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15. Kotobuki Hano, Heitaroh Iwata, and Kunihiro Nakajima : Studies on the Anticancer Activity of Phenazine Derivatives.*1, *2

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It is known that some of phenazine derivatives isolated from the products of certain bacteria have a powerful bacteriostatic action and interfere with the growth of several types of cells[1~5].

Neutral red,[6] which also has a phenazine skeleton in its molecule, was found to show inhibitory effects on the growth of Ehrlich ascites carcinoma in mice. A similar effect was shown with phenazine-di-N-oxide by Furst, *et al.*[7] In 1959, Abe, *et al.*[8] made systematic antitumor screening tests on various phenazine derivatives and found that 10-hydroxydibenzo[*a*,*c*]phenazine were the most effective against the solid form of Ehrlich carcinoma.

In this study, investigations were made on the correlation between the chemical structures and antitumor effects of 16 phenazine derivatives. Further, to investigate their mode of antitumor action, our studies were concentrated mainly on effects on energy yielding systems, especially glycolysis and respiration of malignant tissues, which are quantitatively different from those of normal tissues.

Materials and Methods

Sixteen phenazine derivatives which were synthesized and supplied from Prof. I. Yosioka, Faculty of Pharmaceutical Sciences, Osaka University, were used in these experiments and they were classified structurally into the following four groups.

1) Phenazine-di-N-oxides
Phenazine-5,10-di-oxide (I)

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*1 This constitutes Part XXXVIII of a series entitled "Pharmacological Studies on the Metabolism of Cancer Tissues."

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*3 6-5, Toneyama, Toyonaka, Osaka-fu (羽野 壽, 岩田平太郎, 中島久二瑛).

1) T. T'ung : Proc. Soc. Exptl. Biol. Med., **39**, 415 (1938).
2) F. Wrede, E. Strack : Ztschr. Physiol. Chem., **140**, 1 (1924).
3) H. Akabori : J. Antibiotics. Ser. A., **12**, 17 (1957).
4) J. F. Riley : Cancer Res., 8, 183 (1948).
5) L. Karczag, L. : Biochem. Z., **230**, 411 (1931).
6) H. Lettre : Z. Krebsforsch., **57**, 1 (1950).
7) A. Furst, C. Klausner : Nature, **184**, 908 (1959).
8) M. Abe, D. Mizuno, I. Yosioka : Yakugaku Zasshi, **79**, 1350 (1959).

1,6-Dihydroxyphenazine-5,10-di-oxide (Iodinin) (II)
2-Hydroxyphenazine-5,10-di-oxide (III)
2-Methoxyphenazine-5,10-di-oxide (IV)
2-Chlorophenazine-5,10-di-oxide (V)

2) Phenazine-mono-N-oxides
Phenazine-5-oxide (VI)
1-Bromo-2-methoxyphenazine-5-oxide (VII)
1-Methoxy-4-bromophenazine-5-oxide (VIII)

3) Phenazine-carboxylic acid and its amino acid derivatives[9]
1-Phenazinecarboxylic acid (IX)
N-(1-Phenazinylcarbonyl)glycine (X)
Ethyl N-(1-phenazinylcarbonyl)glycine (XI)
N-(1-Phenazinylcarbonyl)-DL-valine (XII)
N-(1-Phenazinylcarbonyl)-DL-2-aminobutyric acid (XIII)
N-(1-Phenazinylcarbonyl)-DL-alanine (XIV)

4) Phenazinols
1-Hydroxyphenazine (XV)
1,6-Dihydroxyphenazine (XVI)

I. Experiments with the Assay of Antitumor Activity

Animals used in these experiments were male ddO strain mice weighings 20 ± 2 g., obtained from the Central Breeding Station of Experimental Animals, Osaka University Medical School, and they were separated into two groups, experimental and control, containing 5 animals each.

