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QUANTITATIVE MEASUREMENT OF MICROSOMAL SUBFRACTIONS ISOLATED FROM LIVERS OF RATS FED WITH 1,1,1-TRICHLORO-2,2-BIS (*p*-CHLOROPHENYL) ETHANE (DDT)*

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Abstract—Fractions enriched in smooth and rough endoplasmic reticulum were prepared from rat liver microsomal membranes by centrifugation and were characterized by analysis of their protein, RNA and phospholipid contents and by electron microscopy. Upon inclusion of 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT) in the diet, the amount of liver smooth endoplasmic reticulum increased rapidly and transitorily in both weanling and adult rats to approximately twice that of control animals. The increase was more pronounced in males than in females irrespective of age, but the rate of increase (although not the maximum extent of proliferation) in male animals became significantly less as they matured.

THE ADMINISTRATION of a wide variety of chemicals to animals either in the diet or by injection causes increases in a broad spectrum of hepatic microsomal drug-metabolizing enzymes.[1–3] In many instances, enzyme induction is accompanied by proliferation of the smooth endoplasmic reticulum (SER), and among compounds known to induce this latter phenomenon in rat liver are such chemically diverse substances as phenobarbital,[4–8] ethanol,[9] butylated hydroxytoluene,[10] methylcholanthrene,[7] dieldrin[11] and 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT).[12–15]

Although an increase in SER has been documented for the above cases, it has not generally been measured by biochemical techniques. Most investigators have relied upon electron microscopy to demonstrate changes, although in a few instances[15–17] the total microsomal population of the liver has been collected by differential centrifugation and estimated on the basis of its protein content. One study[8] describes changes in nucleic acid and protein content of hepatic microsomal subfractions from male rats which had received intraperitoneal injections of 3,4-benzpyrene, phenobarbital or chlordane up to 4 days previously. In this study, we report the isolation, characterization and quantitation of hepatic SER and rough endoplasmic reticulum (RER) of normal and DDT-treated rats of both sexes and describe the changes which these components undergo as a function of time of exposure of the animals to DDT. Isopycnic sucrose gradient centrifugation was used to effect separation, and membranes were characterized and quantitated by measurement of protein, RNA and phospholipid levels.

EXPERIMENTAL

Animals and diets. Wistar strain rats from the closed colony maintained by the

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Oregon State University, Agricultural Chemistry Department, were weaned at 28 days of age, housed in individual cages, and fed a semisynthetic diet[18] which comprised of 22% casein, 68% cerelose, 5% corn oil, 4% salt mixture and 1% vitamin mixture. Routine analyses of this feed showed it to contain less than 5 ppb chlorinated hydrocarbons. Where appropriate, p,p'-DDT (greater than 99 per cent pure; City Chemical Corp., New York) was added to the corn oil of the diet to give a final concentration of 150 ppm. This level did not alter the dietary intake of the animals, so they were fed *ad lib.*

As the rats grew, the food intake increased less rapidly than did the body weight. Consequently, the oral dose of DDT received per day decreased from an initial value of 20 mg/kg for weanling rats to 10 mg/kg for those 2 months older. In order to eliminate any possible diurnal variations in microsomal constituents, animals were killed at the same time each day (8.00 a.m.) and were starved overnight prior to sacrifice to reduce the liver glycogen content.

Isolation of microsomal subfractions. Rats were decapitated, and the livers were perfused *in situ* for 3–4 min with ice-cold 0·35 M sucrose in TKM (50 mM Tris, 25 mM KCl and 10 mM $MgCl_2$, pH 7·6). All subsequent operations were performed at 0–3°. After perfusion, the livers were removed, weighed, minced and then homogenized with four strokes of a Teflon–glass homogenizer in 4 vol. (milliliters/gram of fresh liver) of 0·35 M sucrose–TKM. The homogenate was centrifuged at 15,000 *g* for 10 min. Pelleted material consisting of organelles and membrane aggregates was gently resuspended with a Teflon pestle in 2 vol. of 0·35 M sucrose–TKM and centrifuged as before in order to recover as many of the microsomes as possible. Further resuspension was found to increase the microsomal yield by only 3–4 per cent and was not performed routinely.

