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EFFECT OF BENZENE ON RAT LIVER POLYRIBOSOMES*

GEORGE P. TRYFIATES

Department of Biochemistry, West Virginia University Medical Center, Morgantown, W. Va. 26506, U.S.A.

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Abstract—The effect(s) of benzene on rat liver polyribosomes was studied. The data show that administration of benzene to rats intraperitoneally resulted in disaggregation of liver polyribosomes, accumulation of ribosomal monomer–dimers and appearance of an intermediate, previously absent, ribosomal peak. The protein synthetic capacity of disaggregated liver polyribosomes as measured by the incorporation of L-[^{3}H]-phenylalanine *in vitro* was significantly impaired (> 50 per cent) but the poly U-directed polymerization of L-[^{3}H]-phenylalanine was not affected. Protein synthetic tests with polyribosomes and pH 5·1 enzyme fractions from benzene-treated and untreated animals showed that treatment with benzene *in vivo* did not affect the pH 5·1 enzyme fraction. Further, other experiments showed that the incorporation of labeled ribonucleic acid precursors into liver polyribosomes during treatment with benzene was considerably impaired while incorporation into total liver ribonucleic acid and the size of the acid-soluble fraction radioactive label pool were not affected.

These results are discussed in terms of benzene action at the molecular level.

SUCROSE density gradient analyses of liver polyribosomes after the intraperitoneal administration of labeled hydrocortisome ([1,3-^{3}H]-hydrocortisone in benzene–ethanol, 10%) to rats showed polyribosome breakdown. Additional experiments revealed that the observed breakdown of polyribosomes *in vivo* was accompanied by loss in ribosomal protein synthetic function and was caused by the presence of benzene.[1]

Experiments reported here show that the intraperitoneal administration of benzene to rats causes disaggregation of liver polyribosomes and decreased ribosomal incorporation of labeled RNA† precursor. In addition, benzene had no effect either on the size of the acid-soluble fraction radioactive label pool or on the incorporation of labeled RNA precursor into total RNA of the liver cell. Furthermore, a considerable increase in the 80S and 120S ribosomal peaks ensues with concurrent appearance of a new ribosomal peak inbetween the monomer–dimer region of the gradient. Endogenous protein synthesis with a subcellular rat liver protein-synthesizing system and purified polyribosomes from benzene-treated animals was considerably impaired (50 per cent) but the poly U directed L-[^{3}H]-phenylalanine incorporation remained unaffected. The possible significance of these results is discussed in connection with benzene toxicity at the molecular level.

* A preliminary report was presented; *Fedn Proc.* **27**, 713 (1968).

† Abbreviations: RNA, ribonucleic acid; tRNA, transfer RNA; sRNA, soluble RNA; mRNA, messenger RNA; DNase, deoxyribonuclease; poly U, polyuridylic acid; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; ATP, adenosine-5′-triphosphate (Na_2); GTP, guanosine-5′-triphosphate (Na); TCA, tricholoracetic acid.

METHODS

Subcellular fractions were prepared at 0–4° from the livers of treated or untreated animals. Wistar strain male rats (Hilltop Lab Animals, Inc., Scottsdale, Pa.) weighing approximately 250 g were injected intraperitoneally with 0·520–5·630 mM benzene (Fisher). [5-^{3}H]-Uridine (sp. act., 15·1 c/m-mole) or 5-[^{3}H]-orotic acid (sp. act., 16·0 c/m-mole) was given intraperitoneally at a dose of 30 and 50 μc per animal respectively.

Preparation of polyribosomes. Cytoplasmic polyribosomes were prepared as described by Wettstein *et al.*[2] The procedure was employed in this laboratory with minor modifications as follows: Homogenization was carried with a Blessing glass homogenizer (L-pestle clearance, 0·006 in.) (12 strokes) in 1 vol. of homogenizing medium. An additional volume of homogenizing medium was added and the whole was re-homogenized with a Blessing "T" pestle (5 strokes). After centrifugation at 36,000 *g* for 30 min the post-mitochondrial supernatant was adjusted to 0·5 per cent with DOC and polyribosomes were prepared by centrifugation through a layer of 0·5 M sucrose over 2 M sucrose at 150,000 *g* for 2·5 hr as described.[2] The ribosomal pellet was gently resuspended in medium A without the sucrose (5 mg ribosomal protein/ml) and was used as a source of mono- and polyribosomes. Preparations were stored at −70° and used within a week.

