4-Aminopyrazolo(3,4-d)pyrimidine: an Inhibitor of the Synthesis of Purines and Proteins in Ehrlich Ascites Cells

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Carcinostatic activity of 4-(substituted amino)-pyrazolo(3,4-d)pyrimidines was found to be confined to compounds whose basic dissociation constants approximated that of adenine (1, 2). In addition, the toxicity of 4-aminopyrazolo(3,4-d)pyrimidine could be reversed by adenine or adenine-containing compounds in a number of biological systems (1, 3-7). These findings led to the hypothesis that the antineoplastic properties of amino pyrazolo(3,4-d)pyrimidines might be due to interference with the utilization of adenine or of an adenine derivative (2).

This hypothesis stimulated interest in the mode of action of 4-APP, and subsequent initial reports from this and other laboratories (7-11) indicated that this compound interfered with purine biosynthesis. These findings supported earlier observations that 4-APP caused a decrease in ribonucleic acid of mouse liver and in deoxyribonucleic acid of Sarcoma 180 (12). The present communication presents the effects of 4-APP on the utilization of preformed adenine, the incorporation of labeled isotopic precursors of nucleic acid purines by the Ehrlich ascites carcinoma in vivo. Evidence is presented for the presence of two metabolic blocks along the pathway de novo to polynucleotide purines and for a marked decrease in the rate of amino acid uptake into cellular protein.

EXPERIMENTAL PROCEDURE

Materials—Swiss female mice, weighing approximately 30 g, were used for these experiments. These animals were inoculated intraperitoneally 6 days before use with $2 \times 10^4$ Ehrlich ascites cells. Glycine-2-C$^14$ (Tracerlab, Inc.) was used at a level of 100 µg (10$^4$ c.p.m. per µg); adenine-8-C$^14$ (California Corporation for Biochemical Research) at a level of 50 µg (14 x 10$^4$ c.p.m. per µg); L-lysine-U-C$^14$ (Schwartz Laboratories, Inc.) at a level of 600 µg (5.8 x 10$^4$ c.p.m. per µg); sodium formate-C$^14$ (Tracerlab, Inc.) at a level of 90 µg (4.7 x 10$^4$ c.p.m. per µg); and hypoxanthine-8-C$^14$ (Nuclear-Chicago Corporation) at a level of 50 µg (9.4 x 10$^4$ c.p.m. per µg).

1 The abbreviations used are: 4-APP, 4-aminopyrazolo(3,4-d)-pyrimidine; azaserine, O-diazocetyl-L-serine; lysine-U-C$^14$, uniformly labeled lysine-C$^14$; AIC, 4-amino-5-imidazolecarboxamide hydrochloride; and AICR, 4-amino-5-imidazolecarboxamide ribonucleotide.

Sixty-eight per cent inhibition of total packed cell volume, by methods previously described (13), was obtained after four consecutive treatments of 20 mg of 4-APP per kg in Ehrlich ascites carcinoma (B. A. Booth and A. C. Sartorelli, unpublished results).

Obtained from Rockland Farms, New York, New York, and from A. R. Schmidt Company, Madison, Wisconsin.

4-APP (Sigma Chemical Company) was employed at a dose level of 30 mg per kg of body weight except as indicated.

Radioactivity was established by elution from a column of Dowex 50-X4 as described by Moore and Stein (14); all the radioactivity was associated with a single ninhydrin-positive peak. When adenine-8-C$^14$ and hypoxanthine-8-C$^14$ were chromatographed on paper with isopropanol-concentrated hydrochloric acid-water (170:41:39) according to Wyatt (15), each gave a single ultraviolet-absorbing spot which contained all the radioactivity. Paper chromatography of lysine-U-C$^14$ with the pyridine-acetic acid-water (50:35:15) system described by Decke and Riffart (16) resulted in a single spot containing all the radioactivity.

