Preparation of Hepatic Microsomal Fraction for Drug Metabolism Studies by Rapid Decompression Homogenization (36395)

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Various methods of homogenization of tissue (Waring Blendor, colloid mill, ultrasonic vibrator, Potter homogenizers, and other grinding equipment) used in the preparation of microsomal fractions have been described in the literature. The Teflon pestle and glass tube (P.T.) or Potter–Elvehjem types of homogenizer have generally been found superior in producing a microsomal fraction of high activity, and these homogenizers are most widely used today. The use of the P.T. homogenizer, however, is not without faults. For reproducible results, the process of homogenization should be rigidly controlled (1). Occasional microscopic examination of the homogenate produced is warranted to assure complete cell disruption. In addition, high shear forces accompanied by significant local heating can occur under conditions which produce the most complete cell disruption (small pestle–tube clearance and high rotor speeds) (2).

The present paper reports on an investigation into the suitability of the rapid decompression (R.D.) technique of homogenization for the preparation of microsomes to be used in studies on the mixed function oxidases and certain other drug metabolizing enzyme systems. The applicability of the method to the isolation of mitochondria from mammalian tissues has previously been described (3). The technique involves the equilibration of an inert gas, usually nitrogen, with tissue mince under high pressure. When the pressure is released rapidly, the change in pressures causes an explosive rupture of the cellular plasma membrane. This method of cell disruption is reported to have the advantages of lack of heat generation during homogenization, mild and selective action (pressure can be varied), and more uniform homogenization of cells of different sizes.

The activity of oxidative, reductive, hydrolytic, and glucuronide-forming enzyme systems of microsomal fractions prepared by R.D. or P.T. homogenization is compared. It is shown that the yield of microsomal enzyme activity per unit weight liver is higher for R.D. homogenization. The greater yield of microsomal fraction is probably related to more complete cell rupture and possibly to differences in the morphology (size) of the microsomal particles.

Methods. Adult male rabbits were sacrificed by decapitation and the livers were removed and placed in ice-cold 1.15% KCl or 0.25 *M* sucrose after excision of the gall bladder. The liver was minced with a hand press (Harvard Apparatus) and a weighed portion was suspended in three volumes of isotonic KCl or sucrose. The crude suspension was then subjected to homogenization with either a Teflon pestle and glass tube homogenizer (Kontes Glass) or a cell disruption bomb (Parr Instruments). A single relatively close-fitting pestle and tube homogenizer was used throughout the study, and tissue was homogenized uniformly with six strokes of the pestle rotating at 800 rpm with the tube in a Dewar flask of ice. Thirty milliliters of suspension was placed in a conical centrifuge tube in a cell disruption bomb sitting in an ice bath. The suspension was subjected to 700–1000 psi of nitrogen for varying periods of time. The homogenate produced upon release of pressure was collected in a cold Erlenmeyer flask. In one experiment, the initial suspension was subjected to

TABLE I. Effect of Varying Pressure and Time on Homogenization of Rabbit Liver in 1.15% KCl.[a]

		Cytochrome P_{450}		Succinate oxidase natoms O/mg microsomal protein/min	Microsomal protein mg/g liver
		nmoles per g wet wt of liver	nmoles per mg of microsomal protein		
Cell disruption bomb					
Pressure (psi)	Equilibration time (min)				
1000	5	85.05 ± 1.73[b]	2.35 ± 1.00	17.6 ± 2.0	35.7 ± 3.6[b]
	15	93.72 ± 1.46[b]	2.53 ± 0.39	15.3 ± 1.3	37.0 ± 2.6[b]
900	5	85.91 ± 1.49[b]	2.49 ± 0.17	14.9 ± 0.7	34.4 ± 2.8[b]
	15	89.47 ± 1.33[b]	2.56 ± 0.23	14.8 ± 1.1	34.9 ± 1.7[b]
800	5	88.44 ± 1.19[b]	2.76 ± 0.26[b]	14.0 ± 1.1	32.0 ± 1.6[b]
	15	82.75 ± 1.36[b]	2.50 ± 0.17	15.0 ± 1.4	32.7 ± 2.4[b]
700	5	87.31 ± 1.57[b]	2.53 ± 0.17	15.4 ± 1.4	33.5 ± 4.0[b]
	15	86.35 ± 1.46[b]	2.63 ± 0.20	14.4 ± 0.9	32.8 ± 2.5[b]
Teflon pestle and glass tube		40.89 ± 0.96	2.13 ± 0.17	17.3 ± 2.1	19.2 ± 2.1

