Summary

To test the antibacterial activity, 2- or 4-[2-(5-nitro-2-furyl)vinyl]pyrimidines were synthesized by the condensation of 5-nitro-2-furaldehyde (Va) with the active methyl group of pyrimidines.

It has been found that Va predominantly condensed with the 4-methyl group of 2,4-dimethylpyrimidine and in the reaction of Va with 2,4-dimethylpyrimidine containing a functional group in the 6-position, the active hydrogen reactivity between the 2-methyl group and 4-methyl group is affected by the presence of group in the 6-position: in 2,4-dimethylpyrimidines substituted by the hydroxy, amino and alkoxy group in the 6-position, the 2-methyl group was founded to be more active, while in the ones substituted by the acetamido, dimethylamino and chloro group, the 4-methyl group was more reactive.

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153. Osamu Tamemasa, Shoji Okada, and Yasuo Wakita : Inhibition and Stimulation of the Biosynthesis of Protein and Nucleic Acid. I.

Effect of Some Phenylalanine Analogs on the Aromatic
Amino Acid Incorporation into Proteins of Ehrlich
Mouse Ascites Tumor Cells *in vitro*.

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It has been reported in recent years that many kinds of chemical compounds show inhibiting or stimulating effect on the biosynthesis of protein or nucleic acid. As a part of those studies, investigations have been undertaken on the inhibition of protein synthesis by amino acid analogs in bacteria, and it has frequently been observed that many structural analogs of amino acid depress bacterial protein synthesis.¹⁻⁴⁾ As for neoplastic cells, however, there have been only few reports on the structural analogs which inhibit the amino acid incorporation.^{2,3,5-9)}

In the present study, the effect of some phenylalanine analogs was investigated on the incorporation of aromatic amino acid into proteins of Ehrlich mouse ascites tumor cells *in vitro* with an emphasis on the difference in the response to the analogs between tumor cells and normal mouse liver cells.

Experimental

Materials—DL-Phenylalanine-2-¹⁴C (DL-Phe-2-¹⁴C), L-tyrosine-¹⁴C (u) (L-Tyr-¹⁴C(u)), and DL-trypto-

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- 1) A. Meister : "Biochemistry of the Amino Acids," 114 (1957), Academic Press Inc., New York.
- 2) W. Shive, C.G. Skinner : Ann. Rev. Biochem., **27**, 643 (1958).
- 3) Y. Miura : Tampakushitsu Kakusan Kôso, **6** (11), 123 (1961).
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- 5) M. Rabinovitz, M.E. Olson, D.M. Greenberg : J. Biol. Chem., **210**, 837 (1954).
- 6) *Idem* : Cancer Research, **17**, 885 (1957).
- 7) *Idem* : J. Biol. Chem., **227**, 217 (1957).
- 8) *Idem* : Cancer Research, **19**, 388 (1959).
- 9) J.E. Wilson, J.R. Irvin, J.E. Suggs, K. Liu : *Ibid.*, **19**, 272 (1959).

phan-3- ^{14}C (DL-Try-3- ^{14}C) were obtained from Daiichi Pure Chemicals Co., Ltd., and were diluted with their stable isomers to 1 mc./mmol. of a specific activity.

4-Nitro-DL-phenylalanine (4- NO_2 -Phe), 4-amino-DL-phenylalanine (4- NH_2 -Phe), DL-phenylglycine (phenyl-Gly), and 3-(1'-naphthyl)-DL-alanine (naphthyl-Ala) were synthesized in the authors' laboratory, 4-fluoro-DL-phenylalanine (4-F-Phe) was obtained from the Nutritional Biochemicals Corp., and 3-(2'-thienyl)-DL-alanine (thienyl-Ala) was kindly furnished by Dr. Tomizawa of the National Institute of Health.

Whole Tumor Cells—Ehrlich mouse ascites tumor cells were obtained from Dr. Mizuno of the University of Tokyo, and have since been maintained in the authors' laboratory by weekly transfers through the intraperitoneal injection of the transplanted ascitic fluid into the female mice of the strain dd/Y (6~7 weeks old, 15~18 g.). Animals inoculated with the tumor cells 7 days in advance were used in the present work. After an intraperitoneal injection of 3 ml. of incubation medium (the modified Krebs-Ringer phosphate buffer (KRP),⁹⁾ in some experiments physiological saline (NaCl)), the ascitic fluid was drained through a large abdominal incision into a flask dipped in ice-water. Subsequent operations were performed at 0~5°. The tumor cells were collected by centrifugation and washed by resuspending in the ice-cold incubation medium. This washing procedure was repeated two or three times, unless stated otherwise, until the supernatant became clear. After final washing, the packed cells were resuspended in the incubation medium to give about 35 mg. of the cells in dry weight per ml. of the suspension.

