Studies on Carbon Tetrachloride Intoxication. III. A Subcellular Defect in Protein Synthesis*

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ABSTRACT: Exploration of the intracellular defect in protein synthesis in rat liver occurring with carbon tetrachloride intoxication was carried into the subcellular apparatus for protein synthesis. It was found that microsomes and ribosomes derived from the livers of rats treated with carbon tetrachloride, when placed in a suitable reconstituted system, have a substantially reduced capacity to incorporate amino acids. The reduced capacity for protein synthesis is a property of the ribosome particle. This appears to be so for the following reasons: Treated animals show no evidence of a reduction in the capacity of s-RNA to accept amino acid, nor reduction in the activating or transfer enzyme activity.

Ionic and cofactor requirements optimal for amino acid incorporation by the ribosomes of treated and control animals are the same. Structurally there is a shift in the pattern of the ribosomes with reduction in the 80 S component, a comparable increase in the 54 S subunits, but no appearance of the 30 S component. The changes in the ribosomes, demonstrable both by measurement of amino acid incorporation and the sedimentation pattern, can be elicited by very low doses of CCl_4 (0.07 ml/100 g body weight) and within a very short time interval (<1 hour). The facts that the changes are so diffuse, occur so early, and at such low doses of CCl_4 are consistent with the hypothesis that the disorder induced in the intracellular apparatus for protein synthesis is one of the very early events in the pathogenesis of CCl_4 injury to the liver. The fact that addition of CCl_4 to polysome preparations does not reproduce the functional and structural changes implies that direct action by solvation is not the explanation of the observed effect.

The earliest morphological change which has been detected following administration of carbon tetrachloride to rats and other laboratory animals is a disorganization of the arrangement of ribosomes on the coarse endoplasmic reticulum (Oberling and Rouiller, 1956; Bassi, 1960; Smuckler *et al.*, 1962; Smuckler and Barker, 1964; Smuckler and Benditt, 1964; Reynolds, 1964). Associated with this is a decrease in the rate of incorporation of amino acids into liver proteins and liver-derived plasma proteins *in vivo*. Our previous studies found no alteration in amino acid uptake or protein release (Smuckler *et al.*, 1961, 1962); hence it was concluded that the decreased amino acid incorporation is due to an alteration in the apparatus for protein synthesis. It seemed logical to examine the functional capacity and the physical and chemical characteristics of certain portions of the subcellular components for protein synthesis. In earlier communications (Smuckler and Benditt, 1963, 1964) some preliminary observations showing changes in microsomes and ribosomes were presented.

Materials and Methods

Male Sprague-Dawley rats weighing between 200 and 250 g were used in the following series of experiments. The animals were fasted for 14–16 hours prior to experimentation, and all experiments were done in the morning in an attempt to minimize the effect of diurnal variation. The animals were intubated with a soft rubber catheter, and CCl_4 dissolved in mineral oil or mineral oil alone was administered through the gastric tube. CCl_4 was used in dosages of 0.07, 0.13, 0.25, and 0.5 ml/100 g body weight. Control animals in each pair received equal volumes of mineral oil. The animals were sacrificed by a sharp blow to the head, and the livers were perfused with ice-cold saline via the aorta. All following operations were performed at 0–4° unless otherwise specified.

In Vivo Incorporation of Amino Acid into Subcellular Particles of Liver Cells. In these *in vivo* studies the animals were lightly anesthetized with diethyl ether (U.S.P.) and [1-^{14}C]glycine (1 mc/mmole dissolved in physiological saline) was administered by tail vein 2 hours following gastric intubation. Amino acid (25 μc) was administered per 100 g body weight. The animals were sacrificed at 0.25, 0.5, 1, and 2 hours following administration of radioactive amino acid, and the perfused liver was quickly isolated and minced in ice-cold 0.25 M sucrose. The liver was homogenized in 4 volumes of 0.25 M sucrose with a Teflon-pestled homogenizer and centrifuged at 800 $\times$ *g* for 20 minutes. The supernatant

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tant liquid was saved for preparation of the microsomes.

This brei was centrifuged at 18,000 × *g* × 10 minutes, and the supernatant liquid was transferred to tubes for a No. 40 Spinco Model L centrifuge rotor and centrifuged at 105,000 × *g* average for 120 minutes. The sediment from this step was suspended by gentle homogenization in 10 volumes of 5% trichloroacetic acid and analyzed for radioactivity as previously described (Smuckler *et al.*, 1962). The 105,000 × *g* supernatant fluid was made 5% in trichloroacetic acid and the precipitating material also prepared for radioactive analysis. The composition[1] of the sediments used for radioactive analysis was analyzed for total nitrogen, RNA, and DNA. Nitrogen was determined by the micro-Kjeldahl technique with nesslerization. DNA and RNA were determined by the method of Webb and Levy (1955) and Mejbaum (1939), respectively. Standards were DNA sodium salt (ex-salmon sperm) grade A from California Corp. for Biochemical Research and yeast RNA preparations from the same source.