Preparation of pH 5·1 enzymes. The preparation of pH 5·1 enzymes was performed as follows: The post-mitochondrial supernatant (36,000 *g*; 30 min, without DOC) was further centrifuged at 145,000 *g* for 2·5 hr. The microsomal supernatant was carefully removed and diluted with 2 vol. of a solution containing 0·90 M sucrose, 4 mM Mg^{2+} acetate, 70 mM KCl and 6 mM mercaptoethanol. It was then passed through a 0·45 μmillipore filter using suction to assure removal of contaminating substances (monosomes). The pH was adjusted to 5·1 with 0·1 M acetic acid. After standing for 30 min it was centrifuged at 36,000 *g* for 10 min. The enzyme precipitate was washed with cold redistilled water and again centrifuged. The pellet was suspended in medium A without the sucrose (20 mg protein/ml). The solution was stored at −70° and used within a week.

Sucrose density gradient analysis. Sedimentation patters of polyribosomes were resolved by logarithmic 7–34 (w/v) per cent sucrose density gradients containing 5 mM tris–HCl, pH 7·6, 70 mM KCl, and 4 mM Mg^{2+} acetate, as described previously.[3] Gradients of approximately 28 ml were centrifuged in the Spinco SW 25·1 swinging rotor at 4° for 2·5 hr at 25,000 rev/min. The absorbance at 260 mμ was continuously registered with a Gilford 2000 recorder by forcing the gradient upward at constant rate (40 ml/hr) a specially designed fractionator connected to a Gilford flow cell. Radioactivity was measured in a Beckman liquid scintillation spectrometer using Bray's solution.[4,5]

Protein synthesis. Incubation was carried at 37° using an *in vitro* protein-synthesizing system containing in 0·6 ml: 1 mg ribosomal protein; 2 mg pH 5·1 enzyme protein; 100 μg poly U, (where applicable); 0·4 μc L-[^{3}H]-phenylalanine (sp. act., 1 c/m-mole); 0·68 μmoles ATP; 0·18 μmoles GTP; 3·6 μmoles phosphoenol pyruvate; 30 μg phosphoenol pyruvate kinase; 28·20 μmoles NH_4Cl; 3·80 μmoles Mg^{2+} acetate; 1 μmole β-mercaptoethanol; 72 μmoles sucrose; 14 μmoles KCl; and 10·25 μmoles tris–HCl, pH 7·6. At specified intervals 0·1-ml aliquots were removed and radioactivity was determined by the Mans and Novelli technique.[6] The protein was precipitated on Whatman No. 3 filter paper discs (2·3 cm), extracted with TCA, alcohol, ether, dried

and placed in scintillation vials to be counted in a Packard Tri-Carb liquid scintillation spectrometer using Bray's solution,[4] as previously described.[1,7]

Isolation of total RNA, polyribosomes and total acid-soluble fraction of rat liver. In these tests, three animals were injected with 5-[^{3}H]-orotic acid for approximately 20–22 min. One-half of the liver homogenate[8] was used to prepare polyribosomes (this section). The remaining homogenate was used to isolate total nucleic acid (RNA) and acid-soluble material. The procedure described by Yu and Feigelson[8] and Feigelson *et al.*[9] was employed. Nucleic acids (including DNA) were precipitated with NaCl-ethanol, as described.[9] RNA was obtained as follows: The NaCl-ethanol precipitate was dissolved in minimum volume of 0·1 M tris–HCl buffer, pH 7·6, containing $1{\cdot}5 \times 10^{-3}$ M $MgCl_2$. One hundred μg of pancreatic DNase was added and the whole was incubated with shaking at 37° for 30 min. RNA was isolated by phenol-sodium dodecyl sulfate (SDS) extraction. After addition of an equal volume of redistilled phenol, saturated with 0·1 M tris–HCl buffer, pH 7·6, containing 0·5% SDS, the preparation was shaken and the aqueous phase obtained by centrifugation was re-extracted with saturated phenol-SDS. After removal of phenol (by ether) and ether (by bubbling N_2), the aqueous phase was made 2 per cent with K acetate and the RNA was precipitated with 2·5 vol. of cold 95% ethanol at −20° overnight.

