Studies on the Mechanism of Action and Anticancer Activity of N-Methylformamide*

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Clarke et al., employing Sarcoma 180, first observed the tumor-inhibiting activity of formamide and its more potent N-methyl derivative. It was pointed out that formamides were, like urethan, hepatotoxic; however, unlike urethan, these agents were not central depressants (1).

Although formamide or N-methylformamide may not prove to be a clinically useful anticancer agent, it is perhaps of importance to study the mechanism of action of these compounds in order to add to our store of knowledge of the biochemistry of cancer.

It has been observed that urethan inhibition of E. coli can be partially prevented by 2,6-diaminopurine of 2,6-diaminopurine riboside (4). In view of the similarity in structure between urethan and formamide,

\[
\begin{align*}
\text{Urethan} & : & \text{NH}_2 & \quad \text{Formamide} & : & \text{NH}_2 \\
\text{OC} & \quad \text{H} & \quad \text{O} & \quad \text{C} & \quad \text{NH}_2 & \quad \text{NH}_2
\end{align*}
\]

it seemed of interest to determine if the actions of these agents were in any way related from a biochemical standpoint and to assay the effectiveness of formamide and N-methylformamide against additional mouse neoplasms.

EXPERIMENTAL

Mechanism studies with E. coli.—The general objective of these experiments has been to search for materials which might prevent the growth-inhibiting effects of formamide on E. coli (ATCC No. 9637). A minimal broth (MB) or minimal agar (MA), described by Davis and Mingioli (2), was employed. Minimal agar plates were seeded with a final dilution of \(10^{-3}\) of a 24-hour MB culture of E. coli. Initial experiments showed that 15–20 mg. of formamide/ml of MA was sufficient to inhibit the organism for 48–72 hours (at 37° C.).

Solutions of candidate reversal agents were sterilized by filtration through bacteria-tight filters. These solutions (approximately 0.015 ml.) were placed on sterile discs of Whatman No. 5 filter paper cut 1 cm. in diameter. The discs were placed on the surface of E. coli-seeded MA plates containing 17.5 mg/ml of formamide. Control cultures with and without formamide were prepared in all experiments. All plates were incubated at 37° C. and examination of each for bacterial growth was made at intervals up to 72 hours. Heavy bacterial growth around a given disc was assumed to denote prevention of the growth-inhibiting effects of formamide.

In a first series of experiments it was observed that solutions containing 1 mg/ml of the following, when placed on discs, failed to reverse the inhibition by formamide: formate, folic acid, citrovorum factor, guanine, adenine, xanthine, hypoxanthine, guanylic acid, adenylic acid, ATP, and TPN. A similar level of 2,6-diaminopurine caused heavy growth for about 3 cm. (measured from the edge of saturated paper discs) on formamide-inhibited plates. Thus approximately 15 µg. of diamino-purine (DAP) allowed growth of E. coli for about the same area as was observed in reversal of minimal levels of urethan by this quantity of DAP (4).

A comparison of the effectiveness of DAP and DAP-riboside\(^1\) in preventing formamide inhibition of E. coli was then made (Table 1).

These results indicate that DAP and DAP-riboside are of the same order of effectiveness in preventing formamide inhibition of E. coli.

In additional experiments it was observed that

\(^1\)2,6-Diaminopurine riboside was kindly provided by Dr. G. B. Brown.
solutions containing 10 mg/ml of formate, glycine, serine, methionine, adenine, hypoxanthine, or 4-amino-5-imidazolecarboxamide on discs failed to prevent inhibition of formamide (20 mg/ml of MA), whereas a 2,6-diaminopurine solution of 0.1 mg/ml provided easily discernible growth around a saturated disc.

By the simple technic of placing adenine-saturated paper discs in close proximity to DAP-containing discs on a formamide-inhibited plate inoculated with E. coli, it was observed that adenine prevents the action of DAP in reversing formamide inhibition. This antagonistic action between DAP and adenine is similar to results obtained with urethan-inhibited E. coli (4).

In other experiments in which liquid media were employed, the growth of E. coli in the presence of combinations of 2,6-diaminopurine with urethan, formamide, or N,N-dimethylformamide was observed. The minimal broth referred to above was used as the basal medium to which the additions were made. The extent of growth was measured by determining turbidity with a Bausch and Lomb monochromatic colorimeter with a filter having maximum transmission at 660 mμ. Results of these experiments are presented in Table 2.

Cross-resistance between urethan, formamide, and N-methylformamide.—Formamide-, N-methylformamide-, and urethan-resistant lines of E. coli were obtained by treatment of E. coli with sufficient penicillin to kill approximately 99 per cent of the population and then plating out the remaining viable E. coli on minimal agar containing high levels of formamide, N-methylformamide, or urethan. The colonies which appeared after 24 hours were resistant, as could be demonstrated by simple gradient plating (5).

