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Use of 10-sec sonication of homogenates to increase microsomal protein yield in liver and lung from young and adult Dutch Belt rabbits

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THE TECHNIQUE for microsomal preparation used in this laboratory has been that described by Fouts and Devereux[1] and Hook *et al.*[2] Until now the number of experiments involving drug metabolism in young animals has often been limited by the small amount of liver or other tissue available. However, by treating tissue homogenates with 10 sec of sonication, one can nearly double the microsomal protein yield per g of liver or lung. The specific activities of the microsomal enzymes, benzphetamine demethylase, aniline hydroxylase and aminopyrine demethylase as well as cytochrome P-450 concentration remained about the same before and after sonication of tissue homogenates, whereas the total protein, enzyme activities and cytochrome P-450 content increased almost 2-fold.

We used 1-week-old (both sexes) and adult (male) Dutch Belt rabbits (Arrow Farms, Statesville, N. C.) in this study. The rabbit housing conditions and homogenate and microsomal preparation were the same as reported previously[1] with the exception of sonication.

After homogenization of the liver and lung tissues, the homogenates were subjected to 10 sec of sonication at 70 W output. A Branson sonicator model W185 was used. The tip of the probe was held about $\frac{1}{4}$ to $\frac{1}{2}$ inch below the surface of 20–30 ml of homogenate in a 50-ml beaker packed in ice. The temperature of the homogenate remained below 10° during sonication.

The amount of protein in the microsomal fraction was determined by the method of Lowry *et al.*[3] Cytochrome P-450 was measured using the dithionite-difference method of Omura and Sato[4] on a Shimadzu model MPS 50 L recording spectrophotometer. Benzphetamine demethylase and aminopyrine demethylase were assayed by measuring the amount of formaldehyde formed.[5] Aniline hydroxylase was measured following the method of Imai *et al.*,[6] assaying the amount of *p*-aminophenol formed. The mitochrondial enzyme, succinate cytochrome *c* reductase, was assayed according to the method of Singer and Kearney.[7] Lipid peroxidation was measured by the thiobarbituric acid method of Hunter *et al.*[8]

For electron microscopy, unwashed microsomal pellets from unsonicated and sonicated homogenates of liver and lung tissues from 7-day-old and adult rabbits were prepared as usual.[1] After the first sedimentation of microsomes, the pellet was resuspended in 1·15% KCl containing 1 mM Hepes and mixed into about 20 vol. of 4% glutaraldehyde solution. This was then spun in an International centrifuge at 1500 rev/min for 40 min in order to form a small, loosely packed pellet. After this centrifugation, the supernatant fraction was removed and a few milliliters of fresh 4% glutaraldehyde fixative was layered over the microsomal pellet and left overnight. The samples were post-fixed in 2% osmium tetroxide, dehydrated, embedded in Epon, and thin-sectioned by standard, accepted techniques. The sections were stained with 5% uranyl-acetate and Reynolds lead citrate stains and were examined in a Phillips 300 electron microscope.

The effect of 10-sec homogenate sonication on microsomal components from liver and lung of 7-day-old and adult rabbits is shown in Table 1. The specific activities or concentrations of the components measured remained about the same, but the total activities or amounts increased nearly 2-fold or more after sonication.

One of the major concerns about this method of increasing microsomal "yield" per g of tissue was the possible increased mitochondrial contamination of the microsomes. Although the mitochondrial contamination (as measured by succinate cytochrome *c* reductase activity) did increase after sonication (Table 1), this increase was only from 1 per cent (of the total homogenate activity) to 2 per cent in the liver microsomes and from about 3–5 per cent in the lung microsomes (Table 2). However, this same 10-sec sonication of the homogenates increased the yield of benzphetamine demethylase, a microsomal "marker" enzyme, to a much greater extent than the increase in succinate cytochrome *c* reductase (Table 2).