Assays of antitumor activity of the compounds tested were made on mice with Ehrlich carcinoma, both in the ascitic and solid form. In the ascites groups, animals were inoculated intraperitoneally with 1×10^6 Ehrlich ascites carcinoma cells which had been harvested from tumor-bearing mice 7~8 days after tumor transplantation and had been diluted with physiological saline. In the groups with solid tumor, Ehrlich ascites carcinoma cells diluted to 5×10^6 were inoculated subcutaneously into the right side in the inguinal region.

The compounds, suspended in 0.5% carboxymethylcellulose solution, were administered intraperitoneally at dose levels of 60 mg./kg. for the ascites groups and of 100 mg./kg. for the solid groups for 5 consecutive days, beginning 24 hr. after tumor inoculation. Control animals received daily intraperitoneal injections of 0.5% carboxymethylcellulose in physiological saline. Daily changes in body weight of the animals were recorded.

In the ascites group, the evaluation of anticancer activity was based on the comparison of the mean survival time of the treated group to the control. Solid tumors were measured daily with calipers in two perpendicular diameters and the mean values were used to evaluate the tumor size. On the 15th day after the tumor inoculation, animals were sacrificed and the tumor weight were measured.

II. Experiments on Energy Generating Systems

The ascitic fluid, harvested from animals 7 days after inoculation of Ehrlich ascites carcinoma cells or ascites hepatoma (AH 130) cells, was centrifuged for 5 min. at 2000 r.p.m. The cells thus obtained were washed three times with physiological saline, and then suspended in Krebs-Ringer phosphate buffer (pH 7.4) for measurement of respiration, or in Krebs-Ringer bicarbonate buffer (pH 7.4) for measurement of glycolysis. All measurement of respiration and glycolysis were carried out at 37.5°. Phenazine derivatives were added to the reaction mixture at final concentrations of $1.66 \times 10^{-3} \sim 1.66 \times 10^{-4} M$ in Krebs-Ringer buffer solutions. In these cases, of the compounds tested, phenazinols, phenazinecarboxylic acid and its amino acid substituted compounds were soluble in water, and they were dissolved in Krebs-Ringer buffer solutions. Di-N-oxides and mono-N-oxides, which were relatively water insoluble, were homogenized with small amount of the buffer, and then adjusted to the indicated concentration. The results were calculated on a "Q" basis of μl./mg. of dry weight per hour, and are given as percentage inhibition of control values.

1) Measurement of respiration: The oxygen uptake of the cells was determined manometrically. The gas phase was air. After 10 min. incubation to allow temperature equilibration, the oxygen uptake was measured at 10 min. intervals for 60 min.

2) Measurement of glycolysis: Glycolysis was determined manometrically by measuring CO_2 production resulting from the action of lactic acid on bicarbonate. Readings were made of the same intervals as in measurements of respiration.

9) I. Yosioka, Y. Morita: Yakugaku Zasshi, 83, 364 (1963).

Result

I. Antitumor Activity

Of the phenazine derivatives tested, di-N-oxides caused a marked prolongation of the life span of tumor-bearers, and iodinin, which is substituted with hydroxyl groups at positions 1 and 6 of phenazine-5,10-di-oxide, was the most effective. However mono-N-oxides caused only a slight prolongation of survival time (Fig. 1a).