Approximately 80 ml of MA was poured into tilted Petri dishes and allowed to solidify. The Petri dishes were then placed on a level table and an additional 20 ml of MA, containing a known amount of the agent under investigation, was poured to provide a gradient plate as indicated in Chart 1. This procedure provided a medium containing a concentration gradient of the agent under study.

The urethan-resistant, formamide-resistant, and N-methylformamide-resistant E. coli were then tested for resistance to urethan, formamide and N-methylformamide as follows:

Wild E. coli and a resistant line were streaked in straight lines from the "low" concentration to the "high" concentration of a gradient plate containing one of the agents of interest. After 24 hours of incubation at 37° C. the extent of growth was observed. A typical growth pattern is illustrated in Chart 2.

In these gradient plate experiments, nine separately isolated formamide-resistant lines of E. coli were tested against urethan and N-methylformamide; seven lines of N-methylformamide-resistant E. coli were tested against urethan and formamide; and five urethan-resistant lines were tested against formamide and N-methylformamide.

Every formamide-resistant line was cross-resistant to N-methylformamide and to urethan; all N-methylformamide-resistant lines were also resistant to formamide and to urethan; and all urethan-resistant lines were cross-resistant to formamide and to N-methylformamide.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COMPARISON OF THE EFFECTS OF DIAMINOPURINE AND DIAMINOPURINE-RIBOSIDE IN PREVENTING INHIBITION OF FORMAMIDE</strong></td>
</tr>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td><strong>(mg/ml)</strong></td>
</tr>
<tr>
<td>DAP</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>DAP-riboside</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*Concentration of formamide in media was 17.5 mg/ml. Approximately 0.01% of solutions indicated applied to paper discs. Distances of growth from the edge of paper discs in cm.

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EFFECTS OF 2,6-DIAMINOPURINE, URETHAN, FORMAMIDE, AND N,N-DIMETHYLFORMAMIDE ON THE GROWTH OF E. coli IN LIQUID MEDIA</strong></td>
</tr>
<tr>
<td><strong>2,6-DIAMINOPURINE</strong></td>
</tr>
<tr>
<td><strong>(mg/10 ml)</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.10</td>
</tr>
<tr>
<td>1.00</td>
</tr>
<tr>
<td><strong>(mg/10 ml)</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.10</td>
</tr>
<tr>
<td>1.00</td>
</tr>
</tbody>
</table>

*Period of growth, 17 hours.
†Period of growth, 44 hours.
leukemia and several drug-resistant lines of L1210 leukemia.—The effects of N-methylformamide and urethan on several leukemias have been investigated. Leukemias L1210 and A-methopterin-dependent (A-meth-D), 8-aza-D, and 6-mercaptopurine-resistant (6-MP-R) lines (obtained from Dr. L. W. Law) were employed. The results obtained on treatment of mice with L1210 leukemia are summarized in Table 3.

N-methylformamide at a level of 400 mg/kg also increased the life span of mice with the A-meth-D, 8-aza-D, or 6-MP-R lines of L1210 leukemia. Urethan, at the maximum tolerated dosage level (600 mg/kg), failed to increase significantly the life span of mice with any of these leukemias, although it has been shown that urethan increases the life span of mice with Chloroleukemia 1584 (a myeloid leukemia) (9).

TABLE 3
THE EFFECTS OF N-METHYLFORMAMIDE ON L1210 LEUKEMIA*

<table>
<thead>
<tr>
<th>EXPER. NO.</th>
<th>AVERAGE LIFE SPAN (DAYS)</th>
<th>LIFE SPAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON. TREAT.</td>
<td>S.D.</td>
<td>S.D.</td>
</tr>
<tr>
<td>1</td>
<td>6.7</td>
<td>0.87</td>
</tr>
<tr>
<td>2</td>
<td>6.6</td>
<td>0.58</td>
</tr>
<tr>
<td>3</td>
<td>7.2</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Treatment was initiated at 24 hours after inoculation of leukemic cells and was continued every other day until death. Groups of ten or more control mice and ten treated mice were used in each experiment. The dosage level was 400 mg/kg. The route of administration was intraperitoneal.

† Standard deviation.

The effects of N-methylformamide at a level of 400 mg/kg also increased the life span of mice with the A-meth-D, 8-aza-D, or 6-MP-R lines of L1210 leukemia. Urethan, at the maximum tolerated dosage level (600 mg/kg), failed to increase significantly the life span of mice with any of these leukemias, although it has been shown that urethan increases the life span of mice with Chloroleukemia 1584 (a myeloid leukemia) (9).

