PRODUCTION OF MEMBRANE WHORLS

IN RAT LIVER BY SOME INHIBITORS

OF PROTEIN SYNTHESIS

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ABSTRACT

Inhibitors of protein synthesis capable of differential effects on nascent peptide synthesis on membrane-bound and free polyribosomes were employed to investigate the structure and function of cellular membranes of liver. The formation of membranous whorls in the cytoplasm and distension of nuclear membranes were induced by inhibitors of protein synthesis (i.e., cycloheximide and emetine) which predominantly interfere with nascent peptide synthesis on membrane-bound polyribosomes *in situ*. Other inhibitors of protein synthesis such as puromycin and fusidic acid, which inhibit nascent peptide synthesis on both free and membrane-bound polyribosomes, and chloramphenicol, which inhibits mitochondrial protein synthesis, did not induce these alterations. Cycloheximide, puromycin, and chloramphenicol produce some common cellular lesions as reflected by similar alterations in morphology, such as swelling of mitochondria, degranulation of rough endoplasmic reticulum, and aggregation of free ribosomes. The process of whorl formation in the cytoplasm, the incorporation of [^{3}H]leucine and of [^{3}H]choline into endoplasmic reticulum and the total NADPH-cytochrome c reductase activity of the endoplasmic reticulum were determined. During maximum formation of membranous whorls, [^{3}H]leucine incorporation into cytoplasmic membranes was inhibited, while [^{3}H]choline incorporation into these structures was increased; maximum inhibition of protein synthesis and stimulation of choline incorporation into endoplasmic reticulum, however, preceded whorl formation. Cycloheximide decreased the activity of NADPH-cytochrome c reductase of rough endoplasmic reticulum, but increased NADPH-cytochrome c reductase activity of smooth endoplasmic reticulum. In addition, cycloheximide decreased the content of hemoprotein in both the microsomal and mitochondrial fractions of rat liver, and the activities of mixed function oxidase and of oxidative phosphorylation were impaired to different degrees. Succinate-stimulated microsomal oxidation was also inhibited. The possible mechanisms involved in the formation of membranous whorls, as well as their functions, are discussed.

INTRODUCTION

The morphology and physiological functions of the endoplasmic reticulum (ER) of normal tissues have been investigated under a variety of experimental conditions. A number of chemical agents have been found to produce disruptive changes in the structure of the ER. Thus, numerous concentric whorls of imbricated paired membranes, as well as other alterations, have been seen in the cytoplasm of parenchymal cells of rats treated with agents such as dimethylnitrosamine, thioacetamide, 3'-methyl-4-(dimethylamino)azobenzene, dioxane, and ethionine (1-8). Concentric whorls of membranes have also been observed in untreated hepatomas, as well as in virus-induced liver tumors of rodents (9-11).

A variety of inhibitors are known which directly interfere with various steps involved in peptide synthesis in the ribosome cycle. These inhibitory effects on the translational machinery result in interference with several cellular functions, such as the maturation and processing of RNA, DNA replication, enzyme induction and degradation, and cellular immunological responses. Recently, several inhibitors of protein synthesis were shown to have differential effects on nascent peptide synthesis in situ on free and membrane-bound polyribosomes, with polyribosomes associated with membranes being the more sensitive (12). Since the two classes of polyribosomes appear to be relatively distinct functionally, e.g. they differ with respect to the protein molecules synthesized (see for example, references 13 and 14), it was of interest to examine the organization of cellular membranes under conditions of differential perturbation of the cellular protein-synthesizing machinery. Cycloheximide, which selectively inhibits nascent peptide synthesis on membrane-bound polyribosomes, and other inhibitors of protein biosynthesis were used in this context, and this communication reports some morphological and biochemical alterations of hepatic cellular membranes after their administration in vivo.

MATERIALS AND METHODS

Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; L-[^{3}H]leucine and [^{3}H]choline from New England Nuclear, Boston, Mass., and emetine hydrochloride and cycloheximide from Sigma Chemical Co., St. Louis, Mo.

Treatment of Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 160-180 g were housed over corncob bedding and maintained on alternating periods of 12-h dark and 12-h light. Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water were available ad libitum. Two injections of each inhibitor dissolved in 0.9% NaCl were given intraperitoneally with a 12-h interval between injections. The following doses were used: puromycin, 25 or 100 mg/kg; cycloheximide, 1-4 mg/kg; emetine, 13 mg/kg; and chloramphenicol succinate, 100 mg/kg. Control animals received an equivalent volume of 0.9% NaCl adjusted to the same pH as the solution of inhibitor. The food for both control and experimental animals was withdrawn at the start of injections. Animals were killed by decapitation at various times after the first injection of drug, and the liver was quickly removed for biochemical and morphological studies.

Preparation of Liver for Electron Microscope Studies

Immediately after the rats were killed, small pieces (about 200 mg each) of liver were quickly immersed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for at least 2 h. The pieces were then washed in 0.1 M cacodylate buffer and sliced into 0.5-mm strips before osmication in 1% osmium tetroxide. The tissues were washed again in buffer and dehydrated in a graded alcohol series, followed by propylene oxide. Slow embedding in Vestopal-W (Madame Martin Jaeger, Geneva, Switzerland) was carried out first at 40°C and then at 60°C. The resulting tissue blocks were cut on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), stained with uranyl acetate and lead citrate, and viewed under a Zeiss 9-S electron microscope. 20 fields/sample were examined under the electron microscope. Each field had an area of 900 μm^2.