Protein. Protein was determined by the method of Lowry *et al.*[10]

Materials. Density gradient sucrose (ribonuclease free) was purchased from Mann Research Labs., New York, N.Y.; adenosine-5′-triphosphate (Na_2), guanosine-5′-triphosphate (Na), phosphoenol pyruvate (Na_3), phosphoenol pyruvate kinase and poly U were purchased from Sigma Chemical Company, St. Louis, Mo.; L-[^{3}H]-phenylalanine (sp. act., 1 c/m-mole) and 5-[^{3}H]-orotic acid (sp. act., 16 c/m-mole) were purchased from Schwarz Bio Research Inc., Orangeburg, N.Y.; [5-^{3}H]-uridine (sp. act., 15·1 c/m-mole) was purchased from New England Nuclear Corp., Boston, Mass. DNase was purchased from Worthington Biochemical Corp., Freehold, N.J.

Corrections. The counts per minute reported here have been corrected for quenching by internal standardization. For example, after all the scintillation vials of an experiment were counted, to each vial was added a standard volume (0·1 ml) of the same isotope recording 20,000 counts (or 40,000 dis./min on a 50 per cent efficiency). The vials were counted again. The original count of each vial was corrected by applying the per cent quenching of the standard sample. In Fig. 1, the $\mu\mu$moles of L-[^{3}H]-phenylalanine were calculated from dis./min by correcting in addition for the efficiency of the instrument (30 per cent for [^{3}H], Packard Tri-Carb, Model 3003).

RESULTS

The effect of benzene administration on the optical density sedimentation behavior of liver polyribosomes and on the incorporation of labeled uridine into polyribosomes was studied. Benzene was given in different dosage (Methods), followed immediately by the injection of the radioactive RNA precursor. The animals were later sacrificed by decapitation at the indicated time.

The sucrose density sedimentation profile of liver polyribosomes from control animals (untreated) as well as the distribution of [^{3}H]-uridine incorporation *in vivo* during approximately 20 min of pulse are shown in Fig. 2. High uridine incorporation