TABLE 4
EFFECTS OF N-METHYLFORMAMIDE ON SARCOMA 180 AND ADENOCARCINOMA E 0771*

<table>
<thead>
<tr>
<th>TUMOR</th>
<th>DOSAGE (mg/kg)</th>
<th>MOR. CHANGE</th>
<th>AV. WT. TUMOR W.T. (GM.)</th>
<th>PER CENT OF CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-180</td>
<td>400 (X4)</td>
<td>+1.4</td>
<td>1183</td>
<td>12</td>
</tr>
<tr>
<td>S-180</td>
<td>400 (X4)</td>
<td>+9.2</td>
<td>821</td>
<td>9</td>
</tr>
<tr>
<td>S-180</td>
<td>300 (X4)</td>
<td>+3.5</td>
<td>1454</td>
<td>33</td>
</tr>
<tr>
<td>S-180</td>
<td>200 (X4)</td>
<td>+5.6</td>
<td>881</td>
<td>47</td>
</tr>
<tr>
<td>E 0771</td>
<td>400 (X8)</td>
<td>-0.1</td>
<td>1376</td>
<td>7</td>
</tr>
<tr>
<td>E 0771</td>
<td>300 (X3)</td>
<td>+0.1</td>
<td>1376</td>
<td>8</td>
</tr>
</tbody>
</table>

* All treatment was initiated 24 hours after tumor implantation and continued every other day for the total number of injections indicated. Experiments with Sarcoma 180 were terminated at 8 days; experiments with Adenocarcinoma E 0771 were terminated at 18 days.

† Standard deviation.

Effects of N-methylformamide on Sarcoma 180 and Adenocarcinoma E 0771.—The effects of N-methylformamide have been tested against Sarcoma 180 and Adenocarcinoma E 0771. Results obtained are summarized in Table 4. These results are in agreement with those obtained by Clarke et al. (1).

DISCUSSION
The results presented in Table 1, when compared with previously published data (4), strongly suggest at least some similarity in the biochemical action of formamide and urethan in E. coli. The rather specific reversal of urethan and formamide inhibition of E. coli by diaminopurine or diaminopurine-riboside would appear to relate the actions of these agents in some way to purine nucleotide metabolism. As was observed with urethan, adenine prevents the reversal of formamide by diaminopurine. However, it should be pointed out that in the intact animal, Clarke et al. (1) were unable to prevent the toxicity or the tumor-inhibiting activity of formamide or N-methylformamide by administration of diaminopurine. Likewise we have been unable to prevent the tumor-inhibiting action of urethan with diaminopurine.

Another and even stronger bit of evidence relating the biochemical actions of the formamides and urethan in E. coli is the present observation of cross-resistance of urethan-, formamide-, and N-methylformamide-resistant E. coli. All three resistant lines of E. coli were shown to be resistant to urethan, formamide, and N-methylformamide. The resistant lines of E. coli were not cross-resistant to A-methopterin and certain other temporary anticancer agents.

It can be seen from results presented in Table 3 that N-methylformamide has slight to moderate activity against L1210 leukemia. N-Methylformamide also increased the life span of mice with three drug-resistant lines of L1210 leukemia, whereas urethan failed to increase the life span of mice bearing any of these leukemias. It is well known that urethan does increase the life span of mice with certain other strains of leukemia and that this carbamate provides temporary remissions in certain cases of chronic myelogenous leukemia in human beings.

Although it is quite clear that in the intact animal there are differences in the action of urethan and formamide, it is of interest to consider what data are at hand which suggest a possible relationship between the biochemical action of these two agents as listed on page 146.

We have considered the possibility that in vivo formamide might be a catabolic product arising by reductive cleavage of urethan. No evidence for this hypothesis is now at hand.

The consistent observations on reversal of urethan and formamide inhibition of E. coli by DAP or DAP-riboside and disappearance of such reversibility in the presence of adenine suggest that urethan and the formamides block synthesis of a
compound or compounds into which DAP can be converted (not guanine) and that adenine competes with DAP (or derivative) for the same apo-

enzyme.

SIMILARITIES AND DISSIMILARITIES IN ACTIONS OF FORMAMIDES AND URETHAN

Similarities
1. DAP or DAP-riboside prevents toxicity of both to E. coli.
2. Adenine prevents reversal of both agents by DAP in E. coli.
3. Cross-resistance between urethan-, formamide-, and N-methyl-formamide-resistant E. coli.
4. Urethan and N-methylformamide inhibit some mouse leukemias.
5. Both agents inhibit solid tumors in mice.
6. Both agents are hepatotoxic (1).

