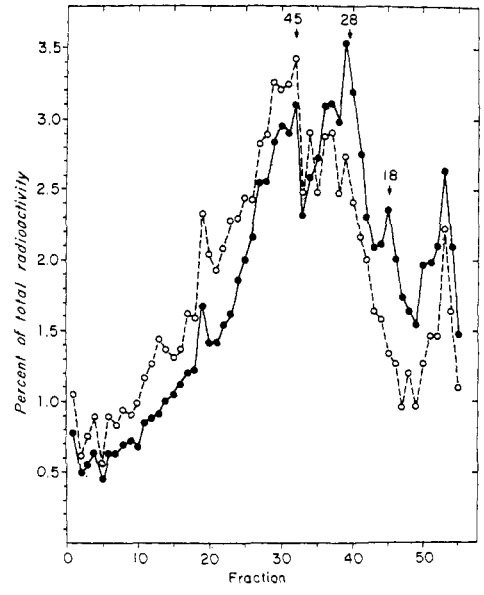

FIGURE 4: Sucrose density gradient profile of RNA synthesized in unstimulated and ConA-stimulated cultures. One culture was incubated with ConA for 5 hr and then with [2-^{14}C]uridine for 1 additional hr. The other (unstimulated) culture was incubated with [5-^{3}H]uridine for 1 hr. The cultures were then mixed and centrifuged and the RNA was extracted as described in the Experimental Section. Sucrose gradients, 5–20%, were centrifuged at 16,000 rpm for 16 hr at 4° with an SW27.1 rotor. Data are plotted as the percentage of total counts recovered on the gradient. ^{3}H-labeled RNA from the nonstimulated culture (○); ^{14}C-labeled RNA from ConA-stimulated culture (●).

and Tabor, 1964). This oxidase rapidly degrades both spermidine and spermine. For example, when [^{14}C]spermine (0.6 nmol/ml) was added to the culture medium in the absence of inhibitor, over 97% was oxidized within 15 min at 37°. The oxidation products eventually decompose to putrescine (in this experiment 40% of the spermine added was recovered as putrescine after 4 hr).

Spermine was not detected in any of the medium samples examined although it would have been detected if more than 3% of the cellular spermine had leaked from the cells during harvest. Only trace amounts of spermidine were found in the medium (Table I) and quantitation of these was subject to considerable error. However, in no case did more than 5% of the cellular spermidine leak to the medium during harvesting and the amount of leakage did not increase in the presence of ConA. Only 1–2 nmol of putrescine were found in 100 ml of the medium from cultures which had been harvested 1 hr after inoculation of cells (Table I). However, after the 24-hr preincubation, this level was up to 20 ± 5 nmol and remained constant until at least 49 hr after the addition of ConA. The putrescine appearing in the medium during preincubation can easily be accounted for by oxidation of the spermidine and spermine released from damaged cells during this period (*vide supra*). Because of the high background level of putrescine in the culture medium, the possibility of putrescine leakage during the harvest of the cells cannot be eliminated. If there was nonuniform leakage during the period of culture, it is possible that putrescine increased at an earlier time. Washing the cells with cold phosphate-buffered saline after centrifuging them from the medium led to substantial leakage of all three polyamines.

Inhibition of S-Adenosylmethionine Decarboxylase by MGBG. *S*-Adenosylmethionine decarboxylase is the first of two enzymes catalyzing the synthesis of spermidine, and subsequently spermine, from putrescine. We have confirmed the report of Williams-Ashman and Schenone (1972) that MGBG inhibits mammalian *S*-adenosylmethionine decarboxylase. When assayed (Fillingame and Morris, 1973c) in the presence of 0.25 mM putrescine, which maximally activates decarboxylation, activity was inhibited by 50% at 0.2 μM MGBG and by more than 98% at 10 μM MGBG. When decarboxylation was measured in the absence of putrescine, higher concentrations of MGBG were required to achieve the same degree of inhibition (2 μM for 50% inhibition). Thus, putrescine facilitated inhibition by MGBG. Similar results have been reported by Corti *et al.* (1973).

