Influence of Formycin B on Polyadenosine Diphosphoribose Synthesis in Vitro and in Vivo


SUMMARY

Formycin B inhibited growth of L5178Y mouse leukemia cells in concentrations of less than twice the concentration that inhibits cell proliferation at 50% by cytostasis; at higher concentrations (more than twice the 50% concentration mentioned), the cells were killed. In cells treated with the concentration that inhibits cell proliferation at 50%, the average cell volume did not change. The formycin B inhibitory effect on cell proliferation was reduced by coincubation with nicotinamide adenine diphosphate or adenosine. The biosyntheses of DNA, RNA, and protein in whole cells were sensitively inhibited by formycin B as checked by incorporation studies with radioactive precursors. In addition, the formation of polyadenosine diphosphoribose was reduced even with higher sensitivity; in particular the extent of adenosine diphosphoribose ribosylation of histone subfraction H1 was reduced.

Formycin B has been shown to be an inhibitor for the polyadenosine diphosphoribose polymerase, isolated from oviduct nuclei of quails. Both the chromatin-bound and the soluble enzyme are inhibited competitively; the relative affinity (K_i/K_m) of formycin B to the polyadenosine diphosphoribose polymerase is 1.5.

INTRODUCTION

Formycin (or formycin A) was isolated in 1964 from filtrates of the microorganism Nocardia interforma by Hori et al. (16). Formycin A has been shown to exert growth-inhibitory effects on various neoplastic cells, on the microorganism Xanthomonas oryzae, and on some viruses (16, 41). Formycin A is phosphorylated in Ehrlich ascites cells to its mono-, di-, and triphosphate derivatives (43). Although an interaction of formycin A triphosphate with the Escherichia coli RNA polymerase reaction has been observed (18), it has been suggested that the main influence of formycin A is to inhibit the synthesis of a-5-phosphoribosyl 1-pyrophosphate (14). Formycin A is deaminated in tumor cells to formycin B (43); this nucleoside can also be isolated from N. interforma (20). The structure of formycin B has been identified as 7-hydroxy-3β-d-ribofuranosylpyrazolo(4,3-d)pyrimidine (19). While formycin B shows only reduced activity in tumor growth inhibition, it is effective against X. oryzae and some viruses (20, 41).

In contrast to formycin A, formycin B is not phosphorylated at all (43). Thus, an inhibition of DNA-dependent DNA polymerase as well as DNA-dependent RNA polymerase by formycin B seems to be unlikely. Hori et al. (17) showed that this nucleoside inhibits the incorporation of radioactive adenosine, uridine, and thymidine into nucleic acids of X. oryzae. Those authors found in an extensive study that adenosine does overcome the inhibitory effect of formycin B to some extent. They claimed that formycin B interferes with some processes essential to the uptake of exogenous nucleosides into the cell. Further on purine nucleoside phosphorylase from human erythrocytes and Sarcoma 180 ascites cells are inhibited by formycin B; the inhibition has been shown to be competitive to inosine and to 6-mercaptopurine ribonucleoside (36).

In a previous paper (28) we demonstrated that formycin B inhibits poly(ADP-Rib) polymerase. This paper seeks to elucidate in more detail the action of formycin B on poly(ADP-Rib) synthesis in isolated enzyme systems as well as in cells (L5178Y mouse leukemia cells).

The polyanion poly(ADP-Rib) found in eukaryotic nuclei was first described by Chambon et al. (5), and was investigated in more detail (e.g., Refs. 6, 8, 15, 30, and 37) later. The biological function of ADP ribosylation of nuclear proteins is only partlyknown. ADP-ribose formation in intact cells seems to be relevant to the control of DNA replication (34, 39) and of transcription (27). From the data of Ueda et al. (42), it is well established that ADP-ribose may be attached to histones (Subfractions H1, H2, and H3) in vivo as monomer and polymer.

