THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 38, Issue of September 20, pp. 23105–23110, 1996

Printed in U.S.A.

Effect of Oxidative Stress, Produced by Cumene Hydroperoxide, on the Various Steps of Protein Synthesis

MODIFICATIONS OF ELONGATION FACTOR-2*

(Received for publication, April 15, 1996, and in revised form, June 19, 1996)

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We have studied the effect of oxidative stress on protein synthesis in rat liver. Cumene hydroperoxide (CH) was used as an oxidant agent. The approach used was to determine the ribosomal state of aggregation and the time for assembly and release of polypeptide chains in the process of protein synthesis in rat liver *in vivo*. The results suggest that the elongation step is the most sensitive to CH treatment. The measurement of both carbonyl groups content and ADP-ribosylatable elongation factor 2 (EF-2), the main protein involved in the elongation step, indicates that under CH treatment EF-2 is oxidatively modified and a lower amount of active EF-2 is present. These results are corroborated by *in vitro* oxidation of EF-2 and could explain for the decline in the elongation step.

The effect of oxidative stress on protein metabolism have been reported by various laboratories (1–13). Most of these studies have focused on protein degradation, the major finding being that oxidatively modified proteins become susceptible to proteolytic digestion (2, 4–7, 9–13). With respect to protein synthesis, the effect of active oxygen species has been less studied and has been addressed by using oxidizing agents (14–16). One of such compounds is cumene hydroperoxide (CH),[1] which has been used as an intracellular source of reactive oxygen intermediates (17–22). Besides its damaging effects such as membrane damage, cell lysis, organ necrosis, tumor promotion (20), and certain aspects of aging (23), it has been reported that this compound and/or cytotoxic aldehydes derived from it inhibit protein synthesis in human skin fibroblasts (14). These *in vitro* results were obtained by measuring the incorporation of radioactive amino acid precursors into proteins, a methodology which is not suitable when using whole animal systems because of the difficulty in measuring the specific activity of the amino acid precursor pool (23). Furthermore, a detailed molecular mechanism of the decline of protein synthesis caused by the oxidant is not available.

Because of the potential importance of decreased protein synthesis in structural and functional deterioration of the cell, the molecular basis for decreased rate of protein synthesis by CH, and hence by oxidative stress, is of interest, especially since this decrease may be an important contributor to certain processes in which free radicals seem to be involved, such as aging (25).

In order to understand the mechanism underlying the inhibition of protein synthesis by CH, we have studied: 1) which of the individual steps in polypeptide synthesis is most affected, and 2) the possible mechanism of how active oxygen species may interact with the protein synthetic machinery.

The effect of CH on the stages of protein synthesis has been studied by investigating the ribosome half-transit time (elongation time) required for the synthesis of an average half-length of a nascent polypeptide chain (26), and the ribosomal state of aggregation (27, 28), which is reflected by the polyribosomal profiles.

The results show that the CH treatment for 7 days produced a decrease in the rate of peptide chain elongation along with a higher state of polyribosome aggregation in rat liver. These findings suggest that the elongation step is the more sensitive to CH treatment. The observed decline in the rate of elongation step seems to be produced by modifications of the EF-2, the main protein involved in this process.

MATERIALS AND METHODS

Experimental Animals and Treatment—Female Wistar rats, 200 g body weight, were used in all the experiments. CH (Sigma, 80%) was dissolved in saline and the solution was sonicated for 5 s at 50 watts. CH-treated rats received an intraperitoneal injection of CH (35 mg/kg body weight/day) at 10 a.m. for 7 days. The control group received NaCl injection. We followed the weight of each animal and found that the rate of increase was similar for all rats used.

Determination of Liver Polyribosomal Profiles—Liver polyribosomal profiles were determined basically as described previously (27, 28). A portion of liver was homogenized in 2 volumes of a media containing 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose (pH 7.3). The homogenate was centrifuged at 20,000 × *g* and 2.3 ml of the supernatant were mixed with 0.3 ml of 20% sodium deoxycholate and 5.2 ml of 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl, 2.3 M sucrose, 7 mM 2-mercaptoethanol (pH 7.3). This mixture was layered over 3.4 ml of a solution containing 2.3 M sucrose, 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl (pH 7.3), and 0.6 ml of a 105,000 × *g* supernatant obtained by 2 h ultracentrifugation of control liver homogenate (1:2) in 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose (pH 7.3). The preparation was centrifuged for 16 h at 150,000 × *g* in the Beckman rotor 65. The pellet was gently suspended in a medium containing 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl (pH 7.3) and centrifuged at 12,000 × *g* in a microcentrifuge. The absorbance of the suspension at 260 nm was monitored and a volume containing 4 units of OD was layered on 10 ml of a 20–40% linear sucrose gradient made up in the same buffer. The gradient was centrifuged in an SW 40.1 Beckman rotor for 35 min at 35,000 rpm and pumped from the tube using a gradient unloader (Nycomed Pharma). The absorbance at 254 nm was continuously recorded using a flow-through cuvette. A blank obtained by measuring the absorbance at 254 nm of 10 ml of the 20–40% sucrose gradient was subtracted from all

