THE CONSERVATION OF RIBONUCLEIC ACID BY EHRLICH ASCITES TUMOR CELLS*

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Experiments with microbial[1,2], mammalian[2-7,9,26], and neoplastic cells[8] have supported the concept of conservation of the constitutents of deoxyribonucleic acid (DNA) throughout the life of the cell. Similar studies on ribonucleic acid (RNA), on the other hand, have yielded a variety of results. Conservation of phosphorus[1,2] and purines[10] of RNA in *E. coli* during log-phase growth has been demonstrated. *In vivo* experiments with mammalian tissues have demonstrated loss of incorporated radioactivity[11] from RNA. SIMINOVITCH[2] has recently shown that suspensions of cells derived from adult mouse connective tissue growing in log phase in tissue culture conserve ^{32}P in the RNA. Working with the Ehrlich ascites tumor, RÉVÉSZ *et al.*[8] have demonstrated an intermediate situation in which labeled cells lose about 30% of the radioactivity in the RNA following transplantation. Following this initial loss, the remaining purine is conserved. MOLDAVE[12,13] has published data which show a loss of nucleic acid purines from labeled cells incubated *in vivo* in sacs of dialysis membrane implanted in the peritoneal cavity of mice. These conditions permit one cell-doubling, but no increase in cell mass. BENNETT AND SKIPPER[14] have suggested that RNA purines of a solid transplantable tumor (Sarcoma 180) are conserved.

In quantitative histochemical experiments we had demonstrated[15] in rat liver a proportionality between the concentration of acid-soluble inorganic plus labile organic phosphate and the concentration of RNA. This proportionality was not observed in tumors arising from the liver under the influence of *p*-dimethylaminoazobenzene and, further, the concentration of RNA appeared to be independent of the acid-soluble phosphate concentration. Reasoning from these data we reached an hypothesis that in these tumors there was little if any turnover of the RNA. Attempts to test this hypothesis with the solid liver tumors were inconclusive for many reasons[16], and we turned to the mouse ascites tumor. In this latter tumor we have found a conservation of pyrimidines of RNA and of DNA.

MATERIALS AND METHODS

Strain A male mice bearing either of two lines of Ehrlich ascites tumor, the hyperdiploid or the tetraploid, have been used. Conditions of inoculation are described under the appropriate experiments. The tumor cells were harvested after various intervals of time by repeated rinsing of the

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abdominal cavity of the host animal with small volumes of cold 0.14*M* NaCl containing approximately 100 USP units of heparin in 25 ml. Recovered cells and washings were pooled and made to volume in a 25 ml volumetric flask kept in cracked ice. Aliquots were removed for cell count and for determination of total RNA and DNA. The remainder of the cells was reserved for determination of specific activity of RNA and DNA and total radioactivity in the acid-soluble fraction. Aliquots for analysis were centrifuged and the supernatant fluid discarded. The pellets were frozen and stored at — 35°C until all samples from a single experiment had been collected.

Aliquots for cell counts were diluted 50- or 100-fold depending on the expected cell number to a final volume of 10 ml with 10% citric acid containing toluidine blue. After thorough mixing the cells were counted in a brightline hemocytometer. The standard error of the counting level was never greater than 15%.

Total RNA and DNA were determined by means of the combined SCHMIDT-THANNHAUSER[17]-SCHNEIDER[18] methods on replicate aliquots. RNA was estimated by the orcinol method[19] and DNA by the diphenylamine reaction[20].

The major portion of the cells was separated into acid-soluble, RNA, and DNA fractions by means of essentially the procedure of HECHT AND POTTER[7]. The procedure was modified by substituting 7% trichloracetic acid (TCA) to precipitate the acid-insoluble fraction and 5% TCA to wash the precipitate. In order to reduce the mass on the planchets used for measurement of radio activity in the acid-soluble fraction, the TCA was destroyed by heating the solution on a boiling water bath for 2 hours.