[^{3}H]Leucine Incorporation into Nascent Peptides

[^{3}H]Leucine (sp act 10 μCi/μmol) in 0.9% NaCl was injected intraperitoneally at a level of 85 μCi/animal 30 min before sacrifice. The liver was rapidly excised and washed twice with 0.25 M sucrose containing 50 mM Tris-HCl (pH 7.4), 50 mM KCl, and 5 mM MgCl$_2$. Livers were homogenized in sucrose (25%) by 10 strokes of a motor driven glass-Teflon tissue grinder at 200 rpm. Homogenates were centrifuged at 12,000 g for 10 min at 4°C to obtain a postmitochondrial (PM) fraction. The microsomal fraction was prepared by centrifugation of the PM fraction at 105,000 g for 60 min. Aliquots of the tissue homogenate, and the PM and microsomal fractions were each precipitated in 4 vol of 10% trichloroacetic acid (TCA). The precipitates were washed twice with

2 ml of cold 5% TCA, and were dissolved in 0.2 N NaOH. Portions were used to determine the protein concentration by the Lowry method (15); radioactivity was measured after addition of 12 ml of Aquasol. No greater than 8% of the radioactivity present in nascent peptides was due to nonspecific precipitation.

Nascent peptide synthesis on free and membrane-bound polyribosomes *in situ* was determined according to the procedure described previously (12). Briefly, inhibitors dissolved in 0.9% NaCl were injected into the portal vein after laparotomy 1 min before administration of [³H]leucine; control animals received an equivalent volume of 0.9% NaCl. [³H]Leucine was allowed to incorporate for 3 min, and the liver was rapidly excised and placed into a beaker of ice-cold 0.25 M sucrose containing 50 mM Tris-HCl (pH 7.8), 50 mM KCl, and 5 mM MgCl₂ (TKM). Liver homogenates (4 g liver in 8 ml 0.25 M sucrose TKM) were prepared using 10 strokes of a motor driven glass-Teflon tissue grinder operating at 2,000 rpm. Homogenates were centrifuged at 10,000 *g* for 20 min at 4°C and the overlying layer of fat was removed. Free and membrane-bound polyribosomes were prepared without the use of deoxycholate according to the method of Blobel and Potter (16) and the radioactivity therein was determined by scintillation spectrometry.

Incorporation of [³H]Choline Chloride

[³H]Choline chloride (sp act 10 μCi/μmol) was diluted with 0.9% NaCl and injected intraperitoneally at 85 μCi/animal. After 30 min, animals were killed and the livers were fractionated into a homogenate, PM fraction, and microsomes as described above. Lipid was extracted twice from these fractions by treatment with 22 vol of CHCl₃/methanol (2:1) and was washed with 0.2 vol of 0.37% KCl according to the method of Folch et al. (17). The lipid extracts were dried at 60°C, the dried residue was redissolved in dried chloroform, the concentration of lipid was determined as described by Bartlett (18), and radioactivity was measured as described above. None of the radioactivity present in phospholipids was due to nonspecific extraction.

## Preparation of Microsomal Fractions and Enzyme Assays

Total, rough, and smooth microsomes were prepared by the procedure of Dallner et al. (19). NADPH-cytochrome *c* reductase was assayed by the modification of the method of Omura et al. (20) described previously (21). The total microsomal P450 content and mitochondrial cytochromes were measured as described in an earlier report (22). Microsomal oxidative demethylation and hydroxylation activities were determined as described previously (23).

Determination of Oxidative Phosphorylation

Liver mitochondria were prepared from 0.25 M sucrose homogenates (2 vol of sucrose/g of liver) after disruption of cells by seven strokes in a Potter-Elvehjem homogenizer with a moderately loose-fitting Teflon pestle using a Sorvall Omnimixer (Ivan Sorvall, Inc.) set at 3 for speed control. The homogenate was diluted to 1:10 with 0.25 M sucrose and centrifuged at 750 *g* for 10 min; the supernate was then centrifuged at 12,000 *g* for 10 min. The pellets were combined and resuspended in 5–10 times the original volume of 0.25 M sucrose and homogenized by hand using 2–3 passes with a loose-fitting pestle. The final pellet was suspended in 0.25 M sucrose 10 mM Tris-HCl (pH 7.4). The ADP/O ratio and respiratory control index was estimated by the procedure of Chance and Williams (24).