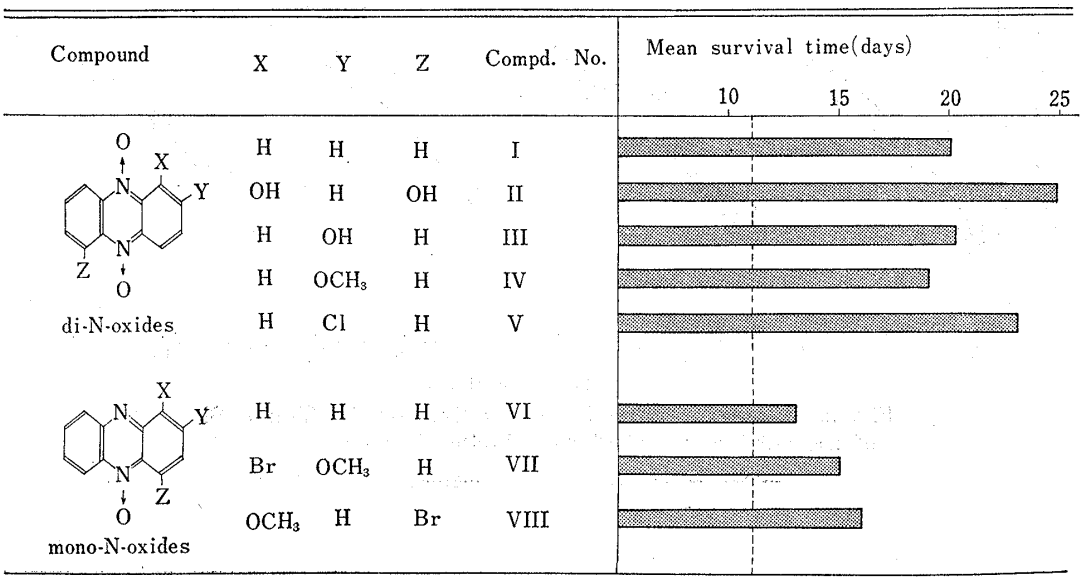

Fig. 1a. Chemical Structures and Antitumor Effects of Phenazine Derivatives

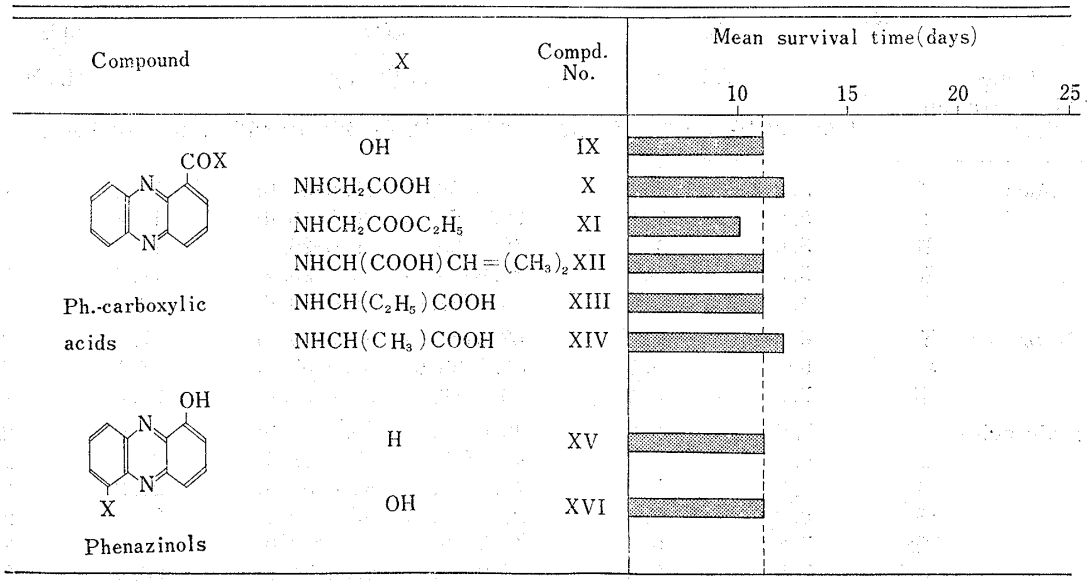

Fig. 1b. Chemical Structures and Antitumor Effects of Phenazine Derivatives

As can be seen from Fig. 1b, phenazine carboxylic acid and its amino acid derivatives and phenazinols, at the dose levels indicated, did not cause a prolongation of life span.

Daily increase in the body weight of the control animals was delayed by treatment with the effective phenazine derivatives.

Fig. 2 shows the daily increase in tumor size of control and treated animals in experiments on the solid forms. As is evident from the data, none of the phenazine derivatives tested inhibited tumor growth.

Furthermore, no significant difference was found in tumor weight between the treated and control animals on the 15th day after inoculation.