Disrupted Tumor Cells—The suspension of the washed whole tumor cells was passed through a French pressure cell at 600 kg./cm². The cells became disrupted by this treatment.

Whole Liver Cells—Normal female mice of the strain dd/Y (6~7 weeks old, 15~18 g.) were stunned, decapitated and perfused through hepatic vein until livers were well blanched with the ice-cold incubation medium. Then liver cells were isolated by the method of Kaltenbach.¹⁰⁾ After washing two or three times with the ice-cold incubation medium, the liver cells were suspended in it to give about 35 mg. of the cells in dry weight per ml. of the suspension.

Incubation and Washing Procedure—Incubations were carried out in two manners: 1) "Pre-incubation"—Two ml. of the incubation mixture contained 1 ml. of the cell suspension and 1 ml. of an amino acid analog solution. After incubation for 60 min. at 37° in air, 1 ml. of the radioactive amino acid solution was added, and further incubation was carried out for 60 min. at 37° in air.

2) "Simultaneous incubation"—Three ml. of the incubation mixture, which contained each 1 ml. of the above suspension and solutions, was incubated for 60 min. at 37° in air.

After incubation, the reaction was stopped by addition of 2 ml. of cold 30% trichloroacetic acid (TCA). The TCA precipitate was washed by suspension and centrifugation once in 5% TCA at 50° for 10 min., twice in 5% TCA at 50° for 5 min., three times in EtOH-ether (3:1) mixture, once in acetone, and finally resuspended in 25% EtOH.

Assay for Radioactivity—The proteins thus obtained by washing and resuspended in 25% EtOH were plated on aluminum disks, dried, and counted for radioactivities in a window-less gas-flow counter. Correction was made for the self-absorption by the use of the absorption correction curve which was previously drawn for glycine- ^{14}C , then counts per min. per mg. protein were calculated.

Results

Fig. 1. Effect of Several Incubation Media on the DL-Phe-2- ^{14}C Incorporation

Effect of Several Incubation Media on the Incorporation of DL-Phe-2- ^{14}C into Proteins of Ehrlich Mouse Ascites Tumor Cells

After washing the tumor cells twice with ice-cold physiological saline, they were suspended in the following six kinds of incubation media; the modified Krebs-Ringer phosphate buffer (KRP), Ca^{++} -free KRP (KRP-Ca), Krebs-Ringer bicarbonate buffer (KRB), phosphate buffer (pH: 7.2) (PB), physiological saline (NaCl), and distilled water (H_2O). Each suspension was incubated with DL-Phe-2- ^{14}C according to

10) J. P. Kaltenbach: Fed. Proc., 11, 237 (1952).

the procedure of "simultaneous incubation" (without analogs), and the incorporated radioactivities were counted.

As shown in Fig. 1, in the case of KRP the highest rate of incorporation was observed. Therefore, in the following experiments, KRP was used as an incubation medium.

Effect of the Pretreatment of the Cells and the Addition of Some Energy Sources or Amino Acids on the Incorporation of DL-Phe-2-¹⁴C into Proteins of Ehrlich Mouse Ascites Tumor Cells

Gotto, *et al.*¹¹⁾ reported that in the presence of a nucleotide or nucleoside the incorporation of leucine or isoleucine into proteins of Ehrlich or Krebs ascites carcinoma cells was stimulated and the data became less variable when the cells were previously incubated for 2 hours at 37°. Gale and Folkes found that, in the case of intact or disrupted Staphylococcal cells, the addition of adenosine triphosphate plus hexose diphosphate increased the incorporation of glutamic acid into proteins,¹²⁾ and the addition of the mixture of amino acids essential for the bacteria had the similar effect.¹³⁾ Furthermore, it was reported by Brown, *et al.*,¹⁴⁾ that the uptake of formate-¹⁴C into TCA insoluble fraction (mainly, nucleic acid fraction) of L-1210 ascites leukemic cells was enhanced by the addition of glucose plus L-glutamine.

