Biogenesiss of an Antitumor Antibiotic Protein, Neocarzinostatin
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A study of the biogenesis of the antitumor protein antibiotic neocarzinostatin (NCS) was undertaken. The production of NCS, as well as the growth of Streptomyces carzinostaticus in a production medium, was sensitive to puromycin, chloramphenicol, and actinomycin D. However, when a 12-hr culture in production medium was transferred to a nongrowth medium consisting of a phosphate buffer with Mg\(^{2+}\) and Ca\(^{2+}\); rapid NCS synthesis and liberation occurred. NCS production in this medium was no longer sensitive to actinomycin D; but was sensitive to puromycin and chloramphenicol. The conversion of a precursor NCS to an active form was shown to occur in this medium. Subcellular analysis suggested that NCS synthesis occurred by a mechanism similar to that of protein synthesis by membrane polypeptides.

The antitumor antibiotic neocarzinostatin (NCS; 7, 10) is a protein with a molecular weight of 11,000 (H. Maeda, personal communication) produced by Streptomyces carzinostaticus. It is highly active against various experimental tumors, including L-1210 (3). Studies on the mode of action of NCS revealed that this antibiotic selectively inhibits the synthesis of deoxyribonucleic acid in Sarcina lutea (11, 12) and HeLa cells (6).

This study was undertaken to reveal the mode of biogenesis of NCS in a liquid culture of S. carzinostaticus. In the biogenesis of the peptide antibiotics so far known, nonribosomal peptide synthesis is generally accepted (1). Recently, Ingram (5) reported that nisin (molecular weight, 7,000) or a related compound, is synthesized through a ribosomal system. Since NCS has a considerably larger molecular size than other peptide antibiotics, the mode of biogenesis is of great interest, as well as its chemical structure, which is being clarified (H. Maeda, C. B. Glaser, and J. Meienhofer, in preparation). In the present study, one of the main objectives was to determine whether the biogenesis of NCS is similar to that of polypeptide antibiotics such as bacitracin (13), gramicidin (15), and tyrocidine (9), which do not go through a ribosomal system, or to that of nisin (5), diphtheria toxin (14), and botulinus toxin (2).

**MATERIALS AND METHODS**

NCS-producing strain. S. carzinostaticus was maintained on Kainsky's agar slants by subcultures made every 2 weeks.

Production of NCS. A 1-week-old culture of S. carzinostaticus on a Kainsky's agar slant was suspended in 10 ml of 0.067 M phosphate-buffered (pH 7.2) saline (0.15 M). A 3-ml amount of this suspension was first grown in submerged culture in a 500-ml Sakaguchi flask at 27 C in 100 ml of medium containing 2.0% starch, 2.0% soybean meal, 0.5% yeast extract, 0.5% NaCl, 0.2% CaCO\(_3\), and 0.0005% each of MnSO\(_4\), CuSO\(_4\), and ZnSO\(_4\) (pH 7.2). After 20 hr, 3 ml of this medium was inoculated into a production medium containing 4.0% glucose, 0.5% Casamino Acids, 0.5% NaCl, 0.2% CaCO\(_3\), 0.1% K\(_2\)HPO\(_4\), and 0.25% MgSO\(_4\) (pH 7.2), and was incubated under the same conditions. After 36 hr, the maximal production of NCS (300 \(\mu\)g/ml) was consistently obtained.

NCS production in nongrowth medium. To analyze the process of liberation of NCS into the fluid, a nongrowth medium (NG medium) was used in which no growth of mycelium occurred. The NG medium was a 0.067 M phosphate buffer of pH 7.2 containing 10\(^{-2}\) M Mg\(^{2+}\) and Ca\(^{2+}\). After the organism had been cultured for 12 hr in production medium, mycelia were spun down and production media were discarded. NG medium was added to the mycelia. In the NG medium, no detectable growth of the mycelium, as determined by protein concentration, was observed up to 12 hr of incubation, but the liberation of NCS into the medium (100 \(\mu\)g/ml) could be detected.

**Bioassay for antibacterial activity.** The activity of NCS was measured by the conventional paper-disc diffusion method with S. lutea PCI 1001 as a test organism. Standard NCS used in this experiment was a gift from Kayaku Antibiotic Research Co. Ltd., Tokyo, Japan. Before the activity of NCS in the production medium and the NG medium was measured, other antibiotics (puromycin, chloramphenicol, and actinomycin) were removed by dialysis against 0.001 M acetic acid solution.