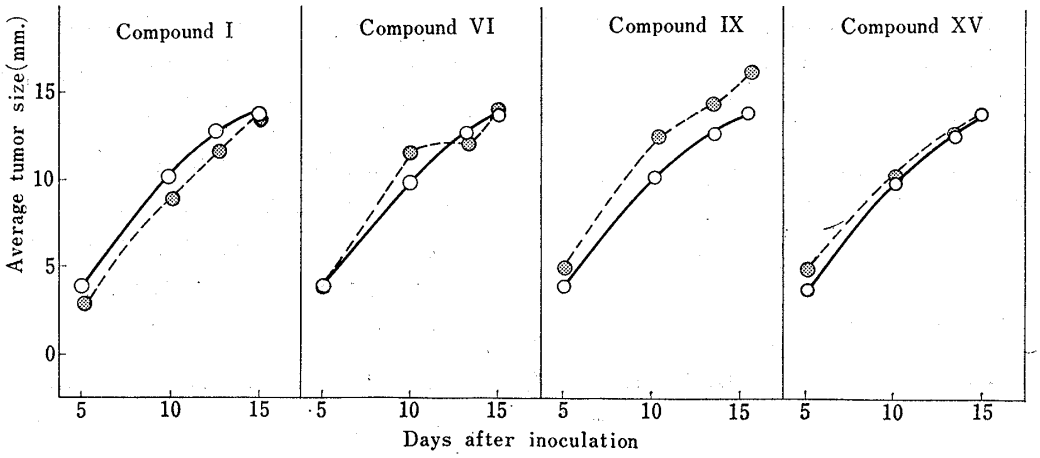

Fig. 2. Effect of Phenazine Derivatives on the Tumor Size of the Solid Form of Ehrlich Ascites Tumor

—○—○— control ···●···●··· treated

TABLE I. Effects of Phenazine Derivatives on the Anaerobic and Aerobic Glycolysis and Respiration of Mouse Ehrlich Ascites Carcinoma Cells and Rat Ascites Hepatoma Cells[a]

Compound No.		Q / Concentration	Q_{O_2} 6.7[b]		$Q^{N_2}_{CO_2}$ 42.7 (39.5)[b]		$Q^{O_2}_{CO_2}$ 24.9[b]	
			$1.66\times10^{-4}M$	$1.66\times10^{-3}M$	$1.66\times10^{-4}M$	$1.66\times10^{-3}M$	$1.66\times10^{-4}M$	$1.66\times10^{-3}M$
Di-N-oxides	I		5.2	3.0	24.3 (38.0)	10.6 (21.0)	19.4	10.6
	II		6.0	4.0	29.5 (36.2)	20.5 (20.4)	18.8	18.0
	III		5.0	2.8	15.2 (30.0)	9.6 (25.2)	18.5	8.5
	IV		5.3	3.1	19.8 (29.3)	9.8 (25.4)	17.6	14.8
	V		4.8	2.5	14.8 (29.8)	4.9 (18.6)	20.6	8.0
Mono-N-oxides	VI		6.8	5.2	48.0 (40.0)	40.2 (35.4)	27.5	22.0
	VII		6.8	5.3	38.0 (38.0)	27.4 (30.2)	28.1	20.2
	VIII		7.0	5.2	40.6 (33.0)	30.6 (30.8)	26.0	19.6
Carboxylic acids	IX		7.6	6.0	40.6 (41.5)	33.5 (41.5)	29.0	22.4
	X		9.0	7.8	48.0 (50.0)	42.5 (47.5)	27.0	26.2
	XI		9.2	7.8	44.8 (45.2)	40.0 (37.5)	28.2	25.0
	XII		8.2	7.6	38.0 (42.2)	37.2 (40.2)	26.0	25.6
	XIII		7.8	6.4	42.4 (43.0)	40.2 (42.0)	27.1	26.1
	XIV		7.8	6.2	44.0 (42.0)	42.4 (41.8)	26.6	25.2
Phenazinols	XV		8.0	6.0	46.8 (41.0)	44.6 (39.2)	29.0	25.0
	XVI		7.8	5.8	48.3 (38.0)	39.4 (32.5)	25.0	22.4

Experimental conditions

Glycolysis: main chamber: 20% cell suspension 1.6 ml., side arm: 0.1*M* glucose 0.4 ml. phenazine derivatives 0.4 ml., gas phase: O_2(95%)+CO_2(5%) in aerobic glycolysis. N_2(95%)+CO_2(5%) in anaerobic glycolysis.

