Effects of 6-Diazo-5-oxo-L-norleucine on the Incorporation of Precursors into Nucleic Acids*

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SUMMARY

The tumor growth-inhibitor 6-diazo-5-oxo-L-norleucine (DON) was shown to inhibit, from 90 to 98 per cent, the incorporation of formate-C$^{14}$ into the purine bases of the nucleic acids from Sarcoma 180 tissue, small intestine, and liver, when given at the same time as the precursor formate. The inhibition was less, but still substantial, when the DON was given in small multiple doses instead of a single large dose. The block caused by the DON was probably the one demonstrated by Buchanan and co-workers, between formylglycinamide ribotide and formylglycinamidine ribotide (FGAR to FGAM); thus, DON had no effect on the incorporation of aminoimidazolecarboxamide-C$^{14}$.

It was observed that DON also inhibited in vivo the conversion of adenine to nucleic acid guanine, a sequence which requires glutamine. By giving large doses of aminoimidazolecarboxamide along with DON, the block from FGAR to FGAM could be minimized without affecting the block from inosinic acid to guanylic acid.

The results are explainable on the basis, reported by other workers, that DON is a strong glutamine antagonist.

The tumor-inhibitory antibiotic, 6-diazo-5-oxo-L-norleucine (DON) has been extensively studied in many experimental systems and clinically in cancer patients (for a tabulation of data and references, see [8]). This compound, as well as structurally similar azaserine (0-diazoacetyl-L-serine), has been of great help in elucidating biosynthetic mechanisms of purines and pyrimidines and therefore has been of interest in biochemistry as well as experimental therapy.

The work reported in this article was performed during the period of greatest interest in this compound (1956-1958). Although DON has not been useful clinically, its interesting biochemical and biological properties make worth while the presentation of the following data, which supplement the previous abstracts (4, 5).

MATERIALS AND METHODS

Sodium formate-C$^{14}$, specific activity of 1 $\mu$C/ $\mu$ mole, was injected in amounts of 1 $\mu$C/mouse, and

4 $\mu$C/rat. Adenine$^{1-8}$-C$^{14}$ had a specific activity of 0.25 $\mu$C/ $\mu$mole; 0.5 $\mu$C/mouse and 2 $\mu$C/rat were administered. Aminoimidazolecarboxamide$^{1-4}$-C$^{14}$ had a specific activity of 0.8 $\mu$C/ $\mu$mole and was given in doses of 4 $\mu$C/rat. Hypoxanthine$^{1-8}$-C$^{14}$ had a specific activity of 30,000 counts/min/ $\mu$mole. Five $\mu$moles were used per incubation vessel in the experiments in vitro.

DON was supplied by Parke, Davis and Company. In single-dose experiments it was administered at a level of 50 mg/kg of body weight. In the multiple-dose experiment it was given at a level similar to that used in therapeutic experiments—100 $\mu$g/kg given twice daily (200 $\mu$g/kg/day).

Most of the in vivo incorporation studies were done on female Swiss albino mice (Millerton Research Farms, Inc.) bearing Sarcoma 180 implants. When rats were used they were male Wistar strain (Carworth Farms) weighing from 125 to 150 gm.

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Incorporation studies with the tumor-bearing mice were performed 6–7 days after implantation, with the exception of experiments illustrated on Chart 2, which were started 3½ days after implantation and terminated 8½ days later. In all experiments the isotope was injected 5–10 minutes after the drug, and animals were sacrificed 5 hours after injection of isotope. All injections were by the intraperitoneal route. Control and experimental

2. Incubations were stopped by adding equal volumes of ice-cold 10 per cent trichloroacetic acid (TCA) and placing the vessels in an ice bath. The residue was centrifuged and washed once with ice-cold 5 per cent TCA. The washing was added to the original supernatant fluid, from which mixture the acid-soluble nucleotides were precipitated as barium salts. The nucleotides were hydrolyzed and the purine bases isolated as above.

