INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE IN RABBIT RETICULOCYTES

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Cycloheximide, an antibiotic obtained from Streptomyces griseus, has been found to inhibit protein synthesis in the yeast Saccharomyces carlsbergensis (Kerridge, 1958), in L cells (Ennis and Lubin, 1964) and in cell-free systems obtained from Saccharomyces pastorianus (Siegel and Sisler, 1964a) and rat liver (Wettstein et al., 1964). Cycloheximide does not inhibit amino acid activation or transfer of activated amino acids to s-RNA in Saccharomyces pastorianus (Siegel and Sisler, 1964b). In the rat liver cell-free system cycloheximide prevents the breakdown of ribosomal aggregates, which is associated with the growth of nascent polypeptide chains (Wettstein et al., 1964). It has thus been suggested that cycloheximide inhibits either the transfer of aminoacyl-s-RNA to the ribosomes or the formation of the peptide bond (Siegel and Sisler, 1964a).

We want to report some experiments on the inhibition of protein synthesis by cycloheximide in intact rabbit reticulocytes and in a cell-free system obtained from reticulocytes.

MATERIALS AND METHODS

Reticulocytes were obtained from phenylhydrazine injected rabbits (Borsook et al., 1957). The reticulocytes were washed twice with a solution 0.14M NaCl, 0.0015M $MgCl_2$ and 0.005M KCl (special saline). The washed reticulocytes were incubated in half the volume of special saline containing 0.6 mg of glucose, 0.36 mg of $NaHCO_3$ and 0.24 mg of $Fe(NH_4)_2(SO_4)_2$ per ml (standard medium). Cold amino acids (Borsook et

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al., 1957) and labeled amino acids were added as indicated in the legends. A cell-free system was prepared by lysing the reticulocytes with an equal volume of water, following the procedure of Lamfrom and Knopf (1964). The components of this cell-free system are indicated in the legend to Fig. 2.

RESULTS AND DISCUSSION

Fig. 1 shows the inhibition of protein synthesis in rabbit reticulocytes by 1.4 x 10^{-5}M cycloheximide. The inhibition occurs very rapidly since it reaches 96% 30" after cycloheximide is added. The final inhibition after 20' incubation at cycloheximide concentration of 10^{-4} and 10^{-3}M has been respectively 97% and 98%.

The inhibition by cycloheximide is reversible. Reticulocytes were incubated 15' in the presence of the antibiotic, washed three times with special saline and reincubated with labeled amino acids. The rate of protein synthesis in these cells was the same as that of control reticulocytes.

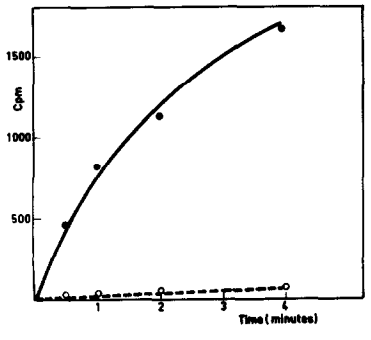

Fig. 1 - Time course of amino acid incorporation by rabbit reticulocytes in the presence of 1.4x10^{-5}M cycloheximide (--O--O--) and in the control (—●——●—). Reticulocytes were incubated at 37°C in standard medium with cold amino acids minus valine. Cycloheximide or saline in the control were added after 15' and 0.15 µc of ^{14}C valine (29 µc/ µM) were added 15" later to each 1 ml. sample.

The inhibition of protein synthesis in the cell-free system from reticulocytes is shown in Fig. 2. The inhibition is lower than in intact reticulocytes; after 20' incubation, 2 x 10^{-4}M cycloheximide inhibits protein synthesis by 60-70 %.