Respiration: main chamber: 20% cell suspension 1.8 ml., side arm: phenazine derivatives 0.4 ml., center well: 20% KOH 0.2 ml., gas phase: air

a) Values are in parenthesis. *b*) Values repesent control level.

II. Influence of Phenazine Derivatives on the Energy-generating Systems of Tumor Cells

The *in vitro* influences of phenazine derivatives on aerobic and anaerobic glycolysis and on the respiration of tumor cells are summarized in Table I.

1) Effect on glycolysis : The anaerobic glycolysis of Ehrlich carcinoma cells was inhibited approximately 70~85% with $1.66\times10^{-3}M$, and 30~60% with $1.66\times10^{-4}M$ phenazine-di-N-oxides. The inhibitory effects of these agents on aerobic glycolysis was somewhat lower, as in the case of anaerobic glycolysis. Similar results were obtained with ascites hepatoma cells.

Mono-N-oxides also inhibited glycolysis to a lesser degree. The other substances tested showed no influence on glycolysis.

2) Effect on respiration : In the presence of phenazine-di- and mono-N-oxides, respiration of Ehrlich ascites carcinoma cells was inhibited. The effects of the various compounds varied in parallel with their effects on glycolysis. Phenazinols and phenazine carboxylic acid derivatives showed no inhibitory effects.

Discussion

The influences of 16 phenazine derivatives with chemically different structures on the mean survival times of mice bearing Ehrlich ascites carcinoma and on the glycolysis and respiration of tumor cells *in vitro* are summarized in Fig. 3.

It is seen from the figure that the survival times of mice bearing Ehrlich ascites carcinoma when treated with phenazine-di-N-oxides were about twice those of controls. Phenazine-mono-N-oxides exerted little effect on the regression of ascites tumors.

An effort was made to increase the transportability of these insoluble phenazine derivatives into the cells. Phenazine carboxylic acid and its amino acid derivatives or phenazinols were also tested, but no favourable results were observed.

As can be seen, among the phenazine derivatives tested, appreciable antitumor activity was only found among the compounds with the $\rangle N\rightarrow O$ moiety. In these compounds, it seems that this moiety plays an important role in carcinostatic activity.

The effective compounds above mentioned, however, showed no influence on the tumor growth in solid form. These findings disagree with the report of Furst,[7] who recognized marked regression of the growth of solid Ehrlich carcinoma on intraperitoneal administration of phenazine-di-N-oxide at a dose of 70 mg./kg. once a day for 7 days.

The fact that phenazine-di-N-oxides were more effective in anticancer activity for ascitic form of Ehrlich carcinoma than any other compound tested seems to be of particular interest, when considered together with their bacteriostatic action[10,11] : namely, the bacteriostatic activities of phenazine derivatives are almost proportional to their antitumor effects.

The energy-generating systems of tumor cells presumably have some abnormal characteristics. Glycolysis (anaerobic and aerobic) is higher than in normal cells, while the levels of TCA cycle group enzymes are depressed. Therefore, studies were made to find which metabolic pathway is affected by phenazine derivatives.

As is evident from the data, only phenazine-di-N-oxides exhibited apparent inhibitory effects both on glycolysis and respiration. Although there is a possibility that these results might be partially arisen from the difference in their water solubility, they are suggestive of the possible correlation between the antitumor effects of phenazine derivatives and the inhibition of glycolysis and respiration. This correlation is also supported

10) Y. Kawakami, I. Yoshioka : J. Antibiotics, Ser. A., 8, 51 (1955).
11) C. N. Iland : Nature, **161**, 1010 (1948).