[a] Mean ± SEM for determination of six animals.

[b] Significant difference ($p < .05$) between the mean values for cell disruption bomb and Teflon pestle and glass tube.

pestle and tube homogenization and then to rapid decompression in the Parr cell bomb.

Homogenates were centrifuged at 9000*g* for 15 min at 2°. The resulting supernatants were used as the enzyme source for assays of the rates of oxidative, reductive, and hydrolytic metabolism or centrifuged at 105,000*g* in a Spinco L-2 ultracentrifuge. The 105,000*g* microsomal pellet was suspended in 0.1 *M* Na–K phosphate buffer, pH 7.35, and was used as the enzyme source for the measurement of phenolphthalein glucuronide formation and for assays of cytochrome P_{450}, cytochrome b_5, protein levels, and cytochrome P_{450} reductase and NADPH–cytochrome *c* reductase activity.

Substrates were incubated with 9000*g* supernatant or 105,000*g* pellet fractions equivalent to 250 mg liver and cofactors necessary to the reaction as described previously (4) except that nicotinamide was omitted from the incubation mixture. The rate of *O*-demethylation of *p*-nitroanisole was determined by measuring the formation of *p*-nitrophenol by a modification (4) of the method of Netter (5). The rates of nitroreduction of *p*-nitrobenzoic acid and ester hydrolysis of procaine were determined by measuring the *p*-aminobenzoic acid formed (6). The rate of azo-reduction of Neoprontosil was determined by assaying the sulfanilamide formed (7) and the rate of formation of phenolphthalein glucuronide was determined by measuring the disappearance of substrate (8).

Cytochrome P_{450} and cytochrome P_{450} reductase were assayed by reduction with NADPH (9) using an Aminco anaerobic spectrophotometric cell. Cytochrome P_{450} was assayed by reduction with sodium dithionite in the studies comparing homogenizing medium, pressure and equilibration time. Cytochrome b_5 was measured using NADH (10) as the reducing agent. The microsomal suspension in the above assays contained the equivalent of 67 mg wet wt liver/ml. NADPH–cytochrome *c* reductase was determined by a modification (9) of the method of Williams and Kamin (10). The microsomal suspension used for this assay contained 0.17 mg of protein/ml.

All of the above assays were performed on a Shimadzu MPS-50L recording spectrophotometer using 1-cm^2 cuvettes. NADPH reductible cytochrome P_{450}, cytochrome P_{450} re-

TABLE II. Effect of Varying Pressure and Time on Homogenization of Rabbit Liver in 0.25 *M* Sucrose.[a]

Cell disruption bomb		Cytochrome P_{450}		Succinate oxidase natoms O/mg microsomal protein/min	Microsomal protein mg/g liver
Pressure (psi)	Equilibration time (min)	nmoles per g wet wt of liver	nmoles per mg of microsomal protein		
1000	5	71.86 ± 1.46[b]	2.16 ± .20	16.6 ± 1.4	33.2 ± 2.6[b]
	15	75.23 ± 1.76[b]	2.23 ± .20	15.8 ± 1.2	33.7 ± 3.9[b]
900	5	72.72 ± 1.30[b]	2.16 ± .21	16.3 ± 0.9	33.6 ± 2.1[b]
	15	70.46 ± 1.46[b]	2.09 ± .23	15.9 ± 1.5	34.3 ± 2.3[b]
800	5	70.89 ± 1.80[b]	2.10 ± .23	14.8 ± 0.9	33.8 ± 3.9[b]
	15	71.53 ± 1.43[b]	2.10 ± .23	15.7 ± 1.1	34.1 ± 1.8[b]
700	5	74.05 ± 1.37[b]	2.20 ± .30	15.0 ± 1.0	33.7 ± 2.4[b]
	15	71.76 ± 1.30[b]	2.26 ± .33	15.2 ± 1.5	31.7 ± 4.4[b]
Teflon pestle and glass tube		29.67 ± 0.69	1.96 ± .30	18.7 ± 1.8	15.1 ± 2.5