MGBG Inhibition of Intracellular Spermidine and Spermine Accumulation. As illustrated in Figure 5, MGBG was capable of inhibiting the accumulation of both spermidine and spermine when added to transforming cultures. Inhibition of

TABLE I: Polyamine Levels in Cells and Medium.[a]

Hr after ConA	mg of DNA/100 ml of Culture	nmol of Spermidine/100 ml of Culture		nmol of Putrescine/100 ml of Culture	
		Cells	Medium	Cells	Medium
−23	2.43	24.7	0.4	12.8	1.5
0	2.11	15.6	0.7	5.5	20.9
6	2.30	18.4	0.7	5.0	22.7
12	2.04	18.4	<0.4	9.9	22.4
24	1.92	35.5	1.2	32.5	24.6
39	1.92	78.9	1.1	61.5	20.6
49	1.98	107.3	1.2	63.5	15.0

[a] Cultures were preincubated with 5 mM iproniazid phosphate for 15 min and harvested as described in the Experimental Section.

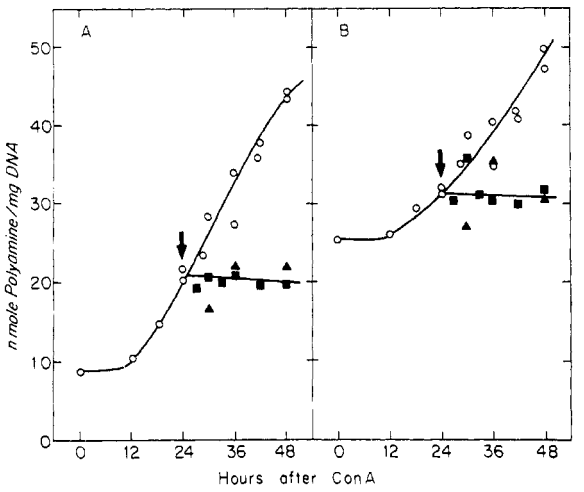

FIGURE 5: Inhibition of cellular spermidine (A) and spermine (B) accumulation by MGBG. ConA was added to lymphocyte cultures at 0 hr and MGBG was added at 24 hr to a final concentration of 8 μM (▲) or 40 μM (■). The controls to which no MGBG was added are indicated by (○).

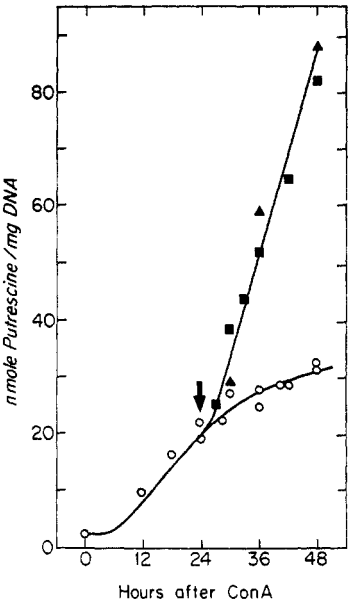

FIGURE 6: Effect of MGBG on cellular putrescine accumulation. Putrescine levels were measured in the same experiment outlined in the legend of Figure 5. Control values are represented by (○). MGBG was added to the other cultures at 8 μM (▲) or 40 μM (■).

spermidine and spermine accumulation was most easily demonstrated over the 24–48-hr interval since both polyamines were accumulating at maximal rates. In this experiment, 8 μM MGBG was equally as effective as 40 μM. In a more thorough study of the concentration dependence of MGBG inhibition, concentrations of 2 μM or greater were shown to completely inhibit spermidine and spermine accumulation and 0.5 μM MGBG was 90% inhibitory. The dose dependence is similar to that observed with *S*-adenosylmethionine decarboxylase (*e.g.*, 0.2 μM MGBG inhibits spermidine accumulation by 60% and the decarboxylase by 50%). When 2 or 4 μM MGBG was added at the time of ConA addition or 6 hr later, increases in spermidine or spermine were not observed for at least 24 hr. However, after about 24 hr the lymphocytes became more resistant to MGBG inhibition and by 40 hr increases of 40% (spermidine) and 20% (spermine) were observed. Addition of more MGBG at 24 hr did not inhibit these relatively small increases.[4]