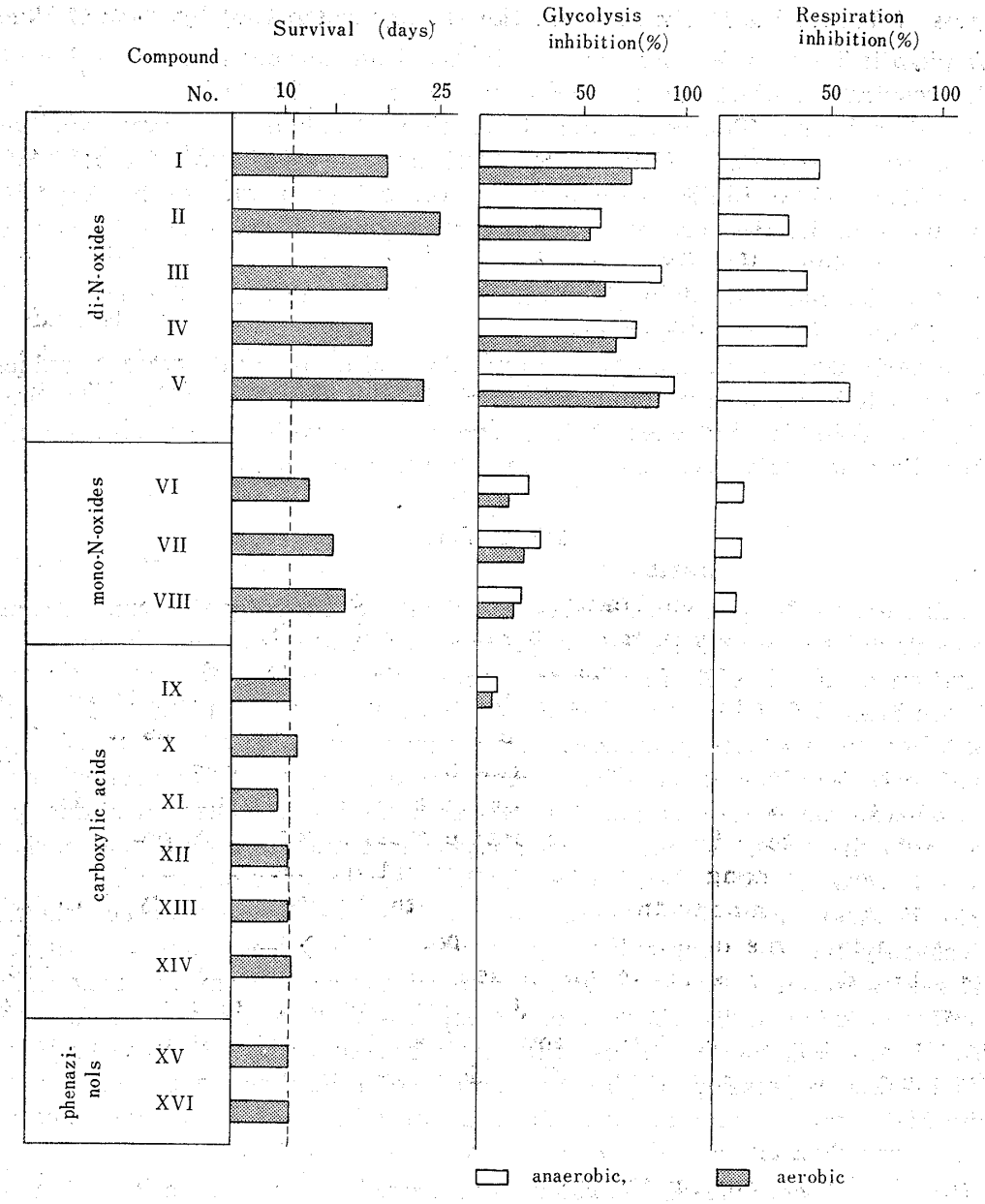

Fig. 3. Comparison of Chemical Structures of Phenazine Derivatives with their Effects on Survival Time and Inhibition of Energy Metabolism

by the findings that chemical agents with antitumor effects may also show pronounced inhibition of glycolysis, as was demonstrated by Fukuoka, *et al.*[12] with quinoline-derivatives against Ehrlich ascites carcinoma cells.

