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Thioguanine in Deoxyribonucleic Acid from Tumors of 6-Mercaptopurine-Treated Mice. (31210)

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One of a number of unsettled questions regarding the mechanism of action of 6-mercaptopurine (6-MP) concerns the nature of incorporation of the compound into nucleic acids. Previous reports(1,2) from this laboratory have shown that radioactivity is associated with nucleic acid fractions isolated from various mouse tissues following administration of either C^{14} or S^{35} labeled 6-MP. Hansen(3,4) has suggested that 6-MP may not be incorporated covalently into nucleic acids but that it may be firmly bound by means of divalent metal ions. Furthermore Brockman *et al*(5,6) found that, although label from 6-MP-S^{35} was associated with ribonucleic acid, none of this label could be found in the mononucleotide fraction of an alkaline hydrolysate. It seems likely, however, that thiol groups would oxidize under these conditions and that this would affect the chromatographic mobilities of such nucleotides. The present report is concerned with attempts to provide a definitive identification of the mercaptopurine-derived material present in tumor deoxyribonucleic acid.

Seven mice of the C57BL strain received transplants of a 6-MP-resistant line of adenocarcinoma 755(2,7). After 18 days each mouse was injected intraperitoneally with 0.5 ml of a solution which contained 1 mg of 6-MP-8-C^{14}, specific activity, 24 μc/mg. Each mouse had also received, 30 minutes previously, 0.25 ml of a solution containing 0.4 mg of 4-hydroxypyrazolo(3,4-d)pyrimidine, a compound which inhibits the degradation of 6-MP by xanthine oxidase(8,9). After 16 hours 10 g of tumor tissue was obtained from the mice and, from this, 55 mg of deoxyribonucleic acid (DNA) were isolated by the method of Kirby(10). The specific activity of this material was 13×10^3 disintegrations per min per mg, corresponding to the incorporation of about 0.2% of the administered dose.

TABLE I. Chromatographic Separation of Purine Derivatives.

Compound	Rf Solvent I	Rf Solvent IIA	Rf Solvent IIB	Recovered radioactivity, % of total*
Deoxyadenosine	.38	.55	.53	20
Deoxyguanosine	.19	.68	.60	10
6-Mercaptopurine	.42	.43	.37	<3
6-Mercaptopurine deoxyriboside	.34	.73	.64	<3
Thioguanine deoxyriboside	.25	.57	.50	60
Thioguanine	.22	.31	.29	—
Adenine	.20	.36	.50	—
Guanine	.08	.40	.38	—

* Calculations based on radioactivities of eluates of spots after 2-dimensional chromatography, calculated as % of the radioactivity. Approximately 70% of the radioactivity of the applied digest was recovered.

Radioactivity was measured in a Nuclear Chicago Liquid Scintillation Counter, Model 8260, at ambient temperature, and corrected for counting efficiency on the basis of a quenching correction curve.

A portion of the DNA was degraded to the constituent deoxyribonucleosides by means of successive treatments with pancreatic deoxyribonuclease and snake venom(11). Aliquots of digests derived from 200 μg of nucleic acid were then submitted to 2-dimensional paper chromatography: solvent I, 86% n-butanol/water(12); solvent IIA, a 5% aqueous solution of Na_2HPO_4; or solvent IIB, a 5% aqueous solution of $(NH_4)_2SO_4$ plus 5% isopropanol(13). Since the level of incorporation of radioactivity into the DNA was sufficient to account for only about 1 deoxynucleoside in 1500, it was not feasible to isolate sufficient quantities of material for direct identification. Consequently it was necessary to use the indirect method of addition of non-radioactive carrier substances. For this purpose the deoxyribonucleoside of 6-MP was synthesized from 6-MP and thymidine by means of a transdeoxyribosidase preparation from *Lactobacillus leishmanii*(14). The chromatograms of the enzymatically degraded deoxyribonucleic acid with added 6-MP and 6-MP deoxyribonucleoside were developed. Areas showing ultraviolet absorption were extracted with water for spectrophotometric identification and measurement of radioactivity. As is apparent from Table I, small amounts of radioactivity were present in the spots identified as deoxyadenosine and deoxyguanosine, but virtually none was present in the spots identified as 6-MP and 6-MP deoxyribonucleoside. About 60% of the radioactivity moved as a single spot to an unidentified area. The deoxyribonucleosides of thioguanine, thioxanthine, xanthine and hypoxanthine were then prepared by using the *L. leishmanii* enzyme (14). Chromatography of the DNA hydrolysate then was performed using these substances as carriers. In all solvents the radioactivity was found to be associated with the deoxyribonucleoside of thioguanine. The other deoxyribonucleosides and the ribonucleosides of 6-MP and of thioguanine were separated from the thiodeoxyguanosine in one or more of the solvents.

