The Incorporation of Radioactive Orotic Acid into the Nucleic Acid Pyrimidines of Animal and Human Tumors

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The incorporation of radioactive orotic acid in vitro into the pyrimidines of the nucleic acid of animal tissues has been studied (6, 7). In this paper data are presented on the incorporation of radioactivity into the pyrimidines of the nucleic acids of animal and human tumors. It is impossible to make any more than tentative conclusions on the differences of tumor tissue from the “normal tissues” for a variety of reasons: the variation from one normal tissue to another, the lack of homogeneity of the specimens studied, and the difficulties in adhering to any rigid microscopic criteria. On the other hand, in this system, as in many others, a certain constancy in the pattern of chemical reaction has been noted regardless of the tissue of origin of the tumor.

METHODS

Among the animal tissues studied were a rat Walker carcinoma 256, a liver from a rat bearing a subcutaneous Walker carcinoma, and a regenerating liver from a normal rat. The human tumors examined were a rapidly growing fibrosarcoma, an adenocarcinoma of the large bowel, a gastric adenocarcinoma, and a teratoma of the testis. Immediately following surgical removal, 6–10 gm. of the tumor were placed on ice, and the following procedure carried out quickly.

Two to 8 gm. of tissue were sliced with a Stadie slicer and suspended in 10 ml. of Krebs saline with added phosphate buffer at pH 7.4; from 1 to 8 mg. of radioactive orotic acid (labeled in the s-position with C14) with a specific activity of 6,000 counts/mg were placed in the medium and incubated at 88° C. for 4 hours in the presence of a 95 per cent O2-5 per cent CO2 mixture. After incubation, the slices were washed with water by centrifugation several times, homogenized, and the proteins precipitated with an equal volume of 10 per cent trichloroacetic acid. The residue was heated with 30-ml. portions of alcohol and ether until the supernatant following centrifugation was clear and colorless.

The residue from this procedure was placed in a large test tube, 10 ml. of 10 per cent aqueous sodium chloride solution was added, and the mixture was heated on a water bath for 24 hours with constant stirring. The solution was filtered and the nucleic acid precipitated by adding 2.5 volumes of absolute alcohol. After the solution had been in the cold room for 24 hours, the precipitate was removed by centrifugation, washed with absolute alcohol and ether, and dried in the desiccator. The nucleic acid was added to 0.5 ml. of 1 N HCl and heated for 1 hour in a boiling water bath. This resulted in a solution containing the free purine bases and the intact pyrimidine nucleotides.

Equal amounts of the hydrolysate were placed in a narrow band near the base of each of four strips of Whatman No. 1 filter paper, 12×40 cm., and allowed to dry. The papers were set up as ascending columns, with a 70 per cent solution of tertiary butyl alcohol (in water) made 0.8 N with HCl and run for 48 hours (5). The bands formed were easily outlined with a pencil when exposed to a “mineralight” lamp that emits most of its light in the region of 350–470 nm. The bands in order from the solvent front down were thymidylic acid, uridylic acid, cytidylic acid, adenine, and guanine. Each band was cut out and eluted with distilled water. The solution was made slightly alkaline and placed on a column of Dowex 1, 300–400 mesh, an anion exchange resin, as employed by Cohn (2). The column was washed thoroughly with water. Ribose cytidylic acid may be eluted from the column with 0.002 N HCl.
unidylic acid with 0.01 N. Thymidylic acid appears in at least two fractions. One portion is eluted from the thymidylic acid band with 0.01 N HCl, the other with 0.1 N HCl. In spleen and regenerating liver it is apparent that they show different specific activities. They gave the same absorption maxima. We assume at present that they represent two types of thymidylic acid. In the other tissues thymidylic acid eluted with 0.1 N HCl was analyzed and reported. Desoxynibose cytidylic acid is eluted from the column with 0.01 N HCl. The previous paper separation prevents contamination with uridylic acid. Further analysis of