Assay for mycelial growth. Mycelia were disrupted...
by grinding with quartz sand in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) containing 10 mM MgCl₂ and 60 mM KCl. The supernatant fluid obtained after centrifugation at 5,000 rev/min for 20 min was used for protein estimation by the method of Lowry et al. (8).

Radioactive amino acids. The ¹⁴C-labeled amino acids used in this experiment were randomly labeled chlorella hydrolysate, obtained from the Institute of Applied Microbiology, University of Tokyo. The specific activity was 8.0 mCi/mole. L-H-3 alanine (3.03 mCi/mole) was purchased from Daiichi Pure Chemical Co. Ltd., Tokyo, Japan. Amounts of 2 or 20 μCi of labeled amino acids in 0.5 ml of phosphate-buffered saline were added to 100 ml of production medium at the desired time, and incubation was continued for an appropriate period.

Purification of radioactive NCS (10). To determine the specific incorporation of labeled amino acids into the NCS molecule, culture filtrates were first precipitated with saturated ammonium sulfate; the precipitate was dissolved in water, dialyzed against 0.001 M acetic acid solution, and freeze-dried. This crude preparation was dissolved in water and passed through a column of Sephadex G-50; bioactive fractions were combined and lyophilized. This preparation was further chromatographed on a carboxymethyl (CM)-cellulose column. Either stepwise or gradient elution of NCS with acetic acid and sodium acetate was made, and 0.5 ml of the highest antibacterial fraction was neutralized with K₂CO₃. The insoluble salt formed was removed by centrifugation, and a sample was dried on a metal planchet for counting.

Density gradient sedimentation analyses. Streptomyces mycelia in NG medium were collected by centrifugation and disrupted by grinding with quartz sand as described above. They were then extracted with a solution containing 10 mM Tris-hydrochloride buffer (pH 7.4) with 10 mM MgCl₂ and 60 mM KCl in 0.25 M sucrose. Quartz sand, intact cells remaining after quartz sand treatment, and cell walls were removed by centrifugation, and the resulting cell-free extract was further fractionated into soluble and particulate fractions by centrifugation at 105,000 × g for 120 min. The yield in the particulate fraction was determined by measuring the total protein contents from the disrupted cells. Samples of 0.3 ml of particulate fraction were layered on 3.6 ml of a 10 to 30% linear sucrose gradient in 10 mM Tris-hydrochloride buffer (pH 7.4) containing 10 mM MgCl₂ and 60 mM KCl, which was then overlaid on a bottom layer which contained 0.6 ml of 50 to 70% sucrose in the same buffer. After centrifugation at 23,000 rev/min for 120 min, three-drop fractions were collected from the bottom of the tube. Each sample after dilution was used for the determination of absorbance at 260 and 280 nm and of radioactivity. The radioactivity was determined after precipitation with an equal volume of 10% trichloroacetic acid solution in the presence of one drop of 1% bovine serum albumin. The precipitates were washed with cold 5% trichloroacetic acid solution and then with a mixture of ethanol:ether-chloroform (2:1:1, v/v). The precipitates were then dissolved in dilute ammonia solution and dried on a metal planchet for counting.

RESULTS AND DISCUSSION

Time course of NCS production in relation to the growth of S. carzinostaticus. A 20-hr seed culture of S. carzinostaticus was inoculated into the production medium, and the culture flask was incubated at 27 C with shaking. Under these conditions, the organisms formed bundles of aggregated myelcelia after overnight culture. The results presented in Fig. 1 reveal that NCS was produced and released into the medium by the organisms at the logarithmic phase of growth (judged by protein content). The production of NCS was almost parallel to the mycelial growth until the mycelium reached maximal growth (20 mg of protein/ml) at 24 hr. The pH of the culture medium reached 6.8 at maximal production. This 3- to 4-hr delayed production curve is quite similar to that of diphtheria toxin (14) but quite different from that of botulinus (2).

Effect of actinomycin D on mycelial growth and production of NCS. Puromycin, chloramphenicol, and actinomycin D were each added to different production media at zero time to give a concentration of 40 μg/ml. In addition to inhibiting the production of NCS, all of these antibiotics inhibited the growth of the organisms completely, although their concentrations were below the lethal level.