* This work was supported by Grant FIS 96-1142 from Ministerio de Sanidad y Consumo. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CH, cumene hydroperoxide; EF-2, elongation factor 2; DT, diphtheria toxin; MDA, malonaldehyde; TBA, thiobarbituric acid; Pn, polyribosomes; Pt, peptides.

polyribosomal profiles. The area under the curve in polyribosomal pattern was quantified using a laser computing densitometer.

Measurement of Incorporation of Amino Acid into Liver Protein—The procedure for the determination of radioactivity in total and nascent proteins was similar to the one described previously (26, 29) and was as follows. The animals were anesthetized with pentobarbital (5 mg/100 g weight). The abdomen of the rat was opened along the ventral midline. The hepatic portal vein was exposed by gently shifting the intestines within the abdominal cavity, and an injection of 20 μCi of L-[3,4-^{3}H]valine in 0.2 ml of NaCl solution was made over a period of 30 s. The needle was kept in the vein to prevent bleeding. Small liver biopsies were taken 0.5, 1, 1.5, and 2 min after the completion of the amino acid administration, taking only two biopsies from each liver (at 0.5 and 1.5 min or at 1 and 2 min). To prevent hemorrhage, liver lobules were ligated immediately before biopsies were taken. Liver samples were frozen by aluminum clamps pre-cooled in liquid nitrogen and stored at this temperature until they were homogenized in 3 volumes of 0.3 M sucrose. A 1.5-ml portion of the homogenate was diluted with 1.5 ml of a medium containing 0.1 M Tris/HCl (pH 7.6) and 2 mM magnesium acetate. The mixture was layered over 1 ml of 0.5 M sucrose containing 1 mM magnesium acetate and centrifuged at 4,000 $\times$ *g* for 15 min. Two ml of the supernatant were mixed with 200 μl of 20% sodium deoxycholate. After 30 min at 4 °C, the solution was centrifuged at 8,000 $\times$ *g* for 15 min. The resulting supernatant was used to estimate the radioactivity of total liver protein and the ribosomal nascent polypeptide chains.

To measure radioactivity of total liver protein, 0.5 ml of the supernatant was mixed with 3 ml of 10% trichloroacetic acid. After sedimenting at 8,000 $\times$ *g* for 10 min, the pellet was washed twice with 3 ml of 10% trichloroacetic acid. After the last wash, the pellet was resuspended in 3 ml 10% trichloroacetic acid and heated for 30 min at 90 °C. The precipitate collected by centrifugation was resuspended successively in 3 ml of ethanol/chloroform/ether (2:2:1, v/v), acetone and ether. The washed pellet was left at room temperature until the ether had evaporated. The dry pellet was suspended in 0.5 ml of protosol, allowed to stand overnight at 60 °C, and after adding 17 μl of acetic acid, transferred to a vial containing 10 ml of toluene-2,5-diphenyl oxazol scintillation mixture.

The incorporation of radioactive amino acid into nascent chains was determined using 1.5 ml of supernatant. This volume was layered over 6 ml of a medium containing 1 M sucrose, 1 mM magnesium acetate, and 1 ml of a 105,000 $\times$ *g* supernatant obtained by 2 h ultracentrifugation of control liver homogenate (1:2) in 50 mM triethanolamine, 5 mM $MgCl_2$, 25 mM KCl, and 0.25 M sucrose (pH 7.3). The mixture was centrifuged at 28,000 rpm for 14 h in a Beckman 42 Ti rotor at 0 °C. The pellet was resuspended in 1 ml of distilled water. This suspension was treated as already described for the determination of radioactivity in total proteins.

Determination of Radioactivity in Plasma—To confirm that the injection of radioactive valine had been done properly, a sample of blood was taken from the heart. Aliquots of 10 μl of serum were transferred to vials containing 10 ml of Formula-989 liquid scintillation mixture (NEN Research Products) for the determination of radioactivity. An average of 2,200 cpm was obtained.

