Reports

Use of Phosphorus-32 in Microassay for Nucleic Acid Synthesis in Escherichia coli

Abstract. A method was developed to facilitate studies of ribonucleic acid and deoxyribonucleic acid synthesis in growing bacterial cultures of small volume. The nucleic acid fractions were separated by means of a modified Schmidt-Thannhauser procedure applied to micro quantities of P^{32}-labeled cells collected on membrane filters. The validity and usefulness of the method are discussed.

There has been a need for a rapid means of following synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in bacterial cultures of small volume. For screening metabolic analogs as inhibitors, and for studying effects of radiation on molecular synthesis, there is an advantage in working with small cultures. Micro techniques involving colorimetric assays have been developed (*1*). By the Keck method, for example, it is possible to detect as little as 0.1 μg of DNA. However, more than 10^7 *Escherichia coli* cells are then required at the limit of sensitivity, and the increment in DNA in a period of several minutes cannot be determined with any accuracy.

The incorporation of isotopically labeled precursors is a very sensitive criterion for molecular synthesis if the different molecular species can be suitably separated, if the amount of "turnover" is known, and if incorporation can be distinguished from adsorption. The Schmidt-Thannhauser procedure has been used to separate P^{32}-labeled bacterial nucleic acids (*2, 3*). Also, an efficient procedure for isolating micro quantities of P^{32}-labeled bacterial cells has been developed by Roberts *et al.* (*4*). It seemed practical to combine these two techniques in order to carry out the separation on a micro scale. Phosphorus conservation in bacterial nucleic acids has been demonstrated by many workers and was verified in these studies (*5*) to a maximum 0.5-percent decrease in activity per hour for P^{32}-labeled log-phase *E. coli* B in nonradioactive medium. Siminovitch and Graham have shown further that there is no redistribution of P^{32}-labeled nucleosides between RNA and DNA (*6*).

Escherichia coli B were grown aerobically at 37°C in 10 ml of Tris synthetic medium (*7*). Five microcuries of P^{32}-orthophosphate were added when the culture had reached a concentration of 10^7 cells/cm³ in log phase. The separation procedure was carried out on duplicate 0.2-ml samples taken by pipette from the culture at 4-minute intervals thereafter.

Step 1. The 0.2-ml samples were placed in small tubes, and 5 ml of ice-cold 5-percent trichloroacetic acid (TCA) was added to each. The tubes were immediately emptied onto pre-wet filters (*8*) on a suction filtration apparatus similar to that described by Roberts *et al.* (*4*). Then, 5 ml of distilled water was passed through the filters as a rinse. After drying in air, the filters were assayed for radioactivity by a Geiger-Müller counter with a 1.4-mg/cm² mica window. The "TCA-insoluble" fraction thus collected contains phosphorus primarily in RNA, DNA, and phospholipids.

Step 2. The filters from step 1 were placed in individual polyethylene cups containing 5 ml of 75-percent ethanol. After 2 hours at room temperature they were again placed on the filtration apparatus and then rinsed with 5 ml of 50-percent ethanol. The alcohol extraction removes over 90 percent of the lipids (*4*). Most of the remainder would be soluble in ether, but this, unfortunately is a solvent for the filter material, so the ether extraction could not be used. The remaining activity on the filters was determined after the filters had dried.

Step 3. The filters were next placed in polyethylene cups containing 3 ml of 1*N* KOH at 35°C for 2 hours and then chilled. The RNA was reduced quantitatively to mononucleotides by this step, but the DNA fraction and the filters were dissolved. It was found that the DNA could be quantitatively reprecipitated by the addition of 3 ml of 10-percent ice-cold TCA to each cup. The cups were then emptied on fresh filters on the filtration apparatus, and the filters were rinsed with 5 ml of 5-percent TCA. The activity on the second filters was then determined as the "DNA-residual" fraction, and the activity in the RNA fraction was obtained by subtracting this from the activity after step 2. No appreciable self-adsorption problems were involved in counting the 1.7 Mev beta particles from P^{32}. When the elapsed time for the procedure was more than a day, a correction was made for P^{32} decay. Since hot TCA was found to destroy the filter, it was impossible to separate the DNA from the residual fraction on the filters. The residual fraction contains about 2 percent of the P^{32} activity incorporated, and this is distributed in proteins, undissolved phospholipids, and adsorbed phosphorus compounds of low molecular weight.

