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Inhibition and Stimulation of the Biosynthesis of Protein and Nucleic Acid. II.[1] Inhibition Sites of 4-Fluoro- and 4-Nitro-phenylalanines on the Incorporation of Phenylalanine into Proteins of Ehrlich Mouse Ascites Tumor Cells *in vitro*

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4–Fluoro– and 4–nitro–phenylalanines inhibited the phenylalanine incorporation in a cell–free system of Ehrlich mouse ascites tumor cells, and their inhibition occurred in two steps, *i.e.* amino acid activation and transfer of phenylalanine to microsomes in protein synthesis. In the over–all incorporation experiment of a cell–free system, there was seen an antagonism of incorporation between phenylalanine and 4–fluorophenylalanine. The phenylalanine analogs did not exert their inhibitions on the incorporation of a few tested amino acids other than phenylalanine. Furthermore, the fluoro and nitro analogs behaved differently on the phenylalanine accumulation into "amino acid pool" of intact cells, the former being stimulative and the latter a little inhibitory.

Discussions were made on several problems encountered in the inhibition of protein synthesis by the analogs.

In the previous paper,[1] it was reported that 4–fluoro– and 4–nitro–phenylalanines inhibited the *in vitro* incorporation of phenylalanine(Phe) or tyrosine(Tyr) into proteins of Ehrlich moues ascites tumor cells. There have been various studies regarding the principle event of the inhibition of protein synthesis by amino acid analogs in different kinds of organisms,[3–12] but to our knowledge, no studies have been reported dealing with the inhibition mechanisms, especially the site of the inhibition in the pathways of protein synthesis.

From the recent developments of the studies on the mechanism of protein synthesis, it has been found that the peptide chains are built up from amino acids through the following steps: (i) Amino acid activation, (ii) formation of aminoacyl–sRNA, (iii) transfer of the aminoacyl group into ribosomal proteins, and (iv) release of the proteins from ribosomes. Besides, in intact cells, amino acids have been found to be accumulated first into their pool of the cells.

The present study was undertaken to determine the sites of the inhibition by the 4–fluoro– and 4–nitro–phenylanine analogs on the incorporation of phenylalanine into proteins of Ehrlich mouse ascites tumor cells and the effect of the phenylalanine analogs on the accumulation of phenylalanine and tyrosine into the amino acid pool in intact cells.

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Experimental

Materials——Radioactive phenylalanine(DL- and L-Phe-2-^{14}C, and L-Phe-^{14}C(U)), alanine(DL-Ala-1-^{14}C) and methionine(DL-Met-1-^{14}C) were obtained from the Daiichi Pure Chemicals Co., Ltd., and used in 0.5—1.0 mCi/mmole of a specific activity in the case of whole cells and 2.0—5.0 mCi/mmole in that of a cell-free incorporating system. 4-Fluoro- and 4-nitro-DL-phenylalanines(FPhe and NO_2Phe, respectively) were obtained from the Nutritional Biochemicals Corp. and 4-fluoro-DL-phenylalanine-3-^{14}C(FPhe-3-^{14}C) from the California Corp. for Biochemical Research. All the concentration data of amino acids and the analogs referred to the L-form only. ATP, CTP, GTP, and phosphoenol pyruvate-kinase were the products of the Sigma Chemical Co. Phosphoenol pyruvate, and creatine phosphate and its kinase were obtained from the Tokyo Kasei Kogyo Co., Ltd.

Whole Tumor Cells——Preparation of the cells was carried out according to the method described in a previous paper,[1] except that the preparation medium (0.14 M NaCl, 0.02 M glucose, 0.04 M tris (hydroxymethyl)aminomethane buffer of pH 8.5) was employed instead of the Krebs-Ringer phosphate buffer or physiological saline.

Accumlation of Phenylalanine into "Amino Acid Pool" in Intact Cells——The determination was conducted essentially according to the method described by Rabinovitz, Olson, and Greenberg.[8]

Preparation of Cell-Free Fractions——The fractions of microsomes, supernatant, and pH 5 enzyme were prepared by the method that followed closely that of Littlefield and Keller.[13]

Amino Acid Incorporation in Whole Cells and a Cell-Free System——The experimental procedure for the whole cells was the same as in the previous paper,[1] and that of a cell-free system followed the method of Littlefield and Keller,[13] unless otherwise stated. In the incorporation experiments, 5-fold amount of the final concentration of corresponding non-radioactive amino acid was added to the incubation mixture before the end of the incubation to reduce the non-specific adsorptoin of radioactive amino acid on the precipitated cells or proteins.