As shown in Figure 6, putrescine continued to accumulate when MGBG was added to transforming lymphocyte cultures. This was anticipated from the block in the biosynthetic pathway after putrescine and before spermidine and spermine. The final rate of putrescine accumulation was 1.5 times the rate predicted from such a block. We have found (Fillingame and Morris, 1973a) that MGBG treatment enhances the level of ornithine decarboxylase about twofold over the usual maximum. This probably accounts for the augmented rate of putrescine accumulation.

Effect of MGBG on RNA and Protein Accumulation. We have previously reported that inhibition of spermidine and spermine accumulation with MGBG between 24 and 48 hr does not affect RNA accumulation over this interval (Fillingame and Morris, 1973a). In the experiment shown in Figure 7, MGBG was added 6 hr after ConA (and before any polyamine accumulation) and RNA accumulation was measured until 40 hr. Accumulation of RNA in the presence of MGBG (and in the absence of spermidine and spermine accumulation) was indistinguishable from the normal control cultures.

Protein accumulation was also unaffected by MGBG (Figure 7). In addition, the normal changes in cell size and morphological character were observed in the presence of MGBG, *i.e.*, the same high proportion of cells was intermediate-sized lymphoblasts with multiple nucleoli at 24 hr.

Rate of RNA Synthesis During Polyamine Limitation. The results in Figure 8 show that the absolute rate of RNA synthesis was elevated upon stimulation with ConA. The rate of adenosine incorporation into RNA in unstimulated cultures was one-ninth that of stimulated cultures. However, the proportion incorporated as GMP was higher in ConA-stimulated cultures than in unstimulated cultures. Thus, the difference in rate of radioactivity incorporated into RNA as AMP was sevenfold (Figure 8). Much of this difference was due to the increased specific activity of the ATP pool in stimulated cultures (insert of Figure 8). The maximal rate of RNA synthesis (calculated from these data) was 35 pmol of AMP/hr μg of DNA in unstimulated cultures and 74 pmol of AMP/hr μg of DNA in ConA-stimulated culture. Thus, most of the large difference in total incorporation was due to changes in the uptake of labeled adenosine into the ATP pool, the absolute rate of RNA synthesis being elevated by only twofold after 20 hr of ConA.

As also shown in Figure 8, adenosine was incorporated into the AMP residues of RNA at the same rate in MGBG-treated, or control, ConA-stimulated cultures. The changes in the specific activity of the ATP pool were also identical in the presence or absence of MGBG (insert of Figure 8). Therefore, the absolute rate of RNA synthesis in the absence of polyamine accumulation was indistinguishable from the control ConA-stimulated culture. Calculation of the maximal rate of

[4] There are two likely explanations for this acquired tolerance to MGBG. It is possible that an enzymatic system capable of metabolizing MGBG was induced and became sufficiently active after 24 hr. An alternative explanation is suggested by the observation that MGBG induced large increases in the cellular level of *S*-adenosylmethionine decarboxylase (Fillingame and Morris, 1973c). After 32 hr of MGBG treatment, adenosylmethionine decarboxylase activity (assayed after removal of MGBG by dialysis) was increased to a level 1500 times that measured in nonstimulated lymphocytes and 65 times the maximally induced level observed during transformation. If MGBG inhibited the intracellular *S*-adenosylmethionine decarboxylase by less than 100%, elevations of this magnitude together with the increased putrescine level could lead to some spermidine and spermine formation.

