Differences between Diploid and Heteroploid Cultured Mammalian Cells in Their Response to Puromycin Aminonucleoside\textsuperscript{1,2}

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SUMMARY

Treatment of several heteroploid cultured mammalian cell lines with suitably low concentrations of aminonucleoside resulted in partial depression of ribosomal RNA synthesis, shown by methylated albumin-Kieselguhr column chromatography of labeled nucleic acids, and a lowering of the cell RNA content. These RNA-depleted heteroploid cell lines continued to proliferate at control rates for several cell generations. Proliferation of diploid cell lines, on the other hand, was inhibited by aminonucleoside without depression in cell RNA content. Methylated albumin-Kieselguhr chromatography of nucleic acids showed that the inhibition of ribosomal RNA synthesis was quickly followed by the inhibition of syntheses of the other types of RNA and of DNA. The inhibitory effects of aminonucleoside on both heteroploid and diploid cells were prevented by simultaneous treatment with inosine, suggesting that the action of the inhibitor was similar in the cells studied, since uptake of labeled aminonucleoside into the cell was not affected by the nucleoside.

These results show that the heteroploid cell has a greater capacity to proliferate when its rate of ribosomal RNA synthesis is depressed, probably because its ability to synthesize DNA has been freed from some of the controls present in diploid cells.

INTRODUCTION

Inexorable progress towards the next cell division is the hallmark of neoplasia. The tumor cell appears to prepare itself for mitosis as soon as the previous division has been completed, and in this process ignores the various regulatory controls which restrain the normal cell from incessant multiplication. As was recently pointed out by Eagle (6) and Hayflick (15), one approach to the study of mechanisms controlling normal and abnormal growth is to compare the properties of mammalian cell cultures which are grossly heteroploid with those which have a strictly diploid chromosome complement. In general, heteroploid cells can be propagated indefinitely in culture, grow rapidly and without marked restraint by cell to cell contact, and show a considerable proportion of abnormalities of mitosis. Diploid cell cultures, on the other hand, have a limited life span in culture, multiply at a slower rate with only a rare abnormal mitotic figure, and their multiplication is subject to contact inhibition by neighboring cells (6, 15, 16). Thus the growth characteristics of heteroploid cell cultures resemble those of tumors, while diploid cell cultures may serve as a model for nonneoplastic tissues, although it must be admitted that some neoplastic cells appear to be diploid (20, 21), that karyotypic changes in cultured mammalian cells do not always signify malignant transformation as judged by their capacity to grow as tumors when implanted into suitable hosts (12), and that it is difficult to find specific indicators of neoplastic conversion in vitro (25).

It has been shown in the first paper of this series (31) that HeLa cells have the unexpected capacity to continue multiplication while their RNA synthesis is partially inhibited by aminonucleoside of puromycin. This compound is the product of hydrolytic cleavage of the molecule of puromycin and is structurally analogous to adenosine (Chart 1). Unlike the parent compound, aminonucleoside is not a specific inhibitor of protein synthesis but inhibits the synthesis of nucleic acids.

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\textsuperscript{2} Some of these results were reported previously in a preliminary form (30), and were presented at the 9th International Cancer Congress, Tokyo, Japan, October 23-29, 1966.

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When the concentration of the inhibitor is suitably low, ribosomal RNA is selectively though incompletely inhibited, allowing near normal synthesis of the other types of RNA and of DNA. The RNA content of the cell is lowered, but if it does not fall below 75% of the control value, the HeLa cell continues to divide at the pretreatment rate. Higher concentrations of aminonucleoside inhibit the synthesis of all species of nucleic acids and after several days of treatment the rate of cell proliferation is retarded or division stops, but in each case the cell shows a marked drop in its total RNA content, the cells which cannot divide having only 50% of the RNA of control cells. When those experiments were repeated on WI38 cells, derived from human embryonic lung and known to be diploid, it was found that aminonucleoside produced stoppage of cell division in these cells without lowering their RNA content. Several other lines of cultured mammalian cells, mostly of human origin, were therefore tested in this way, and it was found that a clear-cut distinction can be made between diploid cells derived from human nonneoplastic tissues and heteroploid cells known to be capable of producing tumors (11, 13, 26, 28) regarding their response to aminonucleoside: only heteroploid cells can continue to divide more than once when their RNA synthesis is partially inhibited.