Dissimilarities
1. N-Methylformamide inhibits L1210 leukemias which urethan fails to affect.
2. The dose of urethan required to inhibit Sarcoma 180 causes toxic manifestations not encountered in mice given inhibitory levels of formamide (1).
3. Urethan is a central depressant; formamide is not (1).

SUMMARY
1. The inhibition of E. coli by formamide was prevented by 2,6-diaminopurine or 2,6-diamino-

purine-riboside. Adenine prevented reversal of formamide by diaminopurine. Similar results have been obtained with urethan.
2. Urethan-resistant, formamide-resistant, and N-methylformamide-resistant lines of E. coli were all cross-resistant.
3. It has been observed that N-methylformamide increased the life span of mice with L1210 leukemia. Urethan failed to affect the life span of mice with this leukemia.
4. N-methylformamide inhibited the growth of the solid tumors Sarcoma 180 and Adenocarcinoma E 0771.

ACKNOWLEDGMENTS
The authors wish to acknowledge the able assistance of Miss Margaret Ann Newton, Miss Anne Bedingfield, Mrs. Barbara Dismukes, and Mr. James Garlington.

REFERENCES
4. Skipper, H. E., and Schabel, P. M., Jr. Reversal of the Growth Inhibitory Action of Urethan by 2,6-Diamino-

Announcements

1955 Gordon Research Conference on Cancer

The Gordon Research Conference on Cancer for 1955 will be held from August 29 to September 2, 1955, at Colby Junior College, New London, New Hampshire. The meetings will be of an informal type, consisting of the scheduled lectures and free discussion groups. The list of discussers is not yet final.

Requests for attendance at the Conference, or for any additional information, should be addressed to Dr. W. George Parks, Director, at the above address.

PROGRAM
Jacob Furth, Chairman
H. S. N. Greene, Vice-Chairman

1. Features of the Cancer Cell and the Cancerous State. Carcinogenesis. (Chairmen: H. P. Rusch, P. Weiss, A. Gelhorn.)
   Biologic Types of Tumors and Tumor Cells.—H. S. N. Greene, H. Stewart.
   Carcinogenesis in Relation to Regeneration and Differentiation.—C. Breidis, S. M. Rose, C. Grobstein.
   Myeloma and Myeloma Proteins.—F. W. Putnam, E. Oserman.
   The Thymic Factor in Leukemogenesis.—L. W. Law, A. Kirschbaum.

2. Hormonal Factors in Origin and Control of Neoplasms. (Chairmen: R. Herts, R. Rawson, J. Aub.)
   a) Tumorigenesis:
   Thyroid and Thyrotropes.—H. Morris, A. Gorbman, H. Isler.
   Somatotropes.—H. Moore.
   Character of Pituitary Tumors in Parakeets.—H. Schultsberger, P. Steiner.
   b) Endocrine Control of Neoplasia:

   Genetics of Tissue Compatibility.—G. Snell, M. Barrett.
   Relation between Genetic and Antigenetic Character of Cells.
   Allergens in Virulence in Relation to Genetic and Immunologic Alterations.—P. Gorer, T. Hauschka.
   Evidence for Immunologic Difference between Cancer and Homologous Normal Cells.—E. Witebsky, B. Bjorklund.
   Extent and Mechanism of Destruction of Malignant Lymphocytes by Guinea Pig Serum.—H. Stoerk.
   Pitfalls Resulting from Use of Immuno-incompatible Grafted Tumors.—J. Syvertsen.
   Acquired Tolerance to Homografts.—L. Brent, N. Kalis, E. Spurway.

4. Viruses Causing Leukemia and Tumors. (Chairman: J. Enders.)
   Leukemia in Fish.—R. Ritchie, M. Gordon.
   Leukemia in Mice.—L. Gross, S. Stewart.
   Salivary Gland Tumors.—J. Gross, L. Lawrence.
   Mammary Tumors.—L. Dmochowski, F. Bang, J. Bittner.

ERRATA

Attention is called to the following erratum in the paper by H. E. Skipper, F. M. Schabel, Jr., V. Binns, J. R. Thomson, and G. P. Wheeler, which was published in the March, 1955, issue of Cancer Research. The structure of urethan should have been shown as:

\[ \text{OC}_2\text{H}_5 \]
\[ \text{O} = \text{C} \]
\[ \text{NH}_2 \]

The title and authors of the abstract printed as by Belkin and Wodinsky, which appeared in Proc. Am. Assoc. Cancer Research, 2 (1): 4, 1955, should be corrected to read:

"Tumor-necrotizing Polysaccharides from Plant Tissues," by Morris Belkin, Adrian Perrault, and Murray J. Shear.
Studies on the Mechanism of Action and Anticancer Activity of N-Methylformamide

Howard E. Skipper, Frank M. Schabel, Jr., Virginia Binns, et al.


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