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Effect of Cycloheximide on Biosynthesis of Rat Liver Catalase[1)]

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It was found that cycloheximide injected into rats inhibited the incorporation of ^{14}C-leucine into catalase *in vivo*, not affecting the concentration of catalase-synthesizing ribosomes and the amount of nascent catalase.

The liver ribosomes of rats given cycloheximide by intraperitoneal injection incorporated less amino acids into catalase than the ribosomes from intact rat liver, when incubated *in vitro* with normal cell sap. The cell sap preparation from livers of cycloheximide-treated rats was also less active for catalase synthesis, and the activity could not be restored by treatment with Sephadex. It may be concluded that both the ribosomes and the cell sap are impaired by the treatment of rats with cycloheximide.

At low concentration, cycloheximide added into the cell-free system inhibited the labeling of soluble catalase, but not that of catalase-synthesizing ribosomes and nascent catalase. The evidence suggests that the low concentration of cycloheximide interferes with the release of completed catalase molecules from the ribosomes without any effect on incorporation of ^{14}C-leucine into nascent catalase. However, at higher concentrations, the labeling of catalase-synthesizing ribosomes and nascent catalase was decreased, as well as that of soluble catalase. A kinetic study also indicated that this antibiotic would inhibit elongation of nascent catalase on the ribosomes.

From these findings, it has been concluded that cycloheximide inhibits the termination, the elongation and possibly the initiation in the course of catalase biosynthesis.

Cycloheximide (Actidione, Naramycin) is an antibiotic isolated from *Streptomyces griseus*, and was first shown to inhibit protein biosynthesis in *Saccharomyces carlsbergensis*.[3)] Similar effect was observed in rabbit liver,[4)] mammalian cell culture,[5)] rat liver,[6)] mouse liver,[7)] rabbit reticulocytes[8)] and human lymphocytes.[9)] It was demonstrated with cell-free system from rat liver and from *Saccharomyces pastorianus*, that cycloheximide does not inhibit the charging of *t*-RNA with an amino acid but it affects some stage of protein synthesis subsequent to the formation of aminoacyl-*t*-RNA.[10)] Wettstein, *et al.*[6)] have shown that this antibiotic blocks the read-out mechanism of *m*-RNA and prevents polysome breakdown during protein synthesis *in vitro*.

Most of these experiments employed the fraction sedimented with trichloroacetic acid into which the incorporation of a radioactive amino acid was investigated. They have given a profound evidence for the effect of cycloheximide on biosynthesis of proteins in total, however, a possibility that the drug may affect in somewhat specific manner the biosynthesis

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3) D. Kerridge, *Gen. Microbiol.*, **19**, 467 (1958).
4) C.W. Young, P.F. Robinson, and B. Sactor, *Biochem. Pharmacol.*, **12**, 855 (1963).
5) L.L. Bennett, D. Smithers, and C.T. Ward, *Biochim. Biophys. Acta*, **87**, 60 (1964).
6) F.O. Wettstein, H. Noll, and S. Penman, *Biochim. Biophys. Acta*, **87**, 525 (1964).
7) A.C. Trakatellis, M. Montjar, and E.A. Axelrod, *Biochemistry*, **4**, 2065 (1965).
8) B. Colombo, L. Felicetti, and C. Baglioni, *Biochem. Biophys. Res. Commun.*, **18**, 389 (1965).
9) A. Korner, *Biochem. J.*, **101**, 627 (1966).
10) H.L. Ennis and M. Lubin, *Fed. Proc.*, **23**, 269 (1964); M.R. Siegel and H.D. Sisler, *Nature*, **200**, 675 (1963); *idem*, *Biochim. Biophys. Acta*, **87**, 83 (1964).

of each individual protein remains to be investigated. In mammalian liver, serum albumin is synthesized predominantly both in the amount and in the rate of synthesis. Therefore, the individual effect of cycloheximide on biosynthesis of each hepatic protein other than albumin including catalase, which is an another specific protein synthesized by liver,[11] may be overlooked in the experiments with acid-insoluble materials.

The present authors have studied the effect of this antibiotic on the biosynthesis of rat liver catalase both *in vivo* and *in vitro*. The experiments consisted of three parts: (i) *In vivo* biosynthesis of liver catalase in rats treated with cycloheximide, (ii) *In vitro* biosynthesis of catalase by a cell-free system employing liver ribosomes and cell sap prepared from rats injected with cycloheximide, and (iii) *In vitro* biosynthesis of catalase in the presence of cycloheximide added to a cell-free system consisting of liver ribosomes and cell sap from intact rats. The biosynthesis of catalase was investigated by assaying the incorporation of ^{14}C-leucine into (i) completed molecules of catalase being in a soluble form, and (ii) nascent catalase being synthesized on the ribosomes. These approaches will provide with a clue to elucidation of the mechanism of catalase biosynthesis in addition to that of the action of cycloheximide.

