The 5-phenylazopyrimidine derivatives were classified into following two groups on the basis of their antagonistic pattern. The first group consists of those compounds whose growth inhibitory activity is reversed by thymine or leucovorin but not by folic acid, and the second group, those compounds whose inhibitory activity is reversed by thymine or leucovorin, as well as by folic acid.

(Received July 3, 1958)

UDC 547.855 : 577.15.025

4. **Minoru Kawashima**: Studies on Nucleic Acid Antagonists. IV.[1] Inhibitory Effect of 5-Phenylazopyrimidines on the Enzymatic Conversion of Folic Acid to Citrovorum Factor *in vitro*.*

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

In the preceding report[1] the author described the inhibitory activity of 5-phenylazopyrimidines upon the growth of *Streptococcus faecalis* and the reversal pattern by a series of metabolites. It was shown that these 5-phenylazopyrimidines are classified into two groups according to their reversal pattern by thymine, leucovorin, or folic acid.

The relatonship between their antagonistic pattern and chemical structure was also demonstrated. The possibility that the inhibitory action of the first group is due to the inhibition of the enzymatic conversion of folic acid to citrovorum factor (CF) was discussed on the basis of results reported by Hitchings.[2]

The present report deals with the inhibitory effect of 5-phenylazopyrimidines on the conversion of folic acid to CF by the chick-liver supernatant solution prepared according to Doctor's method.[3]

Material and Methods

The chemical structure and antagonistic pattern upon the growth of *Str. faecalis* of the 12 kinds of 5-phenylazopyrimidines employed in this experiment are summarized in Table I.

The liver of white Leghorn female chicks of 10 to 12 weeks old were used as the source of enzyme throughout this experiment. The chick-liver supernatant solutions were prepared in a following manner: Preparation of 20% homogenate of chick liver in 0.08*M* sodium potassium phosphate buffer (pH 6.7) was made in a Waring blendor. An aliquot of the homogenate was centrifuged for 30 mins. at 13,000*g*, the clear portion of the supernatant was decanted, and made to the volume of the starting homogenate with the same buffer.

The mixture to be incubated contained 5 cc. of the supernatant solution, 10 mg. of DL-serine, 5 mg. of DL-homocysteine, and 10 mg. of $MgCl_2$ in a total volume of 10 cc. The mixture was incubated with shaking in N_2 atmosphere for 3 hrs. at 37° and autoclaved for 30 mins. at 121°. After cool, the mixture was homogenized in a Waring blendor, made up to an appropriate volume, filtered, and the filtrate was assayed. The CF content of the incubated mixture described above was determined by using CF-assay medium, the composition of which is shown in Table II. *Leuconostoc citrovorum* (ATCC 8081) was used as the test organism and Ca-leucovorin was employed as the standard. The culture was incubated for 24 hrs. at 37° and the growth was measured by using the Coleman Junior spectrophotometer (650 mμ).

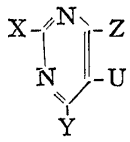
---

* This paper constitutes a part of a series entitled "Studies on Nucleic Acid Antagonists" by K. Tanaka and E. Ohmura. This was read at the Kinki Local Meeting of the Pharmaceutical Society of Japan, October 19, 1957.

** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (川島 實).

1) Part III. M. Kawashima: This Bulletin, **7**, 13(1959).
2) G. H. Hitchings: J. Biol. Chem., **183**, 1(1950).
3) V. M. Doctor: *Ibid.*, **222**, 959(1956).

TABLE I. 5-Phenylazopyrimidine Derivatives and their Antagonistic Properties to Thymine, Folic acid, and Leucovorin by *Str. faecalis*