RESULTS

The administration of two doses of either 2.5 mg of cycloheximide/kg or 12 mg of emetine/kg produced several changes in the ultrastructure of rat parenchymal cells. These alterations involved distension of the nuclear membrane, the appearance of whorls of membrane, vacuoles, and alterations in mitochondria. The time-course of cellular transformations after treatment with cycloheximide was determined to ascertain the onset of drug-induced modification of cellular morphology. The earliest changes were observed in the livers of animals 6–12 h after the first treatment with the antibiotic; these consisted of the formation of both large and small vacuoles containing membranous material and various anomalous cellular elements (Fig. 1). Other prominent features at this time were the dilation of rough endoplasmic reticulum (RER) cisternae, distension of the nuclear membrane, and swollen mitochondria (Fig. 2). In many cases, paired membranous whorls were seen in the perinuclear area (Fig. 3). One end of the membrane whorl appeared to be continuous with the RER membrane, while the other generally surrounded cellular organelles (Figs. 3 and 4). The formation and assembly of the multilayered membrane whorls was not completed after 6–12 h, and their size (Fig. 5) and frequency (Table I) increased progressively with time. Treatment with two doses of cycloheximide 12 h apart increased the frequency of whorl formation; examination of liver lobules indicated that membranous whorls were present at the periphery of the lobule in approximately the same frequency as in the central area of the lobule. Whorls were generally free of polyribo-
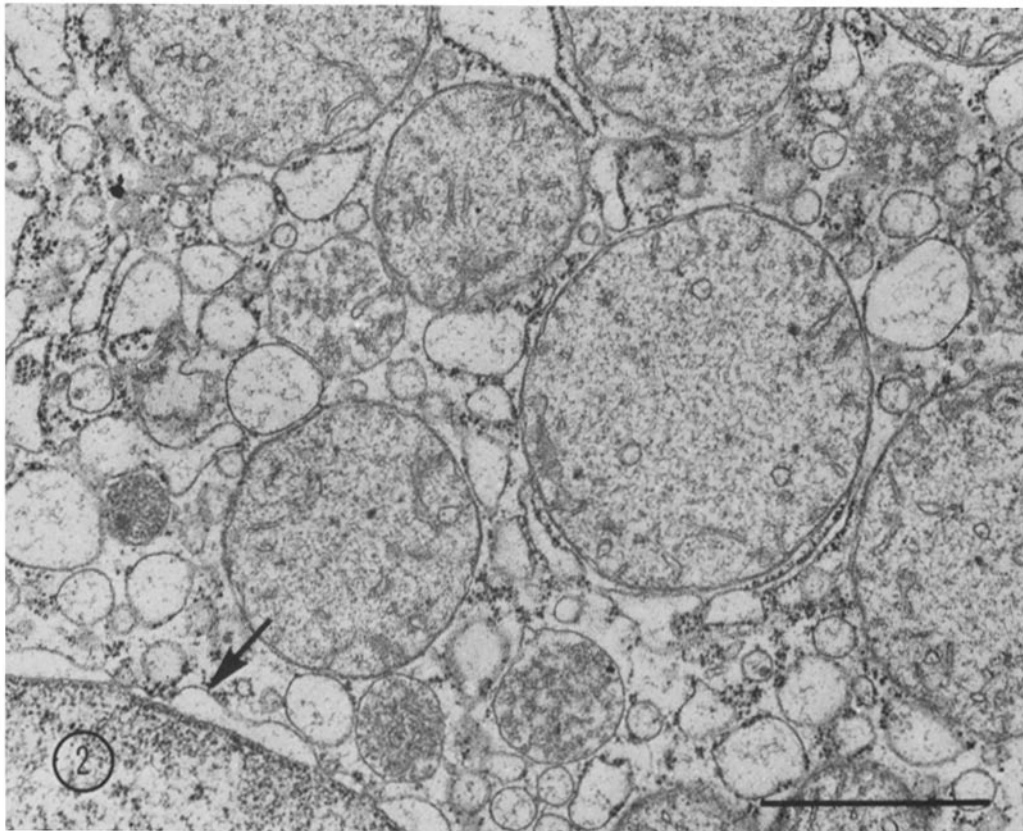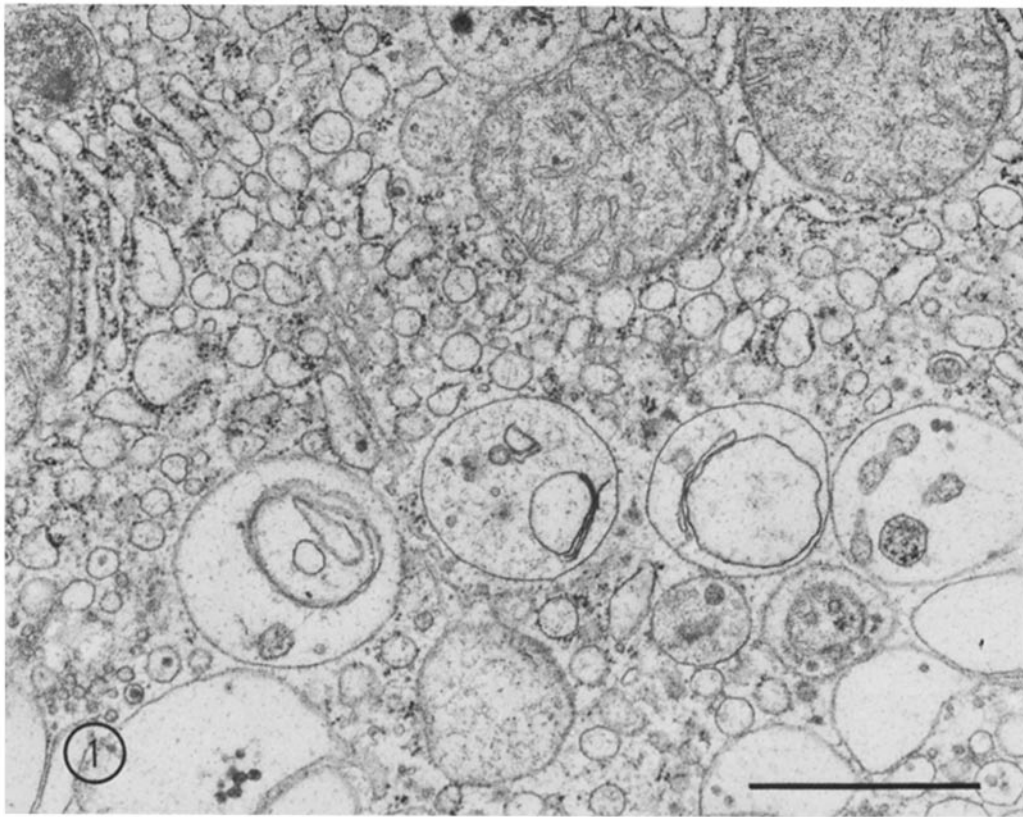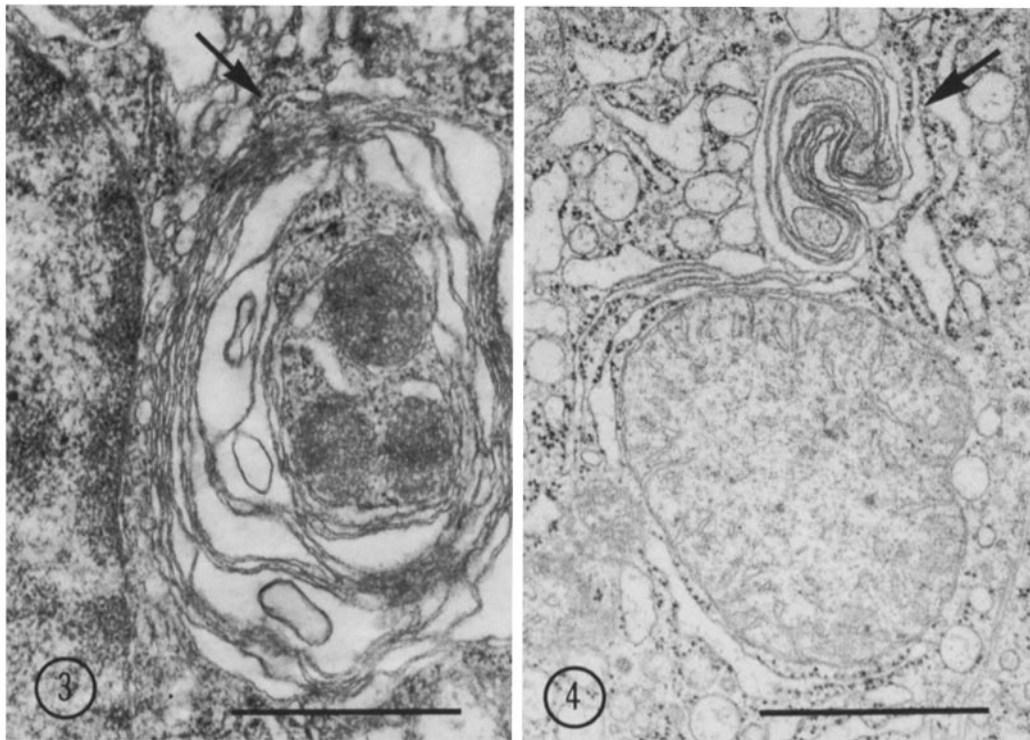
FIGURES 1 and 2   Hepatic cell cytoplasm of rats 12 h after intraperitoneal injection of 2.7 mg/kg of cycloheximide. Many vacuoles containing cellular debris are prominent in the cytoplasm. The RER is distended and the mitochondria are swollen. Note distension of the nuclear membrane in Fig. 2 (arrow). × 30,000.