Rough and smooth endoplasmic reticulum could be effectively separated from each other and from the supernatant proteins by centrifugation over a discontinuous sucrose density gradient. Aliquots (2 ml) from the combined post-mitochondrial supernatants were layered over gradients prepared from 2 M sucrose–TKM (0·7 ml), 1·3 M sucrose–TKM (1·8 ml) and 0·86 M sucrose–TKM (1 ml) and centrifuged for 3 hr at 204,000 *g* using SW50, SW50·1 or SW65 rotors in a Spinco preparative ultracentrifuge at 3°. The method used was basically that of Williams *et al.*[19] with the inclusion of a layer of 0·86 M sucrose, which allows separation of the SER from the supernatant proteins. After centrifugation, the membrane pellicles were removed by pipette and prepared immediately for electron microscopy or stored frozen at −20° for analysis. Pellets of the unattached ribosomes were collected from the bottom of the centrifuge tubes by gentle agitation with a glass rod in three 0·3-ml portions of water.

Chemical and enzymic analysis. RNA was determined by the Schmidt–Tannhauser procedure as modified by Fleck and Munro[20] without lipid extraction. The RNA was digested from the $HClO_4$ precipitate with 0·3 N KOH for 1 hr at 37°; an extinction coefficient of $E^{1\%}_{1cm} = 300$ at 260 nm was used to calculate the RNA content. Protein was measured by the method of Lowry *et al.*[21] using bovine serum albumin as a standard. Phospholipids were extracted according to Bligh and Dyer,[22] and measured by estimation of phosphate according to the method of Smith *et al.*[23] Glucose 6-phosphatase was estimated in the crude homogenate and post-mitochondrial supernatant by a method based upon that of Wilgram and Kennedy.[24]

Electron microscopy. The membrane fractions were removed from the discontinuous

sucrose gradients, diluted slowly with cold TKM solution, and pelleted in an SW25·2 rotor at 48,000 *g* for 40 min. These pellets were fixed with 2% glutaraldehyde for 60 min, embedded in 2% ion agar, washed with 0·25 M phosphate buffer, pH 7·0, and treated with 1% OsO_4. After osmium treatment, they were soaked overnight in acetone saturated with uranyl acetate and embedded in Araldite 6005–Epon 812 mixture. Sections, 60–75 mμ thick, were then cut with a diamond knife on an MT-2 Servall Potter–Blum ultramicrotome. Observations were made using a Phillips EM-300 electron microscope.

RESULTS

Separation and characterization of the microsomal subfractions. A typical separation of microsomal membranes is shown in Fig. 1. The SER banded upon the 1·3 M sucrose and was well separated both from the RER, which banded on the 2·0 M sucrose, and from the soluble proteins of the supernatant, which formed the dark layer visible at the top of the centrifuge tube. This dark layer does not represent particulate material but is a photographic artifact caused by the red color of the supernatant.

Electron micrographs of fractionated material from control and DDT-fed rats confirmed the presence of SER and RER in the appropriate fractions. In all cases, the membranes were present as vesicles, most of whose diameters lay within the range 100–300 mμ, in close agreement with the size distribution of isolated SER and RER reported by Rothschild.[25] Ribosomes were profuse in control RER, scarce in control SER, and could not be detected in the SER from DDT-treated rats. Clearly, the method is highly effective at separating smooth membranes from those with ribosomes attached to them.

The effectiveness of the separation technique was confirmed by measurement of the RNA–protein ratios of the various fractions, illustrated in Table 1. In the control animals, the RNA–protein ratio of the RER was almost six times greater than that of the SER. In the case of DDT-treated animals, it was over eight times greater. A value of 0·058 for the RNA–protein ratio of control SER is consistent with the electron micrograph evidence for some ribosome contamination; and this was confirmed by treatment with 5 mM EDTA and 1·0 M NaCl (a process known to dissociate ribosomes from membranes) which reduced the ratio to 0·011, identical to that reported by Reinert and Davis.[26] The nature of the ribosome contamination is not clear. If it were due to trapping of either free polysomes or of RER within the SER mass, one would expect that the amount of trapped material would increase if the total amount of SER increased. This was not the case, since the RNA–protein ratio of SER from control livers was greater than that of SER from DDT-treated rats. Alternative explanations for the phenomenon are either that the trapping is "internal" and occurs when the tissue is homogenized, or that there is a class of membrane-bound polysomes which are present only in small amounts and which have a much lower density than the bulk of the RER.