Experimental

Animals——Male rats weighing 100—125 g were used after an overnight period of fasting (12—15 hr). Cycloheximide (150 μg per 100 g of body weight) was injected into rats and the treated animals were sacrificed after 3 hr (CH-treated rats).

Media——Medium A is a 30 mM Tris-HCl buffer at pH 7.6 containing 5.0 mM $MgCl_2$, 50 mM NaCl, 80 mM KCl and 6.7 mM 2-mercaptoethanol. Medium B consists of 50 mM Tris-HCl buffer at pH 7.6 containing 25 mM KCl, 10 mM $KHCO_3$, and 0.5 M sucrose.

Preparation of Ribosomes——The ribosomes were prepared as previously reported.[11]

Precipitation of Catalase-synthesizing Ribosomes by Anti-catalase[12]——The ribosome pellet was suspended in medium A and centrifuged at 700 g for 15 min to remove impurities. The suspension was first incubated at 4° for 90 min with anti-catalase rabbit serum followed by addition of 100 μg of purified rat liver catalase. Then, the mixture was incubated for further 60 min. The resulting immunological precipitates were separated by centrifugation at 700 g for 15 min and washed 5 times with cold saline solution by repeated centrifugation. To determine the amount of precipitated ribosomes, the precipitates were dissolved in 0.1 N NaOH and the optical density of the solution was determined at 260 mμ. The radioactivities of the precipitates were measured by the method described below.

Isolation of Nascent Catalase from Ribosomes——Nascent catalase on ribosomes was released by EDTA treatment as follows; ribosomes were suspended in 1 ml of medium A, mixed with one-tenth volume of 150 mM EDTA, layered on 7.5 ml of medium B and centrifuged at 26360 g for 10 hr. About 5 ml from the top of each tube was separated as nascent protein fraction.[13] Nascent catalase in this fraction was isolated by precipitation with anti-catalase and carrier catalase.[14] The amount of the precipitates was assayed by the absorbancy at 280 mμ in 0.1N NaOH, and the radioactivities involved in the precipitates were measured.

Preparation of Cell Sap——Cell sap fraction was obtained from an homogenate of rat liver as reported previously.[11]

Biosynthesis of Catalase in Cell-free System——The reaction mixture contained ribosome, cell sap, ATP and its generating system, GTP and ^{14}C-leucine. The composition has been shown in the previous report.[11] The reaction mixture was incubated at 37° in a water-bath for 60 min under continuous shaking, unless otherwise indicated. After incubation, the mixture was centrifuged at 105000 ***g*** for 1 hr. From the supernatant fluid, labeled catalase was isolated with use of anti-catalase and the radioactivity was assayed. The details of the procedure have been described in the previous paper.[11] On the other hand the ribosomal pellets were subjected to isolation of catalase-synthesizing ribosomes or nascent catalase, the labeling of which was measured.

11) K. Kashiwagi and T. Higashi, *J. Biochem.*, **70**, 785 (1971); T. Higashi and T. Peters, Jr., *J. Biol. Chem.*, **238**, 3982 (1963).
12) T. Higashi and H. Kudo, *J. Biochem.*, **69**, 439 (1971); T. Higashi, H. Kudo, and K. Kashiwagi, *J. Biochem.*, **71**, 463 (1972).
13) M. Takagi, T. Tanaka, and K. Ogata, *Biochim. Biophys. Acta*, **217**, 148 (1970).
14) T. Higashi, K. Kashiwagi, and T. Sakamoto, *J. Japan. Biochem. Soc.*, **43**, 718 (1971).

Incorporation of ^{14}C-leucine into Total Acid-insoluble Materials——In most experiments, besides isolating catalase, catalase-synthesizing ribosomes or nascent catalase by anti-catalase for the assay of ^{14}C incorporated, an aliquot of the same specimen was taken for determining ^{14}C incorporated into total proteins, total ribosomes or total nascent proteins respectively. An equal volume of 10% trichloroacetic acid (TCA) was added and the mixture was heated at 95° for 20 min. Radioactivity in the precipitates was assayed as described below.