MATERIALS AND METHODS

Materials. NAD⁺, adenosine, micrococcal nuclease (from Staphylococcus aureus, 8000 units/mg), DNase I (beef pancreas, 2500 units/mg), and RNase A (beef pancreas, 3000 units/mg) were obtained from Boehringer und Sohne, Mannheim, Germany; phosphodiesterase I

1 We gratefully acknowledge loans from the Fonds der chemischen Industrie.

2 This work was performed in partial fulfillment of the requirements for the degree of Medical Doctor, University of Mainz.

Received May 15, 1975; accepted August 26, 1975.
(Crotalus adamanteus; potency, 0.1/mg) was from Worthington Biochemical Corp., Freehold, N. J.; DEAE-Sephadex (A-25) was from Deutsche Pharmacia, Frankfurt, Germany; formycin B was from Calbiochem, Los Angeles, Calif.; [methyl-3H]thymidine (specific activity, 2 Ci/mmole), [H]uridine (generally labeled; specific activity, 5.3 Ci/mmole), L-[3H]phenylalanine (specific activity, 9.8 Ci/mmole), nicotinamide [U-14C]adenine dinucleotide (specific activity, 260 mCi/mmole), [4(n)-H]NAD+ (specific activity, 50 mCi/mmole), and D-[1-14C]ribose (specific activity, 55 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, England; GF/C filters were from Hormuth and Vetter, Heidelberg, Germany; lysine-rich histone of calf thymus (type III) was from Sigma Chemical Co., St. Louis, Mo.; trypan blue was from E. Merck AG, Darmstadt, Germany; and羧基methycellulose was from Serva, Heidelberg, Germany.

Herring sperm DNA, isolated according to the method of Zahn et al. (48), was a gift of H. Mack, Illertissen, Germany.

Mature, egg-laying Japanese quails (Coturnix japonica), with an average weight of about 120 g, were obtained from H. Linnenschmidt, Wiedenbrück, Germany.

Cell Culture. L5178Y mouse leukemia cells (13) were grown in Fischer’s medium for leukemic cells, supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, N. Y.) in suspension cultures (47). For dose-response experiments, the cultures were usually initiated by inoculation of $5 \times 10^8$ cells/ml and subsequently incubated at $37^\circ$ in roller tubes for 72 hr; the controls reached a population density of about $3 \times 10^5$/ml. At 120 hr the stationary phase with $400 \times 10^5$ cells/ml was attained. The cell size-distribution plotter (Coulter Electronics, Hialeah, Fla.). The “mean window,” a characteristic of a cell volume distribution curve, which represents the window into which all cells would fall if the total cell volume and number of cells were unchanged, was computed according to the method of Brecher et al. (2). The absolute cell volume was determined as described (2).

The number of doublings (n) is calculated with the formula:

$$n = (\log b - \log a):\log 2$$

where a is the number of cells inoculated and b is the number of cells harvested after incubation.

For the determination of cell viability, cells were transferred into a solution of 0.2% trypan blue. After an incubation for 20 min at $20^\circ$, the total number of cells and the number of stained (= dead) cells are counted. The viability was calculated according to the equation

$$\frac{\text{[Total cells} - \text{dead cells}]}{\text{[total cells]}} \times 100\%$$

Isolation of Poly(ADP-Rib). This polymer was isolated by the method described by Ueda et al. (42), which has been slightly modified. Cells (2.5 x 10^6) that had been incubated in [14C]NAD+ or [14C]ribose in spinner cultures in the presence or in absence of formycin B were washed 3 times with ice-cold 0.9% NaCl solution. The sediment obtained by centrifugation was homogenized in 10 volumes of a 0.25 M sucrose solution containing 3.3 mM CaCl2 by 5 strokes in a Dounce homogenizer. After centrifugation (5 min at 1000 x g) the pellet, containing crude nuclei, was suspended in 3 volumes of ice-cold 20% trichloroacetic acid and homogenized (Dounce homogenizer, 10 strokes). Then the homogenate was centrifuged and treated 3 more times with 20% trichloroacetic acid. The acid-insoluble material was suspended in 5 volumes of 0.25 N HCl and stirred at 4° for 120 min. Subsequently, the suspension was centrifuged (60 min at 105,000 x g), and the supernatant (“HCl extract”; see Ref. 42) was collected.