Before quantitative estimates of microsomal subfractions can be considered valid, it is necessary to show that the experimental technique gives reasonable recoveries of the total microsomal membranes present. Further, there should be no significant difference between the recoveries of membranes from tissues of control and treated animals. In order to establish these two points, the ribosomal RNA content of the

Table 1. Composition of rat liver microsomal subfractions*

Fraction	Density	Protein (mg/g liver)	RNA (mg/g liver)	RNA/protein	P-lipid/protein†
Control SER	1·1110–1·1556	13·79 ± 3·15	0·80 ± 0·10	0·058 ± 0·008	0·54 ± 0·06
DDT SER	1·1110–1·1556	31·37 ± 0·82	1·21 ± 0·05	0·038 ± 0·002	0·56 ± 0·04
Control RER	1·1556–1·2534	9·34 ± 0·72	3·20 ± 0·12	0·342 ± 0·020	0·43 ± 0·03
DDT RER	1·1556–1·2534	10·69 ± 0·74	3·28 ± 0·20	0·308 ± 0·008	0·45 ± 0·05
Control ribosome pellet	> 1·2534	0·69 ± 0·04	0·70 ± 0·06	1·02 ± 0·03	
DDT ribosome pellet	> 1·2534	0·47 ± 0·07	0·51 ± 0·03	1·08 ± 0·05	

* Three 42-day-old male rats were fed control diet and three were fed 150 ppm DDT diet for 15 days before their livers were removed. Values represent the mean for three rats ± S.E.M.

† Lipid phosphorus content was assumed to be 4 per cent.

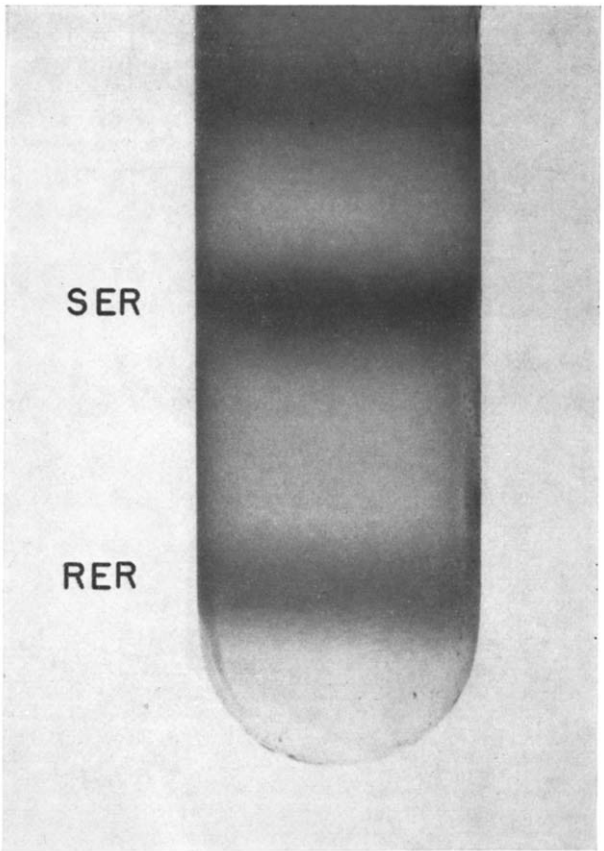

FIG. 1. A discontinuous sucrose density gradient after centrifugation of 2 ml of the post-mitochondria supernatant from the liver of a 28-day-old weanling rat for 3 hr at 204,000 *g*. The composition of the gradient is described in the text.