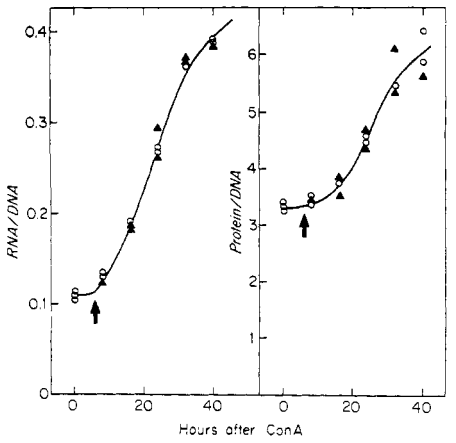

FIGURE 7: Accumulation of RNA and protein in MGBG-treated lymphocyte cultures. MGBG was added at a final concentration of 4 μM 6 hr after the addition of ConA. Accumulation of RNA is shown in the *left* portion of the figure and accumulation of protein in the *right* portion. Control cultures are represented as (○); cultures incubated with MGBG as (▲).

AMP incorporation into RNA gave 70 pmol of AMP/hr per μg of DNA in the presence of MGBG.

Types of RNA Synthesized and Their Processing in the Presence of MGBG. The major classes of stable RNA were synthesized in MGBG-treated cultures, and they were synthesized in the same relative proportion as in control cultures (Figure 9). The synthesis of rapidly labeled RNA species was also identical, as shown in Figure 10 (top). After a 1-hr labeling period, most of the label sedimented in a heterogeneous pattern. Prominent 45S and 28S peaks were also distinguishable and all classes of RNA were synthesized in the same relative amounts.

In order to study rRNA processing, cultures which had been labeled for 1 hr were chased in the presence of actinomycin D (5 μg/ml) and a 100-fold excess of cold uridine. Under these conditions, much of the label was chased from RNA into acid-soluble products and the rate of decay was the same in either the control or MGBG-treated culture (Table II). After a 30-min period, 37% of the label was chased into acid-soluble material. The profile (on sucrose gradients) of the radioactivity remaining in RNA changed significantly during

TABLE II: RNA Turnover in MGBG-Treated and Control Cultures.[a]

Hr of Chase	% Initial Radioactivity	
	Control	+MGBG
0	100	100
0.5	63	63
1	52	51
2	41	40
3	34	34

[a] Cultures were labeled with [5-^{3}H]uridine for 1 hr, 20 hr after the addition of ConA. The (+MGBG) cultures were made 4 μM in MGBG 6 hr after addition of ConA. After the labeling period, actinomycin D (at 5 μg/ml) and a 100-fold excess of cold uridine were added to the cultures. The radioactivity remaining in RNA was followed as a function of time. Each value given is the average percentage obtained from two independent pulse/chase experiments.

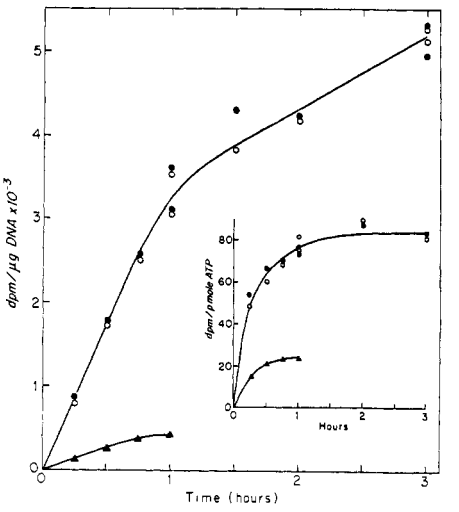

FIGURE 8: Influence of MGBG treatment on the rate of RNA synthesis. The control, ConA-stimulated culture, was incubated with ConA for 20 hr prior to labeling with [8-^{3}H]adenosine (○). The MGBG-treated, ConA-stimulated culture was made 4 μM in MGBG 6 hr after ConA and labeled with [8-^{3}H]adenosine from 20 hr after the addition of ConA (●). The unstimulated culture was not treated with ConA (▲). On the ordinate, incorporation of adenosine into RNA as AMP is plotted as a function of the labeling period. In the *insert*, the specific activity of the ATP pool in the respective cultures is plotted as a function of the labeling period.

a 30-min chase (Figure 10, bottom). The once prominent 45S peak was absent and mature 28S and 18S rRNA peaks were easily discerned. The profile in the MGBG-treated culture was again identical with that in the control culture. After a 1- or 3-hr chase, the profile was still identical (not shown).