Enzyme Preparation. Chromatin-bound poly(ADP-Rib) polymerase was obtained as described earlier (28). Briefly, nuclei isolated from oviducts of egg-laying Japanese quails (C. japonica) according to the method of McGuire et al. (24) were treated with 0.7 M KCl to remove DNA-dependent DNA polymerases, DNA-dependent RNA polymerases, and NNN adenyllytransferase. The chromatin enzyme complex had a specific activity of 45 nmoles [14C]NAD+ per mg protein per 10 min in the test assay, described below.

Soluble poly(ADP-Rib) polymerase was obtained as follows. Nuclei were isolated from oviducts of egg-laying Japanese quails (24). The purified nuclei were suspended in 6 volumes of a Tris-HCl buffer [50 mM Tris-HCl, 1 mM EDTA, 5 mM NaF, and 30% (v/v) glycerol, pH 8.0] containing 1 M NaCl and homogenized by 3 strokes in a Dounce homogenizer with the tight-fitting pestle. After being stirred for 60 min, the preparation was centrifuged in a SW5 rotor of the Spinco L2-65 ultracentrifuge for 60 min at 50,000 rpm and was subsequently dialyzed (15 hr, 2') against Tris-HCl (without NaCl) as described above. The dialysate was centrifuged again in a SW 50 rotor for 60 min at 50,000 rpm. This fraction was subsequently subjected to DEAE-Sephadex chromatography. Soluble poly(ADP-Rib) polymerase was obtained by a stepwise gradient as shown in Chart I. The enzyme eluted from the DEAE column by 1 M NaCl had a specific activity of 120 nmoles [14C]NAD+ per mg protein per 10 min in the test assay described below. This fraction has been used for the experiments.

Enzyme Assays. The chromatin-bound poly(ADP-Rib) polymerase was assayed in a reaction mixture (27) [70 µl] containing 100 mM Tris-HCl (pH 8.5), 6 mM MgCl2, 60 mM KCl, 4 mM dithiothreitol, 150 µM [14C]NAD+ (0.4 mCi/mmole), and 20 µl of the nuclear preparation. The mixture was incubated at 25°, usually for 10 min. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid. After 30 min at 0° the mixture was transferred onto GF/C filters and counted (27).

The poly(ADP-Rib) polymerase assay mixture (50 µl) contained 60 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM EDTA, 4 mM dithiothreitol, 150 µM [14C]NAD+ (0.4 mCi/mmole), 1.7 µg native herring DNA per ml, 0.4 µg lysine-rich histone per ml, and the enzyme sample to be
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**Results**

**Influence on Cell Proliferation.** Formycin B is an effective inhibitor of cell proliferation. In dose-response experiments with L5178Y cells, starting with 5,000 cells/ml and an incubation period of 72 hr, the cell proliferation was reduced to 20% (ED₅₀) by 4.3 ± 0.4 μg/ml (= 16.0 μM) formycin B per ml. To check the influence of formycin B on cell proliferation of exponentially growing cells, the drug was added for 24 hr to cultures grown to a density of 100,000 cells/ml. Under these conditions the ED₅₀ has been determined to be 8.3 ± 0.9 μg formycin B per ml (= 31 μM).

Formycin B effected its cytostatic action only in a certain concentration range. Inhibition of the cell proliferation at ED₅₀ (= 31 μM) in the case of cell concentration of 100,000/ml for a period of 24 hr was completely reversible; after the drug was washed out, cells proliferated again at the same rate as did the controls. The cell viability at the end of the formycin B treatment amounted to 97 ± 3%. At concentrations higher than twice the ED₅₀, cells were killed. Then upon subsequent transfer into a formycin B-free culture medium, the proliferation rate determined after 24 hr was reduced to 53%. The cell viability (by trypan blue) after formycin B incubation was 59 ± 7%.