The mechanism of inhibition of protein synthesis by cycloheximide has been investigated in several ways. Reticulocytes were incubated for 5' at 37°C with ^{14}C-amino acids: 1.2 x 10^{-4}M cycloheximide was then added for 5'. The cells were lysed and the polyribosomes were analyzed by sucrose density gradient centrifugation according to Warner et al., (1963). The polyribosomes pattern in reticulocytes inhibited by cyclo-

heximide was undistinguishable from that of control ribosomes (Fig. 3). Cycloheximide thus, while inhibiting protein synthesis, does not remove growing peptide chains from polyribosomes.

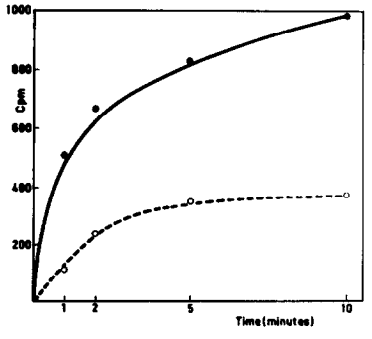

Fig. 2 - Time course of amino acid incorporation by a cell-free system prepared from rabbit reticulocytes, in the presence of cycloheximide (--O---O--) and in the control (—●——●—). Reticulocytes were incubated 30' at 37°C in standard medium with cold amino acids minus valine and then lysed with an equal volume of water. Each sample contained: 0.5 ml. of lysate, 0.6 µM of ATP, 0.15 µM of GTP, 2.5 µM $MgCl_2$, 60 µM CH_3COONH_4, 6 µM tris pH 7.8, 4 µM mercaptoethanol, 2.5 µM phosphoenolpyruvate, 20 µg pyruvate kinase. The final concentration of cycloheximide was 2×10^{-4}M.

The possibility that cycloheximide prevents the release of completed chains from polyribosomes has been investigated in the following experiment. Marks et al. (1964) have reported that NaF inhibits protein synthesis in reticulocytes; they have shown that polyribosomes disappear from rabbit reticulocytes during incubation with NaF. The polyribosomes are reformed if the cells are washed and reicubated in the presence of glucose (P.A. Marks, personal communication).

Reticulocytes were incubated 30' at 37°C with 10^{-2}M NaF. The cells were then washed three times with saline and reincubated with ^{14}C-amino acids in standard medium in the presence or absence of cycloheximide. The results are shown in Fig. 4. Cycloheximide prevents the reassembly of polyribosomes from monoribosomes which have been formed by the NaF treatment, while polyribosomes are reassembled in the control. It seems unlikely that the mechanism of inhibition of protein synthesis by cycloheximide involves the release of completed chains only. If this were the case, polyribosomes could reform in cycloheximide treated reticulocytes after NaF inhibition. Thus, cycloheximide inhibits some step in protein synthesis which is required for the initial binding of the messenger