Compd. No.	X	Y	Z	U	Inhibition reversed by thymine	folic acid	leucovorin
Py-23	NH_2	NH_2	NH_2	—	+	+	+
Py-24	NH_2	NH_2	NH_2	-NO	+	+	+
Py-40	NH_2	NH_2	OH	-N:N-	+	−	+
Py-41	NH_2	NH_2	NH_2	-N:N-	+	−	+
Py-61	NH_2	NH_2	NH_2	-N:N- (-Cl)	+	−	+
Py-62	NH_2	NH_2	NH_2	-N:N- (Cl)	+	−	+
Py-63	NH_2	NH_2	NH_2	-N:N- (NO_2)	+	−	+
Py-72	NH_2	NH_2	NH_2	-N:N- ($-SO_2NH_2$)	+	+	+
Py-74	NH_2	NH_2	NH_2	-N:N- (-COOH)	+	+	+
Py-77	NH_2	NH_2	CH_3	-N:N-	+	−	+
Py-80	NH_2	NH_2	NH_2	-N:N- ($-PO_3H_2$)	+	+	+
Py-91	NH_2	NH_2	NH_2	-N:N- (F)	+	−	+

\+ Growth inhibition, caused by addition of minimal effective concentration of a compound, was reversed by 1 γ/5 cc. of leucovorin or folic acid, or 100 γ/5 cc. of thymine.
− The growth inhibition was not reversed by 10 γ/5 cc. of folic acid.

TABLE II. Basal Medium for CF-Assay by *Leuc. citrovorum*

Casein hydrolyzate	1.0 g.
Glucose	5.0 〃
Sodium acetate	4.0 〃
Ammonium chloride	0.6 〃
Salt A* and B**	1.0 cc. each
Adenine sulfate	2.0 mg.
Guanine	2.0 〃
Uracil	2.0 〃
Xanthine	2.0 〃
Thiamine hydrochloride	100 γ
Pyridoxine hydrochloride	200 〃
Pyridoxamine hydrochloride	60 〃
Pyridoxal hydrochloride	60 〃
Ca pantothenate	100 〃
Riboflavin	100 〃
Nicotinic acid	200 〃
PABA	20 〃
Biotin	0.2 〃

Folic acid	2.0 γ
Tryptophan	40 mg.
Cystine	40 "
Methionine	20 "
Alanine	20 "
Sufficient water to make (double strength)	100 cc.

* 25 g. each of KH_2PO_4 and K_2HPO_4 dissolved in sufficient water to make 250 cc. of solution.

** 10 g. $MgSO_4$, 0.5 g. NaCl, 0.5 g. $FeSO_4$, and 0.5 g. $MnSO_4$ dissolved in sufficient water to make 250 cc. of solution.

For the determination of inhibitory activity on the conversion of 100 γ of folic acid to CF by chick-liver supernatant solutions, 500 γ of the 5-phenylazopyrimidine derivatives was used, and aminopterin was added in the quantity of 10 γ as the positive control.

Since leucovorin has been reported to be half as active as the CF isolated from a horse liver,[4] the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF.

In further experiments, tetrahydrofolic acid (FAH_4) or N^{10}-formylfolic acid (N^{10}-CHO·FA) was also used as the substrate in place of folic acid in the same enzyme system.

Results and Discussion

The results of the first experiment are shown in Table III.

When the complete system mentioned above was used 50 γ of CF per 1 g. of liver was formed, and this enzymatic reaction was completely inhibited by the presence of 10 γ of aminopterin in this system, and the supernatant solution heated previously at 100° for 5 mins. failed to make this reaction proceed.

TABLE III. Effect of Aminopterin, 2,6-Diaminopurine, 8-Azaguanine, and 6-Mercaptopurine on Conversion of Folic Acid to CF by Chick-liver Supernatant Solution

	System	CF formed/g. liver (γ)
Expt. 1	Complete system*	50
	+aminopterin (10 γ)	6.1
	Enzyme heated	0.075
	Without enzyme	0
Expt. 2	Complete system*	138
	Without folic acid	2.7
	+ aminopterin (10 γ)	13.5
	+ 2,6-diaminopurine (500 γ)	160
	+ 8-azaguanine (500 γ)	150
	+ 6-mercaptopurine (500 γ)	170

* System: 5 cc. of liver supernatant solution in 0.08*M* phosphate buffer (pH 6.7), with 5 cc. of the same buffer containing the following amount of substances, to make a total volume of 10 cc.: 100 γ of folic acid, 10 mg. of serine, 5 mg. of homocysteine, and 10 mg. of $MgCl_2$.