[a] Mean ± SEM for determination on six animals.

[b] Significant difference ($p < .05$) between mean values for cell disruption bomb and Teflon pestle and glass tube.

ductase, and NADPH–cytochrome *c* reductase were assayed at 37°. Cytochrome b_5 and dithionite reducible cytochrome P_{450} were assayed at 25°. Extinction coefficients of 18,500, 91,000, and 163,000 cm^{-1} were used to estimate reduced cytochrome *c*, and cytochromes P_{450} and b_5, respectively.

Type I and type II binding spectra were obtained using hexobarbital and aniline, respectively, as substrates as described by Remmer *et al.* (11). The magnitude of spectra obtained using 3.0 m*M* substrate and 2.0 mg of microsomal protein per ml were calculated as the difference in ΔA at 490 nm and 422 nm (hexobarbital) or as the difference in ΔA at 430 nm and 490 nm (aniline).

Succinate oxidase activity was determined on the 105,000*g* microsomal fraction as described by Blair *et al.* (12). Succinate oxidation was measured polarographically with an oxygen electrode (Gilson Medical Electronics Oxygraph) at 30 ± 1°. The 1 ml reaction mixture contained 0.5 ml of a solution containing 2×10^{-4} *M* EDTA in 0.04 *M* phosphate buffer, pH 7.45, 0.02 ml of 1% cytochrome *c* (Sigma, Type III), 0.08 ml H_2O, and 0.3 ml of suspended 105,000*g* pellet containing microsomes equivalent to 250 mg of ml/liver. Microsomal protein was estimated by the method of Lowry *et al.* (13), with crystalline bovine serum albumin as a standard.

Results. The effects of pressure and equilibration time used in R.D. homogenization were studied in tissue mince suspended in 1.15% KCl (Table I) or 0.25 *M* sucrose (Table II). Varying pressure from 700 to 1000 psi had no significant effect on the dithionite reducible cytochrome P_{450} or protein content of the microsomal fraction. In addition, allowing 15 min equilibration time did not increase microsomal yields at any pressure. The cytochrome P_{450} and microsomal protein contents of liver were significantly higher at all pressures and equilibration times than when liver was homogenized with pestle and tube. The specific content of cytochrome P_{450} (cytochrome/mg protein) was only slightly and insignificantly ($p > .05$) higher than that produced by P.T. homogenization in 0.25 *M* sucrose.

Homogenization in 1.15% KCl, however, produced occasionally significant differences in the specific content of the cytochrome. This is in accord with higher *N*-hydroxylation activity in P.T. homogenates of KCl

TABLE III. Comparison of Drug Metabolizing Enzyme Activity, Cytochrome P_{450} Content, and Protein Content of Microsomes Prepared by Teflon Pestle and Tube (P.T.) and/or Rapid Decompression (R.D.) Homogenization.[a]