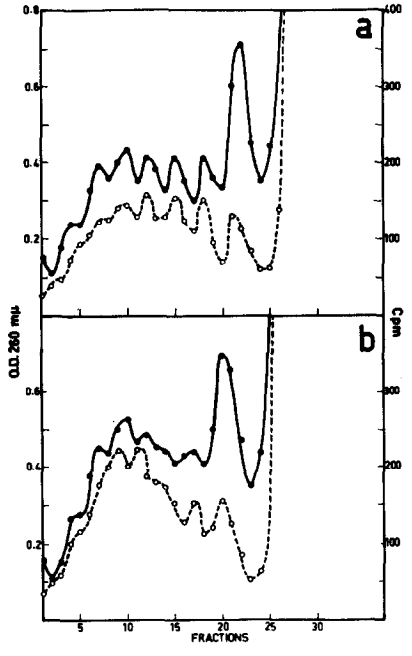

Fig. 3 - Sucrose density gradients of reticulocyte lysate: a) control b) reticulocytes incubated with cycloheximide. For each gradient 0.5 ml. of reticulocytes were incubated 15' in standard medium at 37°; 0.5 μc of ^{14}C-amino acids (Chlorella protein hydrolyzate 1.48 mc/mg) were then added. (a) The incubation (10') was stopped by the addition of cold special saline. (b) Cycloheximide 10^{-4}M was added after 5' incubation with ^{14}C-amino acids. The incubation was stopped 5' after the addition of the antibiotic with cold special saline.
After the incubation the reticulocytes were washed once and lysed in an equal volume of water. The lysate was centrifuged for 10' at 15,000 r.p.m. The supernatant was immediately diluted with two volumes of 0.01 M tris pH 7.8, 0.01 M KCl and 0.0015 M $MgCl_2$. 1.5 ml. of diluted lysate were layered on a 15-30 % sucrose gradient and centrifuged 150' at 24,000 r.p.m. in the S.W. 25 rotor of a Spinco Model L centrifuge. —●—●— , O.D.; --O--O-- cts/min.

to ribosomes in a polyribosomal structure and for the advancement of ribosomes along the messenger.

The mechanism of inhibition of protein synthesis by cycloheximide is different from that of puromycin. Puromycin causes the detachment of growing peptide chains from polyribosomes and a shift toward smaller polyribosome size (Williamson and Schweet, 1964). The action of puromycin on reticulocytes that were inhibited by cycloheximide has been investigated. Reticulocytes were incubated 5' in standard medium with ^{14}C amino acids and then for 5' with cycloheximide; 2.8 x 10^{-4} M puromycin was then added for 30" at 37°C. Polyribosomes prepared from reticulocytes incubated with cycloheximide and puromycin were analyzed by sucrose gradient centrifugation. Approximately 50% of the labeled peptide chains were removed by puromycin from the polyribosomes of cycloheximide

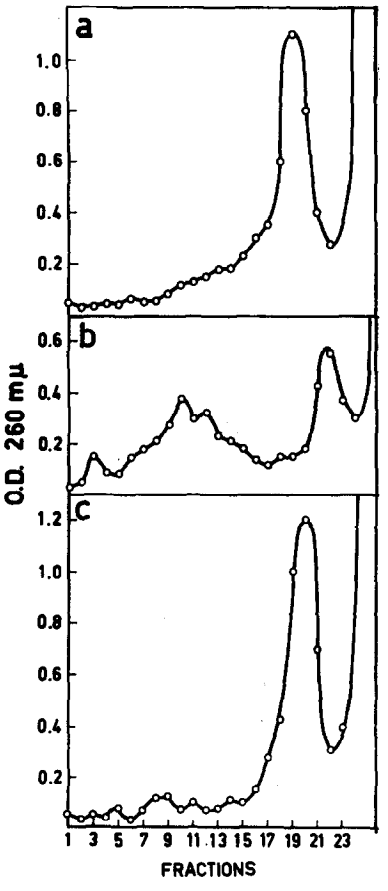

Fig. 4 - Sucrose density gradients of reticulocyte lysate after NaF treatment (a) and reincubation in the absence (b) and in the presence (c) of cycloheximide. For each gradient 0.5 ml of reticulocytes were incubated 30' at 37° with 10^{-2} M NaF. The reticulocytes were washed three times with cold special saline. The reticulocytes were lysed and the lysates analyzed by sucrose density gradient centrifugation as indicated in the legend of Fig. 3.
(a) The reticulocytes were lysed immediately after incubation with NaF. (b) The reticulocytes preincubated with NaF were incubated 10' at 37° in standard medium.
(c) The reticulocytes preincubated with NaF were incubated 10' at 37° in standard medium containing 10^{-4}M cycloheximide.

inhibited reticulocytes; the O.D. profile instead, was almost identical to that of cycloheximide inhibited polyribosomes (Fig. 5). The O.D. profile of polyribosomes prepared from reticulocytes incubated with puromycin only is different, since the amount of polyribosomes is decreased (Fig. 5). If the incubation with puromycin after cycloheximide treatment, is prolonged to 5', all the labeled chains are detached from polyribosomes (Fig. 6). These results suggest that cycloheximide slows down the attachment of puromycin to the carboxyl end of the growing polypeptide chains (Nathans, 1964). The incomplete inhibition of protein synthesis by cycloheximide (97%) may account for the attachment of puromycin to the growing peptide chains.