Formycin B did not alter the average volume of L5178Y cells (exponentially growing cells of a density of 100,000/ml) appreciably. After incubation for 24 hr with the ED₅₀, the average cell volume amounted to 623 cu μm; the value for the corresponding controls was 578 cu μm.

The formycin B inhibitory effect on cell proliferation can be reduced by coinubication of the drug with NAD⁺ or adenosine. In the 72-hr dose-response experiments, starting with 5000 cells/ml, formycin B concentrations of 5.8 ± 0.5 μg/ml (= 21.6 μM), 6.4 ± 0.6 μg/ml (= 23.8 μM), and 8.9 ± 0.7 μg/ml (= 33.1 μM) were needed to reduce cell proliferation to 50% in the presence of NAD⁺, 0.5 μg/ml (= 0.75 μM), 1 μg/ml (= 1.51 μM), and 3 μg/ml (= 4.52 μM). It is remarkable that the dose-response curve obtained for formycin B in the absence of NAD⁺ is parallel to the ones of experiments with formycin B and NAD⁺. The same preventing effect of NAD⁺ was observed in cultures with exponentially growing cells treated with formycin B (Table 1). The number of doubling steps increased considerably in the cultures after coincubation of 10, 40, or 100 μM formycin B with 4.5 μM NAD⁺ compared with formycin B alone. Adenosine reduced the formycin B effect in a way similar to that of NAD⁺ (Table 1). From these experiments, it is evident that the formycin B-reducing effect exerted by NAD⁺ is in the same magnitude as that of adenosine. The formycin B-protecting effect of NAD⁺ in addition can be followed up by comparing the cell volume distribution curves from cultures growing in formycin B alone (Chart 2B) or together with NAD⁺ (Chart 2C). The volume distribution of cells growing in the absence of formycin B and NAD⁺ had a 1st minimum in Channel 5; the “mean window” calculated from the values of the relative frequency distribution graphs between the 1st minimum and Channel 25 amounted to 12.59 ± 3.44 (Chart 2A). The graphs from cell populations treated with formycin B in the absence of NAD⁺ show a spread with no maximum (Chart 2B), while the cultures treated with formycin B and NAD⁺ can be characterized by a pronounced volume distribution curve with a 1st minimum in Channel 6 and a corresponding “mean window” of 12.97 ± 3.51 (Chart 2C). These observations are a 1st clue that NAD⁺ counteracts cytotoxicity of formycin B. Viability tests support this assumption. Incubation of exponentially growing cells (100,000 cells/ml) for 24 hr with 100 μM formycin B alone decreased dye exclusion to 42 ± 4% of the cells, while the corresponding value in assays with 100 μM formycin B and 4.5 μM NAD⁺ is 62 ± 7%.

**Influence on the Synthesis of Macromolecules in Intact Cell System.** The incorporation of radioactive thymidine, uridine, phenylalanine, and NAD⁺ into acid-insoluble material was strongly inhibited during an incubation with formycin B for 24 hr (Table 2). Even the small dose of 7.2 μM formycin B which caused a reduction of the doublings from 2.09 to 1.84 reduced the rate of DNA synthesis (by [³H]thymidine incorporation) to 70%, the rate of RNA synthesis (by [³H]uridine incorporation) to 79%, and the rate of protein synthesis (by [³H]phenylalanine incorporation) to 51%.

As shown earlier by Nolde and Hilz (31), NAD⁺ does not enter the cells as such but only after enzymatic degradation can the NAD⁺ derivatives enter. Therefore, NAD⁺ preparations have been used that are either labeled with ¹⁴C in the adenine moiety or labeled with ³H in the nicotinamide moiety. As shown in Table 1, only after incubation with [¹⁴C]NAD⁺ can radioactive material be detected in the acid-insoluble material. After extracellular degradation of NAD⁺ to adenosine and adenine (31) and their uptake into...
The calculation of cell doublings was performed as described under "Materials and Methods." The reduction of cell growth, caused by formycin B together with different compounds, was evaluated by subtracting the number of doublings with formycin B alone from the number of doublings with formycin B plus either NAD\textsuperscript{+} or adenosine. Each value in the table represents the mean ± S.D. of 10 parallel assays.