Method of preparation	Cytochrome P_{450} nmoles per g liver	Microsomal protein mg/g liver	Enzyme activity (μmoles substrate metabolized/g liver/hr)				
			p-Nitroanisole	*p*-Nitrobenzoic acid	Neoprontosil	Procaine	Phenolphthalein
a. P.T.	36.92 ± 1.26 (2.02)	18.2 ± 2.2	0.961 ± .115 (52.8)	0.352 ± .070 (19.3)	4.087 ± .492 (224.5)	9.729 ± .209 (534.5)	1.572 ± .255 (86.4)
b. P.T. + R.D.[b]	77.80 ± 2.19[c] (2.71)[c]	28.7 ± 4.1[c]	1.349 ± .162[c] (47.0)	0.536 ± .069[c] (18.7)	6.259 ± .661[c] (219.3)	14.834 ± 1.20[c] (516.5)	2.405 ± .282[c] (83.8)
c. R.D.[b]	78.63 ± 1.92[c] (2.70)[c]	29.1 ± 3.6[c]	1.472 ± .157[c] (50.6)	0.573 ± .075[c] (19.7)	6.150 ± .627[c] (211.3)	16.101 ± 1.84[c] (553.3)	2.294 ± .207[c] (78.8)

[a] Values represent mean ± SEM for determination on eight animals. Enzyme activity or cytochrome content per mg microsomal protein is represented in parentheses.

[b] 700 psi pressure and 5 min equilibration time.

[c] Significant difference ($p < .05$) between R.D. and P.T.

TABLE IV. Comparison of Microsomal Electron Transport Cytochromes and Reductases and Substrate Spectral Changes in Tissue Prepared by Teflon Pestle and Tube (P.T.) or Rapid Decompression (R.D.) Homogenization.[a]

Method of preparation	Cytochromes (nmoles/mg protein)		Reductases (nmoles substrate reduced mg protein/min)		Spectral Changes (Δ A per mg protein $\times 10^3$ 3×10^{-3} *M* Substrate)	
	NADPH-reducible P_{450}	NADPH-reducible b_5	Cytochrome P_{450}	NADPH–cytochrome c	Hexobarbital (Type I)	Aniline (Type II)
P.T.	1.36 ± .08	0.67 ± .02	3.62 ± 0.37	89.38 ± 4.32	11.6 ± 0.7	22.1 ± 1.0
R.D.	1.32 ± .10	0.69 ± .01	4.34 ± 0.25	84.30 ± 3.72	9.4 ± 0.8	21.4 ± 1.1

[a] Values represent mean ± SEM for determinations on six animals.

than sucrose reported by von Jagow *et al.* (1). Homogenization by either method in KCl produced significantly higher levels of cytochrome P_{450}/g of liver than in sucrose, while protein levels were affected noticeably only with P.T. homogenization.

Succinate oxidase activity of the suspended microsomal pellet was assayed in order to assess the extent of contamination of this fraction with electron transport particles derived from mitochondrial disruption. No significant differences were found between the pestle and tube preparation and that of the cell disruption bomb at any pressure or equilibration time. Similar results were obtained on a limited number of succinate oxidase assays run on the 9000*g* supernatant fraction, though the values were smaller because of the high protein content of this fraction. This is in accord with the findings of Hunter and Commerford (3) who found no loss of this activity in the mitochrondrial fraction isolated following homogenization with a cell disruption bomb using 1000 psi and 20 min equilibration time. There were no differences in the succinate oxidase activity related to the homogenizing medium.

The substrate metabolizing activity of the 9000*g* supernatant fractions is compared in Table III. The protein and cytochrome P_{450} contents of the 105,000*g* microsomal pellet are included, and the values in parentheses represent cytochrome content or enzymic activity per milligram microsomal protein. The values obtained from tissue homogenized by P.T. and R.D. were not significantly different from those obtained on tissue homogenized by R.D. alone. The nearly identical values obtained indicate: 1) that there was no additive effect of the two treatments, *i.e.*, that prior P.T. homogenization had no positive effect on the R.D. method of preparation; and 2) that P.T. homogenization did not decrease enzymic activity through such factors as heat generation and mechanical shear under the conditions employed. This latter conclusion is further supported by the finding that the substrate metabolizing activity of tissue prepared by all three methods was not significantly different when computed as

TABLE V. Cytochrome P_{450} Content and Weight of 105,000*g* Microsomal Pellets Prepared by Varying Methods.[a]

Source of 105,000*g* pellet preparation	Method of preparation	Cytochrome P_{450} (Δ A per microsomes equiv to 67 mg/liver/ml)	Weight of 105,000*g* pellet (mg)
9000*g* Supernatant	P.T.	0.280 ± .047	434 ± 27
	R.D.	0.540 ± .083	624 ± 34
800*g* Supernatant	P.T.	0.215 ± .034	336 ± 29
	R.D.	0.146 ± .027	289 ± 31

[a] Values represent mean ± SEM for determinations on four animals.
[b] Microsomes reduced with sodium dithionite.