Determination of Hepatic Valine-specific Radioactivity—Fifty μl of homogenates from liver were deproteinized with 1 ml of 20% trichloroacetic acid and the precipitate was removed by centrifugation. Aliquots of the supernatant were transferred to vials containing 10 ml of toluene-based scintillation mixture for the determination of radioactivity.

Determination of Malonaldehyde (MDA) with Thiobarbituric Acid—A portion of liver tissue was homogenated in 5 volumes of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20% glycerol, 0.2 mM dithiothreitol and 0.3 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000 $\times$ *g*. The supernatant was centrifuged again at 105,000 $\times$ *g*. The pellet was gently suspended in homogenization buffer. The sample (liver microsome suspension or 10,000 $\times$ *g* supernatant) was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was used for MDA determination (30).

Production of Polyclonal Antibodies against EF-2—A synthetic peptide (H-CGTRFTDTRKDEQGC-NH_2) corresponding to a particular region of EF-2 (Chiron Mimotopes Peptide Systems, Australia) was used to prepare rabbit polyclonal antibodies against EF-2. The synthetic peptide was coupled to keyhole limpet hemocyanin using a conjugation kit of Pierce. The specificity of the polyclonal antibody was tested by immunostaining of nitrocellulose membranes after electrophoretic transfer of rat liver homogenates. A Western blot of an SDS-gel electrophoresis of liver homogenate yielded a single band at the expected molecular mass of 100 kDa.

Quantification of EF-2—Concentration of EF-2 in the cell-free systems was measured by an enzyme-linked immunoabsorbent assay. A sample of liver extract was diluted in carbonate-bicarbonate buffer (pH 9.0) (100 μg of protein/ml). The wells of a Nunc microtiter plate were coated, in triplicate, with 100 μl and incubated overnight at 4 °C. Unbound sites were blocked with 200 μl of 3% (w/v) bovine serum albumin-Tween. After rinsing, 100 μl of anti-EF-2 solution diluted 1/100 in phosphate-buffered saline-Tween were added to each well and incubated for 1 h at room temperature. The plate was then washed as before and incubated for 1 h at room temperature with a mouse anti-rabbit Ig-peroxidase. After 1 h of incubation at room temperature, 100 μl of substrate solution (10 mg of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)] diammonium salt dissolved in 100 ml of citrate buffer (pH 5.8) containing 0.02% hydrogen peroxide) were distributed into each well. The reaction was left to develop for 60 min at room temperature, in the dark.

Elongation Factor Assay—A sample of liver was homogenated in 3 volumes of homogenization buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 0.25 M sucrose, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was shaken vigorously in charcoal for 5 min to remove endogenous NAD. Charcoal was removed by centrifugation (15,000 $\times$ *g*, 20 min) and the supernatant was centrifuged again (27,000 $\times$ *g*, 15 min) to remove cellular debris and to ensure elimination of all charcoal. All operations were performed at 0–4 °C.

The amount of "active" EF-2 was determined in liver extracts as described previously (31, 32). Briefly, the assay was performed in a final volume of 500 μl in a buffer of 20 mM Tris-HCl, 50 mM dithiothreitol, and 1 mM EDTA, containing 10 μg of diphtheria toxin (DT), 0.18 M histamine, and 0.1 mM [^{14}C]NAD (0.1 μCi). The reaction was started by mixing in an aliquot of liver extract and the assay mixture was incubated 90 min at 37 °C. The reaction was stopped by adding 1 ml of cold 10% perchloric acid. After centrifugation, the radioactivity incorporated into the protein pellet was determined as described above.

Determination of Carbonyl Groups of Proteins in Homogenates—Carbonyl groups were determined by measurement of incorporation of tritium into proteins after reduction with $NaB^3[H]_4$ as described by Lenz *et al.* (33).

Determination of Carbonyl Groups of EF-2—A sample of hepatic 105,000 $\times$ *g* supernatant was labeled with radioactive sodium borohydride (33). After incubation for 30 min at 37 °C, samples were extensively dialyzed in a Microdialyzer system 500 (Pierce) using a 12,000 M_r cut-off dialysis membrane. Polyclonal antibody against EF-2 was incubated with protein A-Sepharose CL-4B (Sigma) for 4 h at 4 °C. The complex was washed three times in 10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, and 0.1% SDS (RIPA buffer). An aliquot of the radiolabeled sample was incubated with the complex protein A-antibody overnight at 4 °C. The samples were centrifuged in a microcentrifuge and the immunoprecipitate was washed three times with RIPA buffer. Radioactivity incorporated into EF-2 was determined using 10 ml of Ready-Protein liquid scintillation mixture (Beckman Instruments).