TABLE IV. Effect of Adenine or Thymine on Inhibited Conversion of Folic Acid to CF by Chick-liver Supernatant Solutions

No.	System	CF formed/g. liver (γ)
1	Complete system*	88
2	+ aminopterin (10 γ)	3.8
3	+ Py-40 (500 γ)	14
4	+ Py-40 (500 γ) +thymine (10 mg.)	14
5	+ Py-40 (500 γ) +adenine (10 mg.)	24
6	+ Py-41 (500 γ)	10
7	+ Py-41 (500 γ) +thymine (10 mg.)	20
8	+ Py-41 (500 γ) +adenine (10 mg.)	16
9	Enzyme heated (100°, 5 mins.)	0.36

4) J. D. Kereztesy: J. Am. Chem. Soc., **73**, 5510 (1951).

* System: 5 cc. of chick-liver supernatant solution in 0.08*M* phosphate buffer (pH 6.7), with 5 cc. of the same buffer containing the following amount of substances, to make a total volume of 10 cc.: 100 γ of folic acid, 10 mg. of serine, 5 mg. of homocysteine, and 10 mg. of $MgCl_2$.

It is also apparent from Table III that 2,6-diaminopurine, 8-azaguanine, and 6-mercaptopurine have no inhibitory effect on CF biosynthesis.

The results of experiments concerning the effect of thymine and adenine on the inhibitory actions of 2,4-diamino-6-hydroxy-5-phenylazopyrimidine (Py-40) and 2,4,6-triamino-5-phenylazopyrimidine (Py-41) on the conversion of folic acid to CF are presented in Table IV, showing that the inhibitory effect was not reversed by 10 mg. each of these two substances.

Leucovorin added to the chick-liver supernatant solution containing 500 γ of each of 5-phenylazopyrimidine derivatives was recovered completely in the microbiological CF assay method described above. This seems to indicate that the presence of the 5-phenylazopyrimidines in the samples tested for CF assay did not exert any effect on the growth of the assay organism. It was shown that the inhibitory activities of the synthetic compounds on the CF biosynthesis may be apparently detectable in these enzymatic systems. Aminopterin was also reported to inhibit the enzymatic conversion of folic acid to CF in rats and chicks.[5, 6]

Then 12 kinds of 5-phenylazopyrimidines in a quantity of 500 γ were applied in these systems and 7 of them were found to be inhibitory towards the enzymatic conversion of folic acid to CF but the others were not effective.

Compounds inhibitory to biosynthesis of CF were 2,4-diamino-6-hydroxy-5-phenylazopyrimidine (Py-40), 2,4,6-triamino-5-phenylazopyrimidine (Py-41), 2,4,6-triamino-5-(4-chlorophenylazo)pyrimidine (Py-61), 2,4,6-triamino-5-(2-chlorophenylazo)pyrimidine (Py-62), 2,4,6-triamino-5-(3-nitrophenylazo)pyrimidine (Py-63), 2,4,6-triamino-5-(3-fluorophenylazo)pyrimidine (Py-91), and 2,6-diamino-4-methyl-5-phenylazopyrimidine (Py-77). These compounds belong to the first group according to their antagonistic pattern described before.[1] The compounds which belong to the first group inhibited the biosynthesis of CF in 500 γ, but the compounds of the second group exerted no influence on the biosynthesis, as indicated in Table V.

TABLE V. Effect of 5-Phenylazopyrimidines on Conversion of Folic Acid to CF by Chick-liver Supernatant Solution

	System	CF formed/g. liver (γ)
Expt. 1	Complete system*	80
	+ aminopterin (10 γ)	4
	+ Py-23 (500 γ)	70
	+ Py-24 (500 γ)	70
	+ Py-40 (500 γ)	10
	+ Py-41 (500 γ)	9
	+ Py-72 (500 γ)	70
	+ Py-74 (500 γ)	80
	+ Py-80 (500 γ)	66
	Enzyme heated (100°, 5 mins.)	0.04
Expt. 2	Complete system*	138
	Without folic acid	2.7
	+ aminopterin (10 γ)	13.5
	+ Py-61 (500 γ)	8
	+ Py-62 (500 γ)	6.3
	+ Py-63 (500 γ)	6.3
	+ Py-69 (500 γ)	13.4
	+ Py-77 (500 γ)	9.4
	Enzyme heated (100°, 5 mins.)	0.01