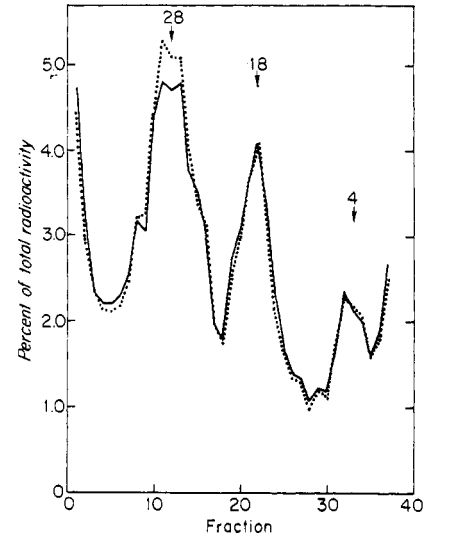

FIGURE 9: Sucrose density gradient profile of the stable RNA synthesized in control or MGBG-treated, ConA-stimulated cultures. The control culture was incubated with [2-^{14}C]uridine for 3 hr, 21–24 hr after the addition of ConA. The MGBG-treated culture was made 4 μM in MGBG 6 hr after the addition of ConA and then labeled with [5-^{3}H]uridine for 3 hr, 21–24 hr after ConA. The cultures were then mixed and centrifuged and the RNA was extracted as described in the Experimental Section. Sucrose gradients, 5–20%, were centrifuged at 23,000 rpm for 20 hr at 4° with an SW 27.1 rotor. The fractions were precipitated with trichloroacetic acid and the radioactivity in alkali-hydrolyzable material was determined. Data are plotted as the percentage of total counts recovered on the gradient: (—) ^{3}H-labeled RNA from the MGBG-treated culture; (. . .) ^{14}C-labeled RNA from the control culture.

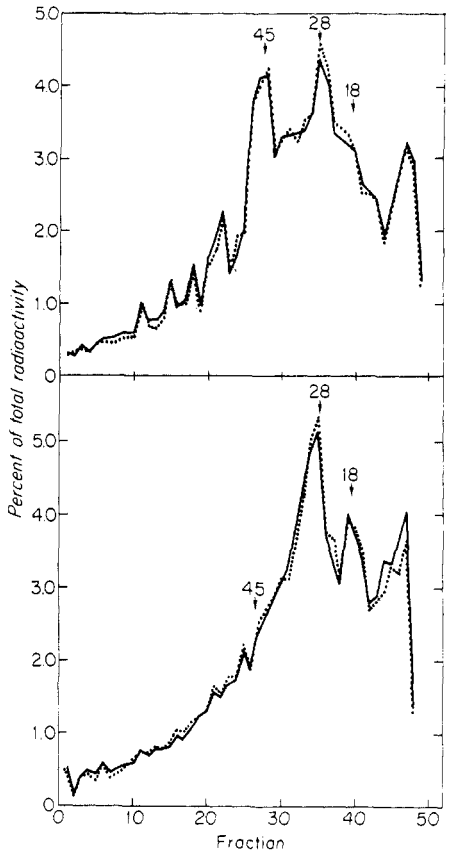

FIGURE 10: Synthesis and processing of rapidly labeled RNA in control or MGBG-treated cultures stimulated with ConA. Control cultures were incubated with ConA for 20 hr and then labeled with [2-^{14}C]uridine for 1 hr. MGBG-treated cultures were made 4 μM in MGBG 6 hr after addition of ConA, and 20 hr after the addition of ConA were labeled with [5-^{3}H]uridine for 1 hr. In the *top portion* of the figure, a control and a MGBG-treated culture were mixed after the 1-hr pulse and the RNA was extracted and centrifuged on a 5–20% sucrose gradient at 16,000 rpm for 16 hr with a SW 27.1 rotor. The labeling pattern shown in the *lower portion* of this figure was obtained with cultures which were labeled for 1 hr and then chased with actinomycin D (5 μg/ml) and a 100-fold excess of cold uridine for 30 min. The data are plotted as the percentage of total counts recovered on the gradient: (—) the ^{3}H-labeled RNA from the MGBG-treated cultures; (. . . .) ^{14}C-labeled RNA from the control cultures.