"In Vitro" Study of CH Effect on Carbonyl Groups and Ribosylatable Diphthamide Content of EF-2—Rat liver homogenate (105,000 $\times$ *g* supernatant) was treated with 1 mM CH for 90 min. After this, CH and endogenous NAD were removed by extensive dialysis. Carbonyl group content of EF-2 was determined using tritiated sodium borohydride (33). For the measurement of ribosylatable-diphthamide content of EF-2, the control and oxidized sample were treated with radioactive NAD plus DT. Radioactivity incorporated into EF-2 was determined as described above.

RESULTS

Oxidative Effect of CH Treatment on Rat Liver—CH is an oxidant, which has been used to assess the effects of free radicals and reactive oxygen intermediates on various biological molecules (17–22). In the present study, the oxidative effect of CH has been determined by measuring MDA and carbonyl groups content in liver extracts. Aldehydes are always produced when lipid hydroperoxides break down in biological systems (29), and MDA is the most abundant individual aldehyde resulting from lipid peroxidation. Its determination by thiobarbituric acid is one of the most common assays in lipid peroxidation studies (30). In addition, the carbonyl groups content in proteins is used as an index of protein oxidative damage (33).

CH treatment produced a significant increase in MDA of 2.7-

TABLE I
Effect of CH treatment on MDA level and carbonyl groups content of proteins and EF-2

MDA was determined by thiobarbituric acid reactive substances. Carbonyl groups in total protein were determined using radioactive sodium borohydride. See "Materials and Methods" for details. Numbers in parentheses indicate number of rats in the group.

	Control	CH
MDA (nmol/mg protein)		
10,000 × *g* supernatant	22.4 ± 2.3 (5)	53.2 ± 18.0[a,b] (5)
Microsomes	28.8 ± 10.5 (5)	97.4 ± 9.6[a,c] (5)
CO content in proteins (nmol CO group/mg protein)		
10,000 × *g* supernatant	155.2 ± 8.1 (5)	249.0 ± 10.7[a,d] (5)
Microsomes	408.5 ± 105 (5)	831.9 ± 37.8[a,e] (5)

[a] Significantly different from level in control rats (multifactor ANOVA followed by Tukey's test, $p < 0.05$).
[b] $F = 32.43$, $p = 0.0047$.
[c] $F = 17.36$, $p = 0.0141$.
[d] $F = 35.4$, $p = 0.0095$.
[e] $F = 14.18$, $p = 0.0197$.

and 3.4-fold in 10,000 × *g* supernatant and microsome fraction, respectively (Table I). A significant increase of carbonyl groups content of liver proteins was also found in both subcellular fractions (Table I).

Effect of CH Treatment on Polypeptide Chain Completion Time—Polypeptide chain completion time was calculated assuming that after injection, the radioactive amino acid will meet the ribosomes in the middle of the translation of a mRNA of average size. When a full cycle is completed, the whole peptide on the ribosomes will be labeled, while the chains that have been terminated and released will be only half-labeled. Thus, the ratio between the radioactivity incorporated into nascent peptides in the polyribosomes (Pn) and that incorporated into total peptides (Pt) will be reduced by 50%, and the time required to reduce Pn/Pt from 50 to 25% is taken as the average polypeptide chain completion time, T_c (26–29). Changes in T_c reflect changes in elongation rate and termination. This seems to be true in as far as the specific radioactivity of the precursor does not change over the 2-min period (28, 29). In our experiments, the total acid-soluble valine specific radioactivity was determined at each experimental time. The results were 368.7 ± 17.8 cpm for control rats and 356.7 ± 19.11 cpm for CH-treated rats (mean ± S.E. of 10 animals), with no significant changes at different times between 30 and 120 s. These small variations were not statistically significant as determined by ANOVA, followed by Tukey's test.

In our experiments, T_c was obtained directly from the slope of a plot of Pn/Pt *versus* time. Fig. 1 summarizes the values of hepatic Pn/Pt at different times after injection of the tracer in control (Fig. 1*a*) and CH-treated rats (Fig. 1*b*). The value Pn/Pt was reduced from 50 to 25% in 60 s in the normal liver and in 101 s in the CH-treated one (see legend to Fig. 1).