In order to confirm such influence in the present experiments, the cells were kept for 2 hours at 37° or for 24 hours at 5° before incubation, and adenosine triphosphate

Fig. 2. Effect of the Pretreatment of Cells and the Addition of Some Energy Sources or Amino Acids on the DL-Phe-2-¹⁴C Incorporation

adenosine triphosphate (ATP); 1.2 μ mole, hexose diphosphate (HDP); 4.0 μ mole, glucose (Glc); 16.7 μ mole, L-glutamine (Glu-NH₂); 5.1 μ mole, mixture of amino acids; the mixture of following amino acids—L-Arg, L-Lys, L-His, DL-Val, DL-Thr, L-Leu, L-Ileu, DL-Met, L-CySH, L-Tyr, DL-Try, L-Glu-NH₂, L-Asp-NH₂, Gly, DL-Ala, DL-Ser, L-Asp, L-Glu, L-Pro, and L-(CyS)₂ (0.03 μ mole as L-form for each)

□ 2 washings

▨ 5 washings

- 11) A. M. Gotto, M. L. Belknode, R. W. Hester, R. Siler, O. Touster : *Biochim. Biophys. Acta*, **80**, 163 (1964).
- 12) E. F. Gale, J. P. Folkes : *Biochem. J.*, **59**, 661 (1955).
- 13) *Idem* : *Ibid.*, **55**, 721 (1953).
- 14) F. U. Brown, M. Klein, T. S. Kandaswamy, H. G. Mandel : *Cancer Research*, **23**, 254 (1963).

plus hexose diphosphate, glucose plus L-glutamine, or the mixture of amino acids were added in KRP.

Furthermore, in order to observe the effect of times of washing cells with ice-cold KRP, one portion of cells was washed twice and the other five times.

As shown in Fig. 2, there was neither remarkable effect of the addition of the energy sources or amino acids, nor particular decrease in the rate of incorporation by repeated washings. The extent of incorporation, however, was much decreased when the cells were previously kept for 2 hours at 37°, and still stronger decrease was found when kept for 24 hours at 5°.

On the basis of these results, all the following experiments were performed without addition of the energy sources or amino acids, and the cells were used for experiments immediately after two or three times washing.

Effect of Amino Acid Analogs on the Incorporation of Aromatic Amino Acid into Proteins of Ehrlich Ascites Tumor Cells

Fig. 3 presents the results of experiments in which the tumor cells were incubated with several kinds of aromatic ^{14}C -amino acids of a concentration of $0.5 \times 10^{-4} M$ as

Fig. 3. Inhibition of the DL-Phe, L-Tyr or DL-Try Incorporation into Ehrlich Ascites Tumor Cell Proteins by their Analogs
 --○-- pre-incubation —●— sim. incubation

L-form and their analogs of several different concentrations; ratios of molar concentrations of the analogs to the amino acids were 1:1, 1:5, 1:10, and 1:100.

It shows that the inhibiting actions of 4-NO₂-Phe and 4-F-Phe on the Phe or Tyr incorporation were, especially at higher concentrations of the analogs, more intensive than those of the other analogs. On the other hand, there was no remarkable difference between the two conditions, *i.e.* "pre-incubation" and "simultaneous incubation."

Time Course of Amino acid Incorporation in the Presence or Absence of Analogs

In order to ascertain that the uptake of radioactivity was dependent on biological incorporation of the labeled amino acids, and to see the time course of the incorporation in the presence of the analogs, the tumor cells were incubated in the presence of DL-Phe-2-¹⁴C or L-Tyr-¹⁴C(u), and with or without 4-NO₂-Phe or 4-F-Phe for various periods.

As shown in Fig. 4, the incorporation curves of the intact cells were clearly different from that of the heated cells (the tumor cells were previously treated for 10 minutes at 100°), this result indicating the occurrence of the biological incorporation of the labeled amino acids in the intact cells. It was found, furthermore, that the rate of inhibition at each incubation period by 4-NO₂-Phe or 4-F-Phe was in a similar degree, and that the extent of incorporation increased for 60 minutes and did not reach the equilibrium by the end of this period in the cases of both Phe and Tyr.