MATERIALS AND METHODS

Tissue Culture. HeLa cells used in this study were cultivated in this laboratory for several years. Originally, they were obtained from the following sources: HeLa-wild from Dr. J. J. Freed; HeLa-S3 from Dr. N. Salzman, HeLa-(af) (Ref. 24) from Dr. W. Henle. L cells (presumptive NCTC clone 929) were also obtained from Dr. W. Henle; FL amnion cells were obtained from Microbiological Associates, Bethesda, Maryland. WI38 and WI20 cells were a gift from Dr. R. Love, who also established their diploid karyotype. The SRO cell strain is a diploid line derived from adult skin fibroblasts by Dr. S. R. Oleinick. The passage numbers of the diploid cultures ranged from 13 to 35. Absence of mycoplasma infection was monitored by mycoplasma culture methods (17) in the laboratory of Dr. L. Hayflick.

All these cells were treated and handled in an identical manner. The cells were propagated as monolayers in plastic bottles but were seeded into Petri dishes 72 hours before each inhibitor experiment. The dishes were placed into a humidified CO₂ incubator. The cell concentration was so chosen that the control cells would be in optimal logarithmic growth during the experimental period. The medium used was in all cases Eagle’s minimal essential medium (5) supplemented with 10% calf serum; the concentration of amino acids and vitamins was doubled for optimal growth of diploid cells, and to ensure uniformity of conditions this enriched medium was also used for heteroploid cells. The medium was routinely supplemented by kanamycin (0.167 mg per ml) or aureomycin (50 µg per ml), but the antibiotics were omitted when the cells were plated out for an experiment. During the inhibitor treatment, the medium was changed daily on all Petri dishes.

Biochemical Methods. Three aliquots of 1.5 × 10⁶ cells each were taken for estimation of nucleic acid content by a scaled-down version of the modified Schmidt-Thannhauser procedure (32). The rates of nucleic acids synthesis were measured using the dual label method previously described (8). Briefly, cytidine-5³H and cytidine-U-14C were used as precursors, one to label the control cells, the other to label the experimental group. After completion of the labeling, the cells were lysed and the nucleic acids extracted. The nucleic acids from each group were redissolved, an aliquot taken for DNA estimation (19), and the control and experimental samples were mixed. The individual types of nucleic acid in the mixed sample were resolved by methylated albumin-Kieselguhr chromatography and the ³H/¹⁴C ratios determined for each. A second batch was processed similarly except that the isotopes were reversed between control and treated cultures. A calculation analogous to taking the geometric mean of the relative rates of synthesis for each nucleic acid in the two batches of each sample gives a precise estimate of the relative rate of synthesis of each type of nucleic acid in the experimental compared to the control culture. By simultaneous chromatography of the control and treated nucleic acids, column to column variation is eliminated, and the effects of tailing and overlapping of fractions is minimized by use of the ³H/¹⁴C ratios of peak fractions (8).