<table>
<thead>
<tr>
<th>Formycin B ((\mu\text{M}))</th>
<th>Additional compound ((\mu\text{M}))</th>
<th>Cell concentration after incubation (cells (\times 10^8/\text{ml}))</th>
<th>Reduction of formycin B effect by additional compound (increase in doubling steps)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>283 ± 26</td>
<td>1.50</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>249 ± 24</td>
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</tr>
<tr>
<td>100</td>
<td></td>
<td>95 ± 8</td>
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<tr>
<td>0</td>
<td></td>
<td>279 ± 26</td>
<td>1.48</td>
</tr>
<tr>
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<td>NAD\textsuperscript{+}: 4.5</td>
<td>273 ± 26</td>
<td>1.45</td>
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<tr>
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<td>Adenosine: 4.5</td>
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<td>100</td>
<td>Adenosine: 4.5</td>
<td>265 ± 24</td>
<td>1.41</td>
</tr>
<tr>
<td>40</td>
<td>Adenosine: 4.5</td>
<td>189 ± 17</td>
<td>0.92</td>
</tr>
<tr>
<td>100</td>
<td>Adenosine: 4.5</td>
<td>120 ± 12</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Chart 2. Volume distribution of cells treated without formycin B (A), with 100 \(\mu\text{M}\) formycin B (B), and with 100 \(\mu\text{M}\) formycin B plus 4.5 \(\mu\text{M}\) NAD\textsuperscript{+} (C). The incubation procedure was the same as described in Table 1. Capillary aperture diameter, 100 \(\mu\text{m}\); reciprocal amplification, 0.7; reciprocal aperture current, 4. Records by Model B graphout.

Thus extracellular NAD\textsuperscript{+} supplies precursors for DNA, RNA, and poly(ADP-Rib) synthesis. It was our aim to find out whether after incubation of L5178Y cells with \(^{14}\text{C}\text{NAD}^+\), which is labeled in the adenine moiety, some radioactivity can be detected in poly(ADP-Rib). For that purpose, cells were incubated with \(^{14}\text{C}\text{NAD}^+\); subsequently, an HCl extract (containing histone subfractions H1, H2, and H3) (3) was prepared from them as described in "Materials and Methods." This HCl extract contained a considerable amount of radioactivity; this material has been characterized with respect to acid insolubility and enzyme sensitivity (Table 3). Eighty \% of the material was acid precipitable. From the DNA and RNA nucleases tested, only the phosphodiesterase I reduced the amount of acid-precipitable material. This finding is a suitable proof (37) that the acid-precipitable material is poly(ADP-Rib). Shuster and Goldin (38) and Ueda et al. (42) showed that ribose is more selectively incorporated into NAD\textsuperscript{+} than into other nucleotides, and thus this sugar seems to be an appropriate precursor for \textit{in vivo} labeling of NAD\textsuperscript{+} and thereby poly(ADP-Rib). With this precursor the amount of incorporation into the phosphodiesterase I-sensitive material, associated with the HCl extract, was considerably higher (76 pmoles = 5760 cpm/assay) compared with the NAD\textsuperscript{+} precursor (1.9 pmoles = 670 cpm/assay) under otherwise identical incubation conditions (Table 3). The \(^{14}\text{C}\text{ribose precursor was used to investigate the influence of formycin B on the synthesis of poly(ADP-Rib) in cells. This can be followed up as radioactivity in the HCl extract. As shown in Table 4 the antibiotic strongly inhibited the incorporation of \(^{14}\text{C}\text{ribose into HCl extract; compared to untreated controls (100%), a decrease of the incorporation rate into the HCl extract to 71% resulted after incubation with 20 \(\mu\text{M}\) formycin B along with a reduction of cellular proliferation rate to 77%. In order to obtain information on the material associated with poly(ADP-Rib), the nuclear HCl extract from cells previously incubated with \(^{14}\text{C}\text{ribose was chromatographed on a|
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Table 2