* System: 5 cc. of chick-liver supernatant solution in 0.08*M* phosphate buffer (pH 6.7), with 5 cc. of the same buffer containing the following amount of substances, to make a total volume of 10 cc.: 100 γ of folic acid, 10 mg. of serine, 5 mg. of homocysteine, and 10 mg. of $MgCl_2$.

5) A. D. Welch: J. Pharmacol. Exptl. Therap., **103**, 403 (1951).
6) V. M. Doctor: Arch. Biochem. Biophys., **48**, 248 (1954).

These results suggest that there seems to be a good agreement between the antagonistic properties on *Str. faecalis* and the inhibitory effect on CF biosynthesis of chick-liver supernatant solution of the two groups of 5-phenylazopyrimidine derivatives.

It was demonstrated by Hitchings, *et al.*[7] that 2,4-diamino-5-*p*-chlorophenoxy-6-ethylpyrimidine inhibited the growth of *Str. faecalis*, and that the growth inhibition was reversed by leucovorin but not by folic acid. Doctor[3] also reported that this compound inhibited the conversion of folic acid to CF in the supernatant solution of chick-liver homogenate. The inhibitory activity of 5-phenylazopyrimidines which belong to the first group and which were employed in the present experiment seems to be characteristic as those of 2,4-diamino-5-*p*-chlorophenoxy-6-ethylpyrimidine.

2,4-Diaminopyrimidine and derivatives of condensed pyrimidine system containing 2,4-diaminopyrimidine are reported to be competitive antagonists of folic acid on the growth of *L. casei*.[8] The diaminopyrimidine which possesses a bulky substituent in the 5-position of the pyrimidine ring have been reported to be more effective antagonists than 5-unsubstituted or 5-alkylated derivatives.[8]

From the facts mentioned above, it was concluded that the compounds which belong to the first group inhibit the enzymatic synthesis of CF from folic acid, while those belonging to the second group disturb the utilization of folic acid in the metabolic pathway of *Str. faecalis* which requires folic acid for growth.

Studies by Blakley[9] suggest that aminopterin may interfere with the conversion of folic acid to tetrahydrofolic acid (FAH_4). Since FAH_4 has been postulated as the first intermediate in the conversion of folic acid to CF by many investigators,[10, 11] it seemed of interest to determine whether the conversion of folic acid to FAH_4 would be inhibited by the 5-phenylazopyrimidine derivatives of the first group in the present enzyme system. The results presented in Tables VI and VII indicate that the conversion of FAH_4 to CF is not influenced by the 5-phenylazopyrimidines belonging to the first group. This may suggest that, under the experimental conditions described above, all of the 5-phenylazopyrimidine derivatives of the first group may inhibit the conversion of folic acid to CF by inhibiting the enzymatic formation of FAH_4.

TABLE VI. Effect of 5-Phenylazopyrimidines on the Conversion of FAH_4 to CF by Chick-liver Supernatant Solution

	System	CF formed/g. liver (γ)
1	FAH_4; complete system*	59.0
2	〃 ; 〃 +aminopterin (10 γ)	49.0
3	〃 ; 〃 +Py-40 (500 γ)	55.0
4	〃 ; 〃 +Py-41 (500 γ)	56.0
5	Without FAH_4	5.5
6	N^{10}-CHO・FA; complete system	11.5
7	〃 〃 ; 〃 +aminopterin (10 γ)	6.5
8	Enzyme heated (100°, 5 mins.)	0.0

* 5 cc. of chick-liver supernatant solution in 0.08*M* phosphate buffer (pH 6.7), with 5 cc. of the same buffer containing the following amount of substances, to make a total volume of 10 cc.: 100 γ of FAH_4 or N^{10}-CHO・FA, 10 mg. of serine, 5 mg. of homocysteine, and 10 mg. of $MgCl_2$.