The authors wish to express their gratitude to Professor Dr. Ichiro Yosioka for kindly supplying phenazine derivatives and for his interest in the work and valuable suggestions.

Summary

Of the phenazine derivatives tested, phenazine-di-N-oxides produced the most marked prolongation of the survival time of mice bearing Ehrlich ascites carcinoma. Phenazine-mono-N-oxides were only moderately effective.

12) F. Fukuoka, H. Naora : Gann., 48, 271 (1957).

The di-N-oxide compounds, however, were ineffective against the solid form of Ehrlich carcinoma.

Other phenazine derivatives exhibited no effect on the growth of tumor cells.

The degrees of inhibition of glycolysis (aerobic or anaerobic) and respiration of tumor cells by these compounds were in the same order as their antitumor activities.

So, the following general structure-activity relationships were observed with the series of compounds tested: appreciable antitumor activity was found only among compounds with the $\geqslant N \rightarrow O$ moiety.

Possible parallels between the antitumor activities of these compounds and the degrees of their inhibition of energy-generating systems of tumor cells were shown.

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16. Masahiro Nakadate, Yoshihiro Takano, Tadamasa Hirayama, Setsuko Sakaizawa, Tadashi Hirano, Kenji Okamoto, Kennichi Hirao, Tadao Kawamura, and Michiya Kimura: Janovsky Reaction of Nitropyridines. II.*1 Preparation of 5-Nitronicotinic Acid and its Related Compounds.

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During the course of an investigation of the color reaction of polynitrobenzene derivatives with active methylene compounds, it was found that the same kind of reaction could similarly occur on the mononitro-benzene derivatives in which the electron-attracting substituents such as CN, $COOCH_3$, COOH occuppied at the *meta*-position of nitro groups.[1] This finding prompted us to explore the Janovsky color reaction of nitropyridine derivatives. The present paper deals with the preparation of several β-nitro-β'-substituted pyridine derivatives.

At the earlier stages of our investigation some attempts to obtain 5-nitronicotinic acid were carried out unsuccessfully as shown in Chart 1. Both nitration and amination of nicotinic acid with the mixture of fuming nitric acid and 30% fuming sulfuric acid and with sodium amide in N,N-dimethylaniline respectively, did not proceed so that the starting material was recovered. Alternatively 8-quinolinol was submitted to the degradation into 2,3-pyridinedicarboxylic acid with fuming nitric acid, which was then converted to the imide through anhydride. Hofmann rearrangement of 2,3-pyridinedicarboximide thus formed gave 3-aminopicolinic acid alone in place of the desired 2-amino isomer.*3 According to Wallis and Lane[2] the Hofmann rearrangement of substituted phthalic imide can afford two kinds of isomer, that is, the one possessing a

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*1 Previous paper, "Synthesis of 3-Nitropyridine and its Related Compounds," Yakugaku Zasshi, **79**, 549 (1959), represents Part I of this series.

*2 Nishi-5-Chome, Kita-12-Jo, Sapporo (中舘正弘，高野良宏，平山忠允，堺沢節子，平野 正，岡元賢二，平尾健一，河村忠男，木村道也).

*3 Sucharda obtained these two isomers from the same reaction using hypochlorite in the yields of 67.2 and 23.2% respectively.[3]

1) M. Kimura, M. Tohma: Yakugaku Zasshi, **78**, 1401 (1958).
2) E. Wallis, J. Lane: "Org. Reactions" Vol. III, p. 277 (1946), John Wiley & Sons inc., New York.
3) E. Sucharda: Ber., **58**, 1727 (1925).