Effects of Inosine on the Uptake of Aminonucleoside. Cells to be used for testing the effects of nucleosides on the uptake of aminonucleoside were grown on coverslips in replicate ring cultures (34). Their growth was checked by harvesting and counting the cells. To estimate the total uptake of aminonucleoside, aminonucleoside-³H was presented to cells, using 4 replicate ring cultures per group, in the presence or absence of the nucleoside. At the end of the incubation period the medium was removed, the steel ring wall taken off the coverslip, and the 4 coverslips of each group passed through 4 washes in ice cold medium, the washing time being five minutes. The coverslips were drained after the last wash and allowed to dry. They were then broken into scintillation vials, the cells dissolved with 0.5 ml of NCS solubilizer (Nuclear Chicago), and, after the addition of a toluene dilution of Liquifluor (Nuclear Chicago), counted in a Nuclear Chicago scintillation spectrometer. Quench correction was effected using the channels ratio method with an external standard. To provide blank levels for isotope uptake, medium containing aminonucleoside-³H was pipetted onto and immediately sucked off a series of ring cultures, which were then processed as above. The counts detectable on these cultures were subtracted from the figures for total uptake.
Cytochemistry. Some dishes contained a small glass coverslip which was removed at the time of cell harvest, fixed in Carnoy's fluid, and stained for 30 minutes with aqueous toluidine blue, 0.5% at pH 3 in McIlvaine's buffer.

Materials. Tissue culture media were obtained from Grand Island Biological Co. Aminonucleoside was purchased from Nutritional Biochemical Co. Radioisotopes were obtained from Nuclear Chicago. Cytidine-5-3H (specific activity 1000 mc/m mole) and uniformly labeled cytidine-14C (specific activity 233 mc/m mole) were used, with unlabeled cytidine (Schwartz Bioresearch Inc.) present at 5 µg/ml to provide saturating conditions for incorporation. Uniformly labeled aminonucleoside-3H had a specific activity of 1550 mc/m mole.

RESULTS

Correlation between Cell RNA Content and the Rate of Cell Proliferation. As has been previously shown for uncloned HeLa cells (31), a concentration of aminonucleoside can be found which lowers the content of RNA in FL amnion cells, yet allows cell division to proceed for several generations at an unimpaired rate (Chart 2). In this case such a concentration of the inhibitor is 3 µg/ml, showing that this cell line is more sensitive to aminonucleoside than HeLa cells. When the level of aminonucleoside is increased to 6 µg/ml, cell proliferation is retarded and eventually ceases, while cell RNA content is markedly decreased. When a similar experiment is performed using human embryonic lung diploid cells (Chart 3), concentrations of 6 µg/ml or less have no apparent effect on the cell RNA content or the rate of proliferation for the first 48 hr, while concentrations of 12 µg/ml or more arrest cell proliferation after a lag of 24-48 hr. In this case the concentrations of aminonucleoside which inhibit cell division do not affect cell RNA content (Chart 3). It is impractical to study diploid cell cultures for more than 72 hr, since such cultures do not grow well when seeded at low cell concentrations.

A series of experiments such as those illustrated in Charts 2 and 3 were performed on different mammalian cell lines and strains, and the results pertaining to the correlation between cell RNA content and the rate of proliferation are summarized in Table 1. Without exception, the diploid cell strains studied in these experiments show virtually complete inhibition of cell division after 48 hours (roughly equivalent to one generation time of these cells) with aminonucleoside at 6 µg/ml or more, and no mitotic figures can be found by microscopic examination of stained cultures. The RNA content of the inhibited diploid cells does not decrease; on the contrary, a slight increase is frequently seen. This contrasts with the results obtained with heteroploid established cell lines. Here similar doses of aminonucleoside produce a lower cell RNA content but allow a control rate of cell proliferation if the decrease in cell RNA is not too severe. It should be noted that the heteroploid lines were chosen so that they would overlap with the diploid series with regard to generation times (JMC1 and HeLa-wild were close in this respect) and their origin; a line derived from adult skin was included in the diploid series.
Chart 3. The rates of cell proliferation and RNA content in lung diploid cells (JMC1) treated with aminonucleoside (AMS). The points shown represent means of 3 separate experiments, each performed in triplicate, and the standard error of the means. The numbers in the chart represent concentration of AMS in µg/ml. Concentrations of AMS less than 6 µg/ml had no effect on cell proliferation or RNA content during the period of study.

(SRO), and L cells, derived from a fibroblast, were among the heteroploid lines. An incidental correlation may be seen in Table 1: the heteroploid lines with shorter generation times appear to be more sensitive to aminonucleoside necessitating the use of lower concentrations of the inhibitor. This is probably a reflection of the fact that a larger number of cell divisions takes place within the experimental period.