Table VII. Effect of 5-Phenylazopyrimidines on the Conversion of FAH_4 to CF by Chick-liver Supernatant Solution

	System	CF formed/g. liver (γ)
1	FAH_4; complete system*	63.0
2	〃 ; 〃 +Py-40 (500 γ)	40.0
3	〃 ; 〃 +Py-41 (500 γ)	63.0
4	〃 ; 〃 +Py-61 (500 γ)	68.0
5	〃 ; 〃 +Py-63 (500 γ)	66.0
6	〃 ; 〃 +Py-77 (500 γ)	75.0
7	〃 ; 〃 +Py-91 (500 γ)	72.0
8	〃 ; 〃 +aminopterin (10 γ)	58.0

7) G. H. Hitchings: J. Biol. Chem., **199**, 43 (1952).
8) G. H. Hitchings: Ann. New York Acad. Sci., **52**, 1318 (1950).
9) R. L. Blakley: Biochem. J., **58**, 448 (1954).
10) V. M. Doctor: Federation Proc., **14**, 204 (1955).
11) G. R. Greenberg: *Ibid.*, **13**, 745 (1954).

9	Enzyme heated (100°, 5 mins.)	0.0
10	N^{10}-CHO·FA; complete system	14.0
11	〃 ; 〃 +aminopterin (10 γ)	8.0
12	Enzyme heated (100°, 5 mins.)	0.0

* 5 cc. of chick-liver supernatant solution in 0.08*M* phosphate buffer (pH 6.7), with 5 cc. of the same buffer containing the following amount of substances, to make a total volume of 10 cc.: 100 γ of FAH_4 or N^{10}-CHO·FA, 10 mg. of serine, 5 mg. of homocysteine, and 10 mg. of $MgCl_2$.

It was also demonstrated by Asahi[12] of this laboratories by polarography of the 5-phenylazopyrimidine derivatives in 40% EtOH solution (pH 6.8) that the compounds belonging to the first group showed a more positive $E_{\frac{1}{2}}$ value than those belonging to the second group, the border being about -1.11 V. The same study on folic acid revealed that the acid exhibited two $E_{\frac{1}{2}}$ values of -0.76 and -1.13 V, which corresponded to the reduction potentials observed at the formation of FAH_2 and FAH_4, respectively. That the $E_{\frac{1}{2}}$ value of the compounds belonging to the first group is more positive than that observed in the formation of FAH_4 seems to mean that in coexistence with folic acid, the compounds act as a competitor of FAH_4 at the stage of formation of the latter in the course of conversion of folic acid to CF.

The present series of experiments failed to explain the mechanism of action of the compounds belonging to the second group by using the microörganisms which do not require folic acid for growth, such as *E. coli*, *L. arabinosus*, and *Leuc. mesenteroides*, because the typical compounds of this group (Py-64 and 80) did not inhibit the growth of these microörganisms at a concentration less than 100 γ/cc.

The author is deeply indebted to Dr. S. Kuwada, Director of the Laboratories, for his permission for this investigation and its publication. The author also expresses his grateful thanks to Dr. A. Watanabe for his encouragement throughout this work. Gifts of N^{10}-CHO·FA from Dr. K. Iwai, Research Institute for Food Science, University of Kyoto, and of FAH_4 from Mr. Y. Sanno, in this Laboratories, are gratefully acknowledged.

Summary

Examinations were made on the inhibitory action of two groups of 5-phenylazopyrimidines, which were classified on the basis of their antagonistic pattern on *Str. faecalis*, on enzymatic conversion of folic acid to the citrovorum factor by supernatant solutions of chick-liver homogenates.

It was demonstrated that the compounds which belong to the first group inhibited the enzymatic conversion of folic acid to the citrovorum factor by interfering in the enzymatic formation of FAH_4, but the compounds of the second group exerted no influence on CF conversion.

(Received July 3, 1958)

12) Y. Asahi: Paper presented at the 78th Annual Meeting of the Pharmaceutical Society of Japan, April 8, 1958.