Inhibition of glycolysis of tumor cells is one of the most conspicuous metabolic effects of carcinostatic alkylating agents.\(^1\)\(^\text{15}\) This inhibition coincides with a profound diminution of the steady-state concentration of DPN of tumor cells, as observed by Roitt,\(^3\) and Holzer et al.,\(^4\) and amply confirmed by numerous investigators.\(^5\)\(^\text{11}\) The mechanism responsible for the decrease in DPN content of tumor cells by carcinostatic alkylating agents has been unknown. Holzer et al.,\(^12\)\(^13\) suggested that carcinostatic alkylating agents inhibit at some point the biosynthesis of DPN. On the other hand, Green and Bodansky\(^14\) as well as Hilz et al.,\(^15\) believe that an activation of DPNase is the primary reason for a decrease of DPN content of tumor cells. A hint regarding the mechanism of carcinostatic alkylating agents on glycolysis has already been provided by the work of Roitt,\(^3\) who found that nicotinic acid amide antagonizes the inhibitory effect of these drugs. Interpretation of this observation remains ambiguous, however, since nicotinic acid amide is a well-known biosynthetic precursor of DPN as well as an inhibitor of DPNase. We have reinvestigated this question with the aid of structural analogues of nicotinamide. In order to eliminate the uncertainty concerning the possible dual action of nicotinamide on DPN metabolism, DPNase inhibitors had to be found which could not serve as precursors of DPN. Two among the nicotinamide homologues tested
(5-fluoroo and 5-methyl nicotinamide) fulfilled this requirement; thus correlation was established between DPNase inhibition and the antagonistic effect of this inhibition on the glycolysis blocking action of a carcinostatic alkylating agent (Trenimon) in Ehrlich ascites tumor cells.

Materials and Methods.—Ehrlich ascites cells were harvested from mice 8–10 days after inoculation, filtered through gauze, centrifuged, and washed free of erythrocytes with 0.9 per cent NaCl. DPNase was extracted from tumor cells according to Hilz and assayed as follows: 1 µmole of DPN, 50 µmole of phosphate (pH 7.2), and 0.3 ml of enzyme extract were incubated in 1 ml vol for 30 min at 37°. The reaction was stopped by 1 ml 6 per cent HClO₄, and after deproteinization ClO₄⁻ was removed by solid KHCO₃. DPN was assayed with alcohol dehydrogenase according to Warburg and with the cyanide test of Colowick, Kaplan, and Ciotti. Manometric experiments were performed under conditions described by Kun, Talalay, and Williams-Ashman. Glucose (0.06 ml 40%) and 0.06 ml 0.1 per cent Trenimon solution were tipped in after 10 min temperature equilibration (37°C; gas phase CO₂; Ringer-bicarbonate buffer of pH 6.0). Paper chromatography of pyridine derivatives was carried out with solvent C of Preiss and Handler, and paper electrophoresis as described earlier. Identification of pyridine nucleotide derivatives was accomplished by conversion to fluorescent derivatives with methyl-ethyl-ketone and NH₃ according to Kodicek and Reddi. Trenimon (2,3,5-tris-ethyleniminobenzoquinone-1,4) was a gift of Bayer A. G. (Leverkusen, Germany); 5-fluoro-nicotinamide and 5-methyl-nicotinamide were received from the Lilly Research Laboratories, Indianapolis; 3-acetyl-pyridine and pyridine-3-sulfonic acid were analytical grade commercial preparations.

Results.—The effects of various homologues of nicotinic acid amide on DPNase activity of extracts of Ehrlich ascites tumor cells are shown in Figure 1. The 5-fluoro and 5-methyl derivatives of nicotinic acid amide and nicotinic acid amide itself were inhibitory to DPNase. The possibility of enzymatic incorporation of nicotinamide derivatives into DPN was also investigated by both enzymatic and chemical methods. In the presence of extracts of tumor cells, 3-acetyl-pyridine exchanges with the nicotinamide moiety of DPN to form 3-acetyl-pyridine-DPN, which upon reduction exhibits a characteristic absorption maximum at 363 µm. No DPN homologues of the other nicotinamide derivatives could be detected by either chemical analysis, including spectrophotometry of CN-adducts, paper
chromatography, and paper electrophoresis, or by testing the absorption maxima after enzymatic reduction. These results show that 5-fluoro- or 5-methylnicotinamide in Ehrlich ascites cells remain unchanged and must therefore exert their biochemical action as inhibitors of DPNase. On the other hand, the conversion of 3-acetyl-pyridine to 3-acetyl-pyridine-DPN had no influence on DPNase activity of tumor cells. Pyridine-3-sulfonic acid was inert in this system.