Measurement of Radioactivity——The precipitates to be assayed were washed more than 5 times, dried in a vacuum desiccator for about 1 hr and dissolved in 0.1—0.15 ml of hyamine solution under heating at 57° for about 30 min. The samples were then transferred into counting vials with use of 12—13 ml of liquid scintillation fluid[11] and their radioactivities were assayed by a liquid scintillation counter (Aloka LSC-502).

Preparation of Antisera——Rat liver catalase was purified by the method of Higashi and Peters.[15] Rabbits were injected intramuscularly with 0.2% solution of the purified catalase, emulsified in an equal volume of Freund's adjuvant (10 mg of catalase per rabbit). After 3 weeks, if necessary, a second injection of 5 mg of catalase was given in the same manner.

Rabbit gamma-globulin was purified according to the method of Utsumi, *et al.*,[16] and injected into goats to yield anti-*gamma*-globulin antiserum. 100 and 40 mg (per animal) of antigen were given at an interval of 2 weeks with complete Freund's adjuvant.

The serum was collected about 6 weeks after the first injection. Pooled antisera were treated at 56° for 30 min to avoid non-specific precipitations.

Assay of Catalase——Catalase enzymatic activity was measured by a modification[15] of the spectrophotometric method of Beers and Sizer.[17]

Radioactive Amino Acid——Uniformly labeled L-leucine-^{14}C (270 mCi/m mole) was obtained from Daiichi Pure Chemicals Co.

Chemicals——Cycloheximide was purchased from Sigma Chemical Co. As to other chemicals see ref. 12).

Result

Effect of Cycloheximide Administration on Biosynthesis of Catalase *in Vivo*

The liver catalase of CH-treated rats was first assayed enzymatically. No difference was detectable in the level of liver catalase between normal and CH-treated rats (Table I).

Table I. Amount and *in Vivo* Labeling of Rat Liver Catalase

	Normal	CH-treacted
Amount of catalase[a] (μg/g liver)	771 (100%)	759 (98%)
^{14}C in catalase[b] (cpm/catalase in 0.1 g liver)	145 (100%)	16 (11%)

Liver homogenates were treated with 1% sodium deoxycholate, followed by centrifugation at 105000 g for 60 min. Catalase in the supernatant was assayed. For counting, catalase was isolated with use of anti-catalase.
a) determined by measurement of enzyme activity
b) determined at 20 min after ^{14}C-leucine injection (10 μCi per 100 g of body weight)

The incorporation of ^{14}C-leucine into liver catalase was then investigated with CH-treated rats. ^{14}C-leucine was injected into the tail veins of the treated rats 160 min after administration of cycloheximide. The rats were sacrificed 20 min after the leucine injection, and the labeling of liver total catalase was assayed. As demonstrated in Table I, a significantly low incorporation was observed when rats were injected with cycloheximide (about 10% of the control).

In the next experiment, the amount of catalase-synthesizing ribosomes was compared between normal and CH-treated rats. Table II indicates that the concentration of such ribosomes remained unchanged after tne cycloheximide treatment. However, the labeling

15) T. Higashi and T. Peters, Jr., *J. Biol. Chem.*, **238**, 3945 (1963).
16) S. Utsumi and F. Karush, *Biochemistry*, **3**, 1329 (1965).
17) R.F. Beers and I.W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).

of those catalase-synthesizing ribosomes in 20 min by the injected ^{14}C-leucine was remarkably impaired (to 20%) in CH-treated rats compared with that in normal rats (Table II). On the other hand, the labeling of total ribosomes was inhibited to 32.4% in CH-treated animals.

Table II. Amount and *in Vivo* Labeling of Catalase-synthesizing Ribosomes

	Normal	CH-treacted
Amount (OD_{260} unit)	0.281 (100%)	0.280 (100%)
Labeling with ^{14}C (cpm)	116 (100%)	24 (20%)

Figures indicate the values for 100 OD_{260} units of liver ribosomes. Labeling was assayed at 20 min after ^{14}C-leucine injection.

Nascent catalase released from ribosomes by EDTA and isolated by the immunochemical method, was assayed both for the amount and for the ^{14}C incorporated *in vivo*. The labeling experiment was carried out as described above. The results are presented in Table III. The incorporation of ^{14}C-leucine into the nascent catalase was found to be significantly lower in CH-treated rats than that in normal ones, whereas the actual amount of the nascent catalase on ribosomes was almost the same in both groups of animal. The labeling of nascent catalase on the ribosomes of intact rats decreased in accordance with the passage of time during the period of the experiment. However, on the contrary, that of CH-treated rats continued to increase until 20 min after injection of ^{14}C-leucine. The evidence indicates that the rate of catalase synthesis was reduced by the treatment with cycloheximide. The inhibitory effect of the drug to total nascent proteins was similar to the effect to nascent catalase (Table III).