### RESULTS

Table I shows the results of incubation of slices of two normal rat livers and a normal cat liver. It will be noted that the uridylic acid had 2–4 times the specific activity of the cytidylic acid. This difference has been consistent in many similar experiments involving brain, kidney, and liver from several different species. In several cases of spleen the uridylic acid was 6–7 times as active as the cytidylic acid, but in all other cases of normal tissue it has been a two- to three-fold difference. All tissues studied in this laboratory have shown degrees of incorporation of orotic acid into the

<table>
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<th>TABLE 1</th>
<th>SPECIFIC ACTIVITIES OF PYRIMIDINE NUCLEOTIDES OF NUCLEIC ACID FROM TISSUE SLICES INCUBATED WITH RADIOACTIVE OROTIC ACID</th>
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<tr>
<td><strong>Tissue</strong></td>
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<tr>
<td>Rat liver (1)</td>
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<tr>
<td>Rat liver (2)</td>
<td>146</td>
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<tr>
<td>Cat liver</td>
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<td>Fibrosarcoma</td>
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<td>126</td>
</tr>
<tr>
<td>Rat liver (tumor-bearing animal)</td>
<td>1,480</td>
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</table>

* Eluted from Dowex-1 column with 0.1 N HCl.
† Eluted from Dowex-1 column with 0.01 N HCl.

these methods will be presented elsewhere. Five- or 10-ml. portions of the eluates were examined for purity and quantity in the Beckman spectrophotometer. Small amounts of material not yet identified have also been obtained.

The reasons for the use of a separation based on a combination of paper and resin have been reported previously (7). It has been shown that contamination with radioactive orotic acid does not occur and that radioactivity occurs only in the pyrimidines and not in the sugar or the purines (7).

Eluates were evaporated to dryness and counted in a windowless counter. Sufficient counts were made to reduce the statistical error of counting to less than 5 per cent, except in several low counts on thymidylic acid in which the possible error is 20 per cent.

Orotic acid labeled in the 2-position was synthesized according to the method of Nyc and Mitchell (8) from cyanate made from C14-NaCN. A-methopterin was kindly provided by the Lederle Laboratories.

The table also shows the ready incorporation of C14 into slices of Walker carcinoma. A considerable difference in degree of incorporation into the uridylic and cytidylic acids may be observed. The ratios of their specific activities rise above normal to from 7 to 10:1. The thymine had an activity 1/25 that of the uracil. In the next experiment, slices from the same tissues used in the previous experiment were incubated with orotic acid plus 1µg. of A-methopterin. No significant effect of A-methopterin on the incorporation of C14 into pyrimidines was noted, but the data from the two experiments illustrate the reproducibility of results in the system.

The patterns of incorporation of the orotic acid into the nucleic acid pyrimidines of the human tumors are shown next. The incorporation of C14 into cytosine is relatively low in all cases. To one portion of slices of the gastric tumor 100 µg. of A-methopterin was added, with no inhibitory effect. The spleen of the patient bearing the gastric
carcinoma was also studied. The specific activities of the uridylic and cytidylic acids bear somewhat the relationship to one another as in the tumor itself.

Table 1 also shows the results of a similar experiment with regenerating liver, 48 hours after surgery. It will be noted that the ribonucleic acid components show little variation from the normal liver, whereas the activity of the thymidylic acid eluted with 0.1 N HCl is relatively much greater. A small amount of thymidylic acid eluted with 0.01 N HCl gives an even higher specific activity. The nature of the difference between the two types of thymidylic acids is not yet clear, and the low counts make accurate evaluation of the specific activities difficult. It is probable that this result is owing to the synthesis of new nuclear material. The results from a liver of a tumor-bearing rat, though slightly lower, are similar to those of the normal liver.