μmoles of substrate metabolized/mg of microsomal protein.

All substrates were metabolized at a higher rate on a per gram liver weight basis when incubated with 9000*g* supernatant but there was no change in the specific activity (units metabolized per mg of protein) of microsomal enzymic activity for any pathway. In contrast, the specific content of dithionite reducible cytochrome P_{450} was significantly ($p < .05$) increased by R.D. homogenization in this experiment, producing a poor relationship between increase in the rate of metabolism of *p*-nitroanisole, *p*-nitrobenzic acid, and Neoprontosil and increase in the content of this oxygen activating enzyme.

No significant difference in the specific content of NADPH-reducible cytochrome P_{450} was found, however (Table IV). Cytochrome b_5 levels proved to be a function of protein content, as did cytochrome P_{450} reductase and NADPH-cytochrome *c* reductase activities. The magnitude of Type I or Type II spectral changes produced at one concentration of hexobarbital or aniline, respectively, were not significantly different either, indicating that the degree of binding of these substrates to cytochrome P_{450} was not appreciably altered by R.D. homogenization.

Several short studies were carried out in an attempt to explain the increases in parameters of drug metabolizing enzyme activity observed on a liver weight basis for R.D. homogenization. Slides of crude homogenates of both preparations were stained with Wright's stain and examined by light microscopy. No whole cells were found in the R.D. homogenate produced using 700 psi and 5 min equilibration time, while numerous clumps of cells were observed in the P.T. homogenate. It was obvious that a failure of P.T. homogenization to disrupt all cells in the minced tissue was a major contributing factor to the differences observed for the two methods of homogenization. This lack of efficiency warranted the examination of other sets of pestles and tubes for apparent "closeness of fit" and production of microsomal protein and cytochrome P_{450}. A limited trial showed that there was considerable variation between three sets of such homogenizers, but that fairly reproducible results were obtained with any one set.

Another possible explanation for the higher yields of dithionite reducible cytochrome P_{450} produced by R.D. homogenization was considered. It was known to early investigators (14) that the sediment from the initial centrifugation contained considerable amounts of microsomal fraction, and it has been shown that the extent of this loss can be varied by altering centrifugation speed and time (1). The possibility that more of the microsomal fraction was sedimented at 9000*g* in the P.T. homogenate was investigated by centrifuging both homogenates initially at 9000*g*, preparing a 105,000*g* pellet from the supernatant, and weighing the pellet and measuring the Δ A (450–490) of the CO-binding pigment in the suspended pellet. The 9000*g* sediment was then suspended in 1.15% KCl and centrifuged at 800*g* to remove whole cells and large debris. A 105,000*g* pellet was prepared from the supernatant and

treated as above. The results presented in Table V show that more cytochrome and more 150,000*g* sedimentable material (microsomes and mitochondria) were washed out of the P.T. 9000*g* sediment than out of the R.D. sediment. This was an unexpected result if one assumes nearly equal dispersion of microsomal particles throughout the 9000*g* supernatant and sediment, as the cytochrome content of the R.D. 9000*g* supernatant was nearly twice that of the P.T. supernatant. This finding suggested that the size of the microsomal particles produced by the two methods of homogenization may not be equal, and specifically, that the size of those produced by P.T. homogenization may vary in size, some being sedimentable at 9000*g* and 15 min centrifugation. Electron microscopy of the 105,000*g* pellets prepared by both methods illustrated that many of the microsomal particles of the P.T. homogenate were larger than those of the R.D. preparation, and that they were in general less uniform in size. While sedimentation constants were not obtained, the observed morphological differences could be responsible in part for the higher activities per gram of tissue weight obtained by R.D. homogenization.