somes and were formed around such cell organelles as mitochondria, peroxisomes, smooth endoplasmic reticulum (SER), and lipid (Fig. 5). Frequently, the peripheral long cisternae of the whorls, at 16–24 h, ended in distended bulbs resembling the Golgi complex (Fig. 6). 24 h after treatment with cycloheximide, the whorls decreased in both size and frequency, and they essentially disappeared by 36 h, after which cytoplasmic organization seemed relatively normal.

Emetine produced membranous whorls which were morphologically similar to those produced by cycloheximide; however, other inhibitors of protein synthesis, which do not produce differential inhibition of peptide synthesis on membrane-bound and free polyribosomes, caused different alterations in membrane structure (i.e., fusidic acid and puromycin). For example, the injection into rats of levels of puromycin (25 and 100 mg/kg), which cause approximately the same degree of total inhibition of protein synthesis as did cycloheximide in this system, did not cause the formation of whorls. Chloramphenicol, which selectively inhibits the mitochondrial protein-synthesizing system, at a dose of 100 mg/kg, also did not produce cytoplasmic whorls of membrane. The effects of puromycin treatment are shown in Fig. 7. The alterations observed were a striking dilation of RER cisternae and clustering of free ribosomes similar to that reported by Reid et al. (25). The mitochondria contained dense granular deposits, similar in appearance to the ribosome particles which are accumulated in the cytoplasm; these particles may be analogous to the mitoribosomal particles described by Beale et al. (26).

In the presence of chloramphenicol, the mitochondria appeared swollen and their content of tubular cristae was decreased as described previously by several authors (27–29). No whorl-like cristae appeared to be present in mitochondria as reported recently for HeLa cells by Storrie and Attardi (30). The changes caused by chloramphenicol in the ER were similar to those induced by puromycin, but appeared to occur less frequently.