The specific and total activities of the three fractions were determined in the following manner. Microscope coverglasses (Corning No. 2915, 22 or 25 mm circles, Thickness No. 2) were used as planchets*. To obtain even spreading of the plated solutions the coverglasses were scrubbed with Shamva**, rinsed with distilled water, and allowed to dry. The glasses were tared and the weighting was repeated after the sample had been dried under a heat lamp. A Nuclear Chicago counter with a Micromil end-window detector was used to measure the radioactivity. The standard error of the counting level was never greater than 3%. All counts were corrected for self-absorption. The total fraction of RNA and DNA was plated in all experiments. After counting, the RNA and DNA on the planchets was eluted with water and the total phosphorus of the eluate was determined by the method of KING[21]. The phosphorus values were converted to mg RNA and DNA using respectively 9.16% P and 9.43% P and were then used to calculate specific activities. Total counts were calculated as specific activity times total amount of RNA or DNA present. In some experiments all the acid-soluble fraction was plated; in others an aliquot was plated. Total activity of the acid-soluble fraction was determined directly.

Orotic acid-6 ^{14}C*** had a specific activity of 1.36 $\mu C/\mu mole$. It was used without added carrier as a neutral solution containing 10 μC/ml.

RESULTS

In Fig. 1 the pooled results of two experiments are presented. In each, a group of 10 animals was inoculated at time zero with equal aliquots of Ehrlich hyperdiploid cells. On the third day each animal received 1 μC of orotate-6-^{14}C. Beginning on the fourth day the animals were killed in pairs, and the tumor harvested. During the six-day interval the increase in cell number was logarithmic§ with considerable variation. In this same interval the total number of counts recovered in RNA and in DNA remained essentially unchanged; again with considerable variation. The lines drawn are the best fit (least squares) for a straight line to the points as plotted. Both lines have a positive slope suggesting a gain in total counts with time, but neither slope is significantly different from zero ($m_{RNA} = + 0.016 \pm 0.11$ c.p.m. (log units)/day; $m_{DNA} = + 0.008 \pm 0.012$ c.p.m. (log units)/day). The total counts in the acid-soluble fraction

* These coverglasses have low but appreciable radioactivity which is not measurably reduced by the range of mass per unit area plated in these experiments.

** A metallographic polishing agent obtained from the Golwynne Magnesite and Magnesia Corporation, 420 Lexington Avenue, New York.

*** Tracerlab, 130 High Street, Boston, Massachusetts.

§ When these data are plotted as $\sqrt[3]{\text{cell number}}$[23], the correlation coefficient for a straight line fit to the points is not significantly different from that for the logarithmic plot used here.

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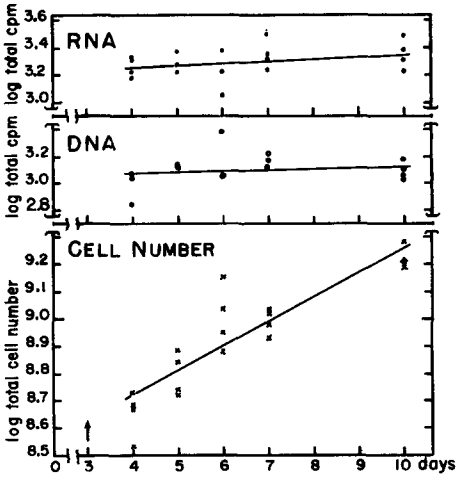

Fig. 1. Retention of labeled nucleic acid pyrimidines by ascites tumor cells in host animals receiving orotate-6-^{14}C. Two sets of 10 mice each were inoculated with equal volumes of Ehrlich hyperdiploid ascites tumor cells at time zero. On the third day each animal received 1 μC of orotate-6-^{14}C intraperitoneally. Animals from each set were sacrificed in pairs at the indicated times. The total tumor cell population was washed from the abdominal cavity of the animals. Total cell number and total radioactivity in RNA and DNA were determined as described in the text. The two experiments were run at separate times.