The effect of actinomycin D (40 μg/ml) at

![Fig. 1. Neocarzinostatin (NCS) production and pH changes in relation to growth of Streptomyces carzinostaticus in production medium. Symbols: ○, mycelial growth as measured by protein content; □, NCS activity; ●, pH.](image-url)
different time periods of cultivation on mycelial growth and NCS production (Fig. 2) was examined in the production medium. The addition at 8 hr resulted in growth inhibition of about 90% as measured by mycelial protein at 36 hr. Mycelial protein content at 8 hr was less than 10% of that at 36 hr. In other words, there was almost no apparent difference in the mycelial protein content at 8 and 36 hr when actinomycin D was added at 8 hr. However, 30% production of NCS was found at 36 hr, in spite of the sustained presence of actinomycin D.

When the drug was added at 12 hr, the growth was almost one-tenth of the control, but the production of NCS measured at 36 hr was almost 60% of the control. The addition of actinomycin D at 24 hr did not have any inhibitory effect on either mycelial protein or NCS production.

In these experiments, it is evident that the addition of actinomycin D at the beginning of exponential growth inhibited NCS production, but drug addition at the late logarithmic phase did not inhibit NCS production.

This observation was further examined by pulse administration of labeled amino acid at various times during logarithmic growth. To test the incorporation of labeled amino acids into the NCS molecule, 20 μCi of \(^{3}H\)-L-alanine per 100 ml was added to the production medium every 4 hr from zero time to 16 hr. After 4 hr of pulse-labeling followed by continued fermentation for 40 hr, the culture was centrifuged at 3,000 rev/min for 15 min to precipitate the mycelia. From this supernatant fluid, NCS was purified by precipitation with 60% ammonium sulfate, molecular sieving with Sephadex G-50, and CM-cellulose column chromatography.

The results in Table 1 clearly show that the most effective incorporation occurred from 4 to 8 hr. However, active NCS was never detected at 8 hr in either culture fluid or mycelia. These results may indicate the formation of an NCS-related protein (precursor protein) through messenger ribonucleic acid (mRNA) at the 4- to 8-hr period and eventual conversion into radioactive NCS as described below.

**Critical period for \(^{14}\text{C}-\text{amino acid incorporation into NCS-related protein}**. The incorporation of \(^{14}\text{C}-\text{amino acids into NCS-related protein and conversion into NCS were further studied by short-period pulse-labeling. Except for the 1-hr period of labeling, the experimental design was the same as that illustrated in Table 1. Pulse-labeling for 1 hr was done at 0 to 1, 4 to 5, 8 to 9, 12 to 13, and 16 to 17 hr after inoculation in the production medium. The results are shown in Fig. 3, in which specific incorporation of \(^{14}\text{C}-\text{amino acids into the NCS molecule determined after 36 hr of incubation is shown by histograms. The results clearly indicate that the greatest incorporation occurred during the period of 8 to 9 hr, followed by that at 4 to 5 hr and 12 to 13 hr, whereas the logarithmic growth of the Streptomyces started at 12 hr. When the activity (counts per minute) of \(^{14}\text{C}-\text{amino acids incorporated into the NCS molecule was divided by the total protein content of mycelia (milligrams), the curve given by the dashed line in Fig. 3 was obtained. The highest yield of specific activity (counts per min per milligram of protein) of the precursor protein (NCS-related protein) is**

**Table 1. Radioactive neocarzinostatin (NCS) formation after the addition of \(^{3}\text{H}-\text{L-alanine at various times}**

| Labeling time (hr) | Radioactivity (counts/min) of NCS/mg |
|--------------------|--------------------------------------|
| 0-4                | \(2.14 \times 10^{3}\)                |
| 4-8                | \(14.74 \times 10^{3}\)               |
| 8-12               | \(10.23 \times 10^{3}\)               |
| 12-16              | \(6.45 \times 10^{3}\)                |
| 16-20              | \(4.44 \times 10^{3}\)                |

*After 4 hr of pulse-labeling with \(^{3}\text{H}-\text{L-alanine}, radioactive NCS was purified from each culture filtrate. Samples of purified NCS were dissolved in water to measure the protein content and radioactivity.*
shown at 4 to 5 hr, without an appreciable increase in mycelial protein content.