Assay for Phenylalanine Activation——The assay was performed essentially according to the method of Lipmann and Tuttle,[14] with some modifications. The reaction mixture was incubated at 37° for 30 min and the reaction stopped by the addition of a color assay reagent containing trichloroacetic acid. The color intensity of the supernatant after removing the precipitated protein by centrifugation was determined by means of a spectrophotometer (540 mμ).

Assay for the Formation of Phenylalanyl-sRNA——The preliminary incubation of phenylalanine-2-^{14}C with a supernatant fraction from the Ehrlich cells and ATP for 30 min at 37° was followed by the addition of sRNA and an analog, and again by incubation under the same conditions. The reaction was stopped by the addition of ethanol and potassium acetate (pH 5). Samples for the determination of radioactivity were prepared according to the method of Rendi and Ochoa.[15]

Assay of the Transfer of Phenylalanine from sRNA to Microsomes——The experiment was made according to the method of Rendi and Ochoa[15] except that the incubation period was 30 min.

Assay for the Release of Proteins from Microsomes into a Soluble Fraction——This was carried out essentially according to the methods of Rendi and Ochoa,[15] and of Littlefield and Keller.[13] The reaction mixture was incubated for 30 min at 37° and cooled in ice water to stop the reaction. Separation of the incubation mixture into microsomal and soluble proteins was carried out by centrifugation for 2 hr at 105,000g. After the purification of each fraction, samples were assayed for radioactivity.

Assay of Radioactivity——The assay was carried out according to the method described in the previous paper.[1]

All experiments were performed in triplicate and the arithmetical mean of determinations was indicated in the tables and figures.

Results

Effects of 4-Fluoro- and 4-Nitro-phenylalanines on the Accumulation of Phenylalanine and Tyrosine into "Amino Acid Pool" in Intact Cells——It is well-known that, in the whole cells, prior to synthesis of proteins, amino acids are accumulated into "amino acid pool" in the cells. Thus, an attempt was made to determine the effect of 4-fluoro- and 4-nitro-phenylalanines on the accumulation of phenylalanine and tyrosine in the pool. As shown

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in Table I, the nitro compound inhibited the accumulation to a small degree in a concentration ratio of Phe:NO_2Phe=1:10, whereas the fluoro analog showed a stimulative effect at the same ratio. Their actions were similar on tyrosine accumulation as on phenylalanine. Such a stimulation by fluorinated phenylalanines on phenylalanine and tyrosine accumulation has also been reported by Hancock,[16] and by Chirigos, Fanning, and Guroff.[17] They have attributed this stimulation to the compensatory increase in amino acid content in the pool owing to the decrease of incorporation into protein.

Table I. Effects of 4–Fluoro– and 4–Nitro–phenylalanines on the Uptake of Phenylalanine into "Amino Acid Pool" and Protein in Intact Cells

Analog	Phe per mg residual protein				Inhibition (%)	
	Accumulation in "amino acid pool"		Incorporation in protein		"Pool"	Protein
	cpm	mμmole	cpm	mμmole		
——	1261	11.4	81	0.73	—	—
FPhe	1692	15.2	53	0.48	−33[a]	34
NO_2Phe	1157	10.4	66	0.60	9	18

a) Stimulation
The incubation mixture contained the cells (*ca.* 40 mg (dry weight)), Phe–2–^{14}C(0.15 mmole), and an analog (1.5 mmoles) in KRP medium of 3.0 ml.
Incubation: 37°, 1 hr

Effect of 4-Fluoro- and 4-Nitro-phenylalanines on Phenylalanine Incorporation into Proteins in a Cell-Free System——i) Over-all effect: As indicated in Table II, inhibitory effect of 4–fluoro– and 4–nitro–phenylalanines was observed on the incorporation of phenylalanine–2–^{14}C in a cell–free system. The extent of the over–all inhibition was about the same when compared with that in the whole cell system (Table I). It is interesting to note that a certain antibiotic inhibits protein synthesis in a cell–free system, but not in the whole cells.

In Fig. 1 results are presented demonstrating the effect of added phenylalanine analogs of the kinetics of phenylalanine–^{14}C incorporation in a cell–free system.