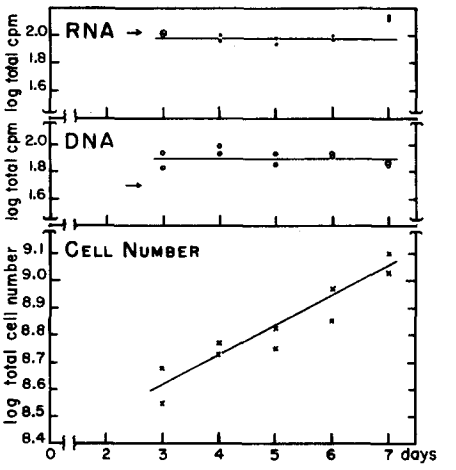

Fig. 2. Retention of labeled nucleic acid pirimidines by ascites tumor cells when transplanted to unlabeled hosts. Three mice bearing a 6-day Ehrlich hyperdiploid tumor received 1 μC of orotate-6-^{14}C intraperitoneally. Twenty-four hours later the cells were collected and pooled. Aliquots were taken for cell count, determination of radioactivity, and analysis as described in the text. Aliquots were also injected into each of 10 animals which were subsequently sacrificed in pairs on the days indicated. The progeny of the injected cells were harvested, counted, and analysed as described. The arrows indicate the total number of counts in the aliquot of cells received by each animal. The acid-soluble fraction contained 8 c.p.m.

were very low and variable, but were of the order of 0.1–1% of the sum of the counts in RNA plus DNA.

The organs, principally the livers[23], of the animals in these experiments, contained labeled nucleic acids as well as did the tumors. The livers contained an average of 3000 counts in RNA on the fourth day and 1700 counts on the tenth day. It is conceivable that activity lost from these organs could be incorporated into the tumor nucleic acids and thus obscure a loss from the tumors. To avoid this uncertainty the following experiment was done. Each of three animals bearing a 6-day Ehrlich hyperdiploid tumor were injected intraperitoneally with 1 μC of orotate-6-^{14}C. Twenty-four hours later the cells were harvested into ice-cold tubes and centrifuged. The cells were resuspended in ascitic fluid in a total volume of 10 ml. Each of 10 animals received a 0.5 ml aliquot of the suspension containing $7.6 \cdot 10^7$ cells. Aliquots were also taken for determination of radioactivity and for analysis. The results are shown in Fig. 2. The total counts in both RNA and DNA remain essentially unchanged during the period 3 to 7 days after injection. There is good agreement between the counts recovered and the counts injected in the RNA fraction. The agreement in the case of DNA is not good and one can only suspect some error in the analysis of the DNA fraction of the aliquots of injected cells, particularly since the range of recovered counts is small and the number of counts recovered is greater than the found number injected. An experi-

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ment of the same general type using the tetraploid tumor yielded the same result. This experiment differed in that 2 animals bearing a 3-day tumor each received 3 μC of orotate-6-^{14}C in 1 μC intraperitoneal doses given at 2-hour intervals. Twenty-four hours after the last injection the cells were collected and treated as in the previous experiment. Each animal received $7.9 \cdot 10^7$ labeled cells. The results are shown in Table I. In these latter experiments the total counts in the acid-soluble fraction of the recovered cells ranged between 0–5% of the total counts in RNA plus DNA.

TABLE I

RETENTION OF LABELED NUCLEIC ACID PYRIMIDINES BY ASCITES TUMOR CELLS

Animal	*Days after transplant*	*Cell number*	*RNA*	*DNA*
			Total counts/min	
1	3	$19 \cdot 10^7$	*	*
2	3	26	*	*
3	4	29	401	655
4	4	29	425	611
5	5	38	530	641
6	5	54	561	818
7	6	60	520	612
8	6	94	635	584
9	7	93	354	811
10	7	102	362	515
Mean			474	656
Injected aliquots**		7.9	457	669

* Samples lost.

** Acid-soluble fraction contained 63 c.p.m. total.

Two mice bearing a 3-day Ehrlich tetraploid tumor each received 3 μC of orotate-6-^{14}C intraperitoneally in 3 doses of 1 μC each at 2-hour intervals. Twenty-four hours later the cells were collected and treated as described in legend of Fig. 2.

DISCUSSION

Conservation of labeled constitutents of DNA previously reported to occur in many cells and tissues (see INTRODUCTION) is confirmed for these ascites tumor cells, at least with regard to pyrimidines.