Amino Acid Incorporation into s-RNA. Perfused livers were minced in 2.3 volumes of 0.35 M sucrose solution containing 0.25 M KCl, 0.005 M $MgCl_2$, and 0.5 M Tris buffer at *p*H 7.6. Materials that sediment at *p*H 5.2 were obtained by the method described by Keller and Zamecnik (1958). The protein content of the *p*H 5 s-RNA solution so prepared was 10–20 mg/ml, measured by the Lowry technique (Lowry *et al.*, 1951).

One ml of this suspension was incubated in an ATP-generating system containing 10 μmoles of ATP, 0.5 μmole GTP, 6 μmoles $MgCl_2$, 10 μmoles phosphoenolpyruvate, and 0.01 mg phosphoenolpyruvate kinase with sufficient $KHCO_3$ to maintain the *p*H at 7.4. To this, 0.1 ml of the solution containing 10 μmoles of [1-^{14}C]valine with a specific activity of 1 mc/mmole was added. The reaction was carried on at 37° for 10 minutes and stopped by the addition of 2 ml of 90% phenol. The amino acid s-RNA was extracted by shaking for 1 hour at room temperature. The aqueous layer was carefully removed and saved and the aminoacyl s-RNA was precipitated from the extract by adding 2 volumes of absolute ethyl alcohol acidified with 1 M acetic acid (*p*H 5.6). The precipitated material was dried, weighed, and dissolved in hydroxide of Hyamine. This solution was diluted with 10 ml of a toluene scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene. The solutions were counted in a Packard Model 314 EX liquid scintillator with a counting efficiency of approximately 40% for carbon 14. Correction for quenching and conversion to decompositions per minute was accomplished by the addition of an internal standard.

In addition further experiments were done in which 105,000 × *g* supernatant prepared in the manner described above was used in place of the purified *p*H 5 s-RNA system. These experiments were stopped at 3, 6, and 9 minutes and the s-RNA was extracted with phenol as before.

Incorporation of Amino Acid into Microsomes. Microsomes were prepared by differential centrifugation from liver homogenate prepared in 0.15 M sucrose, containing 0.005 M $MgCl_2$, 0.0025 M KCl, 0.005 M mercaptoethanol, and 0.1 M Tris buffer, *p*H 7.3. Microsomes were obtained by sedimentation carried out for 60 minutes at 105,000 × *g* (av) in the No. 40 rotor in the Model L preparative ultracentrifuge. The 105,000 × *g* supernatant fluid containing soluble fraction (*p*H 5 enzymes and s-RNA) was saved, and the microsomes were resuspended in a small amount of the original homogenizing medium. The protein content of the microsomal suspension and the 105,000 × *g* supernatant fluid was measured by the Lowry technique (Lowry *et al.*, 1951), using human albumin as a standard. The protein concentration of the microsomal suspension was adjusted to 4 mg/ml, and that of the supernatant fraction to 10 mg/ml.

Amino acid incorporation was measured in an incubation medium containing supernatant fluid (2 mg protein), microsomal suspension (2 mg protein), 1 μmole ATP, 0.25 μmole GTP, 5.0 μmoles phosphoenolpyruvate, 50 μg phosphoenolpyruvate kinase, 5 μmoles GSH, 100 μmoles KCl, 6 μmoles $MgCl_2$, and 0.05 μmole [1-^{14}C]leucine having a specific activity of 8 mc/mmole. The final volume of the incubation mixture was 1.2 ml. In each experiment, tubes containing control or treated microsomes were incubated with isologous as well as heterologous 105,000 × *g* supernatant fluid. In addition tubes were included in which the concentration of each of the above-named reagents was varied.[2]

Incubation was carried on in air at 37° and stopped at 0, 20, and 40 minutes by the addition of 8 ml of ice-cold 5% trichloroacetic acid containing 0.1 M nonradioactive leucine. The protein portion of the precipitate was isolated essentially by the method of Siekevitz (1952), and the dry protein was weighed and dissolved in 1 ml hydroxide of Hyamine. The radioactivity was measured as described previously.

Ribosome Preparation. Ribosomes were isolated by a modified Takanami (1960) procedure. Perfused livers were used and homogenization was carried out in a Sorvall Omni-Mixer in 6.7 volumes of 0.12 M KCl,

[1] The morphology and homogeneity of the sediment was monitored by electron microscopy. A specimen taken through the entire pellet was fixed in s-collidine-buffered 4% osmium tetroxide (Luft, 1961). The specimen was dehydrated by passage through a graded series of alcohols, embedded in epoxy resin, cut with glass knives with a Porter Blum microtome, and examined in an RCA EMU-3F electron microscope. The microsome pellet consisted of ribosomes, free and attached to lipoprotein membranes. No mitochondria were noted.

[2] In a preliminary set of experiments optimal concentrations of ATP, GTP, phosphoenolpyruvate, KCl, $MgCl_2$, and GSH were determined and found to agree with published data. In addition, optimum concentrations of these cofactors were determined using microsomes isolated from CCl_4-treated animals. No difference in optimal concentrations for maximum incorporation was found, but maximum incorporation was less than for control. Also addition of more of any one or combination of cofactors did not restore incorporation to control levels.