FIGURES 3 and 4 Membranous whorls present in the hepatic cell cytoplasm of rats 12 h after the intraperitoneal injection of 2.7 mg/kg of cycloheximide. Paired whorls of membrane surrounding cell organelles are found in all parts of the cell, but most often in perinuclear areas. The whorls appear to be continuous with the RER (arrows). × 30,000.
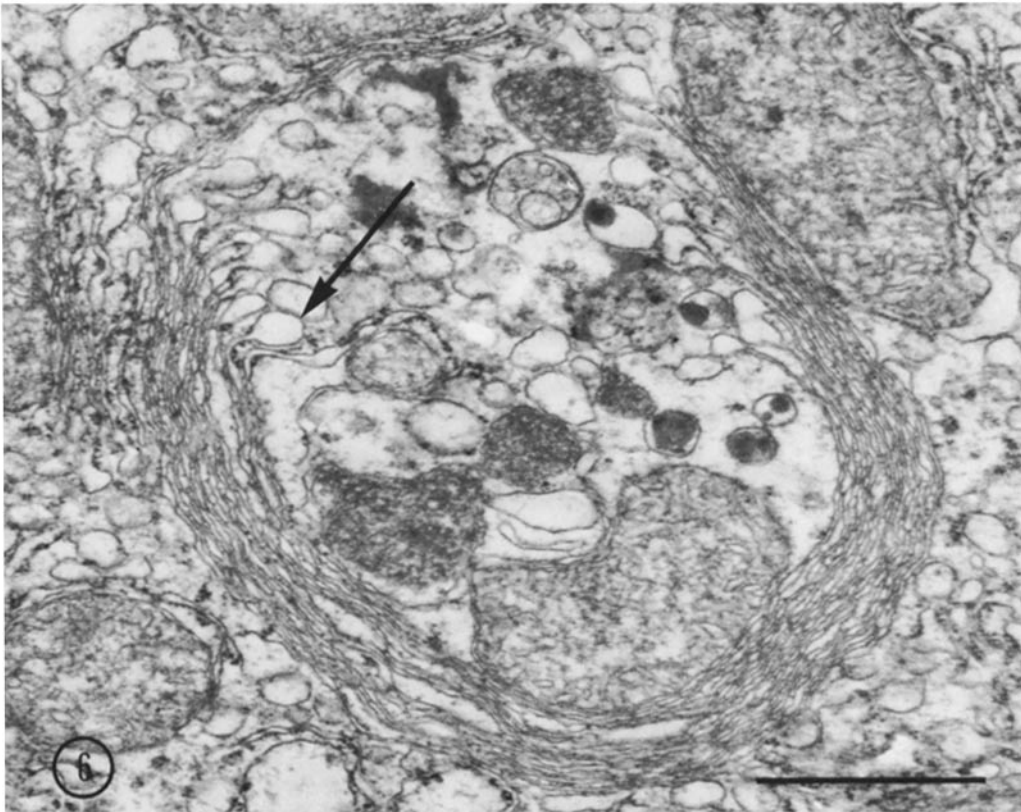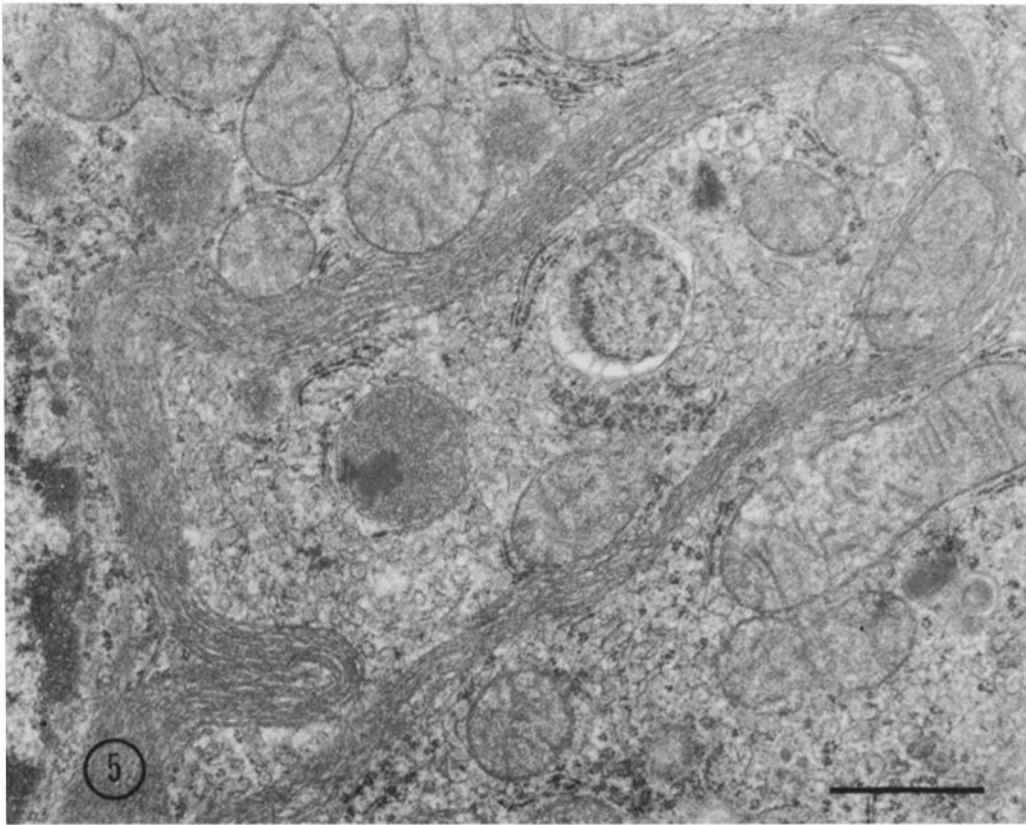
FIGURES 5 and 6   The structure of membrane whorls present in hepatic cell cytoplasm of rats 24 h after treatment with 2 doses of 2.7 mg/kg of cycloheximide given 12 h apart. Whorls surround a very large volume of cytoplasm. The proliferating SER is prominent (Fig. 5). Note the bulbous whorl endings in Fig. 6 (arrow). Fig. 5, × 20,000; Fig. 6, × 30,000.

The Frequency of Appearance of Membrane Whorls in Liver after Treatment with Cycloheximide

Treatment*	Percent of fields with membrane whorls‡					
	6 h§	12 h§	18 h§	24 h§	30 h§	36 h§
Cycloheximide (one time)	38 ± 12	58 ± 10	43 ± 3	22 ± 4	5 ± 2.5	0
Cycloheximide (two times)			75 ± 7	95 ± 3	35 ± 21	2 ± 1.5

* Rats received either a single intraperitoneal dose of 2.7 mg/kg of cycloheximide (one time) or two doses of 2.7 mg/kg each at 12-h intervals (two times).

‡ The percentage of fields in which whorls of membrane were observed in a total of 20 fields from each sample, each of which covered 900 μm²; three samples were taken from different grids of the central area of the liver from each of two rats. The results indicate the average of the six samples ± the standard error.

§ Time after the initial injection of cycloheximide at which sample was obtained.
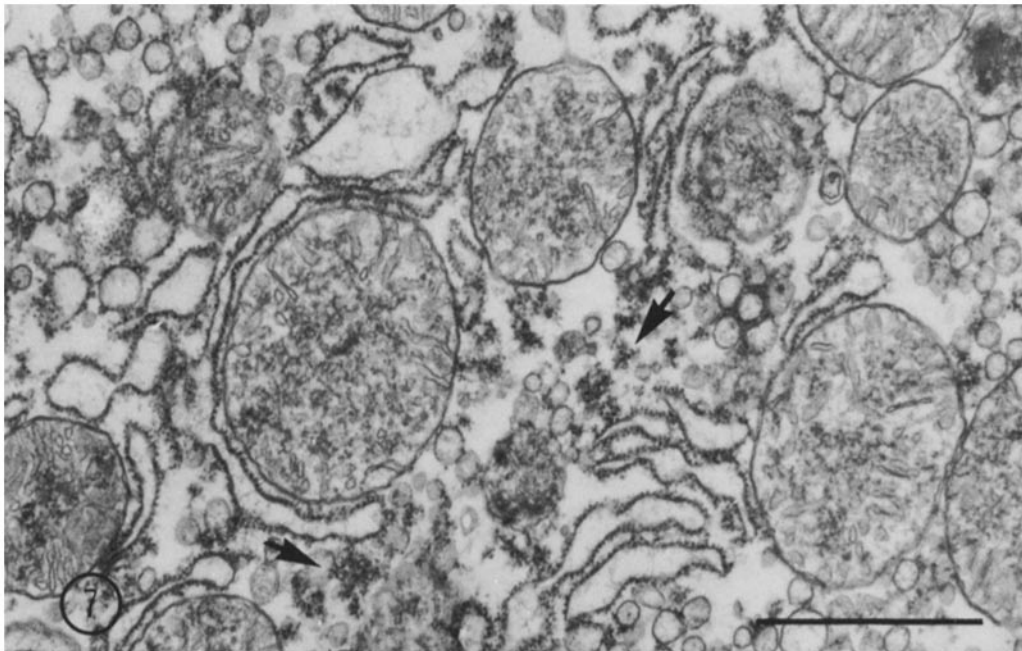

FIGURE 7   Hepatic cell cytoplasm of rats 10 h after treatment with 100 mg/kg of puromycin. Ribosome clusters (arrow) can be seen among the distended RER cisternae. Altered mitochondria contain granular deposits. × 30,000.