### TABLE 1

**EFFECTS OF DON ON INCORPORATION OF PRECURSORS INTO RAT TISSUES**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Precursor</th>
<th>Addition</th>
<th>Tissue</th>
<th>DNA</th>
<th>RNA</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RSA</td>
<td>RSA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(μmol)</td>
<td>(μmol)</td>
</tr>
<tr>
<td>1</td>
<td>Formate-C₁⁴</td>
<td>None</td>
<td>Intest.</td>
<td>1.97</td>
<td>1.77</td>
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<tr>
<td></td>
<td>DON*</td>
<td></td>
<td></td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>2</td>
<td>AIC-4-C₁⁴</td>
<td>None</td>
<td>Liver</td>
<td>4.8</td>
<td>5.2</td>
</tr>
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<td></td>
<td>DON</td>
<td></td>
<td></td>
<td>4.2</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Intest.</td>
<td></td>
<td>12.5</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td></td>
<td></td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>Adenine-³-C₁⁴</td>
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<td>Liver</td>
<td>3.7</td>
<td>0.6</td>
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<td></td>
<td>DON</td>
<td></td>
<td></td>
<td>8.2</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>30</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td></td>
<td></td>
<td>5.5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
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<td>None</td>
<td>Liver</td>
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<td>0.20</td>
</tr>
<tr>
<td></td>
<td>AIC†</td>
<td></td>
<td></td>
<td>0.65</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>AIC+DON</td>
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<td></td>
<td>0.95</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Intest.</td>
<td></td>
<td>6.12</td>
<td>6.84</td>
</tr>
<tr>
<td></td>
<td>AIC</td>
<td></td>
<td></td>
<td>7.36</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td>AIC+DON</td>
<td></td>
<td></td>
<td>3.00</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* DON injected immediately after precursor at a level of 50 mg/kg.
† AIC injected intraperitoneally at a level of 500 mg/kg.

The results given in the tables are expressed as relative specific activities, where

\[
RSA = \frac{\text{counts/min}/\mu\text{mole isolated compound}}{\text{counts/min}/\mu\text{mole injected precursor}} \times 100
\]

In the charts results are depicted as per cent of control values. All the data obtained in the rat experiments are presented in Table 1; the data from mouse experiments are in the charts.

### RESULTS

Experiment 1, Table 1, shows the drastic inhibition of formate incorporation into purines of rat intestinal nucleic acids when the drug and precursor were administered at approximately the same time. The inhibition was almost 100 per cent. At the same time the utilization of formate for thymine synthesis was not disturbed.
In tumor-bearing mice the results of a single dose of DON were the same in all three tissues examined (Chart 1)—i.e., almost 100 per cent inhibition of incorporation into purines, and no effect on the level in thymine.

Administering a large single dose of an antimetabolite is effective in showing a direct effect on a metabolic system but does not approach the true physiological situation present during therapy. Sternberg et al. (15) have reported that repeated injections of DON into normal mice increase the toxicity more than 100-fold over that of a single dose, and Clarke et al. (7) have reported the same for tumor-bearing mice. The latter authors have established a therapeutic dose of 100–150 µg/kg of body weight, such dosage preferably divided into two daily injections. To examine the effect of DON at such a therapeutic level on purine metabolism, mice bearing S-180 were given injections twice daily of 100 µg/kg of DON each time for a total of seven injections. After the last injection of DON, the tracer dose of formate-C¹⁴ was administered, and the mice were sacrificed 5 hours later. Chart 2 depicts the results of such an experiment. In tumor and intestine, the inhibition of formate incorporation into the purines was still profound, though not as complete as that due to a single large dose. The liver showed signs of regenerative activity, especially in regard to the RNA.

At about this time Levenberg et al. (14) demonstrated, by in vitro enzymatic reactions, the effectiveness of DON in blocking the reaction in purine biosynthesis going from formylglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM) and showed that DON (as well as azaserine) was acting as a glutamine antagonist. Since this step is before the cyclization to the purine ring, utilization of precursors situated after FGAM in the biosynthetic sequence should not be affected provided glutamine is not required as a co-factor.

This indeed was the case with aminomidazole-carboxamide (AIC). When tracer doses of AIC-C¹⁴ were administered along with DON, there was no apparent effect on isotope incorporation into the purines (Experiment 2, Table 1).

In a first experiment with adenine-C¹⁴ used as a precursor in normal rats (Experiment 3, Table 1), it was surprising to see substantial inhibition of utilization of adenine for nucleic acid synthesis in intestinal tissue. This may be partly the result of general cellular damage. Particularly noticeable was the great reduction in conversion of adenine to guanine in this tissue.

This experiment was repeated on tumor-bearing mice, and, for comparative purposes, azaserine was used at the same level as DON. In Chart 3, it is readily apparent that DON had a real effect on the conversion of adenine to guanine in tumor tissue. In both DNA and RNA this inhibition was significant. In the DNA, DON also inhibited, though to a lesser extent, utilization of adenine. The same picture was seen in intestinal tissue. In both tissues DON, though not azaserine, inhibited

5 Fragmentation of nuclei of intestinal mucosa cells is sometimes observed when animals are given the dose of DON used here (personal communication from Dr. Stephen Sternberg).
the conversion of adenine to guanine, with intermediate inhibition of adenine incorporation into DNA. In the liver the inhibition of conversion of adenine to guanine was observed with little or no effect on adenine incorporation.