0.005 M $MgCl_2$, and 0.02 M phosphate buffer at *p*H 7.6. The Omni-Mixer had a blade revolution of 6000 rpm. Principal modification of the remaining procedure was the addition of 0.001 M potassium chloride into all solutions. The final ribosomal pellet was suspended in 0.0005 M $MgCl_2$, 0.001 M potassium chloride, 0.005 M Tris buffer at *p*H 7.8. To measure amino acid incorporation the protein concentration of the ribosome suspension was measured by the Lowry technique (Lowry *et al.*, 1951) and adjusted to 4 mg/ml.

Labeling of ribosomal protein was measured utilizing the system identical to the one in the microsomal experiments, with the exception that the 105,000 × *g* supernatant fluid was replaced by 2 mg of *p*H 5 s-RNA enzyme preparation prepared by the method of Hoagland and Askonas (1963). Incubation was carried on in air at 37° and stopped at 0, 20, 40, and 60 minutes by the addition of 8 ml of trichloroacetic acid containing leucine. The proteins were prepared and analyzed in the same method described for the microsomal preparation.

Chemical analysis of ribosomes included measurement of protein by the Lowry method and RNA by the modified Mejbaum (1939) technique. Ultraviolet absorption spectra were recorded in a Beckman DB recording spectrophotometer and analysis of absorption at 235, 260, and 280 mμ was done with a Beckman DU spectrophotometer.[3]

Ribosomal suspensions were analyzed in an analytical ultracentrifuge with rotor speed of 37,000 rpm and a rotor temperature at approximately 20°. Specimens were examined using schlieren optics with a phase angle of 70°. Photographic records were made at 4-minute intervals, and were analyzed on a microcomparator. Sedimentation coefficients were corrected to $s_{20,w}$ at infinite dilutions. No attempt was made to correct for the Johnston-Ogston effect (Johnston and Ogston, 1946) or for buffer viscosity. Concentration dependence of the sedimentation coefficient was identical to that reported previously by Hamilton and Petermann (1959).[4]

Polysome Preparation. Perfused livers were quickly minced in a medium containing 0.15 M sucrose, 0.005 M $MgCl_2$, 0.0025 M potassium chloride, 0.005 M mercaptoethanol, 0.10 M Tris buffer at *p*H 7.3. The resultant suspension was homogenized in a Teflon-glass system at 0° with eight passes of the pestle. The brei was centrifuged at 15,000 × *g* for 10 minutes in a Sorvall SS-1 centrifuge, and the supernatant fluid quickly was made 1% in sodium deoxycholate by the addition of a 7% solution of sodium deoxycholate in 0.1 M glyclglycine buffer, *p*H 7.8.[5]

The resultant suspension of polysomes was analyzed in an analytical ultracentrifuge with a rotor speed of 29,500 rpm and a rotor temperature of 4–8°, with schlieren optics at a phase angle of 60 or 70°. A photographic record was made at 4-minute intervals and analyzed as mentioned previously. No attempt was made to correct for the Johnston-Ogston effect or for buffer viscosity. In one set of experiments the polysomes were incubated at 0° for 30 minutes with 0.1 unit RNAase/10 mg protein prior to analysis in the ultracentrifuge and for amino acid incorporation.

Amino acid incorporation by polysome structures was accomplished in a cell-free system, utilizing 1.5 mg of polysome protein, 2 mg *p*H 5 s-RNA material, 1 μmole ATP, 0.25 μmole GTP, 100 μmoles KCl, 6 μmoles $MgCl_2$, 5 μmoles phosphoenolpyruvate, and 50 μg phosphoenolpyruvate kinase.[6] In addition it was found that the polyribosomes required the presence of 2 μmoles each of 17 amino acids other than the added radioactive labeled acid.[7] Radioactive leucine (or phenylalanine) (0.05 μmole) was added to the mixture and incubated under air at 37° for 0, 20, 40, and 60 minutes. Ice-cold 5% trichloroacetic acid (8 ml) containing 0.1 M unlabeled amino acid was added, and proteins were isolated in the manner described previously.

Absorption spectra of the polyribosome were recorded as mentioned previously. Suspensions of polysomes diluted with 0.0005 M $MgCl_2$, 0.001 M KCl, and 0.005 M Tris, *p*H 7.8, were dried on carbon-coated copper grids and shadowed with platinum at an angle of 11°. The specimens were examined in an RCA EMU-3G electron microscope.

Results

In vivo incorporation of amino acid into subcellular particles in control animals showed a pattern of incorporation similar to that described by Keller *et al.* (1954) and by Mirsky *et al.* (1954) (Figures 1 and 2). The isolated microsomal fraction was most heavily labeled and following this in time the 105,000 × *g* supernatant fluid showed increased radioactivity. The materials isolated from the intoxicated animals uniformly showed significantly less radioactivity. Morphologically the microsomal pellet was homogeneous,

[3] Ribosomes were also analyzed for lipid and polyamine content. No difference was found between those from control and those from treated animals. Also, extracted RNA revealed only the 28 and 18 S structures previously reported for mammalian liver ribosomal RNA (Hall and Doty, 1959).

[4] The principal component in the control preparation had a sedimentation coefficient of 79.6 Svedberg units at infinite dilution, and we shall refer to these as the 80 S ribosome. The principal component from treated rat livers had a sedimentation coefficient of 53.6 Svedberg units, and we shall refer to these as the 54 S subunit.