Effect of CH Treatment on Hepatic Polyribosomal Profiles—The polypeptide chain completion time is an expression of the rate of the peptide chain elongation and termination steps. It is independent of the number of ribosomes engaged in the process and it should not be affected by variations in the initiation step (28). However, in order to determine whether the effect observed by CH treatment was accompanied by a significant effect of the initiation step, the ribosomal state of aggregation was determined by studying the hepatic polyribosomal profiles in control and CH-treated rats.

As shown in Fig. 2, CH treatment increased the ribosomal state of aggregation (Fig. 2*b*) with respect to controls (Fig. 2*a*).

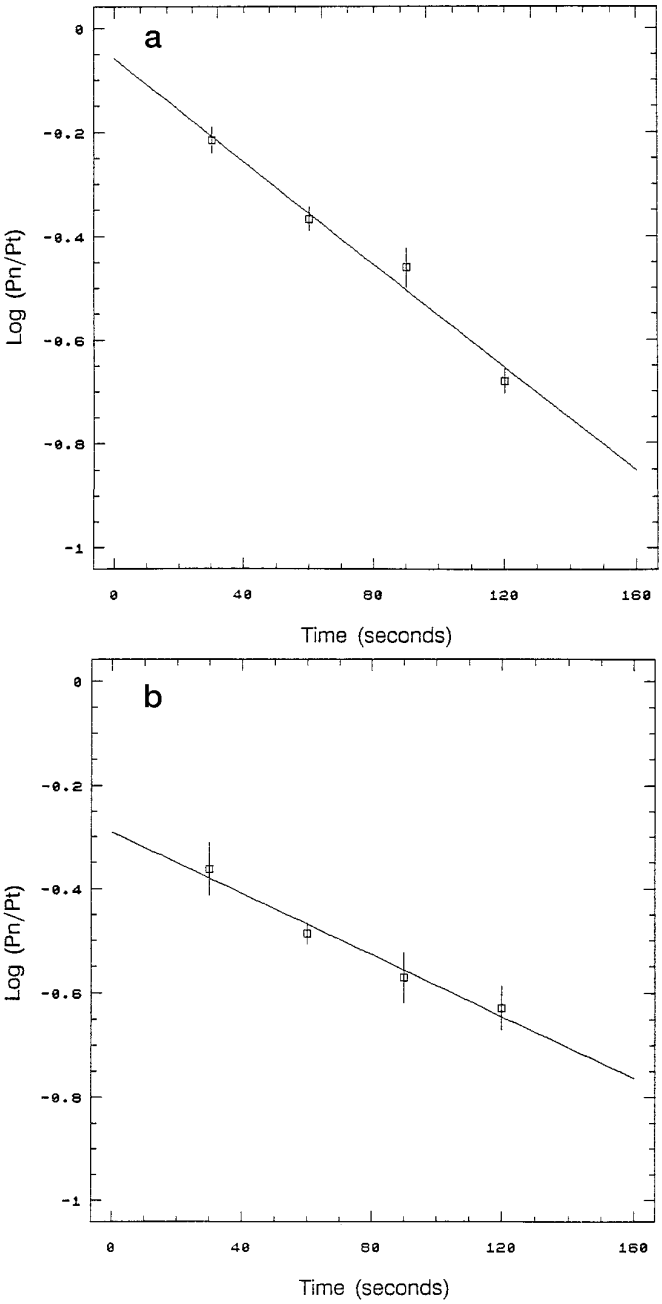

FIG. 1. **Effect of CH on the rate of decay of the ratio between the radioactivity incorporated into hepatic nascent peptides and the radioactivity incorporated into hepatic total peptides (Pn/Pt) in control (*a*) and CH-treated rats (*b*).** Each point represents the mean ± S.E. of four rats. T_c (seconds) was obtained from the slope of the plot (see "Materials and Methods" for details). T_c in control, 60 s; T_c in CH-treated rats, 101 s.

An increase of 69% in the ratio polysomes/monomers was observed in CH-treated rats (see legend to Fig. 2). These results indicate that CH treatment acts preferentially on the elongation or termination step, or both.