Fig. 4. Time Course of Incorporation of Labeled Phe or Tyr into Ehrlich Ascites Tumor Cell Proteins in the Presence of 4-NO₂-Phe or 4-F-Phe

--○-- , --△-- , --□-- , --x-- pre-incubation
 --●-- , --▲-- , --■-- , --x-- sim. incubation
 ○ ● control
 △ ▲ 4-NO₂-Phe (1 × 10⁻³M)
 □ ■ 4-F-Phe (1 × 10⁻³M)
 x heated cells (100°, 10 min.)

Effect of Analogs on the Incorporation of Amino Acids in Whole or Disrupted Cells of Ehrlich Ascites Tumor or the Mouse Liver

Fig. 5 shows that 4-F-Phe inhibited the Phe or Tyr incorporation at nearly the same ratio for the three sorts of cells; the whole tumor cells, the disrupted tumor cells, and the whole liver cells. 4-NO₂-Phe, however, inhibited the Phe or Tyr incorporation into proteins of the whole tumor cells only. In the disrupted tumor cells, no

inhibition was found on the incorporation of Phe or Tyr, while rather stimulation was found in the whole liver cells. Other analogs exerted no remarkable inhibition on the aromatic amino acid incorporation into proteins of whichever cell material was used.

Fig. 5. Inhibition of the DL-Phe, L-Tyr or DL-Try Incorporation into Proteins of Three Sorts of Cells by their Analogs ($1 \times 10^{-3} M$)

	pre-incubation	} whole tumor cells
	sim. incubation	
	pre-incubation	} disrupted tumor cells
	sim. incubation	
	pre-incubation	} whole liver cells
	sim. incubation	

Discussion

It was found that 4-NO₂-Phe and 4-F-Phe inhibited the Phe or Tyr incorporation into proteins by whole cells of Ehrlich mouse ascites tumor. 4-F-Phe acted inhibitorily on the Phe or Tyr incorporation in the whole tumor cells, the disrupted tumor cells, and the whole liver cells, whereas 4-NO₂-Phe behaved differently among the three sorts; there was a little inhibition in the disrupted tumor cells, while stimulation in the whole liver cells.

Such an inhibitory action of 4-NO₂-Phe is interesting in view of the fact that it has a chemical structure, somewhat resembling to that of chloramphenicol which inhibits protein synthesis in bacteria but not in mammals.¹⁵⁾

15) R. Rendì, S. Ochoa : J. Biol. Chem., 237, 3711 (1962).

Richmond⁴⁾ assumed that the inhibition of intracellular protein synthesis was caused by amino acid analogs, having molecular shape and size closely similar to that of corresponding natural metabolites. Under this concept, it is expected that 4-F-Phe inhibits the incorporation of Phe in the intracellular protein synthesis, because of its structural similarity to Phe.⁴⁾ Furthermore, in view of the fact that the inhibitory action of 4-F-Phe was observed in both of the whole and disrupted Ehrlich ascites tumor cells, it is assumed that the action is displayed independently of the integrity of the cell membranes. Although it was reported by Bergmann, *et al.*¹⁶⁾ that 4-F-Phe was not the antimetabolite of Tyr in *E. coli*, of interest is the observation that, in the tumor and liver cells, it inhibits the Tyr incorporation.

It may be considered that the action of 4-NO₂-Phe in the case of tumor cells is partially dependent on the inhibition of active transport of Phe or Tyr across the cell membrane, because of little inhibition in the case of disrupted cells. The inhibition of intracellular protein synthesis by 4-NO₂-Phe is expected less than that of 4-F-Phe, since the molecular size of 4-NO₂-Phe is much larger than that of Phe or Tyr.

In order to clarify these problems, further studies are in progress on the effect of these analogs upon the intracellular protein synthesis and on the permeability of amino acids across the cell membrane.

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Summary

4-NO₂-Phe and 4-F-Phe exert the inhibiting action to the Phe or Tyr incorporation into proteins of the intact Ehrlich mouse ascites tumor cells.

The inhibiting effect of 4-F-Phe is similar in the intact and disrupted tumor cells and the intact liver cells, while the effect of 4-NO₂-Phe is different among these three materials; little inhibition in the disrupted tumor cells, and rather stimulation in the whole liver cells.

Optimum incubation medium and conditions of these experiments were studied. Finally, the site of biological systems, which is affected by amino acid analogs, is discussed.

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16) E. Bergmann, S. Sicher, B.E. Volcani : *Biochem. J.*, 54, 1 (1953).