[5] Preliminary experiments indicated it was essential that the sodium deoxycholate be added to the 15,000 × *g* supernatant fluid. Centrifugation of material without deoxycholate, carried out at 105,000 × *g* (av), resulted in the destruction of the polysome pattern when later analyzed in the ultracentrifuge. Further, the addition of microsomal material to polysome preparations resulted in a loss of polysome structures.

[6] Preliminary experiments indicated that these concentrations resulted in optimum incorporation. Also, the addition of the amino acid mixture increases incorporation two to three times.

[7] These amino acids were threonine, tyrosine, methionine, cysteine, isoleucine, glutamic acid, glutamine, aspartic acid, histidine, proline, lysine, alanine, glycine, tryptophan, arginine, valine, and either phenylalanine or leucine depending upon the tracer used.

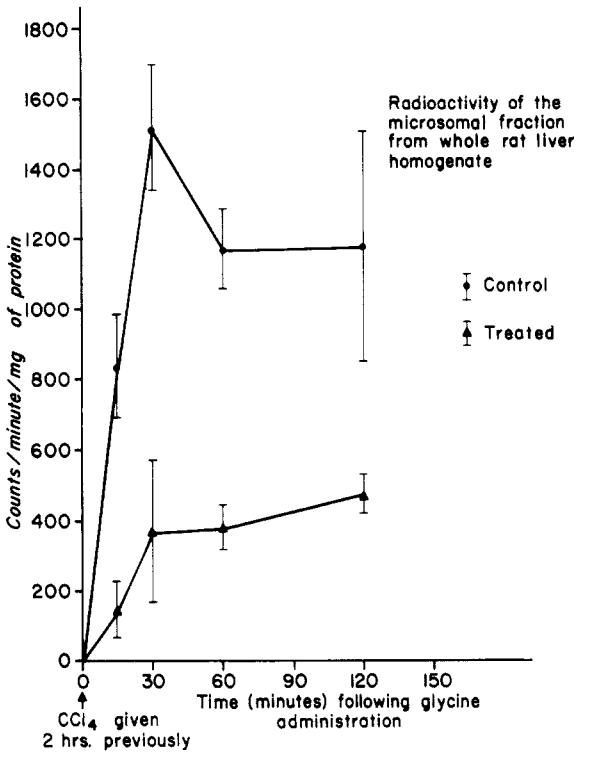

FIGURE 1: Effect of CCl_4 (0.5 ml/100 g body weight) on amino acid incorporation into microsomes *in vivo*. The points represent the mean of three or more determinations; the vertical bars represent 1 standard deviation.

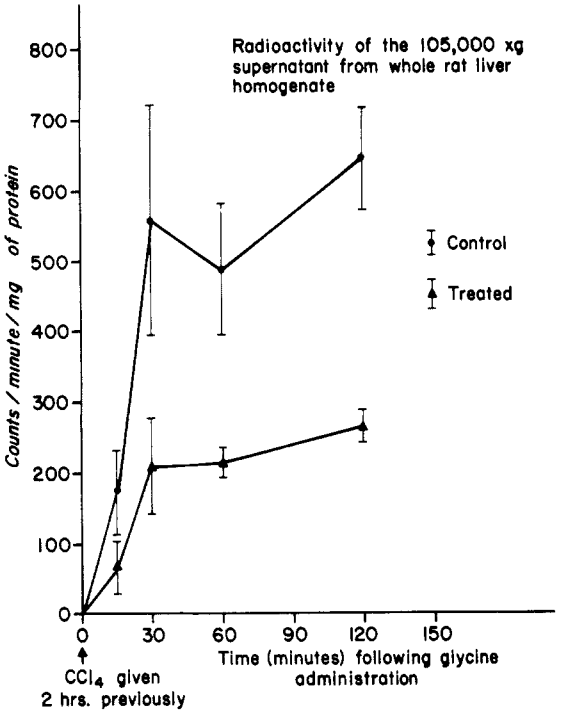

FIGURE 2: Effect of CCl_4 (0.5 ml/100 g body weight) on amino acid incorporation into protein in the 105,000 × *g* supernatant fluid. The points represent mean values of three or more determinations; the vertical bars represent 1 standard deviation.

consisting of small granules free and attached to membrane structures. There was no significant alteration in the RNA/protein ratios of these pellets isolated from livers of treated and control animals.

The isolated *p*H 5 s-RNA materials from both treated and control rats incorporated amino acid in an *in vitro* situation to the same degree. Using valine as the tracer, control *p*H 5 s-RNA had a specific activity of 1726 ± 406 dpm/mg; treated *p*H 5 s-RNA (0.5 ml CCl_4/100 g body weight) had a specific activity of 1770 ± 635 dpm/mg. Similar results were obtained with glycine as the tracer. The time study of incorporation also showed that the curves from treated and control animals were parallel. Furthermore, utilization of crude 105,000 × *g* supernatant also failed to reveal a significant difference between the materials isolated from control animals when compared to those that had received CCl_4.