Methods—Six days after tumor implantation, mice were treated by intraperitoneal injection with a single dose of 4-APP. After the time interval indicated, the appropriate isotope was administered intraperitoneally and allowed to incorporate for 1 hour. In some cases, cells were pretreated with 0.2 mg of azaserine per kg of body weight before drug and isotope administration. Control mice received no drug, but a comparable volume of isotonic saline was injected before the isotopic substrate. At the end of the incubation period, cells were harvested, and the nucleic acid purines and acid-soluble adenine were isolated and analyzed as described by LePage (17), except that all nucleic acid adenine and acid-soluble adenine samples were further purified from contaminating 4-APP by paper chromatography in Wyatt’s solvent system (15). Radioactivity was measured with a Nuclear-Chicago model D47 windowless gas flow counter.

The residue remaining after removal of nucleic acids was washed twice with 0.2 M perchloric acid, once with 70% ethanol, twice (with constant stirring) for 20 minutes with absolute ethanol, and twice with absolute ether. Four volumes of solvent were routinely used. The residual protein was dried overnight in a vacuum desiccator, plated on tared aluminum planchets, and radioactivity was determined as previously described.

RESULTS

Physicochemical similarities between adenine and 4-APP have been described (3). To determine whether growth-retarding properties of 4-APP were accompanied by alterations in the utilization of preformed adenine, the incorporation of labeled adenine into mixed nucleic acid purines and acid-soluble adenine of Ehrlich ascites cells was measured after a dose of 4-APP. No
The duration of inhibition of purine formation de novo by 4-APP was determined and is shown in Table II. In this and in subsequent experiments, an arbitrary level of 30 mg per kg was selected as the test dose. Inhibition of glycine incorporation into polynucleotide purines occurred at the earliest time measured (5 minutes) and was present for up to 6 hours after exposure to drug. By 12 hours, a normal rate of synthesis of purines de novo appeared to have been restored.

To determine whether 4-APP was simulating the demonstrated capacity in vitro of adenine to function as a feed-back inhibitor of purine synthesis de novo in these ascites cells (17, 18), a 30 mg per kg dose of adenine was administered before the radioactive glycine. No inhibition of biosynthesis de novo as measured by glycine incorporation into polynucleotide guanine was found in this test system in vivo.

The essentially equal inhibition of both polynucleotide adenine and guanine when glycine-C\textsuperscript{14} was used suggested a block before formation of inosine-5'-phosphate. In an attempt to localize the site of purine inhibition by 4-APP, other isotopic substrates were employed in the ascites cell system. When radioactive formate, which is utilized for the formylation of both α-N-glycinamide ribonucleotide and AICR, was used as a measure of the pathway de novo, 4-APP treatment resulted in an inhibition of isotope incorporation into nucleic acid and acid-soluble purines (Table III). Treatment with the glutamine antagonist, azaserine, before formate-C\textsuperscript{14} caused essentially complete inhibition of incorporation into purines. Exposure to 4-APP after treatment with azaserine resulted in adenine-containing compounds of a higher specific activity than was found with azaserine treatment alone.

To limit the number of metabolic reactions measured, AIC was injected simultaneously with formate-C\textsuperscript{14} to serve as an acceptor molecule. Retardation of the incorporation of formate into purines in the presence of AIC was obtained after exposure to 4-APP; the inhibition was of the same magnitude as that obtained with glycine-C\textsuperscript{14}. To prevent or minimize the reaction of formate with αN-glycinamide ribonucleotide, cells were pretreated with azaserine before 4-APP treatment and isotope administration. Considerable incorporation of formate-C\textsuperscript{14}, and presumably AIC, into polynucleotide purines was found in vivo.

### Table I

<table>
<thead>
<tr>
<th>Time after 4-APP</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA* guanine</td>
</tr>
<tr>
<td>min</td>
<td>c.p.m./μmole X 10\textsuperscript{3}</td>
</tr>
<tr>
<td>0</td>
<td>7.4 ± 1.0\dag</td>
</tr>
<tr>
<td>5</td>
<td>8.2 ± 1.5</td>
</tr>
<tr>
<td>60</td>
<td>9.0 ± 1.5</td>
</tr>
</tbody>
</table>

* NA, nucleic acid; AS, acid-soluble.
\dag Standard deviation of the mean.