Electron micrographs of microsomal pellets from unsonicated and 10-sec sonicated homogenates from liver and lung of 7-day-old and adult rabbits were prepared in order to see if any microstructural differences could be discerned. Electron micrographs of microsomal preparations from unsonicated and 10-sec sonicated homogenates of adult rabbit liver are shown in Figs. 1 and 2. There were few obvious differences in microstructure among microsomal pellets from the various tissues before or after sonication of homogenates. Almost no mitochondria or mitochondrial fragments were observed in any of the microsomal pellet preparations. The liver preparation from the adult and 7-day-old rabbits differed slightly by the presence of fewer membranes and more free ribosomes in the young than adult tissues (both sonicated and unsonicated). This observation has been reported previously.[9]

TABLE 1. EFFECT OF 10-SEC SONICATION OF HOMOGENATES ON MICROSOMAL COMPONENTS FROM LIVER AND LUNG OF YOUNG AND ADULT RABBITS*

A. Specific activities and concentrations of some components and contaminants of microsomes

	Time sonication (sec)	Benzphetamine metabolism (nmoles HCHO/ mg protein/min)	Aniline hydroxylase (nmoles p-AP/ mg protein/min)	Aminopyrine demethylase (nmoles HCHO/ mg protein/min)	Cytochrome P-450 concn (nmoles P-450/ mg protein)	Succinate cytochrome *c* reductase (nmoles cyt. *c* reduced/ mg protein/min)
Adult liver	0	11·8	0·55	9·5	2·11	4·0
	10	11·5	0·61	10·1	1·80	10·3
7-Day liver	0	1·6	0·88	1·1	0·62	3·4
	10	1·7	0·86	1·1	0·69	10·5
Adult lung	0	15·6	0·40	3·6	0·38	3·6
	10	16·7	0·40	3·8	0·36	6·0
7-Day lung	0	0·7	0·09	0·2	0·04	3·0
	10	0·9	0·07	0·3	0·06	6·1

B. Total amounts of microsomal components

	Time sonication (sec)	Benzphetamine metabolism (nmoles HCHO/ g tissue/min)	Aniline hydroxylase (nmoles *p*-AP/ g tissue/min)	Aminopyrine demethylase (nmoles HCHO/ g tissue/min)	Cytochrome P-450 (nmoles P-450/ g tissue)	Microsomal protein (mg protein/ g tissue)
Adult liver	0	153	7·2	124	27	13
	10	265	14	232	43	23
7-Day liver	0	16	8·8	11	6·2	10
	10	32	16	21	13	19
Adult lung	0	76	2·0	18	1·9	4·9
	10	128	3·1	29	2·8	7·7
7-Day lung	0	3·7	0·4	0·9	0·2	4·5
	10	7·8	0·6	2·6	0·5	8·5

* Preparation of microsomes and assay methods were as described in text and Ref. 1. The results show a single representative experiment.