Incorporation of amino acid into microsomes isolated from animals previously treated with a range of CCl_4 dosage revealed significant depression when compared with microsomes isolated from control animals (Table I). This depression was present regardless of the source of the 105,000 × *g* supernatant fluid (Table II). The depression was noticed quite early in the course of the intoxication, and was of the same order of magnitude as that seen in *in vivo* experiments (roughly 50%). Electrolyte and cofactor requirements for optimal incorporation were identical for microsomes isolated from treated as well as control animals. Variation of cofactor concentrations failed to restore control level activity to microsomes from poisoned animals. Moreover, the

TABLE I: Effect of Dose of CCl_4 on *in Vitro* Microsomal Amino Acid (Leucine) Incorporation.[a]

Dose (ml of CCl_4/ 100 g body weight)	Specific Activity	Per Cent of Control
0	908 ± 120 (one standard deviation)	
0.07	397[b]	47
0.13	194[b]	27
0.25	323[b]	28
0.50	203[b]	29

[a] Microsomes were isolated from rat livers 2 hours following administration of the drug by stomach tubes. [b] Each of these values is compared with the control preparation in the same experiment and not with the mean control value indicated in the table.

TABLE II: Microsomal Amino Acid (Leucine) Incorporation.[a]

Microsome Source	105,000 × g Source	Isolated Protein (dpm/mg)
Control	Control	1486
Control	Treated	1343
Treated	Control	615
Treated	Treated	607

[a] Microsomes isolated from rat liver 2 hours following administration of 0.25 ml CCl_4 per 100 g body weight. It was found in these experiments that the omission of 105,000 × g supernatant fluid reduced incorporation by microsomes from control animals to 33% of the value with this material, and treated microsomes 55%. Omission of ATP and phosphoenolpyruvate produced significant reduction in activity.

TABLE III: Chemical and Spectral Analyses of Ribosomes.

Time in Hours Following Administration of CCl_4[a]	RNA/Protein Ratio[b]	260/280 OD Ratios[b]
0	1.15 (8) ± 0.18	1.91 (8) ± 0.19
0.5	1.49 (3) ± 0.08	2.10 (1)
1	1.07 (2) [1.05–1.08]	1.79 (2) [1.67–1.92]
2	1.21 (1)	1.97 (2) [1.91–2.03]
3	1.02 (2) [0.97–1.08]	1.88 (3) ± 0.06

[a] CCl_4, 0.25 ml/100 g body weight. [b] Number in parentheses indicates the number of experiments from which the average was calculated; standard deviations are indicated by numerals following ±; numerals in brackets indicate the range.

105,000 × g material from the treated animal had no inhibitory effect on microsomes isolated from control animals.

Analysis of ribosomes in the analytical ultracentrifuge revealed that there was a progressive loss in the 80 S ribosome isolated from livers of CCl_4-treated animals (Figure 3). There was concomitantly an increase in a more slowly moving subunit with a sedimentation coefficient of 54 S. In no pattern was there evidence of a 30 S structure. The addition of CCl_4 to liver homogenates failed to alter the pattern of ribosomes observed

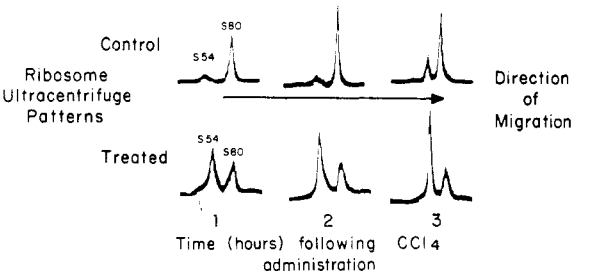

FIGURE 3: Representative ultracentrifuge patterns of ribosomes from control and CCl_4-intoxicated rat liver. The patterns were photographed 16 minutes after reaching 37,020 rpm; a schlieren phase angle of 70° was used.

in the ultracentrifuge. The ultraviolet absorption spectra, particularly the ratio of absorbances at 280:260, were identical in preparations from control and treated animals (Table III). In addition, analysis of protein and RNA from these structures also failed to reveal significant differences.

Amino acid incorporation into these isolated structures was significantly reduced within 0.5 hour following the administration of CCl_4 to the intact animals (Table IV). The degree of depression was of a similar order of magnitude as that found in microsomes. Analysis of cofactor requirements for optimal amino acid incorporation failed to reveal significant differences between those structures isolated from control or from treated animals.

TABLE IV: Effect of Carbon Tetrachloride on Ribosomal Amino Acid (Leucine and Phenylalanine) Incorporation.

Dose (ml/100 g body weight)	Time (hours) Following Administration	Per Cent of Control Specific Activity[a]
0.5	1	62
	2	49
	3	23
0.25	0.25	100
	0.5	53
	2	48
	3	47
0.13	2	75[b]
0.05	1	57[b]

[a] In this total the per cent of control value is obtained by dividing the specific activity of ribosome from CCl_4-treated animals by the activity of ribosomes from control animals and multiplying by 100. All results are the mean value of at least two separate incubations except where indicated. Incubations as indicated in the text. [b] Single determinations.