### Table II

<table>
<thead>
<tr>
<th>Time after 4-APP</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA* guanine</td>
</tr>
<tr>
<td></td>
<td>c.p.m./μmole X 10\textsuperscript{3}</td>
</tr>
<tr>
<td>0</td>
<td>4.5 ± 0.5\dag</td>
</tr>
<tr>
<td>5 min</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>60 min</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>3 hrs</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>6 hrs</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>12 hrs</td>
<td>5.8 ± 0.5</td>
</tr>
</tbody>
</table>

* NA, nucleic acid; AS, acid-soluble.
\dag Standard deviation of the mean.
amino acid. As a determinant of protein synthesis, a more pronounced and significant depression of formate uptake, radioactive isotopes were injected and allowed to be incorporated with 30 mg of 4-APP per kg of body weight as indicated. Azaserine at a level of 0.2 mg per kg was injected when indicated, 30 minutes preceding 4-APP. One hour after 4-APP administration, radioactive isotopes were injected and allowed to be incorporated for 1 hour. The zero time point represents the results obtained with control mice which received an injection of isotonic saline 1 hour before radioactive substrate. Each value represents the average of results obtained from the separate analyses of ascites cells from four to eight animals.

Analyses of residual protein suggested that 4-APP produced an inhibitory effect on protein biosynthesis. The results are shown in Table IV. Glycine-2-C\(^4\) incorporation into protein was decreased 1 hour after administration of 4-APP, with inhibition reaching a maximum greater than 50\% at 6 hours. By 12 hours after administration of 4-APP, the incorporation of labeled glycine was restored to the control level. The possibility that inhibition was a reflection of an altered metabolic disposition of glycine appeared to be eliminated by employing lysine-C\(^4\) as a determinant of protein synthesis. A more pronounced and more prolonged inhibition was found with the use of this essential amino acid.

### TABLE III

Incorporation of labeled precursors into purines of Ehrlich ascites cells treated with 4-APP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>From glycine 2-C(^4)</th>
<th>From lysine-U-C(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate-C(^4)</td>
<td>-64.1 ± 19.7 (\times 10^4) c.p.m./mg</td>
<td>981.2 ± 135</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-34.2 ± 5.0</td>
<td>391.5 ± 51.8</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-1.4 ± 0.2</td>
<td>10.0 ± 1.3</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-1.2 ± 0.3</td>
<td>21.5 ± 3.6</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-71.3 ± 9.1</td>
<td>58.6 ± 8.7</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-4.4 ± 3.9</td>
<td>13.3 ± 2.6</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-66.6 ± 13.7</td>
<td>431.4 ± 60.0</td>
</tr>
<tr>
<td>Formate-C(^4) + AIC</td>
<td>-33.5 ± 5.3</td>
<td>520.8 ± 43.9</td>
</tr>
<tr>
<td>Hypoxanthine-8-C(^4)</td>
<td>-22.1 ± 1.1</td>
<td>353.1 ± 16.5</td>
</tr>
<tr>
<td>Hypoxanthine-8-C(^4)</td>
<td>-26.4 ± 1.9</td>
<td>630.0 ± 42.3</td>
</tr>
</tbody>
</table>

* NA, nucleic acid; AS, acid-soluble.
† Standard deviation of the mean.