The extraction procedure was checked by a parallel sampling (1 ml) from the culture, carried through the same extraction procedure in microtubes; centrifugation (11,000*g* for 2 min) was used to collect the precipitates, and the nucleic acids in the different fractions were determined by colorimetric analysis (*1*). The comparison was made on samples from a culture at a concentration of 10^8 cells/cm³ which had incorporated radioactivity of about 10,000 count/min into the TCA-insoluble fraction. The supernatants were assayed for radioactivity by placing aliquots on planchets for counting. The hot-TCA extraction step from the Schmidt-Thannhauser procedure was used on the microtube samples (*2*). In Table 1 the comparison of P^{32} distributions is shown for three separate runs.

The colorimetric analysis revealed that 94 ± 3 percent of the RNA was recovered in the RNA fraction and that 4 to 5 percent appeared in the alcohol-soluble fraction, but that none appeared in the DNA fraction. Of the DNA, 84 ± 4 percent was recovered in the DNA fraction, 12 ± 3 percent appeared in the RNA fraction, and less than 4 percent appeared in the alcohol-soluble and residual fractions. Since there is 5 to 6 times as much RNA as DNA in the *E. coli* cell, the DNA contamination in the RNA fraction should cause less than 2.5 percent error in that fraction, if the specific activities are approximately the same. At the limit of sensitivity of this check, the RNA contamination in the DNA fraction could cause a maximum of 20 percent error in that fraction, although the actual error is probably less. A comparison of the filtration and centrifugation methods led to the operational definition of the DNA fraction as the DNA-

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Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* 125, 16 (1957)].

Table 1. Comparison of P^{32} distribution in fractions obtained by centrifugation (C) and by filtration (F) (three separate runs). Values are given as percentage of total activity in the TCA-insoluble fraction.

Method	Alcohol-soluble	RNA	DNA-residual	DNA
C	17.8	69.0	13.6	11.8
F	14.3	71.5	14.6	12.6*
C	17.2	68.0	14.5	12.4
F	14.7	72.5	12.8	10.8*
C	16.2	69.0	14.8	13.4
F	13.8	71.1	15.1	13.1*

* Defined in text.

residual fraction minus 2 percent of the total activity in the TCA-insoluble fraction. The validity of this step was further checked by carrying out the procedure on cultures of *E. coli* 15_{T^-} in thymine-deficient media (*9*). In confirmation of Cohen's findings, there was negligible incorporation of P^{32} into the DNA fraction, defined as above, in the absence of thymine. Incorporation into the RNA fraction, however, was linear with time under this condition of no DNA synthesis. The separation procedure has been found useful in further studies of "unbalanced growth" in *E. coli*, induced by the action of ultraviolet light (*10*).

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References and Notes

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5. I wish to thank Professor R. B. Setlow for his encouragement and critical interest in this problem and Dr. Ellis Bolton for helpful advice. The technical assistance of Joanna Hanawalt is appreciated. This study was assisted by U.S. Pubic Health Service grant No. E-1285.
6. L. S. Siminovitch and A. F. Graham, *J. Histochem. and Cytochem.* 4, 508 (1956).
7. Tris synthetic medium contains 0.1*M* tris (hydroxymethyl) aminomethane and 2.25 g of KCl, 1.5 g of NH_4Cl, 0.04 g of $MgCl_2 \cdot 6H_2O$, 0.014 g of Na_2HPO_4, 0.026 g of Na_2SO_4, and 5 g of glucose per liter. The mixture was adjusted to *p*H 7.2 with HCl.
8. Schleicher and Schuell membrane filters (27 mm, coarse porosity) were used.
9. S. S. Cohen and H. Barner, *J. Bacteriol.* 69, 59 (1955).
10. P. C. Hanawalt and R. B. Setlow, *Biophysical Society Abstracts* (1959).
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Leukemogenic Activity of Filtrates from Radiation-Induced Lymphoid Tumors of Mice

Abstract. Cell-free filtrates of x-ray-induced lymphoid tumors of strain C57BL/Ka mice have elicited, on injection into newborn isologous hosts, a lymphoma incidence of 15 to 19 percent. In control mice of the same subline, the incidence of spontaneous lymphoma is about 1 percent. No leukemogenic activity could be detected in filtrates from thymi harvested at 2 to 32 days following completion of x-ray treatment. Activity was evident at 64 days and was perhaps somewhat greater at 128 days. Serial cell-free passage of filtrates in newborn F_1 hybrid mice resulted in a marked increase in lymphoma incidence (69 percent), coupled with a shortening of the median latency. Supplementary x-irradiation failed to enhance the activity of filtrates after neonatal injection.