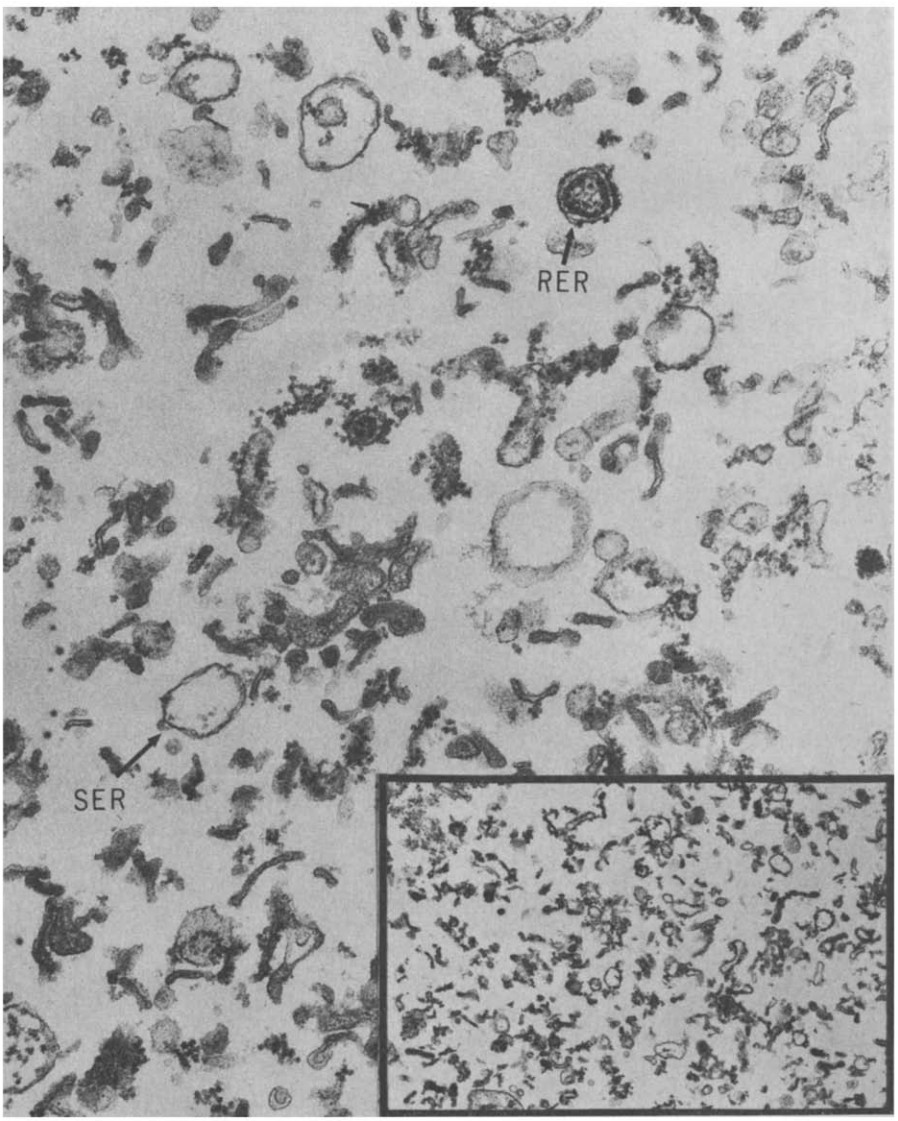

FIG. 1. Electron micrograph of adult rabbit liver microsomal pellet from unsonicated homogenate. Magnification: × 61,560. Insert magnification: × 20,520. SER = smooth endoplasmic reticulum; RER = rough endoplasmic reticulum.

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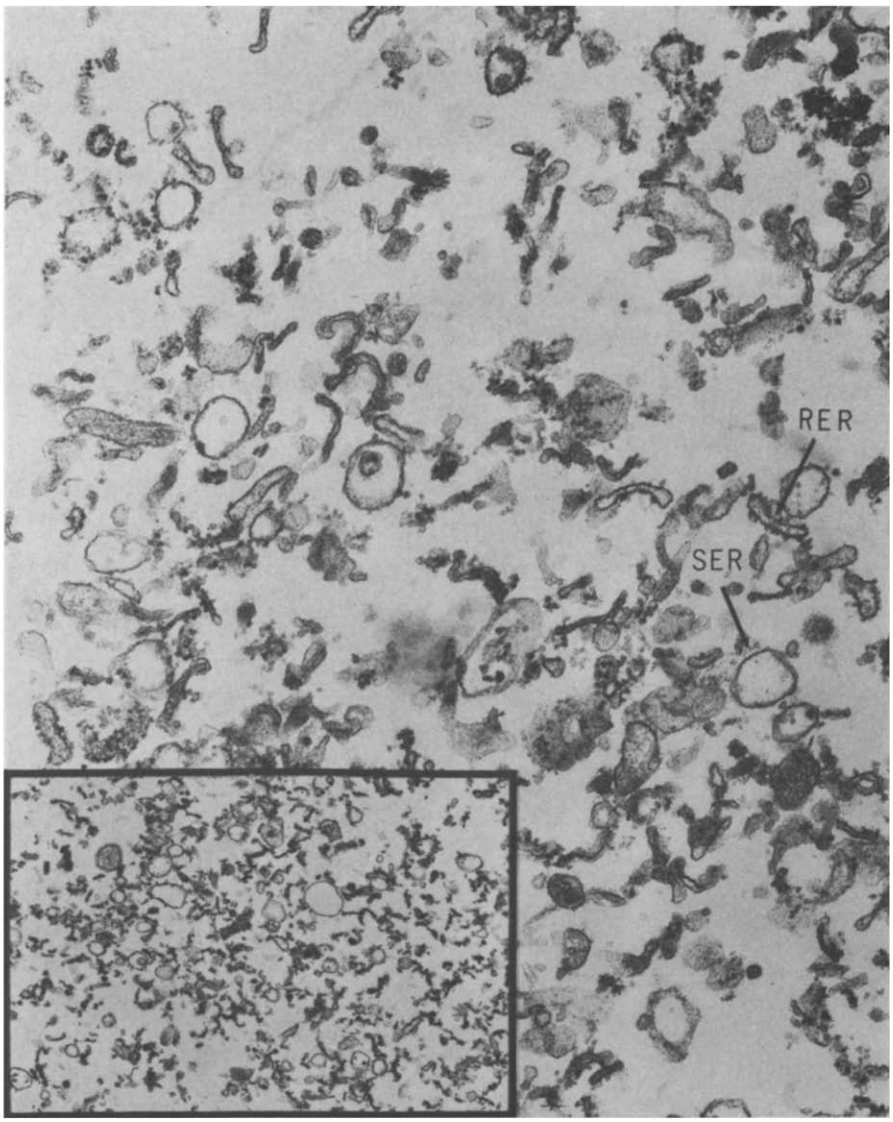