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post-mitochondrial supernatant was compared to that of the crude homogenate and as an index of the amount of total RER liberated. Samples of crude homogenate and post-mitochondrial supernatant were diluted with TKM; subjected to ultracentrifugation, and the total RNA of the pellets was measured. Consistently, 41 per cent of the total RNA was recovered in the post-mitochondrial supernatant. No significant differences were observed between control and treated animals. Assays of glucose-6-phosphatase (a microsomal membrane marker enzyme[24]) supported this conclusion. The results were, as expected, more variable, but showed that approx. 43 per cent of the total microsomes were recovered in the post-mitochondrial supernatant. Total membrane recovery then is reproducible and consistent. The amounts recovered agree closely with those reported by Blobel and Potter.[27]

An increase in the amount of SER as a consequence of DDT feeding (Table 1) was evident at 15 days and is discussed in more detail below. The decrease in free ribosomes in livers of DDT-fed animals was not, however, observed in all experiments and we do not regard the differences shown in Table 1 as significant.

Time course of the effects of DDT treatment on hepatic microsomal subfractions. Changes in SER and RER upon feeding DDT to weanling rats are illustrated in Fig. 2.

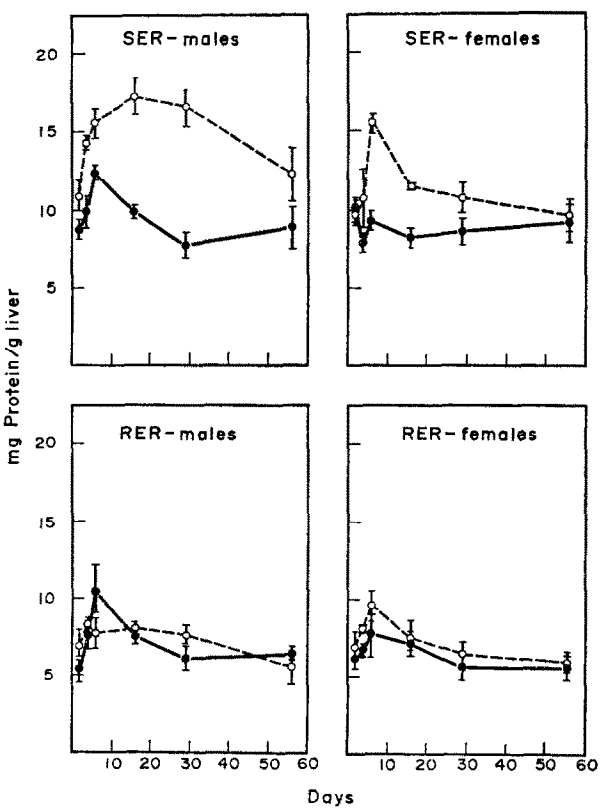

FIG. 2. Effect of DDT on liver microsomal subfractions of weanling rats. Rats at 28 days of age were weaned directly onto either control diet (●——●) or a diet containing 150 ppm DDT (○- - -○). Hepatic SER and RER levels were measured after the time periods shown. Each point represents the mean of three rats ± S.E.M. as indicated by the vertical bars.

Inclusion of 150 ppm DDT in the diet did not affect growth rate but increased liver weight an average of 23 per cent in the males and 9 per cent in the females. In both males and females there was an increase in SER protein per gram of liver to about twice the normal level during the first 2 weeks of DDT treatment and an eventual return toward control SER levels as feeding continued. When phospholipid content (milligrams per gram of liver) was used as a measure of membrane concentration, essentially the same results were obtained. It will be noted that there were temporary increases in SER and RER levels of control male animals when they were weaned on to a solid diet. In order to eliminate these variations, which tended to obscure the effect of the DDT treatment, weanling rats were fed the control diet for 14 days and then placed upon DDT-containing rations for an extended period. Figure 3 shows the results obtained.