Discussion

The experiments presented here were addressed to the question of whether the increased concentration of polyamines observed in growing animal cells was involved in the enhanced synthesis and accumulation of RNA. As described earlier, two roles, related to RNA, had previously been proposed for polyamines: regulation of RNA synthesis, and stabilization of RNA once it had been synthesized. Simply on the basis of the kinetics of RNA and polyamine accumulation, we can conclude that the net cellular levels of polyamines cannot be regulating synthesis of RNA since they increase after RNA. This conclusion is supported by the results with MGBG in that the rate of RNA synthesis increased twofold and a dramatic shift in the types of RNA synthesized occurred in the absence of spermidine and spermine accumulation. The lag between the onsets of RNA and of polyamine accumulation demonstrated here has not been documented in other systems. Firm conclusions concerning the temporal relationship between the accumulation of RNA and of polyamines in regenerating liver (see earlier discussion) have been obscured by large discrepancies in the kinetics of spermidine and spermine accumulation observed by different laboratories (*e.g.*, see Russell *et al.*, 1970, and Raina *et al.*, 1966). However, even in this latter system it is clear, taking into account other independent data, that polyamines cannot be regulating the initial changes in RNA metabolism. Elevations in RNA synthesis (Fujioka *et al.*, 1963), preferentially rRNA synthesis (Chaudhuri *et al.*, 1967), were observed within 2 hr of partial hepatectomy and before any elevation in the putrescine (and polyamine) level (Raina *et al.*, 1970). Similarly, in transforming lymphocytes, events related to RNA accumulation are initiated before there is any evidence of increased polyamine synthesis, *e.g.*, increased ornithine decarboxylase activity (Kay and Cooke, 1971; our unpublished results). Although the increased uridine incorporation observed shortly after stimulation is due primarily to increased uridine uptake and phosphorylation (Lucas, 1967; Hausen and Stein, 1968), it is clear from our data, as well as those of others (see Cooper, 1971 for review), that the proportion of label incorporated into rRNA and its precursors does increase within a few hours of lymphocyte stimulation. In summary, the suggested regulatory role for polyamines is most unlikely since these events precede polyamine accumulation.

As the results with MGBG demonstrate, concomitant accumulation of spermidine and/or spermine is not required during prolonged periods of normal RNA accumulation. Therefore, increases in spermidine and spermine do not seem to serve an essential role in stabilizing RNA. The rate of turnover of rapidly labeled RNA is the same in the presence or absence of spermidine and spermine accumulation (Table II). An independent experimental approach indicates that the rate of degradation of the stable RNA species (28, 18, 4s) must also be the same since their rate of synthesis is not altered and they accumulate at the same rate. It is possible that putrescine, rather than spermidine or spermine, serves a nonregulatory role in RNA accumulation (*e.g.*, by stabilization) since it continues to accumulate in the presence of MGBG. It is also possible that spermidine and spermine normally participate in such a function and putrescine or other cations substitute in this role during MGBG treatment. By definition then, such a role for spermidine and spermine would be nonessential and nonspecific. Such an explanation is less than satisfying since it is not clear why spermidine and spermine should be synthesized if more easily obtainable cations (*e.g.*, putrescine) were capable of serving the same function at equivalent concentrations.