Influence of formycin B on macromolecular synthesis in exponentially growing cells

Five ml of exponentially growing cell suspensions at 60 x 10^6 cells/ml were supplemented with formycin B for 24 hr prior to the addition of the labeled precursors: 25 μCi [3H]hypoxanthine, 25 μCi [3H]uridine, 25 μCi [3H]phenylalanine, 0.6 μCi [14C]NAD+, and 10 μCi [3H]NAD+. They were incubated for 60 min. Then aliquots of 5 ml were analyzed for acid-insoluble radioactivity (29). The values represent the means of 4 samples each. The standard deviation does not exceed 10%.

<table>
<thead>
<tr>
<th>Formycin B (μM)</th>
<th>Incorporation into acid-insoluble material/100,000 cells</th>
<th>Influence on cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>0</td>
<td>11,900</td>
<td>17,400</td>
</tr>
<tr>
<td>7.2</td>
<td>8,300</td>
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</tr>
<tr>
<td>12</td>
<td>7,400</td>
<td>12,400</td>
</tr>
<tr>
<td>36</td>
<td>4,700</td>
<td>8,900</td>
</tr>
</tbody>
</table>

Table 3

Characterization of HCl-extract obtained from exponentially growing cells

Assays (500 ml) were initiated by an inoculum of 15 x 10^6 cells/ml. The spinner cultures were incubated at 37°C for 72 hr, then 49 nCi/ml (= 190 nM) [14C]NAD+ or 10.5 nCi/ml (= 190 nM) [3H]ribose per ml were added. After incubation the HCl-extract was obtained as described in "Materials and Methods." Dialysis and determination of acid-insoluble radioactivity was as described in legend in Table 3.

Table 4

Influence of formycin B on the rate of poly(ADP-Rib) synthesis in intact cells

Exponentially growing cells (500 ml; 10^7 cells/ml) were incubated with different concentrations of formycin B for 24 hr in spinner cultures. During the last 7 hr of the incubation period, 10.5 nCi (= 190 nM) [3H]ribose per ml were added. After incubation the HCl-extract was obtained as described in "Materials and Methods." Dialysis and determination of acid-insoluble radioactivity was as described in legend in Table 3.

<table>
<thead>
<tr>
<th>Formycin B (μM)</th>
<th>Incorporation into HCl-extract</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cpm/assay %</td>
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<tr>
<td>0</td>
<td>5,500 100</td>
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<tr>
<td>20</td>
<td>3,910 71</td>
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<tr>
<td>40</td>
<td>2,310 42</td>
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</table>

<table>
<thead>
<tr>
<th>Cell concentration (cells/ml)</th>
<th>Doublings</th>
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<tbody>
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<td>675,000</td>
<td>0.75</td>
</tr>
<tr>
<td>612,000</td>
<td>0.61</td>
</tr>
<tr>
<td>536,000</td>
<td>0.42</td>
</tr>
</tbody>
</table>

carboxymethylcellulose column according to the method of Ueda et al. (42) (Chart 3). As previously reported by Ueda et al. (42) histone subfraction H1 elutes at 0.17 M sodium acetate (pH 4.2) while with 0.3 M HCl subfraction H2/H3 comes off. According to Chart 3 most of the acid-insoluble radioactivity eluted from the column was found under conditions corresponding to elution of H1. This radioactivity in poly(ADP-Rib) of subfraction H1 is considerably reduced in the fraction derived from formycin B-incubated cells.

The time course of formycin B action at 40 μM on synthesis of DNA, RNA, protein, and poly(ADP-Rib) was investigated with exponentially growing cells. At 40 μM, cell proliferation was reduced to 53%. According to Chart 4 treatment of cells with cytostatic concentrations of formycin B resulted in inhibition of poly(ADP-Rib) synthesis without delay, while synthesis of DNA, RNA, and protein continued normally for another hr. It may be concluded that formycin B primarily interferes with poly(ADP-Rib) synthesis, much in contrast to DNA, RNA, and protein synthesis.