**Nucleic Acid Synthesis in Aminonucleoside-treated Cells.** Nucleic acid synthesis, as measured by incorporation of labeled precursors, was studied in parallel with some of the experiments presented above, and the results are shown in Tables 2 and 3. The data shown in Table 2, obtained using radioactive cytidine on HeLa-(af) clone, are close to those found previously on uncloned HeLa cells using ^32^P as precursor, which necessitated corrections for intracellular phosphate pools (31). In each case, in these heteroploid cells, aminonucleoside inhibits most markedly, though incompletely, the incorporation of the isotope into ribosomal RNA. The incorporation into transfer RNA and 5 S RNA is less depressed, while there is little effect on the uptake of precursors into DNA or DNA-like RNA. Similar selective inhibition by aminonucleoside of ribosomal RNA synthesis was noted by Farnham and Dubin (9) using L cells and sucrose gradient centrifugation for fractionation of RNA, followed by compositional analysis of the RNA formed in the presence of the drug. On the other hand, Table 3 shows that in diploid cells treated with aminonucleoside for 24 h the synthesis of all types of nucleic acids is inhibited, with relative sparing of only DNA-like RNA. The synthesis of DNA is depressed most of all, in complete contrast to the situation in heteroploid cells. To determine whether this shut-down in DNA synthesis is primary or secondary, the effects of aminonucleoside after a shorter period of action on diploid cells was examined. In Table 3 it is evident that inhibition of the synthesis of ribosomal RNA and the low molecular weight RNA's precedes the inhibition of DNA synthesis and that the inhibition of these RNA types is progressive during the 24-hour period. This lack of selectivity in the inhibitory effect of aminonucleoside on DNA and RNA synthesis in diploid cells accounts for the arrest of cell division with undiminished cell RNA content.

**Protection by Inosine against the Inhibitory Effects of Aminonucleoside.** Chart 4 shows that when inosine is administered together with aminonucleoside, both heteroploid and diploid cells continue to divide at the control rate. Deoxyinosine, adenosine, or a mixture of adenosine and guanosine do not have this protective effect on HeLa cells, nor does deoxyinosine protect in diploid cells. Inosine not only allows the normal rate of cell proliferation in the presence of moderate concentrations of aminonucleoside, but also permits the synthesis of the stable forms of RNA to proceed at the control rate both in heteroploid (Chart 5) and diploid cells (Chart 6).

The protective effect of inosine against aminonucleoside is not due to interference with the entry of the inhibitor into the cell. The uptake of aminonucleoside-^3^H into the acid soluble pools of the cell is depressed very little by simultaneous addition of inosine (Table 4).

**DISCUSSION**

The data presented indicate a clear difference between the group of cultures which, for want of a better indication of their non-neoplastic properties, we shall designate as diploid, and the cultures composed of heteroploid cells, which in many cases have been shown to give rise to tumors when injected into suitable animals (11–13, 26, 28). When treated with aminonucleoside, heteroploid cells have the ability to synthesize DNA and DNA-like RNA (which includes "messenger" RNA) at near normal rates, although the synthesis of ribosomal RNA...
Diploid and Heteroploid Cultural Differences