FIG. 2. Electron micrograph of adult rabbit liver microsomal pellet from 10-sec sonicated homogenate. Magnification: × 61,560. Insert magnification: × 20,520.

TABLE 2. COMPARISON OF THE RECOVERY OF A MICROSOMAL ENZYME (BENZPHETAMINE DEMETHYLASE) WITH THE MICROSOMAL CONTAMINATION BY A MITOCHONDRIAL ENZYME (SUCCINATE CYTOCHROME *c* REDUCTASE) BEFORE AND AFTER 10-SEC SONICATION OF HOMOGENATE*

	Time of sonication (sec)	Benzphetamine demethylase (nmoles HCHO/min/g tissue)			Succinate cytochrome *c* reductase (nmoles cytochrome *c* reduced/min/g tissue)		
		Homogenate	Microsomes	Per cent recovery†	Homogenate	Microsomes	Per cent contamination‡
Adult liver	0	212	87	41	1982	14	1
	10	238	156	66	1910	39	2
7-Day liver	0	40	11	28	1456	17	1
	10	37	23	62	1362	43	3
Adult lung	0	125	23	18	191	5·8	3
	10	137	48	48	155	6·2	4
7-Day lung	0	39	3·3	8	141	4·2	3
	10	36	9·8	27	141	7·9	6

* Assay methods are discussed in text and Ref. 1. The results show a single representative experiment.
† Per cent of activity in homogenate that is recovered in 190,000 *g* pellet (microsomal pellet). The remaining activity can be found in the 9000 *g* pellet.
‡ Defined as per cent of homogenate activity found in microsomes.

There was essentially no change in the appearance of the electron micrographs of lung microsomes from 7-day-old or adult rabbits after 10-sec sonication of homogenates. There was no apparent vesicle destruction, no change in relative amounts of attached vs free ribosomes, and no alteration or breakup of the fibrous network discussed by Hook *et al.*[2]

No lipid peroxidation was observed in the homogenates of either sonicated or unsonicated liver or lung when these were tested for such lipid peroxides at the end of the sonication. This is not surprising since rabbit liver microsomes do not peroxidize lipids readily.[10]

The increased microsomal protein yield caused by sonication of homogenates seems to be due to increased cell disruption and release of microsomes rather than any alteration in microsomal structure or increase in contamination of non-microsomal fragments. This conclusion is based on electron micrographs of microsomes before and after sonication of homogenates as well as the fact that there was no change in microsomal enzyme specific activity after sonication.

We favor the use of 10-sec sonication of homogenates as an economic and time-saving step in microsomal preparation when higher yields of microsomal protein are desirable and small amounts of mitochondrial enzyme contamination are not of prime importance. Longer periods of sonication of homogenates of the Dutch Belt rabbit liver and lung tissues were studied and were found to increase the microsomal protein yield slightly but to increase the mitochondrial contamination markedly. In our laboratory the microsomal protein yield from control rabbit lung has been only 2–5 mg/g of lung,[1] and therefore a 10-sec sonication of homogenates has been especially useful in harvesting microsomes from this tissue.

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