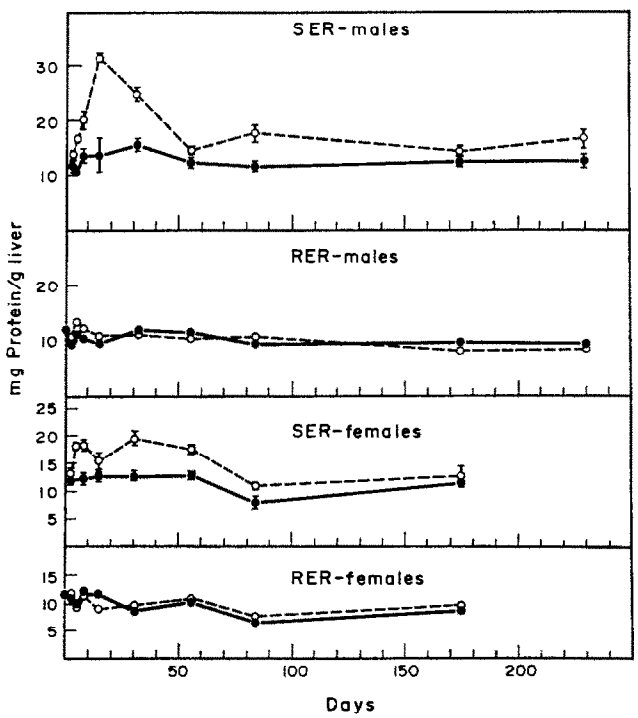

FIG. 3. Effect of DDT on liver microsomal subfractions of weaned rats. Rats at 28 days of age were weaned and fed control diet for 2 weeks. These rats were then fed control diet (●——●) or a diet containing 150 ppm DDT (○- - -○). Hepatic SER and RER levels were measured after the time periods shown. Each point represents the mean of three rats ± S.E.M. as indicated by vertical bars. RER values are not accompanied by the S.E.M., since they overlap at every time point.

The initial increases in control SER and RER levels were eliminated, and the proliferation of SER could be seen more clearly. Moreover, the proliferation of SER followed approximately the same pattern as before, except that the SER in DDT-treated females remained elevated for 2 months before returning to a level slightly above control. In both experiments the DDT-induced SER proliferation was greater in males than females.

Since the onset of sexual maturity occurs between 40 and 60 days, and might be

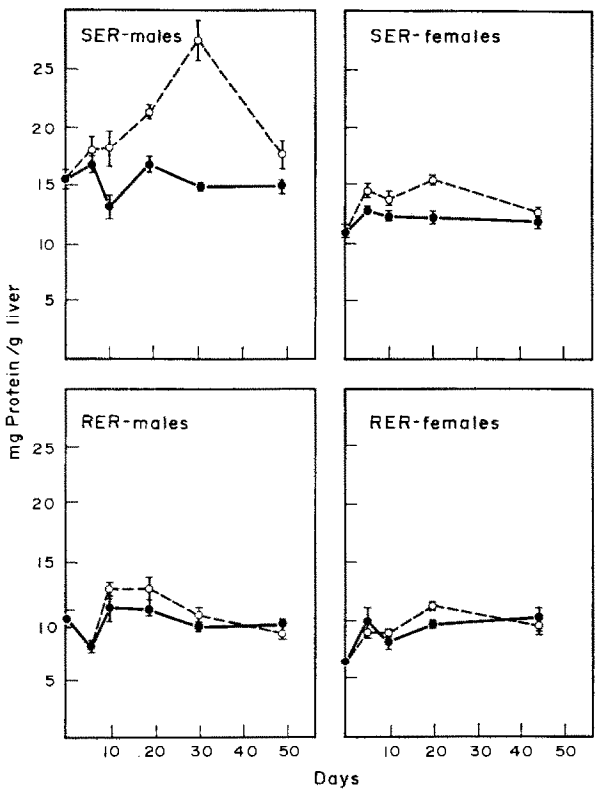

FIG. 4. Effect of DDT on liver microsomal subfractions of adult rats. Rats at 28 days of age were weaned and fed chow diet for 48 days. These rats were then pretreated with control diet for 4 days before they were fed control diet (●——●) or a diet containing 150 ppm DDT (○- - -○). Hepatic SER and RER levels were measured after the time periods shown. Each point represents the mean of four rats ± S.E.M. indicated by the vertical bars.

expected to have some influence upon SER proliferation, the effects of DDT upon mature (80-day-old) animals were also investigated, as shown in Fig. 4. The average weight of the males at the beginning of the experiment was 220 g, and the average was 179 g for the females. Once again there was a transitory build-up of SER in livers of the DDT-treated animals. The rise in SER was slower in the adult males than in the weanling males, and liver SER proliferated to a lesser extent in the adult females than in the immature females of the previous two experiments.