DISCUSSION

Orotic acid is readily incorporated into the pyrimidines of nucleic acids present in slices of normal tissues, regenerating liver, Walker carcinoma of the rat, and human tumors. The incorporation into the uridylic acid is, in the case of the tumors, of the order 7-10 times as great as that into the cytidylic acid, whereas in normal tissues and regenerating liver, the ratio is from 3 to 4 : 1. (Arvidson and Hammarsten found, using N¹⁴-labeled orotic acid, a 7:5 ratio in vivo experiments with rats [1].) In our experiments the reason for the differences in specific activities is not clear. If, in the case of the normal tissues, there were a reservoir of cytidylic acid 3-4 times that of uridylic, and if in tumors there were 10 times the reservoir of cytidylic acid as of uridylic acid, the results could be explained on simple dilution factors alone, assuming an equal rate of synthesis of uracil and cytosine from the orotic acid. In regard to amounts present, a ratio of cytidylic acid to uridylic acid greater than 2 has not been described in most normal tissues. It is possible that the reservoir of cytidylic acid may be somewhat larger than the reservoir of uridylic acid. We cannot be sure of the exact total amounts of the various components present originally, because it is difficult to evaluate the percentage recovery of the various bases. It is interesting to note in the data reported that the amounts of cytidylic acid are roughly twice those of uridylic acid for normal tissues and some of the tumors. Certainly in no case does the ratio approach 10 to 1. It is therefore unlikely that the difference in the specific activities of uridylic and cytidylic acid can be explained by dilution factors alone.

The most common argument used in work with labeled compounds is that the compound with greater specific activity may be the precursor of the compound with the lower specific activity. Using this argument we may say that the uracil group may be the precursor of the cytosine group in our experiment. The argument receives added support in the realization that uracil may be formed from orotic acid by simple decarboxylation. Cytosine may be formed from uracil by one step of amination, or it may be formed from orotic acid by two steps: first, amination, and then decarboxylation. There is no way of deciding which of the last possibilities is correct, but it is apparent that orotic acid forms the uracil group more readily than it does the cytosine group.

The relatively small amount of DNA precludes any explanation based on dilution factors in regard to the figures on thymine. As noted here, and as will be pointed out in other work on the rates of renewal of RNA and DNA, it is important to refer to specific bases, and it is perhaps misleading to draw general conclusions from the renewal rates of a single component such as the combined phosphorus of all nucleotides. Since the tumor is a rapidly growing system it is difficult to reconcile the large amount of cytidylic acid present with the lower rate of incorporation. The factors of renewal, growth, de novo synthesis from nonradioactive precursors, simple exchange mechanisms, and intracellular organization are not easily divorced and evaluated.

The rate of incorporation of radioactivity into the thymine, as compared to the uracil of tumors, is not unlike that found in normal tissue. On the other hand, the rate of incorporation of orotic acid into thymine of regenerating liver is considerably increased over that of non-growing liver.

Skipper (4) has demonstrated the inhibition of nucleic acid synthesis by A-methopterin as measured by the in vivo incorporation of radioactive formate into the nucleic acid purines. The absence of such an effect of A-methopterin in the present work may be owing to lack of permeability or the failure of the single tissue to convert A-methopterin to a more active form. The other possible interpretation of the results is that the rate of renewal of the nucleic acid pyrimidines is independent of the purines and does not involve steps in synthesis dependent upon folic acid.

SUMMARY

The nucleic acid metabolism of rat and human tumors as well as of regenerating liver and normal tissue from the rat has been studied in vivo in
slices by observing the incorporation of C$^{14}$-orotic acid into the pyrimidines of nucleic acid. In most normal tissue the rate of incorporation of the radioactive precursor into the uridylic acid of the nucleic acid was 2–4 times as great as into the cytidylic acid, whereas in all the tumors studied a seven- to ten-fold difference was noted.

ACKNOWLEDGMENTS

I wish to acknowledge particularly the constant help and guidance of Dr. D. Wright Wilson in all this work. I wish to thank Mrs. Natalie G. Aust for her valuable technical assistance. Thanks are due to members of the Department of Surgery for their co-operation in furnishing tissue immediately after its removal from the body, and to the Harrison Department of Research Surgery for preparing the animals with regenerating liver.

REFERENCES

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