Inhibition of Poly(ADP-Rib) Polymerase. Formycin B inhibited the incorporation of [14C]NAD+ into poly(ADP-Rib) by poly(ADP-Rib) polymerase when both chromatin-bound and soluble poly(ADP-Rib) polymerase from quail oviducts were used. The incorporation of label into acid-insoluble material was linear with time up to 10 min for both enzyme preparations, at a rate of 4.5 nmoles [14C]NAD+ incorporated per min per mg protein with the chromatin-bound enzyme and at a rate of 12.2 nmoles per min per mg protein with the soluble poly(ADP-Rib) polymerase (Chart 5).

The value of formycin B-caused inhibition of the enzymatic poly(ADP-Rib) polymerization depends on the NAD+ concentration. Increase of the NAD+ concentration relative to formycin B lowered the inhibition. The inhibition of both enzyme preparations was purely competitive with respect to NAD+ (Chart 6), as shown by double reciprocal plots at 2 inhibitor concentrations. The numerical values for
polymerase is 2.2-fold higher than for the chromatin-bound polymerase; this may be due to the different purity of the enzyme preparations as well as to differing primer (nuclear proteins) and DNA conditions in the reaction mixtures used for assaying chromatin-bound and soluble poly(ADP-Rib) polymerase.

DISCUSSION

One aim in investigations pertinent to rational development of new antitumor drugs is the search for new sites of action for cytostatic agents. Several approaches with promising results have been undertaken, e.g., inhibition of DNA repair processes by chloroquine (25) and inhibition of polyamine formation by methylglyoxal bis(guanylhydrazone) (46). Another possible way to inhibit programmed...
syntheses, leading to a reduction of cell proliferation, may be the possibility of the inhibition of those enzymes that modulate and restrict template capacity of chromatin for DNA synthesis and RNA synthesis by phosphorylation, methylation, acetylation, or poly(ADP) ribosylation of nuclear proteins. In this paper the antibiotic formycin B has been shown to be an inhibitor of poly(ADP-Rib) synthesis in whole cells as well as in isolated enzyme system. Future experiments will decide on poly(ADP-Rib) synthesis as a new biochemical site of action for antitumor agents.

In this paper formycin B action is shown to lower the cell proliferation rate in low concentration by cytostasis and at higher concentrations by cytotoxicity. This drug effect might be due to strong inhibition of programmed synthesis in vivo. Our finding that formycin B reduces DNA synthesis as well as RNA synthesis in intact cells is in accordance with earlier data (17). The observation that protein synthesis is inhibited too is not surprising. It can be explained as a consequence of the observed impairment of RNA synthesis. The finding that formycin B reduces the synthesis of poly(ADP-Rib) is new. The synthetic rate of this poly nucleotide in whole cells cannot be determined in a convenient way since the immediate precursor of enzymatic poly(ADP-Rib) synthesis does not enter the untreated cells as such (31). It is only after treatment of cells with 2 mM ammonium sulfate that NAD* can pass the cell membrane (32). The determination of the synthesized polymer after incubation with either a labeled adenine derivative or ribose is 1 possibility to check the poly(ADP-Rib) synthesis in untreated cells. We used NAD*, labeled with ^14C in the adenine moiety, which after extracellular degradation, uptake, and intracellular resynthesis to NAD* can serve as precursor for poly(ADP-Rib) synthesis (4, 9, 10, 12, 31). Ribose, which readily permeates the cell membrane of intact cells, becomes effectively incorporated into NAD* and subsequently into poly(ADP-Rib) (38, 42). Nevertheless, such an approach requires the evidence of the poly(ADP-Rib) product, since the metabolic pathways starting with NAD* degradation products and ribose include the formation of NAD* as well as deoxyribonucleoside and ribonucleoside triphosphates, the precursors of DNA synthesis and RNA synthesis (10). We determined the extent of poly(ADP-Rib) synthesis occurring on histone subfraction H1 and H2/H3, in relation to formycin B concentration. It has been established that these histone subfractions are covalently bound to poly(ADP-Rib) (42). We found that the formation of poly(ADP-Rib) on histone subfraction H1 became significantly reduced in cells after addition of formycin B.