The influence of nicotinic acid amide and its homologues on glycolysis in presence and absence of the carcinostatic agent, Trenimon (8.7 × 10⁻⁵ M) is shown in Figure 2. While the carcinostatic agent alone virtually stopped glycolysis after 10⁻⁵ min of exposure, combination with nicotinamide, 5-fluoro-, or 5-methyl-nicotinamide at 1.7 × 10⁻² M completely abolished the inhibitory effect of this drug. In presence of 3-acetyl-pyridine and pyridine-3-sulfonic acid, which had no effect on DPNase, Trenimon inhibited glycolysis. At the concentrations used, the nicotinamide derivatives alone had no effect on glycolysis. Analyses for DPN at the end of the experiment showed (Fig. 2) that while DPN content in controls as well as reaction mixtures containing the drug and DPNase inhibitors varied between 0.77 and 1.55 × 10⁻⁴ M, the coenzyme level diminished to 0.1–0.39 × 10⁻⁴ M in presence of the carcinostatic agent alone or in combination with the enzymatically ineffective vitamin homologues. The quantitative relationship between concentration of DPNase inhibitors and protective action on glycolysis in presence of a constant amount of drug (8.7 × 10⁻⁵ M) is shown in Figure 3. A similar relationship was obtained when protective action was correlated with the concentration of DPN remaining in tumor cells at the end of incubation (see Fig. 4). The numerical relationship between the effects of nicotinamide and its 5-fluoro and 5-methyl homologues on DPNase, glycolysis of tumor cells, and DPN concentration, the last two values determined in presence of 8.7 × 10⁻⁵ M Trenimon, is summarized in Table 1.

In order to clarify the specificity of action of Trenimon on tumor glycolysis, its effect on DPNase itself was also determined. A concentration of Trenimon twice as high as needed to inhibit completely glycolysis (1.7 × 10⁻⁴ M) had no appreciable effect on DPNase itself, but reduced DPN content to 7.4 per cent of controls, as shown in Table 2. This table also illustrates the distribution of DPNase in tumor cells, which in preincubated cells is quantitatively accounted for in the microsomal fraction. It is also evident that the carcinostatic drug, when preincubated with tumor cells, apart from having no effect on DPNase, does not alter the intracellular distribution of this enzyme either. These results are in agreement with previous
TABLE 1

RELATIONSHIP BETWEEN THE EFFECTS OF VARIOUS PYRIDINE DERIVATIVES ON DPNase ACTIVITY AND THEIR PROTECTION AGAINST THE ACTION OF TRENIMON ON GLYCOLYSIS AND DPN CONTENT

<table>
<thead>
<tr>
<th>Additions</th>
<th>DPNase activity, %</th>
<th>Glycolysis, % (in the presence of 8.7 × 10⁻⁵ M Trenimon, as expressed in % of controls)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100†</td>
<td>27</td>
</tr>
<tr>
<td>+ Nam 1.7 × 10⁻³ M</td>
<td>23</td>
<td>97</td>
</tr>
<tr>
<td>+ 5-F-Nam</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>+ 5-Me-Nam</td>
<td>1.7</td>
<td>85</td>
</tr>
<tr>
<td>+ 3-Ac-Py</td>
<td>97</td>
<td>31</td>
</tr>
<tr>
<td>+ Py-3-SO₂H</td>
<td>102</td>
<td>20</td>
</tr>
</tbody>
</table>

* Controls = contain no Trenimon.
† In the presence of 4.6 × 10⁻⁴ M Trenimon, this value is 94%.
Experimental conditions are described in legend of Fig. 2 and in Methods.

TABLE 2

INFLUENCE OF TRENIMON (1.7 × 10⁻⁴ M) ON DPNase ACTIVITY AND DISTRIBUTION IN EHRlich Tumor Cells after Preincubation

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>DPNase Activity, %</th>
<th>DPN concentration, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Total cytoplasmin</td>
<td>cytoplasmin</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ Trenimon</td>
<td>85</td>
<td>85</td>
</tr>
</tbody>
</table>

Suspension of Ehrlich ascites cells in Krebs-Ringer bicarbonate (pH 6.0) were incubated in a shaking device in presence (1.7 × 10⁻⁴ M) and absence of Trenimon for 50 min at 37°C. After the cells were separated by centrifugation (500 × g) DPNase was prepared by the method of Hilz et al.,16 and DPNase activity was determined in all fractions. Results are expressed as per cent of total activity. The last column of the table shows the change in DPN content of tumor cells upon incubation with and without Trenimon.

observations of Scriba et al.,22 who could not detect an activation of tumor DPNase by Trenimon.