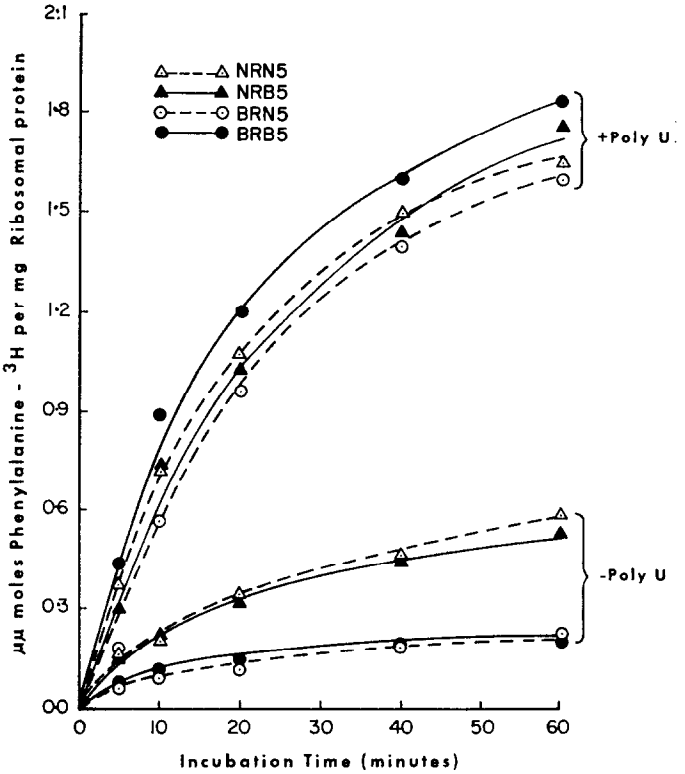

FIG. 1. Time-course of L-[^{3}H]-phenlalanine incorporation *in vitro* with and without poly U and with ribosomal and pH 5·1 fractions from normal and benzene-treated animals. The graph depicts a typical protein synthesis experiment *in vitro*. Incubation of the protein synthesizing system (see Methods) was carried at 37°. Aliquots of 0·1 ml were removed during the indicated time intervals and placed onto Whatman No. 3 filter paper discs (2·3 cm), dried and the protein extracted according to the technique of Mans and Novelli[6] (See Methods). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using Bray's solution.[1,4,7] Protein synthesis experiments were run with and without poly U and with polyribosomes and pH 5·1 fractions from (a) normal animals, (b) benzene-treated (3·37 m-moles benzene/100 g body wt., 20 min), or (c) with cross-reacting subcellular fractions from normal and benzene-treated animals, as indicated. Subcellular fractions were prepared as described in Methods. The results of Fig. 1 were repeated at least four but not more than six times. The results depicted in other graphs were repeated with at least six different subcellular preparations. NRN5 = polyribosomes and pH 5·1 fraction from normal animals NRB5 = polyribosomes from normal and pH 5·1 fraction from benzene-treated animals. BRN5 = polyribosomes from benzene-treated and pH 5·1 fraction from untreated animals. BRB5 = polyribosomes and pH 5·1 fraction from benzene-treated animals.

was found at the light region of the gradient with the remaining radioactivity distributed uniformly over the rest of the gradient.

Figure 3 shows the effect of 3·37 m-moles of benzene administration on the optical density sedimentation pattern of polyribosomes as well as on the ribosomal incorporation of tritiated uridine. In comparison with Fig. 2, Fig. 3 shows breakdown of liver polysomes and appearance of abnormally high monomer–dimer ribosomal peaks. Further, a new ribosomal peak located between the monomer–dimer region of the gradient also appeared. The distribution of tritiated uridine was qualitatively similar