Table III. Amount and *in Vivo* Labeling of Nascent Catalase

		Normal	CH-treated
Amount (OD_{280} unit)		0.138 (100%)	0.139 (101%)
Labeling with ^{14}C (cpm)	after 5 min	233 (100%)	10 (4%)[a]
	after 10 min	134 (100%)	28 (21%)[b]
	after 20 min	105 (100%)	47 (54%)[c]

100 OD_{260} units of liver ribosomes were used for assay.
The labeling of total nascent proteins was inhibited to *a*) 18.8%, *b*) 21.8% and *c*) 57.5% of the control.

Effect of Cycloheximide Administration on Biosynthesis of Catalase in Cell-free System

Attempts were made to determine the impairment of which—the ribosomes or the factors in cell sap—was principally responsible for the depression in biosynthesis of liver catalase of CH-treated rats. Liver ribosomes and cell sap were prepared separately both from normal and CH-treated rats, and the following four combinations were examined:

(i) Ribosomes from normal rats — cell sap from normal rats
(ii) Ribosomes from normal rats — cell sap from treated rats
(iii) Ribosomes from treated rats — cell sap from normal rats
(iv) Ribosomes from treated rats — cell sap from treated rats.

Each mixture, fortified by ATP, GTP and energy generating system, was incubated with ^{14}C-leucine at 37° for 60 min, and the incorporation of radioactivity into the catalase released in the medium (Table IV-A), catalase-synthesizing ribosomes (Table IV-B) and nascent catalase peptides (Table IV-C), was measured. The system consisting of the ribosomes and the cell sap both from intact liver (i) was taken as standard (100%). 47.7, 48.6, and 47.3% incorporation were observed respectively, when the cell sap was derived from treated rats (ii). On the other hand, if the ribosomes were from treated rats (iii) the incorporation was repressed to 36.4, 53.6, and 51.8% respectively. The combination of the ribosomes and the

cell sap both from CH-treated rats (iv) showed an incorporation of only 36.4, 44.7, and 40.6% respectively, the rates being minimum among the four combinations. However, cycloheximide was found to be less effective to the labeling of total TCA-insoluble materials.

Table IV. Impaired Synthesis of Liver Catalase by Cell-free Preparations from CH-treated Rats

Combination		^{14}C incorporated in		
Ribosome	Cell sap	(A) Soluble catalase	(B) Catalase-synthesizing ribosomes	(C) Nascent catalase
N	N	132 (100.0%)	304 (100.0%)	268 (100.0%)
N	C	63 (47.7%)	148 (48.6%)	127 (47.3%)
C	N	48 (36.4%)	163 (53.6%)	139 (51.3%)
C	C	48 (36.4%)[a]	136 (44.7%)[b]	109 (40.7%)[c]

N: Preparations from normal rats C: Preparations from CH-treated rats
Values (cpm) are given on the basis of unit volume of reaction mixture
The labeling of total TCA-insoluble materials was found to be
a) 98.1%, *b*) 87% and *c*) 70.5% of the control, respectively.

These results demonstrate that the machinery for catalase synthesis involved both in the ribosomes and in the cell sap have been impaired by the treatment of rats with cylcoheximide. It should be considered that cycloheximide remaining in the cell sap preparation may exhibit an inhibition on the *in vitro* synthesis of catalase (*cf.* Fig. 1 and 2).

Effect of Added Cycloheximide on Biosynthesis of Catalase in Cell-free System

In the last series of experiments cycloheximide was added into the reaction mixture for *in vitro* synthesis of catalase. Ribosomes and cell sap were prepared from intact livers. Fig. 1 demonstrates the inhibition by various concentrations of cycloheximide of the incorporation of ^{14}C-leucine into soluble catalase, catalase-synthesizing ribosomes and nascent catalase. Even in the presence of a high concentration of the antibiotic (500 μg/ml) the incorporation into soluble catalase occurred to the extent of some 25% compared with that in the control experiment, a portion of the synthetic capacity being apparently resistant to cycloheximide inhibition.