Table 1

<table>
<thead>
<tr>
<th>Cell line or strain and passage number</th>
<th>Origin of the cell line</th>
<th>Approximate cell doubling time (hr)*</th>
<th>Cell RNA content at 48 hr*</th>
<th>Rate of cell proliferation between 48 and 72 hr*</th>
<th>Cell RNA content at 48 hr*</th>
<th>Rate of cell proliferation between 48 and 72 hr*</th>
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<td>Diploid cultures</td>
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<tr>
<td>WI-38 (28)*</td>
<td>Human embryonic lung</td>
<td>48</td>
<td>128</td>
<td>0</td>
<td>132</td>
<td>0</td>
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<tr>
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<td>113</td>
<td>5</td>
<td>127</td>
<td>0</td>
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<tr>
<td>JMC-1 (18)</td>
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<td>0</td>
<td>114</td>
<td>21</td>
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<td>JMC-1 (20)</td>
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<td>93</td>
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<td>103</td>
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<td>JMC-1 (30)</td>
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<td>102</td>
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<tr>
<td>JMC-2 (13)</td>
<td>Human embryonic muscle</td>
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<td>117</td>
<td>0</td>
<td>108</td>
<td>0</td>
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<tr>
<td>JMC-2 (14)</td>
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<td>101</td>
<td>0</td>
<td>107</td>
<td>0</td>
<td></td>
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<tr>
<td>JMC-2 (18)</td>
<td>Human embryonic muscle</td>
<td>101</td>
<td>0</td>
<td>119</td>
<td>0</td>
<td></td>
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<tr>
<td>SRO (20)</td>
<td>Adult human skin fibroblasts</td>
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<td>121</td>
<td>0</td>
<td>109</td>
<td>0</td>
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<tr>
<td>SRO (35)</td>
<td></td>
<td>103</td>
<td>0</td>
<td>100</td>
<td>0</td>
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Established heteroploid lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment</th>
<th>Origin of the cell line</th>
<th>Approximate cell doubling time (hr)*</th>
<th>Cell RNA content at 48 hr*</th>
<th>Rate of cell proliferation between 48 and 72 hr*</th>
<th>Cell RNA content at 48 hr*</th>
<th>Rate of cell proliferation between 48 and 72 hr*</th>
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<tr>
<td>HeLa-wild</td>
<td>Experiment 1</td>
<td>Human carcinoma of the cervix</td>
<td>36</td>
<td>74</td>
<td>117</td>
<td>68</td>
<td>108</td>
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<tr>
<td></td>
<td>Experiment 2</td>
<td></td>
<td>81</td>
<td>109</td>
<td>76</td>
<td>100</td>
<td></td>
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<tr>
<td></td>
<td>Experiment 3</td>
<td></td>
<td>75</td>
<td>107</td>
<td>64</td>
<td>99</td>
<td></td>
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<tr>
<td></td>
<td>Experiment 4</td>
<td></td>
<td>82</td>
<td>120</td>
<td>78</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>HeLa-(-af)</td>
<td>Experiment 1</td>
<td>Cloned from HeLa</td>
<td>30</td>
<td>82</td>
<td>120</td>
<td>78</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td></td>
<td>83</td>
<td>95</td>
<td>73</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td></td>
<td>18</td>
<td>77</td>
<td>77</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>HeLa-S3</td>
<td>Experiment 1</td>
<td>Cloned from HeLa</td>
<td>18</td>
<td>77</td>
<td>18</td>
<td>94</td>
<td>101</td>
</tr>
<tr>
<td>FL cells</td>
<td>Experiment 1</td>
<td>Human amnion</td>
<td>24</td>
<td>81</td>
<td>21</td>
<td>85</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td></td>
<td>69</td>
<td>10</td>
<td>85</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td></td>
<td>52</td>
<td>33</td>
<td>73</td>
<td>97</td>
<td></td>
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<tr>
<td>L cells</td>
<td>Experiment 1</td>
<td>Mouse subcutaneous fibroblast</td>
<td>20</td>
<td>89</td>
<td>102</td>
<td>57</td>
<td>55</td>
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<tr>
<td></td>
<td>Experiment 2</td>
<td></td>
<td>82</td>
<td>97</td>
<td>55</td>
<td>46</td>
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</table>

Correlation between cell RNA content and the rate of cell proliferation. AMS, aminonucleoside of puromycin.

*a Cell doubling time was obtained by enumeration of the cell number per culture every 24 hr and plotting the data on semilogarithmic paper.

*b Percent of the RNA content of untreated cells.

*c Increase in the cell number per culture relative to the cell number at 48 hr, presented as percentage of similarly calculated increase in untreated cultures during this time.