Table II. Effect of 4–Fluoro– and 4–Nitro–phenylalanines on the Incorporation of Phenylalanine into Protein in a Cell–free System

Analog	Phe per mg protein		Inhibition (%)
	cpm	mμmole	
——	401	0.18	—
FPhe	249	0.11	39
NO_2Phe	343	0.15	17

The incubation mixture contained microsomes (3 mg of protein), pH 5 enzyme (4.4 mg), phosphoenol pyruvate (6.7 μmoles) and its kinase (25 μg), ATP (0.25 μmole), GTP (0.04 μmole), mercaptoethanol (40 μmoles), L–Phe–2–^{14}C (0.1 μmole), an analog (1.0 μmole), 19 amino acids (each 0.5 μmole) except for Phe, KCl (100 μmoles), and Tris buffer, pH 7.8, in a final volume of 1.0 ml. Incubated at 37° for 15 min.

ii) Effect of 4–fluoro– and 4–nitro–phenylalanines on phenylalanine activation: To elucidate the site(s) of the inhibition on protein synthesis, the analogs were tested for each

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of the four above-mentioned steps in protein synthesis. As the result of an experiment on amino acid activation (Table III), both 4-fluoro- and 4-nitro-phenylalanines inhibited phenylalanine activation. In Table III, the increase of aminoacyl-AMP's (A) in the presence of 4-fluoro-phenylalanine is due to the behavior of the fluoro analog in the activation; the nitro analog itself was not activated by a pH 5 enzyme fraction from Ehrlich mouse ascites tumor cells, while the fluoro analog was activated by the enzyme, and actually incorporated into the proteins of Ehrlich cells.[18] Such activation of 4-fluorophenylalanine has already been detected in *E. coli*.[19,20] Accordingly, net degree of inhibition of 4-fluorophenylalanine can be calculated from the amount of phenylalanine activated in the presence or absence of the fluoro compound; assuming that the degree of activation of 4-fluorophenylalanine was unaffected in the presence or absence of phenylalanine, and that there may be little difference in color intensity between

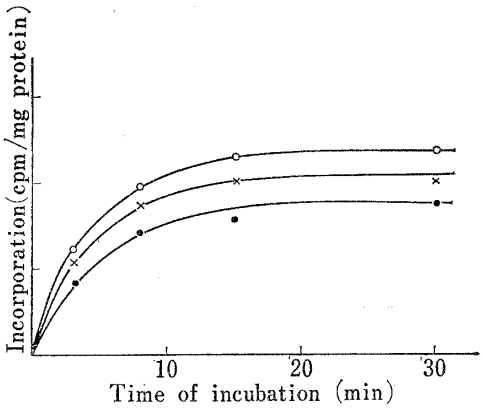

Fig. 1. Time Course of Phenylalanine Incorporation into Proteins of Ehrlich Ascites Tumor Cells in the Presence of 4-Fluoro- or 4-Nitro-phenylalanine in a Cell-Free System

The incubation mixture was the same as indicated in Table II and incubated at 37°.
—⊙— Control (L-Phe-^{14}C(U), 1×10^{-4}M, 2 mCi/mmole)
—●— FPhe (1×10^{-3}M, as L-form)
—×— NO_2Phe (1×10^{-3}M, as L-form)

Table III. Effect of 4-Fluoro- and 4-nitro-phenylalanines on Phenylalanine Activation

Amino acid and/or analog (Final conc., M)	Aminoacyl-AMP's from Phe and analog (A) (mμmole)	Aminoacyl-AMP from Phe or analog (B) (mμmole)	Phenylalanyl-AMP calculated (A−B) (mμmole)	Inhibition (%)
Phe (1×10^{-4})	—	64	—	—
FPhe (1×10^{-3})	—	131	—	—
Phe (1×10^{-4}) + FPhe (1×10^{-3})	176	—	45	31
NO_2Phe (1×10^{-3})	—	0.0	—	—
Phe (1×10^{-4}) + NO_2Phe (1×10^{-3})	55	—	55	14

The incubation mixture for the inhibition test contained Phe (0.1 mmole), an analog (1.0 mmole), ATP (10 μmoles), pH 5 enzyme (10 mg), Tris buffer (200 μmoles), pH 7.8, and NH_2OH (salt-free) (1000 μmoles) in a final volume of 1.0 ml. Incubated at 37° for 15 min.

phenylalanyl- and 4-fluorophenylalanyl-AMP's in the determination of the activation reaction. The net rate of the inhibition was calculated on such a consideration; consequently, it was found that 4-fluoro- and 4-nitro-phenylalanines inhibited the activation in certain degrees.

iii) Effect of 4-fluoro- and 4-nitro-phenylalanines on the formation of phenylalanyl-sRNA: As shown in Table IV, it was revealed that both analogs had little effect on this step of protein synthesis. Recently, Unger and DeMoss have reported that thioproline, an analog of proline, inhibited the formation of prolyl-sRNA from proline in *E. coli*.[21]