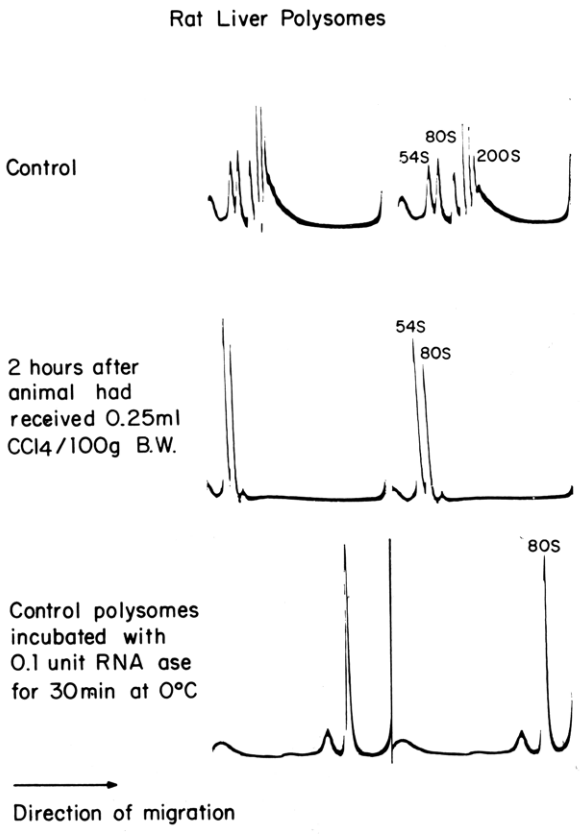

FIGURE 4: Representative ultracentrifuge patterns of rat liver polysomes. The photographs of the control liver polysomes and those from treated animals were made at 12 (left) and 16 (right) minutes after reaching 29,500 rpm. The patterns following RNAase treatment were made 28 and 32 minutes after reaching 37,020 rpm. Phase angle used in all of these pictures was 60°. Please note the resemblance between the patterns of RNAase-treated polysomes and ribosomes prepared by the Takanami (1960) procedure. The RNAase preparation used has an activity of 40 units/mg.

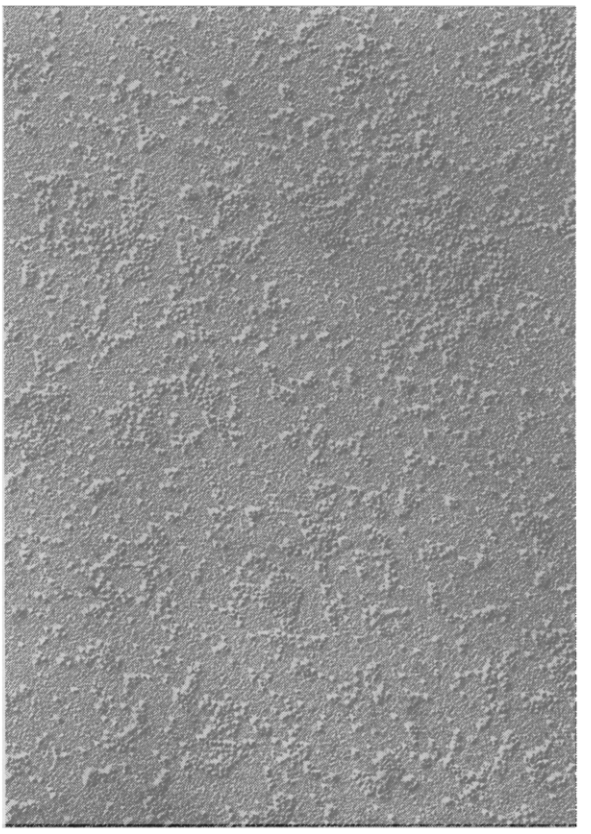

FIGURE 5: An electron micrograph of rat liver polysomes shadowed with platinum at an angle of 11°. Magnification 72,800x.

Polysomes isolated from livers of control animals by the methods described revealed a large group of heavy structures with sedimentation coefficients (uncorrected for concentration) ranging between 140 and 200 Svedberg units, constituting 60–70% of the preparation (Figure 4). There was also a small amount of even heavier material present in addition to 80 and 54 S structures. As early as 30 minutes following CCl_4 administration, in a variety of doses, from 0.1 to 0.5 ml/100 g body wt, there was a total loss of the heavy sedimentating polysome structures in addition to the change in the ribosome previously described; that is, an increase in 54 S subunits and decrease in 80 S ribosomes. There was no evidence of a slower-moving material with a sedimentation coefficient of less than 54 S. Brief treatment with RNAase at 0° resulted in the formation of 80 S units.

There was no clearly defined difference in absorption spectra or in RNA/protein ratio in these polysome preparations. Shadowed preparations examined with the electron microscope revealed aggregates (polysomes) in material from control livers, whereas material from treated animals has a more dispersed pattern with more ribosomes existing singly (Figures 5, 6).

Amino acid incorporation of polysome was particularly active in control preparations, varying between 200 and 400 mμmoles of leucine/mg of protein/hour. Following CCl_4 administration there was a progressive decrease in the incorporation to approximately 50% of the control level. This is of the same order of magnitude as the changes seen previously in ribosomes and microsomes (Table V).

Discussion

The major apparatus for final assembly of proteins seems to be located in the coarse endoplasmic reticulum of mammalian cells. Here ribosomes, presumably together with messenger RNA (m-RNA), appear in juxtaposition to the membranes of the endoplasmic reticulum. In this location the final assembly of amino acids (activated and coupled with s-RNA in the cytoplasmic matrix) into peptides in the proper sequence occurs (Rich *et al.*, 1963).