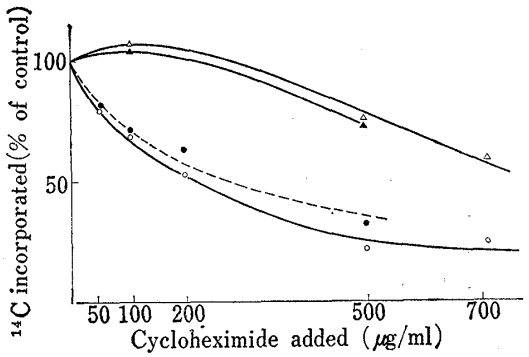

Fig. 1. Inhibition by Cycloheximide of ^{14}C-Leucine Incorporation into Catalase *in Vitro*

○: into soluble catalase
△: into nascent catalase
▲: into catalase-synthesizing ribosomes
A broken line indicates the incorporation into TCA-insoluble proteins in medium.

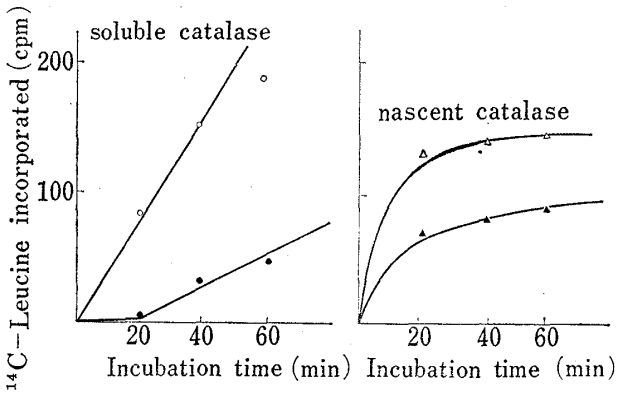

Fig. 2. Kinetics of in *in Vitro* Incorporation of ^{14}C-Leucine into Catalase in the Absence or Presence of Cycloheximide

○△: without cycloheximide
●▲: with cycloheximide (700 μg/ml)

With 100 μg/ml of cycloheximide which caused a 30% reduction in the synthesis of newly completed and released molecules of catalase, the level of ^{14}C in catalase-synthesizing ribosomes and in nascent catalase at 60 min incubation was the same as that found in the absence of the antibiotic. As much as 500 μg/ml of cycloheximide yielded a 22—23.5% decrease in this amount of ^{14}C incorporated, the inhibition still being less apparent than that in the case of soluble catalase (77.0%). As seen in Fig. 1, the antibiotic showed an inhibition for the synthesis of total proteins at almost the same extent as that for the synthesis of catalase.

A kinetic study on the labeling of both nascent catalase and of completed catalase molecules was carried out in the presence of 700 μg/ml of cycloheximide. The results, as shown in Fig. 2, have confirmed the fact that added cycloheximide affects the labeling of soluble catalase released in the medium more extensively than that of nascent catalsae on the ribosomes. No radioactivity was observed in the soluble catalase until about 20 min after the begining of incubation.

Discussion

The results obtained in the present study clearly demonstrate that cycloheximide inhibits the biosynthesis of rat liver catalase as of many other proteins. Substantially the same extent of inhibition was observed with cycloheximide in the labeling of catalase (isolated by anti-catalase) and in that of total proteins (precipitated with TCA). The evidence has been accumulated that the incorporation of ^{14}C-leucine into catalase or its intermediates has been repressed by cycloheximide in a short period of time after injection, when the amount of liver catalase still remains unchanged. Such a rather rapid effect of the cycloheximide for reducing protein-synthesizing activity has been observed by several other investigators.[8,9,18]

The catalase-synthesizing ribosomes and the nascent catalase peptides of CH-treated rats did not differ from those of normal rats in their quantities, but were much less labeled by the injected radioactive amino acid (Tables II and III). This fact would suggest that the rate of translating *m*-RNA on catalase-synthesizing ribosomes has been decreased and/or the release of catalase molecule from the ribosome is being interfered in CH-treated rats. As shown in Table III, the labeling of nascent catalase in intact rats seemed to reach the maximum around 5 min after administration of ^{14}C-leucine, followed by a rapid decrease, whereas in CH-treated rats the label increased gradually until at least 20 min after the injection. The time required for completing a soluble catalase molecule from amino acids appears to be remarkably lengthened by the treatment with cycloheximide. The *in vitro* studies on the kinetics of the labeling of soluble and nascent catalase in the presence of added cycloheximide have also confirmed this evidence, including a lag for about 20 min in the appearance of radioactivity in the completed and released molecules.