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Table IV. Effect of 4-Fluoro- and 4-Nitro-phenylalanines on the Formation of Phenylalanyl-sRNA

Analog	Phenylalanyl-sRNA per mg sRNA fraction		Inhibition (%)
	cpm	mμmole	
——	1244	0.22	—
FPhe	1254	0.23	−4[a]
NO_2Phe	1207	0.22	0

a) Stimulation

A mixture containing supernatant fraction (protein: 8 mg) and sRNA (2 mg) from the cells, ATP (10 μmoles). CTP (0.3 μmole), $MgCl_2$ (10 μmoles), mercaptoethanol (5 μmoles), Tris buffer (100 μmoles), pH 7.9, "Lubrol" (5 mg), L-Phe-^{14}C(U) (0.1 μmole), and an analog (1.0 μmole) in a final volume of 1.0 ml was incubated at 37° for 30 min.

iv) Effect of 4-fluoro- and 4-nitro-phenylalanines on the transfer of phenylalanine to microsomal proteins: Table V shows the inhibitory effect of the two analogs. This observation of the inhibition is interesting from the point that phenylalanine and its analog, 4-nitro-phenylalanine, have some similarity in their chemical structures with chloramphenicol which is an inhibitor on this step.

Table V. Effect of 4-Fluoro- and 4-Nitro-phenylalanines on the Transfer of Phenylalanine from sRNA to Microsomes

Analog	Phe per mg protein in microsomes		Inhibition (%)
	cpm	mμmole	
——	1270	0.23	—
FPhe	991	0.18	22
NO_2Phe	1022	0.18	22

The incubation mixture contained microsomes (4 mg), pH5 enzyme (4 mg), ATP (0.5 μmole), GTP (0.08 μmole), phosphoenol pyruvate (13.4 μmoles) and its kinase (50 μg), $MgCl_2$ (10 μmoles), mercaptoethanol (80 μmoles), Tris buffer (100 μmoles), pH 8.6, ^{14}C-phenylalanyl-sRNA (0.5 mg), and an analog (1.0 μmole) in a final volume of 1.0 ml. Incubated at 37° for 15 min.

v) Effect of 4-fluoro- and 4-nitro-phenylalanines on the release of protein from microsomes: From the result indicated in Table VI, it was concluded that both analogs showed no effect on this step, since the distribution of the radioactivity between microsomes and supernatant was unaffected in the presence or absence of the analogs.

Table VI. Effect of 4-Fluoro- and 4-Nitro-phenylalanines on the Release of ^{14}C-Peptide from Microsomes

Analog	Radioactivity of phenylalanine in			
	microsomal protein		supernatant protein	
	Absolute (cpm/mg protein)	Relative (% of total)	Absolute (cpm/mg protein)	Relative (% of total)
——	58	47	52	53
FPhe	37	51	39	49
NO_2Phe	48	45	40	55

The incubation mixture was the same as in Table II. Incubated at 37° for 15 min.

Effect of Phenylalanine on the Incorporation of 4-Fluorophenylalanine into Proteins in Intact Cells——It is well-known that in a variety of organisms, 4-fluorophenylalanine is

incorporated into proteins in place of phenylalanine,[19,22-33] and we also found that the fluoro derivative was incorporated into proteins of Ehrlich mouse ascites tumor cells,[18] and also that 4-fluorophenylalanine was virtually activated by pH 5 enzyme fraction (Table III). The rate of 4-fluorophenylalanine-3-^{14}C incorporation at a constant concentration was determined in the presence of phenylalanine in variable concentrations. Fig. 2 shows that there might be a competitive antagonism of the incorporation between phenylalanine and its 4-fluoro analog as clearly demonstrated in a case between phenylalanine and 2-fluorophenylalanine.[7]

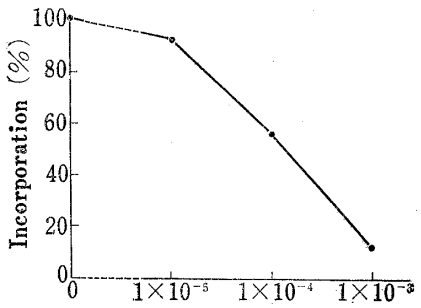

Fig. 2. Effect of Phenylalanine on the Incorporation of 4-Fluoro-phenylalanine into Protein in Intact Cells

The incubation mixture and condition were the same as in Table I.