Differential inhibitory effects of various antibiotics on nascent peptide synthesis on membrane-bound and free polyribosomes in situ have been reported previously by this laboratory (12). The dosage level of 20 mg/kg of emetine used in this previous investigation was too toxic for the present studies, causing the death of rats within 2 days. However, the animals survived for the period of time employed in the present investigation using a level of 12 mg/kg of emetine; this dosage was, therefore, employed to study the synthesis of nascent peptide in situ as previously described. The results were in accord with previous findings (12) that the action of emetine on the synthesis of protein is similar to that of cycloheximide; thus, both drugs caused about 5 ± 3% inhibition of the incorporation of [³H]leucine into free polyribosomes and about 65 ± 5% inhibition of incorporation of the amino acid into membrane-bound polyribosomes 3 min after intraportal administration. Puromycin was also employed at larger doses (i.e., 25 and 100 mg/kg) than those used by Glazer

and Sartorelli (12); inhibition of nascent peptide synthesis by this agent occurred on both free and membrane-bound polyribosomes.

A kinetic study was carried out to determine the effects of cycloheximide and puromycin on the incorporation of [³H]leucine and [³H]choline into the protein and phospholipid, respectively, of ER. The effects of cycloheximide on the incorporation of [³H]leucine into the protein of the ER of liver are shown in Fig. 8 B and D. A marked and similar decrease in the rate of incorporation of [³H]leucine into the ER occurred at 3 h in rats treated with either 2.7 mg/kg of cycloheximide or 100 mg/kg of puromycin (data not shown). The inhibition of protein synthesis by puromycin was rapidly reversible, with incorporation of the amino acid reaching 90% of the control level by 6 h. In contrast, however, recovery from inhibition by cycloheximide was considerably delayed, requiring more

than 24 h (Fig. 8 B). Food deprivation, which was carried out to insure that possible differences in food intake between treated and untreated rats was not a factor, caused a progressive decrease in the rate of incorporation of [³H]leucine into protein of the ER (Fig. 8 D); maximum decrease due to withdrawal of food occurred between 24 and 48 h. During this period recovery from the inhibitory effects of cycloheximide occurred (Fig. 8 B). Thus, under conditions in which whorls of membrane appear after treatment with cycloheximide, the protein synthetic processes are under stress of inhibition by this agent. Furthermore, the rate of [³H]leucine incorporation into protein does not return to normal levels until after the whorls of membrane have disappeared and cellular membranes assume normal conformation.

The incorporation of [³H]choline into ER membrane was also measured after treatment with
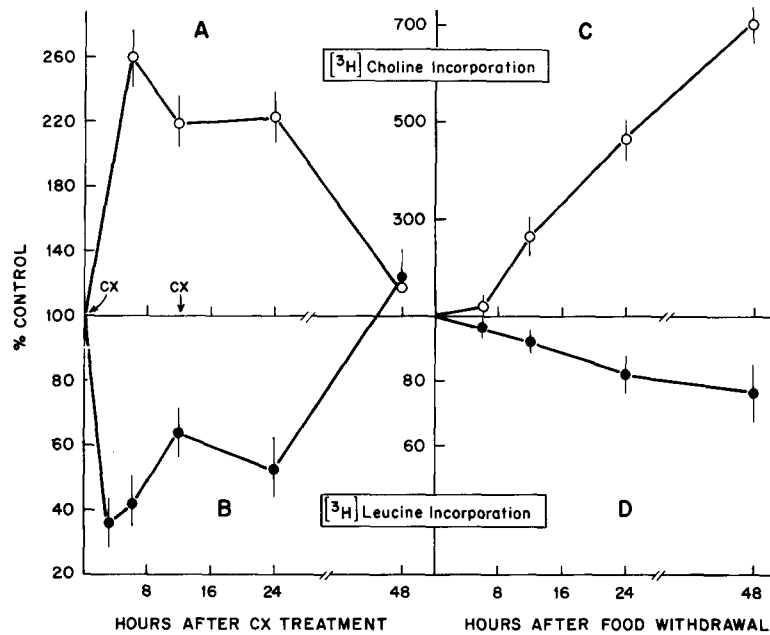

FIGURE 8   The effect of cycloheximide on the incorporation of [³H]leucine and [³H]choline into the total ER of rat liver. [³H]Leucine or [³H]choline at a level of 0.85 μCi/animal (sp act 10 μCi/μmol) was injected intraperitoneally into rats at various times after the intraperitoneal injection of cycloheximide (2.7 mg/kg, 12 h apart). Food was withdrawn at the initiation of the experiment from both untreated and cycloheximide-treated animals. The animals were killed 30 min after exposure to the radioactive materials and the incorporation was determined according to the procedure described in Materials and Methods. Each point represents the mean ± the standard error of separate determinations from three to five rats and is expressed as a percent of untreated nonfasted animals. (A) [³H]choline incorporation into ER after cycloheximide treatment, (B) [³H]leucine incorporation into ER after cycloheximide treatment, (C) [³H]choline incorporation into ER of untreated rats after food withdrawal, and (D) [³H]leucine incorporation into ER of untreated rats after food withdrawal.

cycloheximide; the results are shown in Fig. 8 A and C. Incorporation of [³H]choline into phospholipid increased markedly as a result of the withdrawal of food. Treatment with cycloheximide also caused an increase in the rate of [³H]choline incorporation into phospholipid at 6 h; however, no further increase in the degree of utilization of choline occurred in animals treated with the antibiotic even though food deprivation continued, and by 48 h after cycloheximide the rate of incorporation of [³H]choline into phospholipid was essentially equivalent to that of untreated fed animals.