**Synthesis of mRNA for NCS production.** The data presented in Fig. 2 illustrate the fact that addition of actinomycin D at 12 hr allowed almost 60% production of NCS at 36 hr, irrespective of the restricted mycelial growth. This may indicate that mRNA for NCS-related protein was already synthesized before 12 hr; however, mRNA for mycelial protein seems to be synthesized at a much later stage. In the experiment shown in Fig. 3, NCS-related protein was formed even as early as 4 hr.

The effect of actinomycin D was further investigated in the following experiment. Actinomycin D was added to the production medium at 4, 6, 8, and 10 hr, and at 12 hr the mycelia were transferred into NG medium without actinomycin D. The latter medium did not allow the increase of mycelium protein content. Results obtained by this procedure (Fig. 4) indicate that the addition of actinomycin D to the production medium at 8 hr (i.e., just at the beginning of the logarithmic growth phase) allows the production of NCS in the NG medium.

These results show (Fig. 4) that liberation of NCS occurred in this NG medium as early as 6 hr without further supply of extracellular amino acids. The activity of NCS in this NG medium [100 µg/ml in the control (Fig. 4)] is almost one-third of that in the production medium. These result may indicate that the mRNA for NCS was made before 8 hr.

**Effect of various inhibitors on NCS production at the actinomycin D-insensitive phase.** After cultivation of the organisms in the production medium for 12 hr, precipitated mycelia were transferred to NG medium and incubated as described above. The effect of various concentrations of puromycin, chloramphenicol, and actinomycin D on NCS production in NG medium was examined (Fig. 5). An inhibitory effect on NCS production was found with puromycin and chloramphenicol (Fig. 5A and 5B), but not with actinomycin D (Fig. 5C). Although radioactive amino acids are incorporated into the inactive form of NCS (NCS-related protein) at an early stage in the production medium, the inhibitory effect of puromycin and chloramphenicol in Fig. 5A and 5B indicate that further new protein synthesis may be involved in the activation process of NCS-related protein. It may be suggested that the organisms transferred into NG medium have already finished the synthesis of mRNA for NCS or NCS-related protein (precursor protein; Fig. 3), whereas the conversion of precursor protein into active NCS in NG medium requires further
protein synthesis, as evidenced by the inhibitory effect of puromycin and chloramphenicol at this later stage (actinomycin D-insensitive stage).

**Intracellular mechanism of NCS synthesis.** From the above experiments, it may be concluded that NCS-related protein (NCS precursor) is synthesized in the mycelium before 8 hr in the production medium, and the conversion to the active form occurs in the NG medium and requires protein synthesis de novo. To clarify the latter process further, the following two experiments were conducted.

The first was conducted to clarify the nature of the protein excreted from mycelium into NG medium. For this purpose, $^{14}$C-amino acids (2 $\mu$Ci/100 ml) were added at 0, 4, and 8 hr after the inoculation of *Streptomyces* into the production medium, and cultivation was continued. All cultures were harvested at 12 hr. The mycelia were transferred to NG media and incubated for a further 4 hr. The liberation of radioactivity into the NG medium was examined with a sample of culture fluid at 0.5 and 4 hr. The specific biological activity of NCS was also examined. All three experimental results illustrated in Table 2 show that 70% of the radioactivity liberated into the medium was due to pure NCS, which was proven by isolating radioactive NCS. From this result and that of Fig. 3, it is evident that the conversion from precursor protein (bioactive) to NCS (bioactive) occurred in the NG medium without further supply of amino acids.

In the next experiment, the site of NCS production in the mycelium was investigated. $^{14}$C-amino acids (2 $\mu$Ci/100 ml) were added 4 hr after inoculation, and incubation was continued for 12 hr. The collected mycelia were divided into three portions and incubated in NG medium: one portion received 40 $\mu$g of chloramphenicol/ml, and the other two received none. After incubation periods of 0.5 and 4 hr without chloramphenicol, and after 4 hr with chloramphenicol, each culture was centrifuged in the cold to separate the mycelia. These mycelia were disrupted by grinding with quartz sand and were extracted with 10 mM Tris-hydrochloride buffer (pH 7.4) containing 10 mM MgCl$_2$ and 60 mM KCl in 0.25 M sucrose. Quartz sand, undisrupted cells, and cell walls were removed by centrifugation at 7,000 rev/min for 60 min, and the resulting cell-free extracts were further fractionated into soluble and particulate fractions by centrifugation at 105,000 $\times$ g for 120 min. This particulate fraction was used for sucrose density gradient analysis to separate membrane fractions from microsomal fractions.