*d Passage number.

and (to a lesser extent) transfer RNA and 5 S RNA are depressed. As a consequence, the heteroploid cells continue to divide for several generations in the face of decreasing content of RNA. In diploid cells, on the other hand, aminonucleoside produces marked inhibition of the synthesis of both DNA and ribosomal RNA, and cell division ceases within one cell generation time. In these cells RNA content is not lowered.

A more precise analysis of this difference between diploid and heteroploid cells cannot be made at this time. The principal obstacle to fuller understanding of the molecular events taking place in cells treated with aminonucleoside is the lack of agreement on the mode of action of this antimitabolite, although it is clear that unlike its parent compound, puromycin, aminonucleoside is not a specific inhibitor of protein biosynthesis (2, 23). Dickie et al. (3) found that in E. coli aminonucleoside acts as a pseudofeedback inhibitor of purine biosynthesis, but in subsequent work (4) they withdrew an earlier suggestion that this mechanism also explains the induction of nephrotic syndrome in rat kidney (1). Farnham and Dubin (9) suggested that aminonucleoside may act in L cells as an analog of uncharged transfer RNA, which is thought to inhibit ribosomal RNA synthesis in nutritionally deficient bacteria (20, 29). In more recent studies with L cells (10), these workers have shown that inhibition of RNA synthesis cannot be attributed to incorporation of aminonucleoside into RNA and that this antimitabolite does not significantly inhibit the action of RNA polymerase. They found that adenosine and guanosine do not prevent aminonucleoside action and were...
Table 2

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Aminonucleoside at 12 μg/ml</th>
<th>Aminonucleoside at 6 μg/ml</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Set I*</td>
<td>Set II</td>
</tr>
<tr>
<td></td>
<td>(control culture 14C-labeled)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% synthesis</td>
<td>% synthesis</td>
</tr>
<tr>
<td>tRNA</td>
<td>$^{3}$H (dpm)</td>
<td>$^{14}$C (dpm)</td>
</tr>
<tr>
<td>sRNA II/</td>
<td>13,073°</td>
<td>1,260°</td>
</tr>
<tr>
<td>DNA</td>
<td>12,459°</td>
<td>1,276°</td>
</tr>
<tr>
<td>rRNA*</td>
<td>19,006°</td>
<td>2,396°</td>
</tr>
<tr>
<td>peak tube</td>
<td>33,314°</td>
<td>5,719°</td>
</tr>
<tr>
<td>35° D-RNA*</td>
<td>272,015</td>
<td>18,496</td>
</tr>
<tr>
<td>80° D-RNA</td>
<td>14,131°</td>
<td>1,127°</td>
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</table>

Rates of synthesis of the different nucleic acids of HeLa (-af) cells treated for 24 hours with aminonucleoside. tRNA, transfer RNA; sRNA II, eluted peak from methylated albumin-Kieselguhr composed of tRNA and 5 S RNA; rRNA, ribosomal RNA; D-RNA, DNA-like RNA.

*The actual counts (after correction to dpm) for a typical experiment are presented in this table to illustrate the method of calculating the relative rates of synthesis of the nucleic acid fractions in the treated cells. The methods are dealt with in full detail elsewhere (8). Set I is made up of a pair of cultures, one a control labeled with cytidine-U-14C for 60 min, and the other, a replicate culture labeled with cytidine-5-3H at the same time, 24 hours after exposing it to medium containing 12 μg/ml aminonucleoside. After labeling, the nucleic acids were extracted and handled as described in Methods and Ref. 8. The mixed nucleic acid samples were chromatographed together. Set II is another pair handled similarly but in which the isotopically labeled precursors were reversed.

The nucleic acids are listed in the order of elution from methylated albumin-Kieselguhr.

* Figures are the mean dpm of duplicate aliquots of the pooled fractions of the designated nucleic acid fraction. All samples were counted for either 40 min or until 20,000 counts were recorded in the 14C channel. Dpm were calculated after the sample quenching had been ascertained with an external standard. The approximate efficiencies of counting were: $^{3}$H, 30% with <0.02% falling in the 14C channel and 14C, 65% with 15% in the $^{3}$H channel.