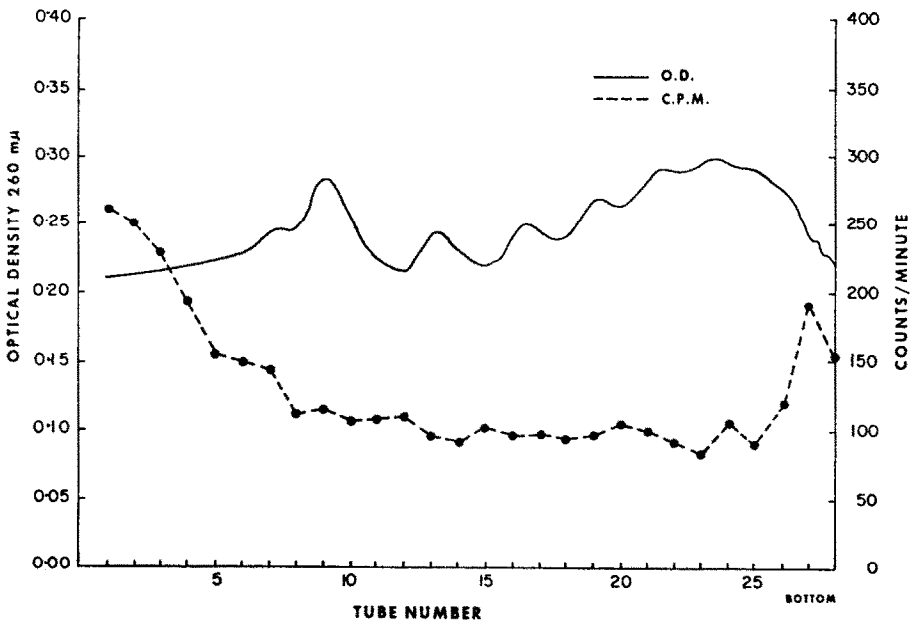

FIG. 2. Sucrose density sedimentation pattern of liver polyribosomes. Polyribosomes were prepared from normal rat livers after a 20-min pulse with [5-^{3}H]uridine (Methods). Polyribosomes were resolved on 7 to 34% logarithmic sucrose density gradients[1,3]. The gradients were centrifuged in the Spinco SW 25·1 swinging rotor at 4° for 2·5 hr at 25,000 rev./min. The absorbance at 260 mμ was continuously recorded with a Gilford 2000 recorder. Radioactivity of each fraction was measured in a Beckman liquid scintillation spectrometer using Bray's solution[4,5,7] (Methods). The figure shows a representative run.

with that of the control (Fig. 2); the amount of radioactivity incorporated in the presence of benzene was, however, diminished considerably (Table 1, sp. act. values). Similar results (Fig. 3) were obtained with 1·13 and 2·25 m-moles of benzene administration. Further, administration of a high dose of benzene (5·63 m-moles/100 g body weight) for 30 min did not alter either the sedimentation pattern of liver polyribosomes or the distribution of incorporated radioactivity. The results were as in Fig. 3.

TABLE 1. EFFECT OF BENZENE ON [5-^{3}H]URIDINE INCORPORATION INTO RAT LIVER POLYRIBOSOMES *in vivo*

Benzene injected (m-moles)	Time (min)	[5-^{3}H]Uridine incorporation		
		counts/min	O.D.$_{260}$	Specific activity (counts/min/O.D.$_{260}$)
0·00	20	3680	6·00	670
3·37	20	1540	8·38	184
5·63	30	1810	11·95	151

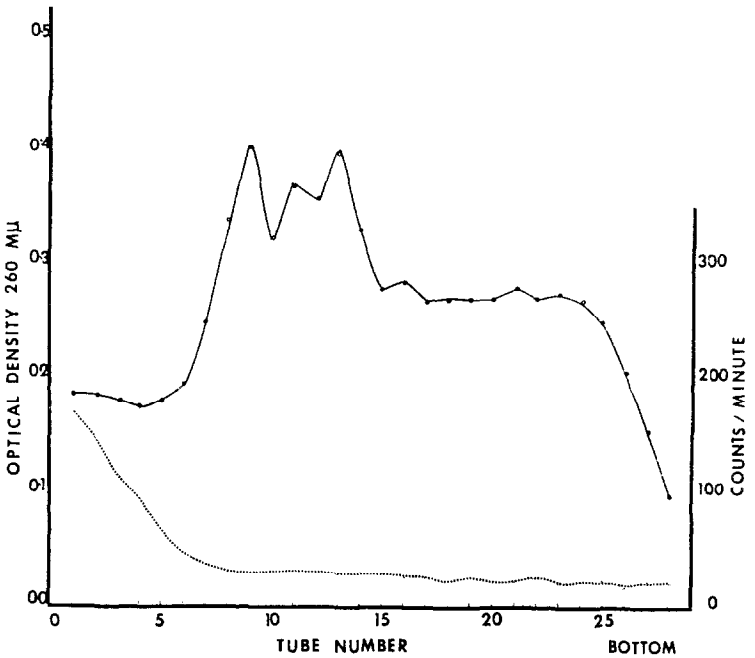

FIG. 3. Effect of benzene administration on the sedimentation behavior and [5-^{3}H]uridine labeling of liver polyribosomes. Polyribosomes were prepared from rat livers after a 20-min pulse with [5-^{3}H]uridine given immediately after the intraperitoneal administration of 3·37 m-moles of benzene per 100 g body wt. Other details as in Fig. 2. Solid line, O.D. at 260 mμ; dotted line, radioactivity.

The data of Figs. 2 and 3 are summarized in Table 1. The results show that increasing the dosage of benzene decreases total uridine incorporation into rat liver polyribosomes (sp. act. column).