When the particulate fraction from the mycelium was subjected to linear sucrose gradient centrifugation by the procedure of Hallberg and Hauge (4), the results illustrated in Fig. 6 were obtained. The heaviest fraction in Fig. 6 was thought to be a membrane-associated fraction, and the average ratio of $A_{280}/A_{400}$ was 0.8. The light fraction, assumed to be ribosomal, possessed an average $A_{280}/A_{400}$ ratio of 0.5. After cultivation for 4 hr, almost 45% of radioactivity in the membrane fraction was lost (Fig. 6B). The radioactivity lost from the membrane fraction was almost equal to that liberated into the medium (Table 2). However, the radioactivity in the ribosomal fraction was almost unaltered. When a sufficient amount of chloramphenicol to inhibit peptide synthesis was maintained for 4 hr, the radioactivity of the membrane fraction was not lost and NCS production was inhibited (Fig. 6A). These results suggest that the synthesis of NCS-related protein (precursor protein) was carried out on this membrane fraction which contains the polysome system. Consequently, from this and the other findings mentioned above, it appears reasonable to deduce that the site of NCS protein synthesis in

**Table 2. Incorporation of $^{14}$C-amino acids into neocarzinostatin (NCS)**

| Time of growth with $^{14}$C-amino acids (hr) | Time of chase in NG medium (hr) | Radioactivity in culture fluid $^a$ | Radioactivity in NCS $^a$ |
|--------------------------------------------|---------------------------------|-----------------------------------|--------------------------|
| 0-12                                       | 0.5                             | 220                               | 2,204                    |
|                                            | 4.0                             | 2,270                             | 11,716                   |
| 4-12                                       | 0.5                             | 260                               | 2,218                    |
|                                            | 4.0                             | 3,710                             | 13,282                   |
| 8-12                                       | 0.5                             | 140                               | 680                      |
|                                            | 4.0                             | 1,250                             | 9,316                    |

$^a$ Total radioactivity (counts per minute) in 10 ml of NG medium.

$^b$ Counts per minute per milligram.
the Streptomycetes mycelia must be closely associated with the membrane fraction.

The question of whether or not membrane-bound ribosomes are directly involved as the actual site in the NCS synthesis is not resolved by these results, although the inhibition by actinomycin D indicates necessary synthesis of mRNA for NCS production.

The above results can be summarized as follows:

(i) NCS production by S. carzinostaticus has been divided into two different stages. The first stage is de novo protein and mRNA synthesis, as shown by the effective incorporation of the labeled amino acids into NCS-related protein and by the susceptibility to the action of actinomycin D at this stage (Fig. 2) which indicates the necessity of mRNA for NCS production.

The second stage is the activation stage of NCS-related protein into NCS, and this stage was insensitive to the action of actinomycin D, but was sensitive to the action of puromycin and chloramphenicol (Fig. 4). These results may indicate that early synthesis of mRNA for NCS or NCS-related protein is necessary before a substantial increase of mycelial protein content can be attained.

(ii) NCS production is inhibited in the second stage by the late addition of chloramphenicol and puromycin, although NCS-related protein has been synthesized by this time (0.5 to 12 hr). These results may reveal that the biosynthetic mechanism for NCS requires a further activation stage which is sensitive to chloramphenicol and puromycin.

(iii) When a 12-hr-old culture was incubated in NG medium, activation of NCS-related protein and liberation of NCS into the medium were observed. Using this system, together with subcellular fractionation and isotopic techniques, we attempted to determine which fraction of the mycelium is directly associated with NCS peptide synthesis in S. carzinostaticus. The results showed that the membrane-associated fraction was responsible for the peptide synthesis. Bodanszky and Perlman (1) suggested that peptide antibiotics are made by a different type of mechanism from that of protein. Biosynthesis of peptide antibiotics is generally insensitive to inhibitors of protein synthesis such as puromycin and chloramphenicol. Ingram (5) reported that a polypeptide antibiotic produced by Streptococcus lactis, a compound related to nisin, was synthesized by a ribosomal mechanism, as the incorporation of amino acids into the antibiotic molecule was inhibited by puromycin and chloramphenicol. The present results may also suggest that NCS-related protein and NCS are synthesized similarly at a site closely associated with the membrane, by use of mRNA and a mechanism of biosynthesis different from that of bacitracin (13), gramicidin (15), and tyrocidine (9).

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