Discussion. These studies have demonstrated that homogenization of liver by the rapid decompression technique produces a larger microsomal fraction per unit weight of liver than a comparable fraction produced by homogenization with Teflon pestle and glass tube. The drug metabolizing enzyme activity per milligram of microsomal protein of the 9000*g* supernatant, however, is not increased by this technique. Spectral binding studies and measurement of cytochromes and reductases involved in the mixed function oxidase electron transport system revealed no significant differences in the microsomal fraction related to the method of preparation. The increase in specific content of dithionite reducible cytochrome P_{450} in two experiments was unexpected in view of the lack of increase in the NADPH-reducible cytochrome. The lack of parallelism between increases in the dithionite reduced cytochrome and substrate metabolizing activity indicates that NADPH is the preferred reducing agent in this assay on microsomes prepared by the R.D. method.

Cell disruption by the R.D. method appeared to produce smaller and more uniform microsomal particles than P.T. homogenization. This may have resulted in a smaller loss of microsomal particles in the initial centrifugation employed in producing the microsomal fraction. Additional significance of this morphologic difference was not apparent.

It would appear that the major advantages of R.D. homogenization are: 1) that it produces a microsomal fraction which more accurately reflects tissue content of endoplasmic reticulum; 2) that, since all cells are disrupted, there is probably less variation in the amount of microsomal fraction produced than with different sets of pestles and tubes; and 3) that the specific activity of certain commonly studied parameters of microsomal enzyme activity is at least equal to that produced by P.T. homogenization. This method thereby allows optimal enzymic activity to be obtained from a given piece of tissue, as it circumvents enzyme degeneration associated with procedures by which P.T. homogenizers yield high microsomal fractions (2).

Summary. The applicability of rapid decompression as a method for homogenization of liver in the preparation of a microsomal fraction has been studied. It was shown that this method is more efficient and probably less variable than homogenization with a Teflon pestle and glass tube. Increased efficiency was related to more complete cell disruption and probably to the production of smaller microsomal particles. The specific content and activity of components of the mixed function oxidase system of the microsomal fraction were similar to those produced with pestle and tube.

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1. Von Jagow, R., Kampffmeyer, H., and Kiese, M., Naunyn–Schmiedebergs Arch. Exp. Pathol. Pharmakol. **251**, 73 (1965).

2. Dounce, A. L., Chargaff, E., and Davidson, J. N., "The Nucleic Acids," Vol. II. Academic Press, New York (1955).

3. Hunter, M. J., and Commerford, S. L., Biochem. Biophys. Acta **47**, 580 (1961).

4. Short, C. R., and Davis, L. E., J. Pharmacol. Exp. Ther. **174**, 185 (1970).

5. Netter, K. J., Naunyn–Schmiedebergs Arch. Exp. Pathol. Pharmakol. **238**, 292 (1960).

6. Fouts, J. R., and Brodie, B. B., J. Pharmacol. Exp. Ther. **119**, 197 (1957).

7. Hernandez, P. H., Gillette, J. R., and Mazel, P., Biochem. Pharmacol. **16**, 1859 (1967).

8. Talalay, P., Fishman, W. H., and Huggins, C., J. Biol. Chem. **166**, 757 (1946).

9. Gigon, P. L., Gram, T. E., and Gillette, J. R., Mol. Pharmacol. **5**, 109 (1969).

10. Williams, C. H., and Kamin, H., J. Biol. Chem. **237**, 587 (1962).

11. Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhulu, J., Cooper, Y., and Rosenthal, O., Mol. Pharmacol. **2**, 187 (1966).

12. Blair, P. V., Oda, T., and Green, D. E., Biochemistry **2**, 756 (1963).

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **19**, 265 (1951).

14. Schneider, W. C., J. Biol. Chem. **176**, 259 (1948).

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