Effect of CH Treatment on the Amount of Absolute and Active EF-2 and on Its Oxidation State in Rat Liver—We have measured the total content of EF-2 using the polyclonal antibody against EF-2. As can be seen in Table II, there is no change in the total amount of EF-2 in CH-treated rat with respect to control rats. Moreover, we have used the diphtheria toxin-mediated 1:1 stoichiometric ADP-ribosylation of the diphthamide residue of EF-2 in liver extract in the presence of radioactive NAD, to determine the amount ADP-ribosylatable EF-2

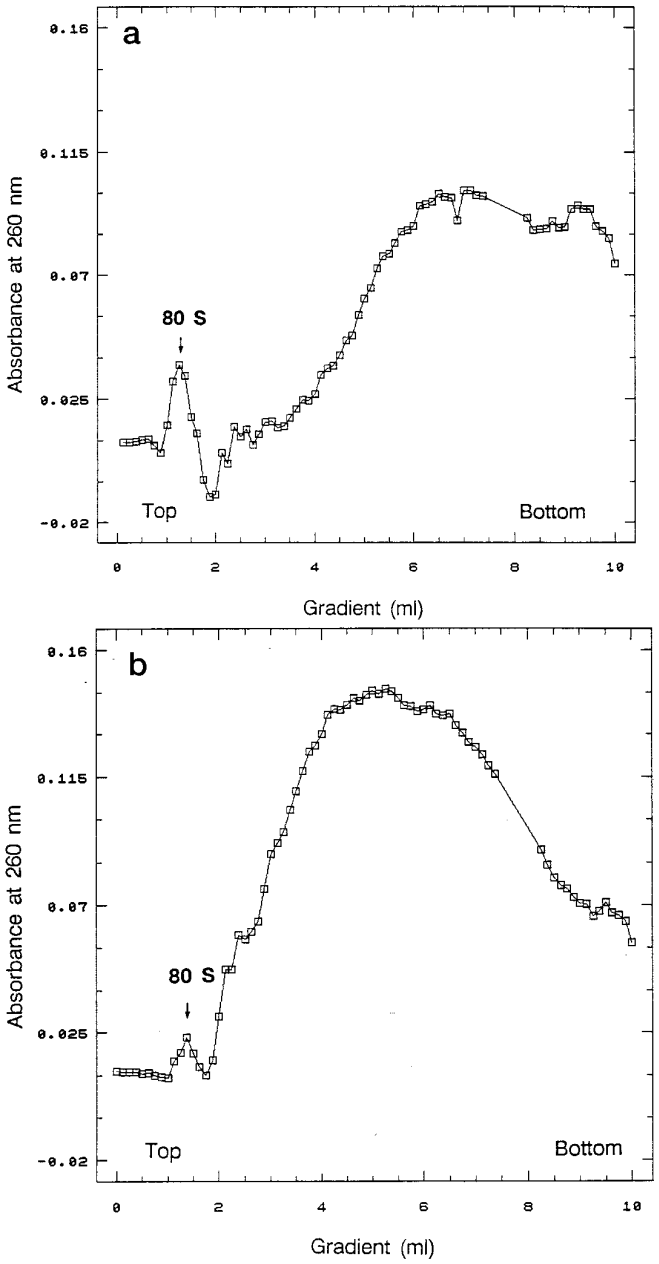

FIG. 2. **Sucrose gradient analysis of ribosome preparations from livers of control (*a*) and CH-treated (*b*) rats.** The polyribosomal profiles were obtained as described under "Materials and Methods." The ratio polysomes/monomers was calculated as described under "Materials and Methods." This ratio was 17.25 ± 2.9 in control rats and 29.30 ± 2.8* in CH-treated rats. These results are means ± S.E. of five animals. *, significantly different from level in control rats (multifactor ANOVA followed by Tukey's test, $p < 0.05$; F = 13.37, $p = 0.0106$).

and, hence, of active EF-2 (31, 32). Our results show that the amounts of ADP-ribosylatable EF-2 are reduced significantly (32% reduction) during the treatment of CH as measured by the content of diphtheria toxin-mediated ADP-ribosylatable EF-2 (Table II).

CH treatment also modified the oxidation state of EF-2. For this experiment a polyclonal antibody against EF-2 was used. The carbonyl content of immunoprecipitated EF-2 from rats treated with CH was significantly increased (2-fold) (Table I), which suggests that EF-2 is highly affected by oxidative damage.

In Vitro Effect of CH on EF-2 Present in Control Liver Homogenate—A control experiment was done to study the effect of

TABLE II
Effect of CH treatment on the amount of total and active EF-2 and on its oxidation state

Total amount of EF-2 was measured by ELISA. Active amount of EF-2 was measured using radioactive NAD plus DT. Carbonyl groups in EF-2 were determined using radioactive sodium borohydride. Results are means ± S.E. of 4–5 rats. See "Materials and Methods" for details.

