Guanine in Cancer*

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It is abundantly clear that the ultimate precursors of the nucleic acids in the Metazoa are to be found among the amino acids. Neither nucleic acids nor purines and pyrimidines are essential nutrients in these species. Whether or not the free bases are intermediates in the biological synthesis of nucleic acids from amino acids is undecided. Adenine alone of the several nitrogenous moieties of nucleic acid is incorporated into the cellular polynucleotides. The incorporation of adenine is more complex than it might appear; when N15-labeled adenine was administered to rats by Brown, Roll, Plentl, and Cavalieri (5) and the nucleic acids subsequently harvested from the tissues, the isotope label was found in the guanine fraction as well as in the adenine fraction. It is probable that the incorporation of adenine in such circumstances is a reflection of a special type of nucleic acid metabolism involving an equilibrium with adenine-containing coenzyme systems rather than an indicator of the steps involved in the biosynthesis of the nucleic acids.

While free adenine is converted in part to polynucleotide-bound guanine, guanine is not utilized at all, according to Brown, Roll, Plentl, and Cavalieri. The latter conclusion was brought into question, however, when Kidder and co-workers (10) discovered the carcinostatic action of guanazolo (8-azaguanine). From the finding that guanazolo inhibits the growth of the protozoan, Tetrahymena geliti, for which guanine is a dietary essential, it was suggested that guanazolo is a competitive inhibitor of a unique metabolism of guanine by cancer tissues. Since the compound also inhibits the growth of a number of types of experimental cancer, the assumption was made that neoplasms, unlike normal tissues but like the protozoan, require free guanine for their development.

The pertinent question as to whether or not guanine is in fact utilized by cancer tissues was studied by Brown, Bendich, Roll, and Sugira, (4) but with equivocal results. They reported that mouse tumors did, indeed, incorporate guanine but that rat tumors did not, and, furthermore, that, contrary to earlier observations in the laboratory (5), the normal mouse tissue nucleic acids (black mice) also fixed labeled guanine.

This report describes our investigation of the same problem, in which guanine-8-C14 was employed to determine if the purine is incorporated into the cell-bound polynucleotides of either normal tissues or tumor tissues—whether they are susceptible to inhibition by guanazolo or not. It was found that guanine is not incorporated in either instance, whereas adenine is readily incorporated into both types of tissue under similar conditions.

Adenine-8-C14 in one instance, and guanine-8-C14 in another, was administered parenterally to groups of animals bearing spontaneous (virus-induced) breast carcinomas or transplanted breast Carcinoma 755, both of which are readily inhibited by guanazolo. A similar experiment for comparison was carried out on mice bearing Sarcoma 180, which is not inhibited by 8-azaguanine (7). The animals were sacrificed at intervals, and the mixed nucleic acids isolated from the dissected organs were examined radio metrically.

Adenine-8-C14 was synthesized according to the scheme:

\[
\text{NaCN} \rightarrow \text{BrCN} \rightarrow \text{NH}_2\text{CN} \rightarrow \text{NH}_2\text{CSNH}_3
\]

\[
(N\text{H}_3)_2\text{CS} + \text{NH}_2\text{C} \rightarrow \text{NH}_2\text{CSNH}_3
\]

\[
\text{NH}_2\text{CSNH}_3 \rightarrow \text{NH}_2\text{CSNH}_3
\]

* This work was aided by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and the Damon Runyon Fund. The radiocarbon used in these experiments was obtained on allocation from the United States Atomic Energy Commission, and the sodium cyanide-C14 was produced by Tracerlab, Inc.

Received for publication January 17, 1951.
Guanine-8-C\textsuperscript{14} was prepared similarly by condensing 2,5,6-triamino, 4-pyrimidol with thiourea-C\textsuperscript{14}.

Details of the synthesis of the isotopic purines, the transplantation of tumors, the administration of the isotopic compounds, the isolation of the nucleic acids, and the radiometric comparison are described in the experimental section below.

Thiourea is a versatile intermediate in the synthesis of either purines or pyrimidines, entering into not only the pyrimidine ring but also the imidazole ring. Although the reactions employed in the synthesis of thiourea are familiar, the incorporation of C\textsuperscript{14} involves certain modifications of procedure in order to obtain adequate yields in high purity on a small scale. The method of Baum (2), with some modification, was employed for the conversion of sodium cyanide to cyanogen bromide. Cyanamide was prepared in a manner similar to that of Bloch, Schoenheimer, and Rittenberg (3), except that the ethereal solution of cyanogen bromide, which has an appreciable vapor pressure, was not filtered free of drying agent before treatment with methanolic ammonia. Ammonium sulfide rather than antimony sulfide was used for conversion of cyanamide to thiourea to simplify the purification process.