### DISCUSSION

4-APP has been shown to be capable of interfering with the biosynthetic formation of purines in several test systems (7-11). The data presented in this paper, obtained with the use of the Ehrlich ascites carcinoma, demonstrated the presence of enzymes sensitive to 4-APP on the pathway de novo of purine biosynthesis. An essentially equal depression of the rate of glycine-2-C\(^4\) incorporation into the nucleic acid purines adenine and guanine was obtained, suggesting the presence of a 4-APP-induced block on the pathway de novo before the formation of the key intermediate, IMP. These findings were in agreement with the observation by Cote and Golub (8) that the adenine analogue decreased the rate of AICR formation by Escherichia coli strain B-96. In an attempt to localize the site of 4-APP inhibition on this pathway, other radioactive substrates capable of measuring purine synthesis de novo were used. Since one of these labeled substrates, formate-C\(^4\), is utilized at two sites in the sequence de novo, low levels of azaserine were used to prevent the participation of metabolic reactions before \(\alpha-N\)-formylglycinamide ribonucleotide. Azaserine treatment caused an almost complete suppression of the incorporation of labeled formate into the polynucleotide purines, suggesting that only a low level of exchange occurs between formate and IMP in these cells. The addition of AIC to serve as an acceptor molecule for the formate-C\(^4\) restored the rate of formate incorporation into purines in azaserine-treated cells, suggesting that AIC was being utilized for purine formation. Administration of 4-APP after azaserine pretreatment resulted in an inhibition of incorporation into polynucleotide guanine, whereas no significant decrease in the rate of formate utilization for adenine nucleotides was obtained. These data are consistent with the presence of two metabolic blocks on the pathway de novo; one before the formation of AICR, and the second presumably after the formation of IMP. The fallibility of this interpretation was apparent when it was observed that 4-APP did not inhibit the conversion of hypoxanthine-8-C\(^4\) to nucleic acid guanine or adenine. It was con-
ceivable that hypoxanthine and glycine were not converted to the same hypoxanthine-containing nucleotide or that one proceeded to inosine before apportionment to adenine and guanine. It was also possible that both substrates did proceed through IMP and that the results obtained were an expression of cellular compartmentalization. A third possibility was that 4-APP prevented the conversion of AICR to IMP. A metabolic bypass by which the condensation product of AICR and aspartic acid could be formylated and converted to AMP without passing through IMP would explain the presence of inhibition of formate-C\textsuperscript{14} incorporation into guanine without concomitant inhibition of adenine in cells treated with azaserine and 4-APP. Such a pathway in pigeon liver homogenates was suggested by Carter and Cohen (19).

The inhibitory effect found on incorporation of labeled amino acids into protein of Ehrlich ascites cells is in contrast to that reported by other workers in a bacterial system (7). The results obtained in the present study with both a nonessential and an essential amino acid would suggest that 4-APP is interfering with protein synthesis rather than altering the metabolic disposition of a given amino acid. The action of 4-APP on residual protein differed from the effect of the drug on purine formation in that inhibition of purine synthesis reached a maximum 1 hour after the drug dose, whereas maximal retardation of protein formation occurred 6 hours after the drug.

Finally, it cannot be concluded from these data that 4-APP per se is the active inhibitor. The adenine analogue is extensively metabolized by the intact rat (20) and by enzyme preparations from a number of mammalian tissues (9, 21, 22). Indeed, one of these metabolites, 4-amino-6-hydroxypyrazolo(3,4-d) pyrimidine has been reported to be a more effective inhibitor of xanthine oxidase than is 4-APP (23).

SUMMARY

Experiments carried out with the Ehrlich ascites carcinoma to study some of the metabolic effects of 4-aminopyrazolo(3,4-d)pyrimidine have been described. It has been demonstrated that this compound exerts an inhibitory effect on both purine biosynthesis de novo and protein biosynthesis.

Sites of inhibition by 4-aminopyrazolo(3,4-d)pyrimidine on the pathway de novo of purine formation have been partially localized by measuring the effect of the analogue on the rate of utilization of isotopic precursors of polynucleotide purines. A site of inhibition occurs before the formation of 4-amino-5-imidazolecarboxamide ribonucleotide and is characterized by an essentially equal decrease in the uptake of glycine-C\textsuperscript{14} and formate-C\textsuperscript{14} into both polynucleotide adenine and guanine. A second site of inhibition, measured by limiting synthesis de novo with O-diazoacetyl-L-serine, occurs after the formation of 4-amino-5-imidazolecarboxamide ribonucleotide. The block is characterized by a decrease in the incorporation of formate-C\textsuperscript{14} into polynucleotide guanine in the presence of 4-amino-5-imidazolecarboxamide with no concomitant decrease in its incorporation into polynucleotide adenine.

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REFERENCES

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