* The figure in parentheses is the difference between the $^{3}$H/14C of the two duplicates expressed as a percentage of their mean dpm.

* These values are the relative rates of synthesis of the nucleic acids of the treated cells as a percentage of the rate in control cells. They were calculated using the following expression: $100 \times \sqrt{\frac{H_1}{H_2}} \times \frac{C_1}{C_2}$ and were corrected for the size of the several cultures used on the basis of the DNA content of the aliquots mixed for chromatography (8). The use of this DNA correction factor introduces an error related to the precision of the assay used to measure the DNA. This error affects each of the nucleic acid values within an experiment by the same amount; the error determines the statistical significance of the amount of the deviation of the relative rate of synthesis from 100%. This error does not influence the significance of the differences between the values for individual nucleic acids within an experiment. The 95% confidence interval for the DNA correction of this experiment was ±10.6% and was similar for the other experiments quoted.

Errors based on the statistics of counting were estimated as the 95% confidence interval of the calculated rate percent for the sample with the least number of counts (sRNA II) was ±0.8% of the quoted value. For the 6 μg aminonucleoside the maximum 95% confidence interval was ±1.0% of the quoted.

The formula used for calculating the 95% confidence interval of the product $\left(\frac{^{3}\text{H}}{^{14}\text{C}}\right)_1 \times \left(\frac{^{14}\text{C}}{^{3}\text{H}}\right)_2$ was $\alpha = 100 \times \sqrt{2 \left(\frac{H_1}{C_1} + \frac{H_2}{C_2}\right)}$ where $H_1$, $H_2$, $C_1$, and $C_2$ are the means of the counts recorded for the duplicate aliquots for $^{3}$H and $^{14}$C in the two series. Since the relative rates of synthesis are the square root of the product, the 95% confidence interval for them = $100 \sqrt{1+a} - 100$, which for small values of $\alpha$ is closely approximated by $\frac{\alpha}{2} \times 100$. This error expresses the significance of the differences between the values of the individual nucleic acids within an experiment.

*sRNA II consists of a mixture of tRNA and 5 S RNA (e.g., Ref. 8).

 Tubes containing eluate in the rRNA zone of the chromatogram were counted individually and the tube corresponding to the peak incorporation (Q RNA, the high molecular weight rRNA precursors) was used to calculate the rRNA values since it is least subject to tailing and cross contamination (8).

The tenaciously bound D-RNA was eluted in two fractions with 2% sodium dodecyl sulfate; one at 35°C, followed by the second at 80°C. In these experiments there were no significant differences noted between their values, so that they are presented as a single value in the other data.
Incorporation of labeled cytidine into nucleic acids of human embryonic lung diploid cells treated with aminonucleoside. tRNA, transfer RNA; sRNA II, eluted peak from methylated albumin-Kieselguhr composed of tRNA and 5 S RNA; rRNA, ribosomal RNA; D-RNA, DNA-like RNA; C.I., confidence interval. Labeling conditions were exactly as described in Table 2. Only one concentration, 12 μg/ml, was used.

* Nucleic acids as in Table 2.
* Expressed as percent of the control value and calculated as described in Table 2.
* The specimens (duplicate aliquots) were counted for either 20 min or 20,000 counts in the 14C channel. The 95% confidence interval which is quoted was calculated for the samples with the least recorded counts (sRNA II). The other fractions have smaller 95% confidence intervals.

led to believe that the drug does not act primarily as a pseudofeedback inhibitor of purine biosynthesis. However, our finding that inosine does protect against the inhibitory effects of aminonucleoside calls for a reevaluation of this possibility. Furthermore, it is possible that the effects of aminonucleoside are really caused by some phosphorylated derivatives of it, rather than by the parent nucleoside (10). It is thus possible that these active metabolites of aminonucleoside may inhibit the RNA polymerases and may inhibit the phosphorylation of exogenously supplied purine nucleosides as well as acting as feedback inhibitors of purine synthesis.