In most strains of mice the thymus is essential for the development of lymphatic leukemia and lymphosarcoma, either arising spontaneously or induced by ionizing radiation, hydrocarbons, or estrogen (*1*). In the case of the radiation-induced disease, however, it has been demonstrated that such tumors may develop from nonirradiated cells of thymic grafts implanted into irradiated hosts (*2*). This phenomenon of indirect induction may be interpreted in terms of the existence of a latent subcellular agent, analogous to the filtrable agent of spontaneous mouse leukemia demonstrated by Gross (*3*), which, having been "activated" by radiation, initiates malignant transformation in susceptible lymphoid cells. For the past 5 years, a series of experiments designed to test this possibility has been in progress in our laboratory (*4*).

Lymphoid tumors were induced in C57BL/Ka mice of both sexes by fractionated whole-body x-irradiation (four weekly doses of 168 r each) started at age 33 ± 3 days (*5*). The incidence of disseminated lymphomas resulting from this treatment is 80 to 90 percent, with an average latency of about 200 days (*6*). The animals were sacrificed and autopsied when unquestionable symptoms of the disease became apparent.

Cell-free extracts of the involved tissues were prepared by a modification of Gross' technique (*3*). Thymus, spleen, liver, and lymph nodes were removed and weighed, and iced Locke's solution was added to make a 20-percent suspension. This was homogenized and centrifuged at 7000*g* for 15 minutes. The supernatant was recentrifuged, and the cycle was repeated for a total of four centrifugations. The entire procedure was carried out at 4°C. The final supernatant was passed through a UF fritted glass filter. Impermeability of the filter to cells was ascertained by retention of *Escherichia coli*.

Filtrates were injected in 0.1-ml amounts either subcutaneously or intraperitoneally into C57BL/Ka or reciprocal F_1 (C57BL/Ka × BALB/c) hybrid mice of both sexes, aged 16 hours or less. The animals were maintained under standard laboratory conditions and were sacrificed when moribund, or when older than 600 days. Sections of the involved tissues were stained with hematoxylin and eosin. Only typical lymphocytic lymphosarcomas, identical to those arising after irradiation of strain C57BL (*7*), were tallied as "lymphomas."

Filtrates from isologous, x-ray-induced lymphoid tumors yielded ten (17 percent) disseminated lymphomas among 59 long-term survivors of neonatal injection (Table 1). In a second experiment, filtrates from tumor C43, a radiation-induced thymic lymphosarcoma of C57BL mice which has been carried by serial isologous transplantation for the past 7 years, gave a similar tumor incidence, indicating preservation of activity through some hundred successive transplant generations.

The only other neoplasms observed were reticuloendothelial tumors, ovarian tumors, and hepatomas, all of which occurred with about the expected frequency for aged, untreated mice of this strain.

In our subline of the C57BL strain there has been only one spontaneous lymphoma (1.3 percent) in a total of 74 untreated or saline-injected mice of both sexes maintained for 600 days or longer. A group of 25 C57BL/Ka mice injected when newborn with filtrates from isologous, spontaneous reticuloendothelial tumors and hemangiomas, and another group of 24 which had received filtrates from strain AK lymphoid tumors all remained free of lymphoma for over 600 days. Filtrates from normal tissues of C57BL/Ka mice injected into newborn isologous hosts have elicited no lymphomas to date (9 months) (*7a*).

The possibility that supplementary irradiation might be required to bring out the full potential of the latent leukemogenic agent was tested in another experiment. Newborn C57BL/Ka mice received lymphoma filtrate (the controls received saline) at birth, and a single, whole-body x-ray exposure of 200 r given concurrently or at 2, 6, 14, or 30 days of age. The incidence of lymphoma after the combined treatment was reduced to the level resulting from x-ray treatment alone (0 to 8 percent), except in the group irradiated 30 days after filtrate injection (17 percent).

Pooled thymus glands (from strain C57BL mice) obtained at serial intervals (2, 8, 32, 64, or 128 days) after fractionated, systemic irradiation were used for the preparation of filtrates and assayed to determine the time of first ap-