At present it is not possible to establish the mechanism of cycloheximide inhibition of protein synthesis. It is quite unexplained why

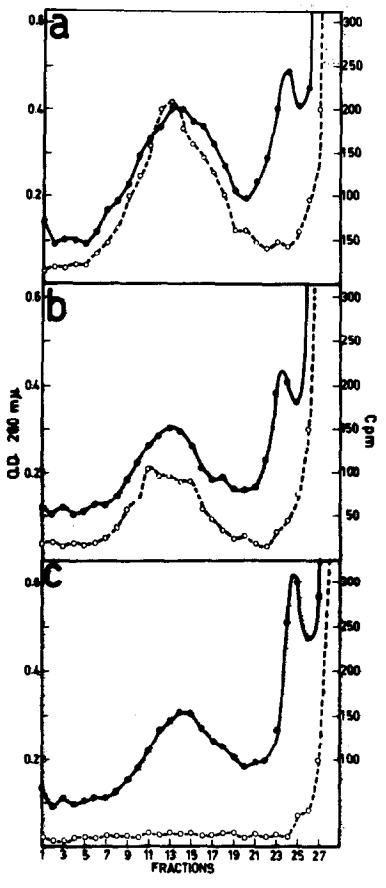

Fig. 5

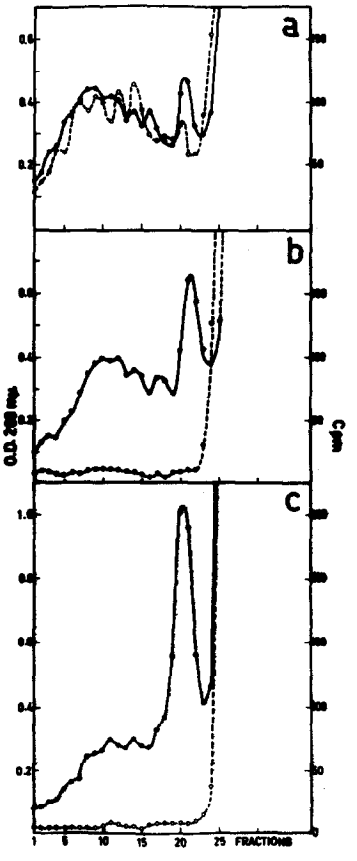

Fig. 6

Fig. 5 - Sucrose density gradients of reticulocyte lysate: (a) incubated with cycloheximide, (b) incubated with cycloheximide plus puromycin and (c) incubated with puromycin. For each gradient 0.5 ml. of reticulocytes were incubated 15' at 37° in standard medium. 0.5 μc of ^{14}C-amino acids (Chlorella protein hydrolysate 1.48 mc/mg) were then added. After 5' the antibiotics were added as follows: (a) 10^{-4}M cycloheximide for 5'; (b) 10^{-4}M cycloheximide for 5' and afterward 2.8 x 10^{-4} M puromycin for 30"; (c) 2.8 x 10^{-4}M puromycin for 30".

Fig. 6 - The experimental conditions are the same as in Fig. 5. (a) 10^{-4}M cycloheximide for 5'; (b) 10^{-4}M cycloheximide for 5' and afterward 2.8 x 10^{-4}M puromycin for 5'; (c) 2.8 x 10^{-4}M puromycin for 5'. —●—●— O.D.; --O---O-- cts/min.

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cycloheximide inhibits less in the cell-free system than in intact reticulocytes. It may be supposed that cycloheximide acts in intact cells at the level of some polyribosomal structure, which no longer

exists in lysed reticulocytes. Further investigations are in progress to clarify the site and the mechanism of action of cycloheximide. The inhibition of protein synthesis in anucleated cells, where synthesis of RNA and DNA does not occur, suggests that the toxicity of this antibiotic is primarily due to the inhibition of a biochemical reaction necessary for protein synthesis.

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