Effect of 4-Fluoro- and 4-Nitro-phenylalanines on the Incorporation of a Few Amino Acids Other Than Phenylalanine in Whole Cells——Finally, we were interested in knowing whether or not the effects of the analogs could be extended to the incorporation of amino acids other than phenylalanine. The previous paper[1] on this problem has indicated that these analogs exerted a little inhibition on the incorporation of tyrosine in Ehrlich ascites tumor cells. The present result showed that the analogs had little influence on the incorporation of alanine and methionine (Table VII); inhibition by the analogs seems to be specific for the incorporation of phenylalanine and tyrosine.

Table VII. Effect of 4-Fluoro- and 4-Nitro-phenylalanines on the Incorporation of Amino Acids Other than Phenylalanine (Inhibition, %)

Analog (Final conc., M)	Amino acid (Final conc., M)	
	Alanine (1×10^{-4})	Methionine (1×10^{-4})
FPhe (1×10^{-3})	9	3
NO_2Phe (1×10^{-4})	−6[a]	—
NO_2Phe (1×10^{-3})	−8[a]	5

a) Stimulation

The incubation mixture and condition were the same as in Table I, except that DL-Ala-1-^{14}C and DL-Met-1-^{14}C were used for the incorporation experiment.

Discussion

In the present experiment it was found that 4-fluoro- and 4-nitro-phenylalanines exerted their inhibitory effects on the steps of amino acid activation and of the transfer reaction

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of phenylalanine into microsomes in the pathway of protein synthesis. And it was also revealed that the inhibitory effects of the analogs on the incorporation of amino acid were specific for that of phenylalanine and tyrosine which is also analogous to 4-fluoro- and 4-nitro-phenylalanines.

As 4-nitrophenylalanine was not activated by a pH 5 enzyme fraction and consequently AMP- and/or sRNA-derivative(s) from 4-nitrophenylalanine should not be formed during the period of incubation, the natural conclusion is that the nitro analog *per se* is responsible for its inhibitions on the activation and transfer steps. The ratio of the inhibitions on the two steps in the cell-free system is estimated to be approximately 4:6 (activation: transfer) at 1×10^{-3} M of the final concentration of 4-nitrophenylalanine. However, it is difficult to estimate, in whole cells, the probable degree of each inhibition on the two steps, because 4-nitrophenylalanine also affects the accumulation of phenylalanine into its pool.

On the other hand, in the case of 4-fluorophenylalanine, it should be assumed that, as the result of amino acid activation and incorporation into protein, some amount of 4-fluoro-phenylalanyl-AMP and -sRNA is formed during the incubation period, and that the AMP and/or sRNA derivative(s) might be inhibitory on the activation and/or transfer step(s) of phenylalanine; therefore, the exact share of the inhibition by 4-fluorophenylalanine itself on the two steps remains obscure. We have already been familiar with such a situation of inhibition in an example of 5-fluorouracil as an inhibitor of nucleic acid synthesis from which certain nucleoside and nucleotide analogs were successively formed during the period of incubation,[34,35] and moreover it was incorporated into RNA.[35] Accordingly also it is obscure whether 4-fluorophenylalanine affects the transfer step.

The degree of inhibition by 4-fluoro-phenylalanine in a cell-free system is approximately two times higher than by 4-nitrophenylalanine, as well as in whole cells reported previously by us.[1]

The similarity of 4-fluorophenylalanine with phenylalanine in biological and biochemical responses, and the effects of introduction of several radicals into amino acid molecules have been precisely discussed by several authors from the point of view of chemical structure.[4,6]

During the preparation of the present paper, a study of Unger and DeMoss[21] was reported; thioproline, an analog of proline, inhibited the formation of prolyl-sRNA in *E. coli* by an application of chloramphenicol known as an inhibitor of the transfer step of protein synthesis in microorganisms. Thioproline has not as yet been submitted to an investigation on the inhibitions of amino acid activation and of the transfer reaction of proline into ribosomes.

From the recent developments on the studies of inhibition mechanism of protein synthesis by antibiotics, we have obtained a large amount of information concerning the site and mode of the inhibition, as has been shown in a large number of examples such as chloramphenicol, puromycin, cycloheximide, streptomycin, *etc.*[37,38] Consequently, the results of such studies have given many contributions and stimulations to the studies of inhibition mechanisms of protein synthesis inhibitors. It is of interest that the 4-nitrophenylalanine related in chemical structure of chloramphenicol which is an analog of phenylalanine in certain biological responses, and that, in addition, the nitro analog inhibits the transfer of phenylalanine into microsomes, as well as does chloramphenicol.

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