The effects of cycloheximide and puromycin on some functional parameters of microsomal and mitochondrial membranes of rat liver were measured. The effect of cycloheximide on the phenobarbital-induced formation of a component (NADPH-cytochrome c reductase) of the microsomal mixed function oxidase system was measured and is shown in Fig. 9. The activity of
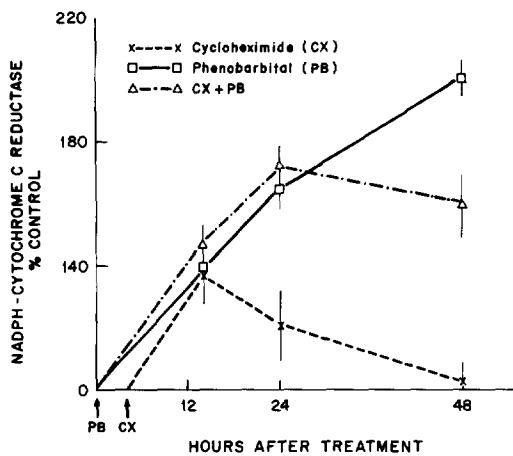

FIGURE 9 The activity of total NADPH-cytochrome c reductase of rats treated with either cycloheximide, phenobarbital, or these agents in admixture. Cycloheximide, 3.5 mg/kg (X---X); phenobarbital, 100 mg/kg (□———□); or the two drugs in admixture (△-·--△) were injected intraperitoneally into rats, and the activity of NADPH-cytochrome c reductase was determined at various times after administration of drugs. When the two drugs were employed in combination, phenobarbital was given first and cycloheximide administered 4 h later. Each point represents the mean ± the standard error of separate determinations from three to five animals. P <0.1 for 12 h cycloheximide compared to untreated control. P <0.1 for 24 h cycloheximide compared to untreated control.

NADPH-cytochrome c reductase was increased slightly initially by cycloheximide and then declined to normal levels by 48 h. Treatment of rats with cycloheximide after administration of phenobarbital did not greatly affect the barbiturate-induced synthesis of NADPH-cytochrome c reductase until 48 h after the barbiturate; this corresponded to the finding that phenobarbital treatment did not affect the formation of membrane whorls by cycloheximide. The increase in NADPH-cytochrome c reductase activity by cycloheximide was confined under these conditions to SER (Table II); this phenomenon again is similar to that produced by phenobarbital in this system. The NADPH-cytochrome c reductase levels in RER, however, were significantly lowered by cycloheximide, but not by phenobarbital. Although the total NADPH-cytochrome c reductase activity of liver was increased by treatment with cycloheximide, the P450 content was considerably decreased (Table II). This suppression of the level of P450 also was accompanied by inhibition of oxidative demethylation and aromatic hydroxylation as shown in Table III. The potentiation of the activities of the microsomal drug-metabolizing enzymes of liver homogenates by succinate, an indication of interaction with mitochondrial metabolism (22, 23), was greatly suppressed by both cycloheximide and puromycin. The mitochondrial respiratory chain was also affected by treatment with inhibitors of protein synthesis. Thus, mitochondria isolated from rat liver 24 h after treatment with cycloheximide tended to lose the intactness of oxidative phosphorylation as shown by a decrease in the P/O ratio and the respiratory control index (RCI). The rate of state 4 respiration was increased in rats treated with both antibiotics (Table III).

DISCUSSION

The administration of sublethal doses of inhibitors of protein synthesis to adult rats produced several definable structural and functional modifications of cellular membranes. The formation of prominent whorls of membranes in hepatic cells was characteristic of animals treated with the group of inhibitors which act only on nascent peptide synthesis on membrane-bound polyribosomes (i.e., cycloheximide and emetine). Inhibitors of protein synthesis such as puromycin, which interferes with nascent peptide synthesis on both free and membrane-bound polyribosomes, and chloramphenicol,

TABLE II

The Effects of Cycloheximide on the Content of Microsomal and Mitochondrial Hemoprotein and NADPH-Cytochrome c Reductase Activity of Rat Liver

	Hemoprotein content*		NADPH-cytochrome c reductase‡ (mmol/mg/min)		
Treatment	Microsomal P450	Mitochondrial a³	SER	RER	Total microsomes
	µM	µM			
None	1.16	0.63	150 ± 15	140 ± 10	145 ± 15
Cycloheximide	0.77	0.19	225 ± 18	60 ± 20	180 ± 10

* Hemoprotein was determined by spectral methods described in Materials and Methods. Cytochrome P450 was measured using the difference spectrum of CO plus dithionite minus dithionite alone in a solution containing 42 mg of liver/ml. The extinction coefficient for 450–490 nm is 91 $mM^{-1} cm^{-1}$. Mitochondrial a³ was determined by difference spectra with an identical concentration of liver; the extinction coefficient for 444–455 nm is 60 $mM^{-1} cm^{-1}$.

‡ NADPH-cytochrome c reductase was assayed by a modification of the method of Omura et al. (20). The assay mixture contained 1 × 10^{-4} M NADPH, 5 × 10^{-5} M cytochrome c, 0.1 M phosphate buffer, pH 7.4, and approximately 30 µg of microsomal protein in a total volume of 1.0 ml. Substrate concentrations were found to be saturating under the experimental conditions employed. Activity was measured at 550 nm with a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a waterjacketed cuvette chamber maintained at 37°C.

TABLE III

The Effect of Cycloheximide and Puromycin on Microsomal-Mixed Function Oxidase Activity of Rat Liver and Its Activation by Succinate

Experiment	Control	Cycloheximide*	Puromycin*
APD‡	222	150	170
APD + succinate§	311	184	245
EMD	235	77	172
EMD + succinate	378	127	271
AH	30	6.5	23
ADP/O (succinate)‖	1.7	1.1	1.3
RCI (succinate)	2.1	1.4	1.9
Rate of state 4 respiration (succinate)	0.06	0.1	0.08

* Two doses of either cycloheximide or puromycin were injected intraperitoneally at a level of 2.5 mg/kg or 100 mg/kg, respectively, 12 h apart and assays were performed at 24 h after the initial dosage.