In each of these experiments there is also conservation of the pyrimidines of RNA during a six-fold logarithmic increase in cell number. This latter finding appears to be at variance with the findings of some other workers (see INTRODUCTION). It should be emphasized at this point that our observations have been confined to pyrimidines while others have followed purines. The work of RÉVÉSZ *et al.*[8] is most interesting in this regard, in that with essentially the same protocol* as that used in the latter two experiments, they observed a 30% loss of RNA purines between the time of injection of the labeled cells and the time of first sampling of the progeny 48 hours later. Thereafter the purine label was conserved at the 70% level. This striking difference in the behaviour of the purines and pyrimidines is most interesting in the light of recent

* These authors estimated the amount of label retained by the cells during the experiment on the basis of the product of specific activity of nucleic acid purines multiplied by cell number. This procedure implies that the amount of RNA as well as the amount of DNA per average cell remains constant during the experiment. Results reported in the present paper are independent of this variable.

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implication of adenosine triphosphate in the activation of amino acids[24], and of RNA and guanosine polyphosphate in the incorporation of amino acids into the protein of microsomes in *in vitro* systems[25].

The experiments described in this paper were based on an hypothesis derived from the interpretation of results obtained on solid, primary tumors. Attempts to carry out experiments in which labeled ascites cells are implanted subcutaneously have so far been unsuccessful because of the early destruction of cells and the early onset of central necrosis after growth begins. BENNETT AND SKIPPER[14] have stated that during a six-fold increase in mass of Sarcoma 180 growing subcutaneously in mice which had received formate-^{14}C there is no significant decrease in the ^{14}C-content of the purines of RNA or DNA of the tumor. Although this experiment apparently suffers from the same ambiguity as the first experiment described above (Fig. 1), it strongly suggests that conservation of RNA purine-labeling also occurs in solid tumors.

In cases of conservation of labeled components of nucleic acids, it should be possible to determine by examination of the acid-soluble fraction of the cell whether the RNA and DNA molecules are stable once formed or whether there is breakdown and very efficient reutilization of the components. In our experiments the activity in the acid soluble fraction has ranged between 0–5% of the total counts found in RNA plus DNA. The results were highly variable between animals. The possibility of enzymic breakdown or acid hydrolysis during harvest of the cells or the early stages of fractionation cannot be eliminated in the experiments reported, and the results are therefore inconclusive.

In a population of cells in logarithmic growth each component of the cell, considered statistically, must be synthesized at a rate which is proportional to the amount of that component present. In these experiments the ratio of total activity in RNA to total activity in DNA is always less than the ratio of total mass of these compounds. The fact that orotic acid is available only for a short time interval[23], suggests that the immediate precursor pool for DNA pyrimidines differs in specific activity from that for RNA pyrimidines and that it reaches a higher specific activity.

Experiments done by others (see INTRODUCTION) using mammalian tissues have consistently indicated a loss of labeled constitutents from RNA. This has been called "renewal" or "turnover" of these constituents, and, with some reservations[11], has been taken to indicate renewal or turnover of the whole of the RNA studied.

The loss from mammalian tissue cells stands in sharp contrast to the essentially complete retention of label in RNA by many microorganisms in rapid growth. This same conservation of RNA-components can also be found in mammalian cells growing logarithmically in suspension in tissue culture[2]. Our results, those reported by BENNETT AND SKIPPER[14], and, at least in part, those reported by RÉVÉSZ *et al.*[8], taken together demonstrate a conservation of pyrimidines and purines of RNA by neoplastic cells, both ascitic and solid.

In all instances where conservation of RNA has been demonstrated the cells studied have been in rapid growth. It might be argued that the conservation of cellular constituents is simply a kinetic matter associated with the rapid synthesis of new cells. However, rapid growth of the liver of 4-week old rats and of regenerating rat liver does not prevent loss of labeled pyrimidines from RNA[26].

One of the outstanding features of the malignant cell is a fundamental independence of whatever mechanisms act to regulate the growth of normal cells. In a sense,

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cells growing in suspension *in vitro* are also independent of such influences. It is suggested that the conservation of RNA may be more than a quantitative consequence of high growth rate and further that it is a reflection of some qualitative difference in cells which are independent of growth-regulating mechanisms.

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SUMMARY

Essentially all of the ^{14}C of orotic acid-6-^{14}C incorporated into the pyrimidines of RNA and DNA of Ehrlich mouse ascites tumor cells (hyperdiploid and tetraploid lines) is retained for periods of 7–10 days *in vivo* during which the cells are increasing six-fold in number at a logarithmic rate. This result is observed whether the cells are maintained in the animals in which the labeling is effected or whether the labeled cells are removed to hosts which have received no radioactivity.

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