After incubation of cells for a longer period (24 hr; Table 2) at low (cytostatic) concentrations of formycin B, the syntheses of all 3 types of polynucleotides [DNA, RNA, and poly(ADP-Rib)] are strongly reduced. However, during the 1st 4 hr (Chart 4), the effect is differential, formycin B acting primarily by inhibition of poly(ADP-Rib) synthesis. Thus an effect of formycin B on purine biosynthesis or ATP formation may not exist at all. Since formycin B is not phosphorylated (43), an interference with the synthesis of DNA and RNA on the level of the polymers is unlikely. The interesting observation that formycin B is an inhibitor of purine nucleoside phosphorylase in isolated enzyme systems as well as in erythrocytes and sarcoma cells (36) does not explain its mode of action in L5178Y cells used in the present studies. This conclusion might be drawn from the fact that the purine nucleoside phosphorylase is involved in the salvage pathway of the purine (deoxy) nucleotide synthesis (10), which does not seem to be essential for L5178Y cells (e.g., Ref. 44). Formycin B does not alter cell volume. From the fundamental studies of Cohen and Barner (7), it is known that “unbalanced growth” is characterized by inhibition of DNA synthesis and cell division while RNA synthesis and protein synthesis go on. From the data about the formycin B effect on cell volume, it can be suggested that the formycin B-caused inhibition of cell proliferation is universal inasmuch as it affects synthesis of DNA, RNA, and protein in such a way that no growth occurs out of proportion to residual DNA synthesis (21, 26). Therefore, we must conclude that inhibition of poly(ADP-Rib) synthesis is the primary inhibitory effect of formycin B.

ADP ribosylation of histone subfraction H1 in vivo is strongly inhibited by formycin B. The physiological function of poly(ADP-Rib) in general and also in the bound state on histone H1 is largely unknown. The presented data suggest that cell division and/or gene expression may be controlled by variations of the poly(ADP-Rib) content of histone H1, the important role of which in modulating cellular functions has been well established (1, 40).

Poly(ADP-Rib) synthesis is blocked by formycin B in vivo as well as in an isolated enzyme system. The 2 forms of poly(ADP-Rib) polymerase, the chromatin-bound and the soluble enzyme, used for the inhibition tests, have identical K_m’s for NAD* and identical K_i values for formycin B. The

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Form of the enzyme</th>
<th>V_max (nmole/mg protein/10 min)</th>
<th>K_m (µM)</th>
<th>K_i (µM)</th>
<th>K_i/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ADP-Rib) polymerase</td>
<td>Chromatin-bound</td>
<td>96.0</td>
<td>43.7</td>
<td>68.9</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>210.7</td>
<td>52.8</td>
<td>75.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Kₘ's of 44 and 53 μM, respectively, determined for the 2 enzyme forms isolated from quail oviduct nuclei are in the same range as the Kₘ for the calf thymus enzyme (11). However, they are considerably lower than for the poly-(ADP-Rib) polymerase from mouse fibroblast nuclei from Physarum polycephalum, from rat liver, or from Ehrlich ascites (35). As shown in this paper, the relative affinity (Kᵢ/Kₘ) of formycin B towards the poly(ADP-Rib) polymerase is 1.5; the corresponding value from the assays with purine nucleoside phosphorylase is 2 (36), signifying a lower affinity of the drug to this enzyme.

In contrast, the antibiotics formycin A and coformycin, as well as adenosine, which are structurally related to formycin B, have been shown not to affect poly(ADP-Rib) polymerase reaction (28, 33). Thus by affecting poly(ADP-Rib) (Ki/Kₘ) of formycin B towards the poly(ADP-Rib) polymerase, formycin B might become a valuable compound, interfering with cellular regulation at the nuclear level.

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