	Control rats	CH-treated rats
Amount of total EF-2 (absorbance @ 405 nm)	0.173 ± 0.010	0.197 ± 0.016
ADP-ribosylatable EF-2 (pmol/mg protein)	13.54 ± 1.48	8.92 ± 1.03[a,b]
CO content in EF-2 (nmol CO group/mg protein)	3.58 ± 0.53	5.60 ± 0.39[a,c]

[a] Significantly different from level in control rats (multifactor ANOVA, Tukey's test, $p < 0.05$).
[b] F = 6.96, $p = 0.0386$.
[c] F = 85.46, $p = 0.0008$.

CH on control EF-2 present in liver homogenates. As shown in Fig. 3, pretreatment with CH resulted in a 45% decreased incorporation of [^{14}C]ADP-ribose, which suggests that CH specifically produced a decrease in the amount of active EF-2. In addition, treatment of rat liver homogenates with CH produced a 1.8-fold increase in the carbonyl group content of EF-2.

DISCUSSION

The present study was undertaken to elucidate the mechanism of protein synthesis inhibition produced by the oxidative stress mediated by CH. CH is an oxidant that has been used as a model compound to assess the effect of oxidative stress on various biological systems (17–22). The evidence that CH produces oxidative damage is that treatment with CH resulted in an increase of MDA, as measured by thiobarbituric acid-reactive substances (Table I). This result agrees with previous reports (34, 35). In addition, CH produced oxidative modification of proteins (Table I) as the carbonyl groups content increased.

Besides the damaging effect of CH on biological molecules, it has been reported that CH inhibits protein synthesis in cell cultures (14, 15). These reports were based on the incorporation of radioactive amino acid into proteins. The application of such a method "*in vivo*" is more limited because of the lack of reliable information on the specific activity of the precursor pool *in vivo* (36–39). In order to overcome this limitation when studying the effect of CH on the various steps of protein synthesis, we have determined the average polypeptide chain assembly time along with the study of the polyribosomal profiles. The time constant for assembly and release of polypeptide chains in rat liver *in vivo* has been determined by a technique described previously (26–29), which is independent of most sources of variation and error in amino acid incorporation experiments. Valine was selected as the amino acid for measurement of protein synthesis because of the poor capacity of the hepatic cells to metabolize it (40). Our results show that the time required to reduce Pn/Pt from 50 to 25% was 1.7-fold higher in CH-treated rats (Fig. 1). This increase in T_c would cause a 40% drop in protein production per unit time if all other factors were equal. These results indicate that CH treatment affects the elongation and/or termination step.

The possible involvement of initiation step has been addressed by studying the ribosomal pattern of aggregation. Under normal conditions, initiation and elongation are synchronized in such a way that any perturbation in only one of them would produce variations in the ribosomal pattern of aggregation (28, 29). Thus, inhibition of peptide chain initiation would produce an accumulation of monomeric ribosomes and polyribosomal breakdown (28). When only the elongation process is inhibited (or it is inhibited to a greater extent than initiation),

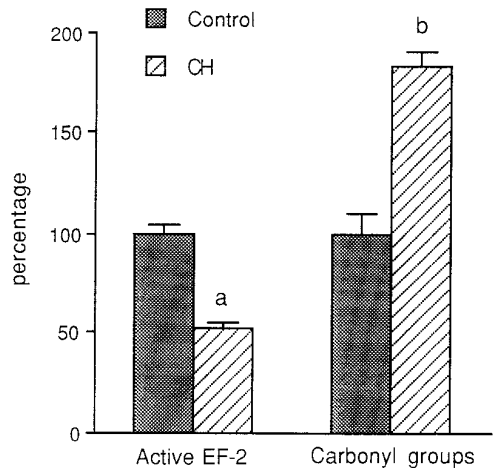

FIG. 3. ***In vitro* effect of CH on the level of ribosylatable control EF-2 and carbonyl groups content.** The results are expressed as percentage of control and are means ± S.E. of six experiments. Active EF-2 was determined as described under "Materials and Methods" and is expressed as picomole of EF-2/mg of proteins. Carbonyl groups content was determined using radioactive sodium borohydride and is expressed as nanomole of CO/mg of protein. *a*, significantly different from level in control homogenate (multifactor ANOVA followed by Tukey's test, $p < 0.05$, $F_{(1,7)} = 91.05$, $p = 0.0024$). *b*, significantly different from level in control homogenate (multifactor ANOVA followed by Tukey's test, $p < 0.05$, $F_{(1,7)} = 23.52$, $p = 0.0085$).

an increased proportion of polyribosomes and a depletion of the monomeric ribosome pool is obtained (28). No change of the ribosomal aggregation patterns would be found if elongation and initiation are completely inhibited. In our case, the polyribosomes displayed a higher state of aggregation in CH-treated rat liver (Fig. 2). These results indicate that changes in peptide chain elongation may be an important mechanism in altering the rate of protein synthesis by CH. The possibility that the chain release mechanism may be rate-limiting (and hence, modulated by CH) cannot be excluded by the model used for determination of T_c.