Cycloheximide added *in vitro* at the concentration of 100 μg/ml showed a considerable inhibition in labeling of soluble catalase without any effect on that of nascent catalase or ribosomes synthesizing catalase (Fig. 1). This would be an indication for the inhibitory effect of cycloheximide on the release of completed molecules of catalase from ribosomes. However, above this concentration, a second effect became evident—the decrease in the incorporation of ^{14}C-leucine into the intermediates to catalase as well (Fig. 1 and 2). Similar effect of the antibiotic was observed by Rajalakshmi, *et al.*,[18] and these authors have concluded that cycloheximide has at least two inhibitory effects on protein synthesis; a potent effect on chain termination or release at low concentration and an effect on amino acid incorporation into nascent protein at higher concentrations. The pattern of *in vivo* labeling of

18) S. Rajalakshmi, H. Liang, D.S.R. Sarma, R. Kisilevsky, and E. Farber, *Biochem. Biophys. Res. Commun.*, **42**, 259 (1971).

nascent catalase in CH-treated rats—*i.e.*, the label continued to increase for 20 min at much lower level than that in normal rats (Table III)—also supports the evidence for the bifunctional effect of cycloheximide on elongation and release of catalase.

Compared with the system consisting of the ribosomes and the cell sap both from intact rat liver, replacement of either one by the preparation from CH-treated rats resulted in rather consistently 50 to 60% reduction in the incorporation of radioactive amino acid into both soluble and nascent catalase (Table IV). It may be concluded that the ribosomes as well as the cell sap factors are impaired by cycloheximide. In this connection, Trakatellis, *et al.*[7] reported a lowered activity of the cell sap factors of CH-treated mouse liver with a finding that the ribosomes maintained to be intact. On the other hand, based on his experimental results with rat liver, Korner[9] claimed that the ability of the ribosomes to synthesize liver proteins was actually repressed by injection of cycloheximide, but the apparent damage or alteration of cell sap factors was merely due to the antibiotic dissolved in the cell sap preparation from CH-treated animals. The concentration of cycloheximide in the cell sap preparations from CH-treated rats used in the present study, is reasonably estimated to be much lower than that to give 100 μg of the antibiotic per ml of the reaction mixture for cell-free synthesis. Therefore, the *in vitro* effect of cylcoheximide, if any, would not be counted on the labeling of nascent catalase (*cf.* Table V), and a significant decrease in the incorporation observed with the cell sap from CH-treated rats thus evidently indicates a drug-induced damage of the cell sap factors. Gel filtration with Sephadex could not remove the inhibitory principle from the cell sap preparation. Furthermore, it would be noted that cycloheximide when given *in vivo*, exhibited a more extensive effect than that demonstrated *in vitro*. This would suggest that cycloheximide inhibits catalase synthesis through impairing the ribosomes and the cell sap factors.

As to the factors in the cell sap that may be impaired by cycloheximide, Baliga, *et al.*[19] pointed out transferase II. According to their results, the inhibitory action of cycloheximide on peptide chain elongation would be accounted for by inactivation of this SH-enzyme. However, McKeehan and Hardesty,[20] and Obrig, *et al.*[21] concluded that transferase II was not inactivated by cycloheximide because a guanosine triphosphatase activity of this enzyme was scarcely reduced, and that the inhibition in transferase II-dependent translocation might due to damage of the ribosomes at a certain site, possibly the donor site of 60 S subunit.

Besides elongation and release of peptide chains, the initiation of protein synthesis would also be interfered with by this antibiotic. Lin, *et al.*,[22] with a reticulocyte system, reported a lowered incorporation of ^{14}C-valine into the amino terminal of hemoglobin in the presence of cycloheximide. The formation of a initiation complex from ribosome, *m*-RNA and *t*-RNA,[21] and the reaggregation of run-off ribosomes[19] were found to be inhibited by cycloheximide. Although it is difficult to explain the present results only by the inhibition in chain initiation, such an effect of the cycloheximide in biosynthesis of rat liver catalase can not be excluded.

19) B.S. Baliga, A.W. Proncznk, and H.N. Monro, *J. Biol. Chem.*, **244**, 4480 (1969).
20) W.L. McKeehan and B. Hardesty, *Biochem. Biophys. Res. Commun.*, **36**, 625 (1969).
21) T.G. Obrig, W.J. Culp, W.L. McKeehan, and B. Hardesty, *J. Biol. Chem.*, **246**, 174 (1971).
22) S.Y. Lin, R.D. Mosteller, and B. Hardesty, *J. Mol. Biol.*, **21**, 51 (1966).