While this work was in progress, Kay and Pegg (1973) reported that addition of MGBG to lymphocyte cultures prevented the increase in [^{3}H]phenylalanine incorporation normally observed during phytohemagglutinin-induced transformation and that it eventually reduced the stimulated rate of [^{3}H]uridine incorporation. Unfortunately these experiments were done with concentrations of MGBG (1 mM final) that were 500–1000 times that required for complete inhibition of spermidine and spermine accumulation. As shown here, when MGBG was added at less than 10 μM, no aberrations in RNA synthesis were observed and protein accumulated at the same rate as in control cultures. The reduced rate of [^{3}H]-phenylalanine incorporation observed by Kay and Pegg was probably due to secondary, pharmacological effects of these excessive doses of MGBG. With concentrations of less than 5 μM MGBG, we have not observed diminished incorporation of [^{3}H]leucine (unpublished data).

This discussion has been limited specifically to the role of the increases in polyamine concentration during growth.

Because spermidine and spermine are initially present in unstimulated lymphocytes, *nonregulatory* participation of these compounds in RNA or protein synthesis cannot be ruled out. Other studies from this laboratory (Morris, 1973; Morris and Hansen, 1973) have led to the conclusion that in bacteria, spermidine could possibly play a cofactor, but definitely not a regulatory role in the synthesis of RNA and/or protein. However, if the basal level of polyamines in lymphocytes was capable of fulfilling this hypothetical cofactor role, then the increased levels must be fulfilling another function. These increases may be required for, or may be regulating, later events in the transformation process. We have found that treatment of lymphocyte cultures with MGBG, an antileukemic drug (Mihich, 1963), does reduce the rate of both DNA synthesis ([^{3}H]thymidine incorporation) and cell division. MGBG also inhibited division of fibroblasts in culture (unpublished data). Unfortunately, at this time we do not know whether these effects are mediated *via* inhibition of polyamine synthesis or by other pharmacological effects. However, it is tempting to speculate that the striking increases in polyamines observed in this system are not involved in growth of the cell *per se*, but rather are required in an event unique to proliferation, *e.g.*, DNA synthesis of chromosome condensation. Apparent aberrations in DNA synthesis have been observed in bacterial mutants during starvation for polyamines (Dion and Cohen, 1972; Morris and Jorstad, 1973).

Added in Proof

We have now confirmed that the high doses of MGBG used by Kay and Pegg (1973) inhibit [^{3}H]leucine incorporation into protein. Whereas 4 μM MGBG inhibited incorporation by only 6%, the concentration used by these workers, 1 mM, inhibited incorporation by more than 50% (incorporation measured 20 hr after ConA ± MGBG). Since both concentrations inhibit polyamine accumulation to the same extent, the inhibition must be due to a pharmacological effect unrelated to polyamine synthesis.

References

Bailey, J. L. (1967), Techniques in Protein Chemistry, 2nd ed, Amsterdam, Elsevier, p 340.

Bender, M. A., and Prescott, D. M. (1962), *Exp. Cell Res. 27*, 221.

Brandhorst, B. P., and Humphreys, T. (1971), *Biochemistry 10*, 877.

Burton, K. (1956), *Biochem. J. 62*, 315.

Caldarera, C. M., Barbiroli, B., and Moruzzi, G. (1965), *Biochem. J. 97*, 84.

Chaudhuri, S., Doi, O., and Lieberman, I. (1967), *Biochim. Biophys. Acta 134*, 479.

Cohen, S. S. (1971), Introduction to the Polyamines, Englewood Cliffs, N. J., Prentice-Hall.

Cooper, H. L. (1971), *in* The Cell Cycle and Cancer, Baserga, R., Ed., New York, N. Y., Marcel Dekker, p 197.

Corti, A., Schenone, A., Williams-Ashman, H. G., Dave, C., and Mihich, E. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 512.

Dion, A. S., and Cohen, S. S. (1972), *J. Virol. 9*, 423.

Dykstra, W. J., Jr., and Herbst, E. J. (1965), *Science 149*, 428.

Emerson, C. P., and Humphreys, T. (1971), *Anal. Biochem. 40*, 254.