Discussion.—Based on relatively simple experimental results presented in this report, it appears that the mode of action of a carcinostatic alkylating agent is directly related to its influence on the steady-state level of DPN of tumor cells. Inhibitors of DPNase counteract the glycolysis depressing action of this drug. Isolated DPNase itself is uninfluenced by the carcinostatic agent. The mechanism of intracellular activation of DPNase, presumably due to a loss of intracellular nicotinamide elicited by the carcinostatic agent, as proposed by Hilz et al.,16 is improbable because the intracellular concentration of nicotinamide (5–9 × 10⁻⁵ M) is too low to inhibit DPNase activity (see Fig. 1). It is apparent from Figure 4 that this concentration of nicotinamide, if present in the medium, cannot protect against a
decrease in DPN content caused by Trenimon. Thus it can be concluded that the selective toxic action of the drug must be localized in the area of control of biosynthetic pathways of this coenzyme. Inhibition of DPN synthesis in tumor cells exposed to a carcinostatic alkylating drug has been previously observed, but the direct relationship between this inhibition and the cessation of a metabolic pathway of fundamental importance (glycolysis) can now be formulated with greater precision. It is probable that the turnover of DPN in tumor cells and the regulation of this pathway play a central role in the chemotherapeutic action of carcinostatic alkylating agents and perhaps of X ray. The mode of action of DPNase inhibitors as apparent antagonists of Trenimon on tumor glycolysis can be readily visualized. Inhibition of DPN breakdown merely stabilizes the steady-state level of DPN, which is sufficient to maintain glycolysis, even though DPN biosynthesis is inhibited by Trenimon. The alkylating drug acts rapidly in vitro, causing a large diminution of DPN content within 10–20 min after exposure. This indicates a very high turnover of DPN in tumor cells. This experimental model does not identify the molecular mechanism of action of the carcinostatic agent; it merely provides clues to the biochemical area which is susceptible to carcinostatic (and perhaps carcinogenic) influences. It is noteworthy that selective toxic action of many antibiotics could be localized in specific biosynthetic processes of microorganisms, thus providing very profitable models for the study of regulation of microbial growth and metabolism. We suppose that elucidation of control mechanisms of DPN biosynthesis and turnover in normal and malignant animal cells involving all levels of control (cf. ref. 23) may provide a molecular basis for tumor chemotherapy.

Summary.—A carcinostatic alkylating agent Trenimon (2,3,5-tris (ethylenimino)-benzoquinone 1,4) inhibits anaerobic glycolysis of Ehrlich ascites tumor cells. Marked diminution of DPN content accompanies this effect. DPNase inhibitors counteract the inhibitory effect of the drug on glycolysis and also maintain normal levels of DPN. Based on kinetic and chemical analyses, the mechanism of action of the alkylating agent was localized in some area of control of DPN biosynthesis in tumor cells.

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† Research Career Awardee of the USPHS; on sabbatical leave from the University of California between June and October, 1963.

1 Holzer, H., Medizinische, 15, 576 (1956).
13 Ibid., 333, 155 (1960).

AN ELEMENTARY THEORY OF THE CATEGORY OF SETS*

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We adjoin eight first-order axioms to the usual first-order theory of an abstract Eilenberg-Mac Lane category to obtain an elementary theory with the following properties: (a) There is essentially only one category which satisfies these eight axioms together with the additional (nonelementary) axiom of completeness, namely, the category $\mathcal{S}$ of sets and mappings. Thus our theory distinguishes $\mathcal{S}$ structurally from other complete categories, such as those of topological spaces, groups, rings, partially ordered sets, etc. (b) The theory provides a foundation for number theory, analysis, and much of algebra and topology even though no relation $\subseteq$ with the traditional properties can be defined. Thus we seem to have partially demonstrated that even in foundations, not Substance but invariant Form is the carrier of the relevant mathematical information.

As in the general theory of categories, our undefined terms are mapping, domain, codomain, and composition, the first being simply a name for the elements of the universe of discourse. Each mapping has a unique domain and a unique codomain,