TABLE V: Effect of Carbon Tetrachloride on Amino Acid (Leucine) Incorporation into Rat Liver Polysomes.[a]

Time after Administration	Per Cent of Controls (with dose in ml/100 grams body weight): 0.1 ml	0.25 ml	0.5 ml
0	100	100	100
1 hour	53 ± 10	55 ± 18	65
2 hours	67 ± 23	40 ± 10	36

[a] Values are given as means ±1 standard deviation when three or more determinations were done.

The observations presented indicate that the amino acid activation and the amino acid transfer systems in the liver cells, including s-RNA, are not impaired during the first few hours following CCl_4 intoxication. Activities of the soluble factors in the 105,000 × *g* supernatant fluid derived from treated animals were not impaired when assayed with microsomes derived from control animals. Also, studies testing specific activation showed no difference in the rate of hydroxamate formation with glycine and leucine (E. A. Smuckler, unpublished data), and no difference in the coupling of valine and glycine to s-RNA was found.

Microsomes from livers of intoxicated rats did not incorporate amino acid to the same degree as those from controls, despite the fact that optimum concentrations of Mg^{2+}, K^+, ATP, and GTP required for amino acid incorporation were the same for microsomes of normal or intoxicated animals. Also RNA/protein ratios were the same for the mirosomes isolated from poisoned animals and controls.

The preparation known as microsomes consists of free ribosomes, ribosomes attached to lipoprotein membranes, and membranes without attached ribosomes (Palade and Siekevitz, 1956). Intact m-RNA is probably not present in this fraction but fragments of messenger may be present.[8] Analysis of the ribosome component of this complex showed two striking features in the treated animals: (1) Amino acid incorporation into these structures isolated from poisoned rat liver was significantly reduced and the relative decrease of incorporation was of the same magnitude as that seen in microsomes from CCl_4-intoxicated rats. (2) Ribosomes from intoxicated rats showed an altered sedimentation pattern, when examined in the ultracentrifuge, consisting of a significant decrease in structures with sedimentation coefficient of 80 S. A nearly corresponding increase in 54 S units was found without appearance of the expected 30 S component.

[8] Our evidence for this statement is inferred from the following observations: (1) Polysomes cannot be isolated from the microsomes prepared from mammalian livers; (2) microsomes added to polysomes cause a loss of polysomes and the concomitant appearance of 80 S ribosomes; (3) RNAase treatment of polysomes results in an 80 S ribosome to which fragments of messenger are attached (Takanami and Zubay, 1964).

FIGURE 6: An electron micrograph of polysomes prepared from the liver of an animal that had received 0.25 ml CCl_4/100 g body weight 2 hours previously. This material was prepared in the same manner as those in Figure 5, and was of the same concentration. Note the difference in aggregation of these samples. Magnification 72,800x.

In the light of observations on microbial, plant, and animal ribosomes (Petermann, 1962; Ts'o, 1962), indicating each 80 S unit to be composed of a 54 and a 30 S subunit, the absence of a 30 S unit is puzzling. The absence of the 30 S particles is not artifactual of the preparative procedure since ribosomes from control animals prepared by this procedure can be dissociated and reassociated in the usual way by alternating the Mg^{2+} concentration, and 30 S as well as 54 S particles do appear. Furthermore (*vide infra*), the preparative technique which preserves polysomes as well as unit ribosomes and subunits showed no evidence of a 30 S subunit.

While individual ribosomes (80 S) are capable of amino acid incorporation, current evidence indicates that ribosomes operate in association with m-RNA in the synthesis of polypeptide chains (Rich *et al.*, 1963). These aggregates of ribosomes attached to m-RNA (i.e., polysomes), when isolated from normal rat liver,

vary in size. They have sedimentation coefficients of 110, 140, 160, 170, 180, 200 S, and greater; 60–70% of the ribosome population appears in these aggregates. As shown previously, these "polysome" aggregates are almost absent from CCl_4-poisoned rat liver; small amounts of 110 S material exist, but most of the ribosomes appear as 54 S units. As might be expected, the capacity of these preparations from CCl_4-poisoned rat liver to incorporate amino acid is also significantly decreased to the same degree as ribosome and microsome preparations.

This constellation of changes (reduction in polysome size and numbers, degradation of ribosomes to the 54 S subunit without the appearance of the 30 S structure, and the decreased rate of amino acid incorporation) appears to be the immediate cause of the decreased rate of hepatic protein synthesis. Considering the loss of polysome formation in CCl_4 intoxication, the following possibilities could explain these observations: (1) Messenger destruction is increased. (2) Messenger formation is reduced. (3) An alteration in the m-RNA prevents association of ribosomes with the nucleotide strand. (4) An alteration in the ribosome prevents association with m-RNA.