‡ APD, EMD, and AH represent aminopyrine demethylase, ethylmorphine demethylase, and aniline hydroxylase, respectively. Activities of the first two enzymes are expressed as nmol/6 min/mg protein and the third enzyme, as nmol/10 min/mg protein. Assays were conducted as reported in Materials and Methods. The concentration of the substrates was 21 µmol in a volume of 3 ml of incubation mixture.

§ Succinate (30 µmol) was added to the final incubation mixture (3 ml).

‖ Isolated liver mitochondria were prepared as described in the Materials and Methods. 2 mg of protein were added to 2 ml of an incubation medium consisting of sucrose (0.25 M), $MgCl_2$ (10 mM), Tris-phosphate (5 mM, pH 7.0), and Tris-succinate (10 mM). The temperature was maintained at 25°C and oxygen uptake was monitored with a Clark-type electrode. In the measurement of the ADP/O ratio or the RCI, Tris-ADP (1 µmol) was used.

which alters the mitochondrial protein-synthesizing system, did not produce membranous whorls with the relatively wide range of doses employed or with multiple administrations of the inhibitors. Puromycin and chloramphenicol, however, caused a number of similar structural alterations in the ER, such as dilation of cisternae of the RER, partial degranulation of RER, disaggregation of free polysomes, as well as various degrees of mitochondrial swelling. The production of membrane whorls by cycloheximide and emetine, under conditions in which membrane-bound, but not

free, polyribosomes are prevented from synthesizing protein *in situ*, suggests that the formation of the protein components of membrane whorls may be a function of free polyribosomes. Such membranes are presumably deficient in protein components formed by membrane-bound polyribosomes and, thereby, accumulate as incomplete membranes. Nevertheless, it seems clear that other metabolic alterations can induce whorl formation, since diverse agents such as dimethylnitrosamine, thioacetamide, 3'-methyl-4-(dimethylamino)azobenzene, dioxane, and ethionine can cause the formation of similar structures.

Since similar structures of membranous whorls have also been observed in normal and regenerating processes, such as in embryonic formation of egg membrane during vitellogenesis of *Lytla mutall* (31), hypertrophic kidney (32), regenerating liver (33), basal cytoplasm of pancreatic acinar cells (34), parathyroid cells (35), and sympathetic ganglion cells (36), it is conceivable that whorl-like membrane structures may be indicative of a normal transient stage of membrane formation and/or membrane repair whose progression has been arrested by treatment with either cycloheximide or emetine.

During the early period after administration of cycloheximide the integrity of the membrane-forming system is altered by a decrease in protein formation and concomitant increase in phospholipid synthesis, as measured by the incorporation of [^{3}H]leucine and [^{3}H]choline, respectively, into the total ER fraction (Fig. 8). The observed reciprocal pattern of the biosynthesis of protein and phospholipid was maintained throughout the entire period of whorl formation. An increase in phospholipid synthesis appears to be involved in the induced proliferation of SER (37). During the period in which disappearance of whorls occurred, the biosynthetic pattern reversed, i.e., leucine incorporation into protein increased and choline incorporation into phospholipid decreased. These findings suggest that formation of the membrane whorls is linked to a desynchronization of the nascent synthesis of protein and lipid components of membranes or other biochemical lesions involved in lipid-protein interaction. Since cycloheximide inhibits protein synthesis about 40–60% (Fig. 8) through the entire process of whorl formation and phospholipid synthesis appears to be enhanced, it is to be expected that the membrane whorls will be in a relative state of protein

deficiency and rich in phospholipid. The formation of this incomplete membrane caused by treatment with cycloheximide, however, may be due to the perturbation of other factors which regulate the functional integrity of membranes, such as the pool of membrane precursor proteins or lipids in the cytoplasm, the rate of turnover of membrane components (20, 38–40), and the function of lysosomes (41).

During the period in which the structure of microsomal membranes is altered, a series of changes in membrane-associated functions exist. This alteration of membrane function is not due to a general destruction of ER membrane, since some of the ER remained either relatively unchanged or contained larger amounts of components such as NADPH-cytochrome *c* reductase; some components and activities, however, are strongly decreased, such as oxidative hydroxylation and demethylation activities and cytochrome P450. These findings suggest that cycloheximide exerts differential inhibition of the synthesis of membrane components of the ER. The fact that only the fraction of NADPH-cytochrome *c* reductase located in RER was apparently susceptible to inhibition by cycloheximide, and that cycloheximide only inhibited nascent peptide synthesis on membrane-bound polyribosomes (12) suggests that the NADPH-cytochrome *c* reductase present in the RER is synthesized by the membrane-bound polyribosomes, while enzyme molecules existing in the SER may be formed on the free polyribosome synthetic machinery. This suggestion is in accord with the findings which indicate that NADPH-cytochrome *c* reductase is synthesized on both membrane-bound and free polyribosomes (42, 43), but is incompatible with the concept that envisions that the reductase enzyme is exclusively synthesized on the RER, is then incorporated into the membrane nearest to the site of its synthesis, and is reversibly transferred to SER (44–47).

Recently, Schenkman and his associates (22, 23) demonstrated the existence of an interaction between hepatic mitochondria and microsomes. This interaction is based on the observation that drug metabolism by the microsomal mixed function oxidase system is partially controlled by an as yet undefined flow of reducing equivalents or energy, produced by the oxidation of some intermediates of the citric acid cycle, to the microsomal electron transport system. The present observations provide further support for the concept that interac-

tion between these two cellular compartments exists. Cycloheximide appears to attack both organelles, since oxidative phosphorylation and oxidative drug metabolism linked to the electron transport system in both organelles are affected. At present, it is not known whether inhibition of the oxidative processes of these two organelles is due to concomitant or sequential events.

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