Fillingame, R. H., and Morris, D. R. (1973a), *in* Polyamines in Normal and Neoplastic Growth, Russell, D. H., Ed., New York, N. Y., Raven Press, p 249.

Fillingame, R. H., and Morris, D. R. (1973b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 621.

Fillingame, R. H., and Morris, D. R. (1973c), *Biochem. Biophys. Res. Commun. 52*, 1020.

Fisher, D. B., and Mueller, G. C. (1969), *Biochim. Biophys. Acta 176*, 316.

Forsdyke, D. R. (1967), *Biochem. J. 105*, 679.

Fujioka, M., Koga, M., and Lieberman, I. (1963), *J. Biol. Chem. 238*, 3401.

Hausen, P., and Stein, H. (1968), *Eur. J. Biochem. 4*, 401.

Hausen, P., Stein, H., and Peters, H. (1969), *Eur. J. Biochem. 9*, 542.

Kay, J. E. (1968), *Eur. J. Biochem. 4*, 225.

Kay, J. E., and Cooke, A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 9.

Kay, J. E., and Korner, A. (1966), *Biochem. J. 100*, 815.

Kay, J. E., and Pegg, A. E. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29*, 301.

Ling, N. R. (1968), Lymphocyte Stimulation, Amsterdam, North-Holland Publishing Co.

Loeb, L. A., and Agarwal, S. S. (1971), *Exp. Cell Res. 66*, 299.

Lucas, Z. J. (1967), *Science 156*, 1237.

Mihich, E. (1963), *Cancer Res. 23*, 1375.

Morris, D. R. (1973), *in* Polyamines in Normal and Neoplastic Growth, Russell, D. H., Ed., New York, N. Y., Raven Press, p 111.

Morris, D. R., and Hansen, M. T. (1973), *J. Bacteriol.* (in press).

Morris, D. R., and Jorstad, C. M. (1973), *J. Bacteriol. 113*, 271.

Morris, D. R., Koffron, K. L., and Okstein, C. J. (1969), *Anal. Biochem. 30*, 449.

Novogradsky, A., and Katchalski, E. (1971), *Biochim. Biophys. Acta 228*, 579.

Nowell, P. C. (1960), *Cancer Res. 20*, 462.

Pohjanpelto, P., and Raina, A. (1972), *Nature (London), New Biol. 235*, 247.

Powell, A. E., and Leon, M. A. (1970), *Exp. Cell Res. 62*, 315.

Puck, T. T., Cieciura, S. J., and Robinson, A. (1958), *J. Exp. Med 108*, 945.

Raina, A. (1963), *Acta Physiol. Scand. 60, Suppl. 218*, 1.

Raina, A., and Jänne, J. (1968), *Ann. Med. Expt. Biol. Fenn. 46*, 536.

Raina, A., and Jänne, J. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 1568.

Raina, A., Jänne, J., Hannonen, P., and Hölttä, E. (1970), *Ann. N. Y. Acad. Sci. 171*, 697.

Raina, A., Jänne, J., and Siimes, M. (1966), *Biochim. Biophys. Acta 123*, 197.

Robbins, J. H. (1964), *Science 146*, 1648.

Russell, D. H. (1970), *Ann. N. Y. Acad. Sci. 171*, 772.

Russell, D. H., and Lombardini, J. B. (1971), *Biochim. Biophys. Acta 240*, 273.

Russell, D. H., and McVicker, T. A. (1973), *Biochem. J. 130*, 71.

Russell, D. H., Medina, V. J., and Snyder, S. (1970), *J. Biol. Chem. 245*, 6732.

Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.

Tabor, H., and Tabor, C. W. (1964), *Pharmacol. Rev. 16*, 245.

Tabor, C. W., Tabor, H., and Rosenthal, S. M. (1954), *J. Biol. Chem. 208*, 645.

Torelli, U. L., Henry, P. H., and Weissman, S. M. (1968), *J. Clin. Invest. 47*, 1083.

Williams-Ashman, H. G., and Schenone, A. (1972), *Biochem. Biophys. Res. Commun. 46*, 288.