It is desirable that the isotope be introduced at the latest possible step in the synthesis of adenine-8-C\textsuperscript{14} for reasons of economy. Pyrimidines were first prepared by methods previously reported, and the imidazole moiety of the purine ring was then formed by ring closure involving ortho amino groups and a single carbon atom compound. Traube's (11) condensation of 2-thiol-4,5,6-triaminopyrimidine with formic acid is not feasible for C\textsuperscript{14} syntheses, since a large excess of formic acid is required. For the preparation of adenine containing N\textsuperscript{3} in the pyrimidine ring, Brown et al. (5) adapted Baddiley, Lithgoe, and Todd's (1) condensation of 4,5,6-triaminopyrimidine with sodium dithioformate, a compound which is both unstable and difficult to purify. In another modification of that condensation involving the use of a mixture of formic acid and anhydrous formamide, Cavaliere, Tinker, and Bendich (6) reported a 3.8 per cent yield of adenine sulfate-4,6-C\textsuperscript{14}.

The utility and adaptability of thiourea for these syntheses were suggested by the work of Johns (9), who had prepared several 8-thiopurines by the condensation of molten thiourea with 0-diaminopyrimidines. Employment of approximately equimolar quantities of thiourea and substituted pyrimidines results in the best yields of 8-thiopurines, from which the purines can be obtained by desulfurization with hydrogen peroxide.

The ultraviolet absorption in neutral, acid, and alkaline medium served for identification, since the maximum absorption of 8-thioladenine is in the region of 300 m\textmu, while that of adenine is at 265 m\textmu. Adenine-8-C\textsuperscript{14} was, therefore, clearly distinguishable from its antecedent, since its absorption curve showed no significant contribution from 8-thioladenine.

EXPERIMENTAL

Cyanogen bromide-C\textsuperscript{14}.—One-half ml. of bromine, 49 mg. (one mm) of sodium cyanide-C\textsuperscript{14}, and 7 ml. of water were stirred magnetically in a 25-ml. distilling flask immersed in ice water. Sodium cyanide solution (100 mg/ml) was added dropwise, until the bromine color disappeared (4.88 ml.). The cyanogen bromide was distilled after 15 minutes into a three-necked 200-ml. flask containing 10 ml. of ether, and cooled in an ice-water bath. To assure complete transfer of the cyanogen bromide, 10 ml. of ether was added to the generator and distilled into the receiver. After being stirred magnetically, the ether layer was removed by a siphon. The extraction was repeated with four 20-ml. portions of ether and the extracts combined and dried over anhydrous sodium sulfate. The yield was determined by treatment of an aliquot of the ether solution with excess standard NaOH solution and back titration with standard HCl solution. It was 84 per cent.

Cyanamide-C\textsuperscript{14}.—Five ml. of 6 per cent methanolic ammonia was added to the ethereal solution of cyanogen bromide-C\textsuperscript{14}. The solution was refrigerated overnight, filtered, and the filtrate evaporated to dryness. The last traces of solvent were removed under vacuum. Crystalline cyanamide-C\textsuperscript{14}, 331 mg., was obtained in 80 per cent yield, based on sodium cyanide.

Thiourea-C\textsuperscript{14}.—Three ml. of concentrated ammonium hydroxide was added to 331 mg. cyanamide-C\textsuperscript{14} and saturated with hydrogen sulfide for 1 hour. The white, pasty cake which formed was allowed to stand overnight, diluted with 10 ml. of water, and boiled until the sulfur agglomerated. The yellow solution was filtered and evaporated to dryness. The residue was leached with boiling water, and the extract decolorized with Norite, evaporated to dryness, and recrystallized from propanol. Thiourea, 587 mg., m.p. 174°-177° C. was obtained in 77.5 per cent yield, based on sodium cyanide.

The specific activity of the thiourea was determined by evaporating an aqueous solution of radioactive thiourea under an infrared lamp after 500-fold dilution with nonradioactive thiourea. The area of the dish was 5 cm\textsuperscript{2} and contained more than 25 mg/cm\textsuperscript{2} of thiourea after being dried to constant weight in a vacuum desiccator. By use of a 1.55 mg/cm\textsuperscript{2} mica end window G.M. counter, 1,350 cts/min above background was obtained. The activity of the diluted sample was 68.4 cts/min/mg C, and the specific activity of the undiluted thiourea was 54,200 cts/min/mg C.