Additional evidence for identification of the radioactive nucleoside was obtained by hydrolyzing it to the aglycone. A solution of the DNA was adjusted to a pH value of 1.6 with formic acid and heated at 100°C for 5 minutes(15). Aliquots of this hydrolysate plus carrier thioguanine were submitted to chromatography in systems IIA, IIB or to electrophoresis at pH 2.0. The radioactivity was found to be associated with thioguanine, adenine and guanine, again in the proportion of 6:2:1. When hypoxanthine, xanthine, thioxanthine, 6-MP or the 8-hydroxy derivatives of the thiopurines were added as carriers, no appreciable radioactivity was found to be associated with any of them.

A chromatographic anomaly in the case of butanol-water solvent should be mentioned. In this solvent, it was found that a major portion of the radioactivity was associated with virtually any purine used as a carrier. When such a spot was cut into sections the radioactivity varied independently of the ultraviolet absorption, and only when thioguanine was

used as carrier was there a constant specific activity throughout the spot. In all other cases the radioactivity was concentrated in either the leading or the trailing edge. 6-Mercaptopurine also showed this tendency to co-chromatography. When a solution containing radioactive 6-MP and 1000 times as much of a non-radioactive carrier purine was submitted to butanol/water chromatography, all of the radioactivity moved with the carrier compound. Co-chromatography did not occur in system IIA or B.

It is apparent from this work that 6-MP is extensively anabolized in the mouse tumor. Since it has already been shown(16,17) that radioactive sulfate ion appears in the urine following administration of 6-MP-S^{35}, it is not surprising that the purine ring should find its way into deoxyribonucleic acid as adenine and guanine. The enzymatic conversion of 6-MP into thioxanthylic acid(18,19) by way of thioinosinic acid also has been shown to occur. One need only postulate the amination of thioxanthylic acid to thioguanylic acid and its subsequent anabolism along pathways known for guanylic acid to account for the eventual conversion of 6-MP to DNA-thioguanine.

The level of incorporation of 6-MP into DNA as thioguanine is within an order of magnitude of that reported under comparable conditions for incorporation of thioguanine into DNA(20), where this incorporation is presumed to be the mechanism of action of the compound. In the present case a 6-MP-resistant tumor was used; the sensitive strain tumor incorporates only one-half as much radioactivity. It remains to be shown that the form of the incorporated substance is the same in both tumor strains.

Summary. Deoxyribonucleic acid was isolated from a mercaptopurine-resistant adenocarcinoma 755 after treatment of the mice with 8-C^{14}-labeled 6-MP. The DNA was degraded to the deoxyribonucleoside level with pancreatic deoxyribonuclease and the digest was subjected to 2-dimensional chromatography. Deoxyadenosine and deoxyguanosine contained 20% and 10%, respectively, of the total radioactivity. The material of the major spot (60% of total radioactivity) migrated with authentic thioguanosine deoxyriboside, and after acid hydrolysis, with thioguanine. No 6-MP derivative could be detected.

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