EF-2 is an essential factor involved in the regulation of the elongation step of protein synthesis (41–43). In order to study the possible implication of EF-2 in causing the decline of elongation step by CH, we studied the effect of CH treatment on the amount of total EF-2 in liver of control and CH-treated rats. No change in total EF-2 was observed in CH-treated rat with respect to control rats (Table II). This result indicates that the observed decline in protein synthesis rate is not due to a decrease in the steady-state level of EF-2, and suggests that changes in protein synthesis and the elongation step could be due to post-translational modification of EF-2. In order to test this possibility, we have studied the amount of active EF-2 and its oxidation state.

EF-2, which catalyzes the translocation of peptidyl-tRNA from the site A to the site P of the ribosome, contains an unusual amino acid named diphthamide, whose importance for the function of EF-2 has been reported (43, 44). The diphthamide residue is specifically ADP-ribosylated by fragment A of DT, which catalyzes the transfer of ADP-ribose from NAD, thus inactivating it (42, 44). The level of ADP-ribosylatable EF-2, which has been used as an indirect measurement of the amount of active EF-2 in extracts (31, 32), can be determined by using the assay of ribosylation of the diphthamide mediated by DT. Although the diphthamide residue as such is not essential for the function of EF-2 in protein synthesis, it has been suggested that the role of this residue is to allow the cell to regulate the rate of elongation by ADP-ribosylation (43, 45–51).

A statistically significant reduction of the amount of ADP-ribosylatable EF-2 (32% reduction) is observed during the treatment of CH (Table II). At the same time, our results show that hepatic EF-2 from rats treated with CH was oxidatively damaged, as indicated by the increase in the carbonyl groups content (Table II). Although the oxidative modification may occur in a region of the EF-2 remote from the catalytic site, this result does prove that EF-2 is oxidatively modified by CH treatment, although it does not prove that EF-2 is inactivated.

Taken together, these results show that the inhibition of protein synthesis by CH could be produced by changes in the elongation step and that this decline in the elongation step could be due to the post-translational modifications of EF-2, which result in an increased oxidative damage and less active form of EF-2.

In order to relate the post-translational modifications found *in vivo* with the oxidative stress damage, a control experiment was carried out to test the *in vitro* effect of CH on control EF-2 (Fig. 3). The result of this experiment showed that CH also affects the level of ribosylatable control EF-2 and carbonyl groups content in a similar manner to that found *in vivo*.

The reason for the decrease in the amount of active EF-2 observed in CH-treated rats can be due to various factors: 1) a chemical modification of diphthamide by oxyradicals, which would abolish the DT-mediated ribosylation; 2) an activation of endogenous ribosylation of the diphthamide by ribosyltransferases. This ribosylation seems to regulate the rate of protein synthesis as part of normal cellular metabolism (46–51) and could be activated by oxidative stress. 3) EF-2 amino acid modification by oxidation: EF-2 has a histidine, which is essential for its activity (44). Because histidine is one of the amino acid residues most susceptible to oxidative damage (52–53), it is possible that free radicals produced by CH could affect the activity of EF-2 through a mechanism involving histidine inactivation by oxidation. 4) conformational changes in EF-2 induced by oxidative modification mediated by free radicals.

The second possibility is not unreasonable considering a previous report showing that oxidative stress activates protein ADP-ribosylation in the catabolism of one major route of pyridine nucleotide (54). If this is the case, ADP-ribosylation activated by CH could be responsible for the decline of the elongation step, as this reaction is a nonreversible phenomenon. Although the ADP-ribosylation does not distinguish between active and inactive EF-2 for reasons other than ADP-ribosylation, such as phosphorylation, it has been suggested by Bodley's group (43, 55–57) that diphthamide residue serves as the recognition element on EF-2 for the calmodulin-dependent protein kinase. Therefore, a lower level of diphthamide could also affect the regulation of the activity of EF-2 by phosphorylation.

Acknowledgment—We are grateful to Dr. Ayuso-Parrilla for valuable information about the methodology.

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Effect of Oxidative Stress, Produced by Cumene Hydroperoxide, on the Various Steps of Protein Synthesis: MODIFICATIONS OF ELONGATION FACTOR-2

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J. Biol. Chem. 1996, 271:23105-23110.
doi: 10.1074/jbc.271.38.23105