A number of other biochemical differences between diploid and heteroploid cells have been described. Eagle et al. (7) found that cultured human diploid and near-diploid cells, unlike grossly heteroploid cells, were deficient in cystathionase activity, necessitating a more stringent nutritional requirement for cystine by diploid cells. They suggested that enhanced cystathionase activity was a consequence of chromosomal aberrations. In a series of correlated spectrophotometric, interferometric, and time-lapse photographic studies, Seed (27) showed that in cultures freshly explanted from normal tissues (embryo human and embryo mouse), the net syntheses of DNA, nuclear protein, and nuclear RNA are closely associated during interphase, but in HeLa and ascites tumor cells the synthesis of nuclear RNA and protein are dissociated to a significant extent from DNA replication. It is also perhaps worth noting in this general context that the modified proposal of Warburg that neoplasia represents a shift to an anaerobic metabolism is not yet ruled out (33), and it is argued that an increase in glycolytic capacity may be a necessary, though not the only, change necessary for the neoplastic transformation (35). More recently, it was reported that there is a differential response to inhibitors of oxidative phosphorylation on protein synthesis in Ehrlich ascites tumor cells and normal thymocytes (18). The authors suggested that a high energy intermediate of oxidative phosphorylation is required for protein synthesis in intact mammalian cells but not in tumor cells.

In all these instances the heteroploid, transformed cell has been shown to acquire increased biosynthetic capacities and thus to become emancipated from the limitations imposed on its growth and division. These limitations may well be the con-
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trol mechanisms which regulate cell division in nonneoplastic tissues, but the relative importance of the known differences between normal and neoplastic cells cannot be at present assessed. It is possible that they are all consequences of a more basic alteration within the cell which is the crux of the neoplastic transformation.

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We are grateful to Miss Jeannie L. Schweitzer and Miss Agnes T. Masse for excellent technical assistance. Mr. Robert A. Cohen of the Jefferson Medical College and Medical Center Management Services generously provided computer facilities, and Mr. Kenneth Brossoie is to be thanked for writing the program.

Chart 5. Protection by inosine against the inhibitory effect of aminonucleoside (AMS) on cell proliferation and RNA accumulation in HeLa(-af) cell cultures. The numbers in the chart show the concentration of the compound in μg/ml. Each point is the mean of three determinations.

Chart 6. Protection by inosine against the inhibitory effect of aminonucleoside (AMS) on cell proliferation and RNA accumulation in WI38 diploid cells. The numbers in the chart show the concentration of the compound in μg/ml. Each point is the mean of three determinations.
REFERENCES


30. Studzinski, G. P. A Difference in Growth Controlling Mech-

Table 4

<table>
<thead>
<tr>
<th>Concentration of aminonucleoside-3H (μg/ml)</th>
<th>Concentration of inosine (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>1,432 ± 190 (100)</td>
</tr>
<tr>
<td>3.0, Experiment 1</td>
<td>2,544 ± 169 (81)</td>
</tr>
<tr>
<td>3.0, Experiment 2</td>
<td>943 ± 173 (75)</td>
</tr>
</tbody>
</table>

Effect of inosine on the entry of aminonucleoside into HeLa (-af) cells.

* Total uptake (cpm) of aminonucleoside-3H (4 μc/ml) into cells on cover slips, during 60 minutes at 37°C. The counts are the mean of 4 replicates ± S.D. The values in parentheses indicate the amount of aminonucleoside taken up by the cells as a percentage of the control value in the absence of inosine.

* The average number of cells per coverslip in Experiment 1 was 2.99 × 10³, and in Experiment 2 was 1.2 × 10⁵.

* This is the only value which is significantly different from the control at the 5% level of significance (0.02 < P < 0.05).
Differences between Diploid and Heteroploid Cultured Mammalian Cells in Their Response to Puromycin Aminonucleoside

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