Destruction of m-RNA in preparation of polysomes results in decreased numbers of heavy aggregates and increased numbers of 80 S ribosomes without significant change in the population of 54 S units. This can be observed following brief RNAase treatment of polysomes at 0°. In this particular the injury after CCl_4 is significantly different and suggests that messenger loss alone is not the cause of the observed defect.

m-RNA has been defined as a short-lived polynucleotide, carrying genetic information from the nucleus to the cytoplasm (Jacob and Monod, 1961). In pulse labeling studies using RNA precursors in bacterial systems, a rapidly labeling polynucleotide has been discovered. The failure to find a similar rapidly labeling substance in mammalian cells was initially puzzling (Hiatt, 1962). It has been pointed out recently that the short half-life, and hence the rapidity with which radioactive precursors are incorporated, may not be characteristic of mammalian systems (Revel and Hiatt, 1964). The reticulocyte is a mammalian cell in which the machinery for messenger synthesis has disappeared, yet it carries on synthesis of a particular protein, hemoglobin, efficiently for a number of days, indicating the presence of stable messenger. Also the generation time of mammalian cells is longer by a significant factor than bacteria; for example, the cells of mature rat liver (Post *et al.*, 1963) divide less than once a year whereas *Bacillus subtilis* reproduced in 100 minutes (Levinthal *et al.*, 1962). The quantity of protein synthesis differs widely, the liver cell forming an amount of protein equivalent to its own weight in 6 days whereas bacteria do this in a matter of hours. It might be expected that m-RNA turnover would reflect these less rapid processes and that mammalian m-RNA would have a longer life and also a slower rate of synthesis than the better-known microbial one. However, rapid synthesis of a particular messenger could occur, but the amount might constitute so small a part of the RNA that its detection would be difficult. Evidence for rapid formation of some liver m-RNA comes from enzyme induction studies: Guidice and Novelli (1962) and Greengard and Acs (1962) found decreased formation of inducible enzymes following actinomycin treatment; however, there was no alteration of amino acid incorporation *in vitro*. These findings are quite compatible with long-lived as well as small amounts of inducible short-lived m-RNA in mammalian liver. If the bulk of the messenger RNA is long-lived (days), a decreased rate of formation of m-RNA in CCl_4 poisoning would not explain the observed polysome changes for the following reasons:[9] Decreased numbers and size of polysomes (nearly complete) occur within less than 1 hour following administration of the toxin, too short a time for changes of the magnitude found to occur to result from decreased messenger synthesis. Furthermore, if only m-RNA formation were reduced, we would expect to find free 80 S ribosomes and not 54 S subunits.

The mechanism involved in the binding of ribosomes to the m-RNA is not known; consequently, experimental testing and discussion of the last two possibilities is difficult. Alteration of messenger and/or ribosomes of their association by direct action of CCl_4 appears not to be the mechanism, since addition of CCl_4 to the liver homogenates during preparation of polysomes and ribosomes did not reproduce the change in sedimentation behavior. The failure of effect of added CCl_4 implies that the intact cell plays a role in causing the alterations, particularly the loss of 80 S ribosomes.

Destruction of the 80 S ribosome unit could occur either as a sequel to the loss of m-RNA or to an alteration in the interaction between m-RNA and ribosomes or as the result of a direct and possibly independent effect upon the ribosomes. The absence of 30 S subunits and the appearance of the 54 S subunits may be of great interest. The first question to which we must address ourselves is: what is the significance of the disappearance of the 30 S units? Are these destroyed by digestion or fragmentation; do they change in shape and/or density and sediment more rapidly; or might they aggregate (dimerize) and disappear somewhere in the 54 S peaks? There is evidence (Tashiro *et al.*, 1964) that the 30 S component is more labile and more easily destroyed during several degradation procedures. At present it does not seem likely that the RNAase of the whole liver or lysosomes plays a significant role in degradation of ribosomes, since changes in activities of this enzyme do not occur until later in the course of the intoxication (Dianzani, 1963; T. F. Slater, personal

[9] Measurement of orotic acid incorporation into nuclear and cytoplasmic and into organelle RNA was done. No differences were found in treated and control animals. We cannot presently interpret this to mean that no alteration in m-RNA formation occurred, since the amounts of m-RNA formed may be small and, therefore, undetectable. It does imply no gross alteration in the ability of the liver cell to convert orotic acid to uracil and incorporate it into polynucleotides.

communication). However, it is quite clear that there is a turnover of ribosomes under ordinary conditions in mammalian cells and that some mechanisms for their catabolism must be present. Activation of the degrading system by the toxin as either a first-order or a second-order event could account for the picture which we have observed. Indeed it appears that administration of a variety of toxins (i.e., actinomycin D, dimethylnitrosamine, thioacetamide, and ethionine [Smuckler and Barker, 1964]) will result in loss of polysome structures and ribosome degradation to 54 S subunits.

The presence of aggregates of ribosomes in orderly arrays associated with ergastoplasmic membranes has now been observed repeatedly. All the evidence points to the necessity of an ordered spatial relation of these parts of the apparatus for protein fabrication in mammalian cells. Fibroblasts producing collagen have an ordered array of ribosomes on membranes, but scorbutic fibroblasts, which do not produce collagen, do not show the grouping (Ross and Benditt, 1964). Early following CCl_4 poisoning there is disorganization of this apparatus. From the foregoing it is now clear that this disorganization of the ribosome-membrane relationship extends not merely to the polysomes, but to the ribosome itself. The regular occurrence of these changes, appearing very early after oral administration and at low doses of the drug, suggests, although it does not prove, that somewhere in this system is the primary lesion in CCl_4 poisoning.

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