8-Thioladenine-8-C\textsuperscript{14}.—Two hundred and twenty-eight mg. (8.0 mm) of thiourea-C\textsuperscript{14} (specific activity 17,100 cts/min/mg C) and 385 mg. (3.08 mm) of 4,5,6-triaminopyrimidine (1) were intimately mixed and heated in a stream of nitrogen at 180° C. The mixture melted and then solidified after 30 minutes. The reaction product was dissolved in hot 2 per cent NH\textsubscript{4}OH. The solution was boiled with Darco, filtered, acidified with 6 N HC\textsubscript{1} solution. It was 84 per cent.

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The ether-salt-tissue suspension was dissolved in 14 ml. of hot 1 n sulfuric acid, the sulfate separated on cooling. An elementary analysis indicated the composition to be: 

(C$_{6}$H$_{12}$N$_{4}$)$_{2}$·H$_{2}$SO$_{4}$·2H$_{2}$O.

The solution was brought to a boil and filtered after the addition of 1 ml. 10 N sulfuric acid. The red insoluble residue was then added, and heating continued for another half hour. After 1 hour of heating, the contents of the tube were washed out with hot dilute alkali and filtered. The filtrate, including 3 N acetic acid. The light brown precipitate which formed was collected in a centrifuge tube, washed with water, alcohol, and ether, and then dried in vacuo over P$_{2}$O$_{5}$. The yield of crude thioladenine-8-C$^{14}$ was 464 mg. (77.8 per cent).

Guanine-8-C$^{14}$—Ten ml. of a fresh 3 per cent solution of hydrogen peroxide was added to 400 mg. of 8-thioguanine-8-C$^{14}$ in a small Erlenmeyer flask which was immersed in a boiling water bath for 4 hour. An additional 2 ml. of hydrogen peroxide was then added, and heating continued for another half hour. The solution was brought to a boil and filtered after the addition of 1 ml. 10 n sulfuric acid. The red insoluble residue was washed with hot 0.1 n sulfuric acid and with water. The hot combined filtrate (75 ml.) was made alkaline with concentrated ammonium hydroxide. Crude guanine-8-C$^{14}$ precipitated. The yield was 294 mg. (77.5 per cent).

Guanine-8-C$^{14}$ sulfate.—The crude guanine-8-C$^{14}$ (294 mg.) was dissolved in 30-55 ml. of 0.2 n sulfuric acid and heated under reflux with a small amount of Darco for 10 hours, filtered hot, and the residue in the funnel washed with 0.2 n sulfuric acid. Pure guanine-8-C$^{14}$ sulfate separated from the filtrate on cooling. The yield was 304 mg.; the specific activity was 3,800 cts/min/mg C.

The tumors used in these experiments were the spontaneous mammary carcinoma (virus-induced) in our own highly inbred Paris R III strain (8), transplanted Adenocarcinoma 755 in our highly inbred C57 strain, and Sarcoma 180, also transplanted in C57 mice. The spontaneous breast carcinoma mice were selected females with well developed tumors, relatively firm and without visible evidence of necrosis. This tumor is susceptible to guanazolo. Adenocarcinoma 755, originally received from Bagg and also sensitive to guanazolo, has been transmitted through 50 passages in this laboratory. It is particularly useful for isotope incorporation experiments, since it grows with fair regularity and does not become necrotic early. Sarcoma 180 has not been maintained in this laboratory since 1914. It grows rapidly and is distinctive for its complete resistance to 8-azaguanine.

One or more young, vigorously growing tumors was dissected under nearly sterile conditions, and small fragments were introduced by trocar intra- muscularly in the flank into 48 female recipients. The tumors took in all cases and were allowed to develop until they had attained a size approximating 1 gm., but without visible necrosis. The 755 tumor grew to this convenient size in about 14 days; Sarcoma 180 required about 10 days. Half of each group, 24, were given 1 ml. each of a solution containing 0.5 mg. adenine-8-C$^{14}$ sulfate intraperitoneally; the remaining 24 received 1 ml. of a slightly alkaline solution containing 0.5 mg. guanine-8-C$^{14}$ sulfate. Eight animals from each group were sacrificed 6 hours later, eight more in 24 hours, and the remaining eight at 48 hours. Tumors, livers, kidneys, and small intestines were dissected out and pooled. The nucleic acids were isolated from these pooled organs.

Fractionation and isolation of the individual nucleic acids will be discussed in a forthcoming publication. The immediate purposes of this experiment, however, were served by the isolation of the mixed nucleic acids without partition of the pentose and desoxypentose types. Thus, specimens prepared from different tissues contained different proportions of the two or more nucleic acids. Preparation of the mixed nucleic acids consisted of simple expedients to dissociate possible protein-nucleic acid linkages and to salt out or otherwise denature the proteins, while retaining the nucleic acids in solution.