To gather information regarding the effect of benzene on the liver radioactive label pool, the RNA precursor 5-[^{3}H]-orotic acid was given intraperitoneally to untreated animals and to animals injected with benzene. The amount of specific radioactivity incorporated into total liver RNA, liver polyribosomes and that found in the acid-soluble fraction were estimated and the results are presented in Table 2. Approximately 11 per cent of labeled precursor was incorporated into total liver RNA by either the untreated or benzene-treated groups. The amounts of specific radioactivity incorporated into the polyribosomes of the untreated animals was approximately 40 per cent of that incorporated into total liver RNA. In contrast, only 20 per cent of total liver RNA radioactivity was incorporated into the polyribosomes of the benzene-treated groups. The data of Table 2 indicate that benzene treatment does not affect the liver radioactive label pool. However, benzene significantly diminishes (50 per cent) the processing of RNA precursor incorporation into ribosomes.

Figure 1 shows a time-course graph of L-[^{3}H]-phenylalanine incorporation in the absence and presence of poly U and with polyribosomes and pH 5·1 fractions from normal and benzene-treated animals. Endogenous L-[^{3}H]-phenylalanine incorporation with fractions from benzene-treated animals is approximately 50 per cent less than that obtained with fractions from normal animals. pH 5·1 fractions from either normal or benzene-treated animals are equally effective with polyribosomes from either source.

TABLE 2. EFFECT OF BENZENE ON THE INCORPORATION OF 5-[^{3}H]-OROTIC ACID INTO TOTAL RNA, POLYRIBOSOMES AND ACID-SOLUBLE FRACTION OF RAT LIVER*

	Specific radioactivity (counts/min/O.D.$_{260}$ mμ)					
	Untreated†		(%)	Benzene		(%)
Total RNA	226‡	(200–250)	10·76	454	(380–510)	10·68
Polyribosomes	90	(60–110)	40·00§	98	(77–133)	21·60§
Total acid soluble fraction	1874	(1500–2100)	89·24	3801	(3050–4560)	89·32

* Benzene (3·37 m-moles/100 g body wt.) was given intraperitoneally followed by the injection of the RNA-labeled precursor. Each animal received 50 μc of 5-[^{3}H]-orotate (sp. act., 16·0 c/m-mole).

† As a precaution, paraffin oil was also injected intraperitoneally to a group of four animals (0·4 ml/animal) and the distribution of specific radioactivity (5-^{3}H-orotate) into various fractions was determined. In our hands, no significant variation from the untreated group was found.

‡ All figures represent the average of three experiments. Three animals were used for each experiment. The numbers in parentheses represent the range.

§ Per cent of total RNA radioactivity incorporated into ribosomes, i.e. 90/266 × 100 = 40 per cent.

Furthermore, approximately the same L-[^{3}H]-phenylalanine incorporation is obtained in the presence of poly U when the subcellular fractions are derived from either normal or benzene-treated animals. Similar results are obtained when cross-reacting the polyribosomes and the pH 5·1 fractions from normal and benzene-treated animals.

DISCUSSION

The data presented here show that the intraperitoneal administration of benzene to rats (a) causes disaggregation of liver polyribosomes which results in accumulation of ribosomal monomer–dimers and appearance of an intermediate, previously absent, peak; (b) diminishes considerably the capacity of the disaggregated liver polyribosomes to make protein *in vitro* as measured by L-[^{3}H]-phenylalanine incorporation but does not interfere with the poly U-directed incorporation of the amino acid; (c) inhibits considerably the incorporation of labeled RNA precursors into liver polyribosomes but does not interfere with their incorporation into total liver RNA and does not alter the size of the acid-soluble fraction radioactive label pool.

The altered sedimentation pattern of liver polyribosomes from treated animals and their considerably diminished ability to make protein *in vitro* using endogenous mRNA[1] support the contention that benzene affects the protein synthetic machinery of the liver cell. A decrease of approximately 50 per cent in amino acid incorporation was observed with polyribosomes from benzene-treated animals. Further, results of hybrid experiments with polyribosomes and pH 5·1 fractions from treated and unteated animals show that the toxic agent has no effect on sRNA and soluble enzymes. On the other hand, liver polyribosomes obtained from benzene-treated or untreated animals and "deprogrammed" by preincubation[11–15] are equally active in the poly U stimulated incorporation of L-[^{3}H]-phenylalanine (Fig. 1). These observations indicate that benzene affects endogenous mRNA resulting in disaggregation of polyribosomes and loss of endogenous *in vitro* protein synthetic function.