DISCUSSION

Since 1960, the fractionation of microsomal membranes has been accomplished by a variety of procedures[19,25,28–33] based upon isopycnic sedimentation in a discontinuous sucrose density gradient. In our hands, the technique of Williams *et al.*,[19] modified by the inclusion of a layer of 0·86 M sucrose to ensure separation of SER from the supernatant fraction, gave good separation. Soluble protein, SER, RER and free ribosomes were all well separated by a single centrifugation on a three-step discontinuous gradient.

Juchau and Fouts[8] noted a 17 per cent increase in SER after 72 hr of treatment with

phenobarbital or chlordane. We found greater increases in hepatic SER upon inclusion of DDT in the diet for a longer time period. At the peak of proliferation, after 20 days on the diet, the SER content per gram of liver was approximately double that of the control animals. However, the experiments revealed that the increase was transitory. In males, where proliferation was most extensive, the levels declined from a peak at 20–30 days of feeding and, after 60 days on the diet, were only marginally elevated over controls. This is in contrast to the findings of Ortega,[12] who, using much larger doses of DDT (1000 and 2500 ppm), was able to show by electron microscopy markedly elevated SER levels in rats fed DDT for 6 months. The decrease in SER subsequent to the peak of induction observed here at a lower dose level may be accounted for by a variety of explanations, including reduction in the effective dose rate with time (as animals aged they consumed less diet in proportion to their body weight), increased metabolism and clearance of DDT, or increased storage in organs other than the liver.

It would be instructive to be able to compare the time couse of DDT induction of microsomal drug-metabolizing enzymes described in the literature[34–38] with the change in SER described here. Unfortunately, in most of the studies in which dietary DDT has been used to induce microsomal enzymes, either the DDT levels have been different from those fed here, or enzyme activities were not measured until after 6 weeks of feeding had taken place, so that valid comparison is difficult. Thus, several enzymes have been shown to be induced to constant levels by feeding rats a diet containing 50 ppm DDT.[34,36] Aminopyrine demethylase behaves typically in that it is elevated to two[34] or three times[36] the control levels after 2 or 3 months of feeding. In our work (with 150 ppm DDT) the SER levels have fallen by this time to levels only slightly above those of control animals, so that nothing can be said as to possible changes in enzyme specific activity or distribution among the microsomal fractions.

Although Kinoshita *et al.*[34] measured total aminopyrine demethylase in crude liver homogenates as early as 1 week after administration of DDT, in only one instance has the specific activity of a microsomal drug-metabolizing enzyme been measured early in the induction process. Gillette[38] fed weanling male rats with a diet containing 25 ppm DDT and examined the activity of aldrin epoxidase in the first 6 weeks of feeding. The specific activity of the enzyme (expressed on the basis of total microsomal protein) was found to increase continuously until at 6 weeks it reached a value 250 per cent greater than that of the controls. In the light of our demonstration that SER proliferation (induced by 150 ppm DDT in rats from the same colony) peaked at 20–30 days and then diminished, it may be that the final high specific activity of aldrin epoxidase is due to enrichment of the SER population with a class of membranes containing much greater amounts of the enzyme. If this is true, then the initial increase in SER would be a reflection of the synthesis of new sets of endoplasmic reticulum containing detoxifying enzymes whose increase in true specific activity would not be apparent until later.

Until accurate measurements of specific activities of several microsomal enzymes, including DDT-metabolizing enzymes, are made in both SER and RER both early and late in the induction process, this point must remain unresolved.

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