The freshly harvested tissues were dispersed in 10 volumes of cold saturated NaCl solution with additional solid NaCl approximating the tissue in weight. A Waring Blender adapted to small volumes was used. The viscous, creamy suspension formed was stirred vigorously by motor in the cold for 1 hour. Small portions of ether were then added to the cold suspension at intervals until the ether phase exhibited a tendency to separate quickly when stirring was stopped. After stirring for about 1 hour more, the ether-salt-tissue suspension was centrifuged at room temperature for 15 minutes at 3,000 r.p.m. Three distinct, separable phases were formed: the bottom layer, a solid phase composed
of salt and insoluble tissue components; the middle layer, a saturated solution of the nucleic acids and a number of other solutes; and the uppermost layer, an ether-protein gel. The middle layer was most conveniently removed by a syringe and large-bore needle and clarified by centrifugation in the Sorval SS1 centrifuge at 10,000 r.p.m. for 15 minutes. The water-clear supernatant solution was ejected into 3 volumes of 95 per cent alcohol. The nucleic acid salt which precipitated was collected from the solution by rapid dialysis with tube-shaped collodion bags, each containing a large glass bead. The contents of the bag were agitated by end-over-end rotation of the bag in a large volume of ice water. Almost all the salt was removed in about $\frac{1}{2}$ hour by vigorous agitation and three changes of water. The solution in the bags was transferred to pyroxylin tubes and recentrifuged at 10,000 r.p.m. in the Sorval for 10 minutes before reprecipitation in 3 volumes of alcohol. This precipitate was most conveniently collected and dehydrated in the centrifuge tube. The preparations were adequate for the purpose when they dissolved readily and completely in the 1 per cent sodium chloride solution and when the absorption curve coincided with that of nucleic acid.

“Infinite” thickness specimens of the sodium nucleate preparations were measured under a mica-window Geiger-Müller counter. The counts observed have only approximate significance, since particle size and distribution were not ideally uniform in these specimens. The data for adenine are recorded in ratio of specimen radiation to background radiation (Table 1). The data for guanine are not recorded in the table, since in no instance was any radiation significantly above background detected. Sufficient counts to produce a statistical precision of 5 per cent were taken. The background varied between 0.30 and 0.40 counts per second.

**DISCUSSION**

The data show that cancer tissues do not uniquely utilize guanine in the synthesis of polynucleotides, and there is no evidence that the biosynthesis of nucleic acids in tumors is distinguishable from that in normal tissues which do incorporate adenine but not guanine. Carcinostasis by 8-azaguanine does not involve synthesis of nucleic acid from guanine.

The test compounds employed to demonstrate these facts were administered intraperitoneally, because nucleic acids, purines, or pyrimidines are not essential to the mouse diet, and, if the purines were to be intermediary synthetic metabolites, it seems probable they would be formed in close proximity to or within the cell rather than be transferred from or formed within the alimentary tract. That the guanine so administered did reach the vicinity of the cells is indicated by the fact that alcohol-dehydrated organs from similarly treated animals were radioactive. This radioactivity could be decreased by extraction of the tissue powder with cold trichloroacetic acid. The nucleic acids subsequently isolated from the tissue powder, however, were devoid of radioactivity. It seems probable that minute deposits of guanine-8-C$^{14}$ or xanthine-8-C$^{14}$ were the source of the whole tissue radioactivity. Indeed, in another experiment in which very large doses of guanine-8-C$^{14}$ were administered (4 mg. per day in divided doses for 3 days), multiple plaques of practically pure guanine were visible in the viscera. The organs of these animals were highly radioactive, and the radioactivity was leached out by cold trichloroacetic acid only slowly and with difficulty. As before, the nucleic acids exhibited no significant radioactivity above background. The resistance to trichloroacetic acid extraction suggests that, had the purines rather than nucleic acids been extracted from the tissue, as is feasible following hydrolysis of untreated tissue, it is probable that opposite and erroneous conclusions would have been reached.

The adenine data, confirming Brown, Roll, Plentl, and Cavalieri’s original finding, and extending it to include tumors, are of additional interest in that the rates of incorporation of adenine

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<td>RATIO OF SPECIMEN RADIATION TO BACKGROUND RADIATION, INFINITE THICKNESS, IN THE NUCLEIC ACIDS ISOLATED AT INTERVALS AFTER THE ADMINISTRATION OF RADIOACTIVE ADENINE</td>
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in different tumors and different organs approximate one another. This point and its implications will be discussed in a forthcoming report from this laboratory.

SUMMARY

1. Guanine is not incorporated into the cellular polynucleotides of either normal tissues or cancers.
2. Adenine is incorporated into cancer polynucleotides at a rate approximating its incorporation in normal tissue polynucleotides.

REFERENCES

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