The above interpretation is further supported by the results of experiments *in vivo*. Treatment with benzene for 20 or 30 min causes breakdown of polyribosomes and decreased uptake of RNA labeled precursor (Figs. 2, 3; Tables 1, 2). Since no ribosomal RNA appears in the cytoplasm until 30–45 min after the administration of label,[16–18] the labeled RNA in the polyribosomes and top of the gradient after 20 min labeling must be mRNA. The possibility, however, of turnover of end groups in tRNA is also not excluded. Further, benzene did not affect the incorporation of RNA-labeled precursor into total liver RNA but did affect precursor incorporation into liver polyribosomes (Table 2). This observation excludes a direct action of the aromatic on RNA synthesis, i.e. on the DNA-depended RNA polymerase reaction. Since the size of the radioactive label pool is not affected by benzene treatment (Table 2), it is unlikely that the observed decreased incorporation of label into polyribosomes is because of such factors as altered peritoneal absorption of the tracer or altered hepatic blood flow.

Breakdown of liver polyribosomes and accumulation of higher monomer–dimer concentration was also found upon administration of actinomycin D,[19,20] puromycin and ethionine,[20,21] and carbon tetrachloride and dinitrophenol.[20,22–24] Furthermore, large amounts of ribosomal monomer–dimers were also found in certain neoplastic tissues as different human leukemias[3] and minimal deviation hepatomas.[25]

Disaggregation of polyribosomes after treatment with toxic agents is not completely understood and it appears that the causative factors differ in each instant. It has been suggested that actinomycin causes depletion of mRNA[19] (which is necessary for aggregation), puromycin increased mRNA decoding[21] and that ethionine[21] and dinitrophenol[24] cause disaggregation of polyribosomes probably by depletion of cellular ATP and hence by inhibition of mRNA synthesis. On the other hand, the occurrence of high monomer–dimer concentration could perhaps result from specific ribosomal aggregation.[26,27] Moreover, other workers[19,26] have also observed the intermediate ribosomal peak and have suggested that it might arise from the combination of the smaller ribosomal subunit and a monomer.[19]

The results of experiments *in vivo* as well as those of *in vitro* point to benzene action on the mRNA-ribosome level. The exact mechanism of action is, however, not understood. It has been reported that after its synthesis in the nucleus mRNA is transported to the cytoplasm where it becomes associated with polyribosomes.[16–18] The results of this study suggest that the toxic agent interferes in some way with its association with polyribosomes. Several investigators[11–13,15,19,22,23,28,29] have reported depression in the endogenous synthetic capacity of polyribosomes from animals treated with toxic agents and enhancement in poly U directed L-phenylalanine polymerization. It has also been suggested that toxic agents increase the availability of ribosomal surface for interaction with the coding agent[13] (mRNA). Since disaggregation of liver polyribosomes because of benzene is also observed *in vivo*,[1] it would be reasonable to deduce that benzene affects the disorganization of endoplasmic reticulum in a manner similar to that thought to occur in CCl_4 poisoning.[11,22–24] This interpretation is not contrary to benzene action at the mRNA-ribosome level (which is suggested by the present data) and is in accord with well documented reports[11,22–24,29–31] that liver poisons produce disruption of endoplasmic reticulum and detachment of membrane-bound ribosomes. It is, however, also conceivable that treatment with benzene may damage cellular structure in such a way as to liberate

ribonuclease activity which in turn acts on mRNA causing disaggregation of polyribosomes and decrease in endogenous ribosomal protein synthetic function.

Although a precise interpretation of the nature of benzene action cannot be offered, the varied effects of this agent both *in vivo* and *in vitro* make it attractive for further studies regarding toxicological effects at the molecular level.

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