If there are indeed two sites of blockage caused by DON—FGAR to FGAM, and adenine to guanine—addition of an intermediate after the first block but before the second should show a favorable differential recovery toward adenine synthesis when formate is used as the precursor. This is in fact shown in Experiment 4, Table 1, and in Chart 4. In the normal rat liver AIC completely overcame the inhibition of formate incorporation caused by DON (Experiment 4). In intestinal tissue, one sees the anticipated differential effect (cf. Experiment 1). In the DNA recovery from DON inhibition of formate incorporation into adenine was of the order of 50 per cent; in the adenine of RNA recovery was complete. However, inhibition of incorporation of formate into guanine of DNA was almost total, and into guanine of RNA about 55 per cent. Repetition of this type of experiment on tumor-bearing mice (Chart 4) showed the same pattern.

Lagerkvist (12), with pigeon liver extract, and Abrams and Bentley (1, 6), with rabbit bone marrow, had reported enzymatic conversions in vitro of inosinic acid to guanylic acid which required glutamine as the amino group donor. It seemed probable to us that this was the reason for the DON effect on the conversion of adenine to guanine. In an in vitro incubation of S-180 tumor brei with hypoxanthine-C14, this was substantiated. In Table 2, Experiment 2, added DPN, with nicotinamide to inhibit the active DPNase of tumor, gave an RSA of 11.4 for the conversion of hypoxanthine to guanine. When DON was added, this figure was reduced by 40 per cent. Preincubation of the mixture for 30 minutes with glutamine, followed by addition of DON, prevented completely the inhibition previously caused by DON. DON also inhibited conversion of hypoxanthine to adenine; this must not have resulted from glutamine antagonism, since this inhibition was unaffected by preincubation with glutamine.
DISCUSSION

The almost complete block of formate utilization for purine synthesis caused by DON is an in vivo result forecast by the work of Buchanan and associates on the in vitro blockade of enzymatic synthesis of inosinic acid caused by azaserine (11, 13) and DON (14). The establishment of a block at the stage of formylglycinamide ribotide would drastically reduce the available pool of aminomimidazolecarboxamid ribotide, which is the next one-carbon recipient in the purine biosynthetic scheme.

There has been reported a second, and earlier, block in the synthetic pathway caused by azaserine (9). This block is between 5-phosphoribosylpyrophosphate and 5-phosphoribosylamine, which requires glutamine as an amino group donor. This block appears before the first of the two formate

small doses (Charts 1 and 2) of DON we believe can be explained by our previous work with hepatotoxic agents (3)—namely, a regenerative response of the liver tissue to a biochemical lesion caused by the repeated injections.

The finding of the DON effect on the conversion of adenine to guanine is again readily explained by the reports of Lagerkvist (12) and Abrams and Bentley (1, 6) referred to earlier. Their work has established the sequence

\[
\text{DPN glutamineinosinic acid} \rightarrow \text{xanthylic acid} \rightarrow \text{guanylic acid}
\]

both in fowl and mammalian tissue. Since the conversion of adenylic acid to guanylic acid is known

incorporating steps, and thus could contribute equally to the over-all inhibition of formate incorporation. However, as has been pointed out by Levenberg et al. (14), the "correspondence of enzymatic and physiological response in comparison of DON and azaserine" led them to postulate that the effect of azaserine and DON on animal tissues is the result of their effect on the enzymatic reaction FGAR \rightarrow FGAM. In any event, the absence of any effect of DON on AIC incorporation (Table 1) seems to indicate that the main block or blocks in purine biosynthesis caused by DON is before the cyclization of FGAM to give the imidazole ring.

The similarity in the extent of inhibition in tumor tissue caused by a single large dose (50 mg/kg) and therapeutic doses (seven injections of 100 \(\mu\)g/kg, see Charts 1 and 2) of DON may be cited as evidence that inhibition of purine biosynthesis is a major factor in the prevention of tumor growth caused by this agent. The difference in the response of the liver to a single dose and multiple

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formed purines, the experiments with large amounts of added AIC (Experiment 4, Table 1, Chart 4) are of great interest. The comparatively new technic of antimetabolite infusion directly into an artery supplying cancer tissue with concomitant administration of a metabolite to protect normal tissues has had some success in the case of methotrexate (antimetabolite) and citrovorum factor (metabolite) (16). It is known that azaserine and DON are irreversible inhibitors of glutamine (10, 14), and glutamine will not protect against them when given at the same time or after the drug (7). Since we have shown that AIC given at the same time does significantly reverse the inhibition caused by DON, it may be useful in protecting normal tissue while flooding the tumor with the antimetabolite.

REFERENCES
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