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Preparation of active, drug-metabolizing, microsomal enzymes under optimal conditions and by iso-electric precipitation

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The influences of homogenization technique and of relative centrifugal force on the enzyme activity of hepatic microsomes have been investigated to establish optimal conditions for preparation. In general, a glass or Ultra Turrax homogenizer provided a satisfactory homogenate having maximal enzyme activity when nuclear and mitochondrial material were removed by centrifugation at >6500 *g* for 20 min. Esterase, UDP-transglucuronylase, reductase and oxidase activities of microsomes prepared from liver breis under optimal conditions from guinea-pig, rabbit and rat have been compared. The enzyme activities in microsomes prepared by iso-electric precipitation at pH 5·0 were similar to those prepared by differential centrifugation except for a 50–75% reduction in glucuronyl transferase activity observed in rabbit and guinea-pig.

Microsomes are currently prepared by modification of the method of Siekevitz (1962), the initial tissue breis being prepared by a variety of homogenization procedures. However, in spite of much published work on microsomes, the effect of the preparative procedure on microsomal yield and enzyme activity is poorly documented.

Lathe & Ricketts (1964), working with neonatal tissue and requiring maximum microsomal yield, established "conditions for maximal recovery of the microsome fraction from rabbit liver homogenates", but investigated only the influence of the time for which the microsomal fraction was centrifuged at 114 400 *g*.

Many enzymes are known to be stable between pH 4–10, and this stability has been exploited in the purification of some enzymes (Strelitz, 1944). Claude (1946) isolated microsomes by precipitation at an acid pH, but pH precipitation for preparing subcellular fractions has subsequently received little attention although the procedure is still occasionally used (Görlich & Heise, 1963).

I have therefore investigated the influence of the homogenization technique and the fractionation procedure (relative centrifugal force) on the microsomal yield and enzyme activity, and compared this with the yield and activity of microsomes prepared by iso-electric precipitation at pH 5·0.

A preliminary report of this work was presented at the Fourth Meeting of the Federation of European Biochemical Societies (Mitchard, 1967).

Subsequently, Karler & Turkans (1968) also investigated the use of precipitation at acid pH for the preparation of microsomes for drug metabolism studies, and they

reported that precipitation at pH 5·0 produces a preparation which metabolizes hexobarbitone at a similar rate to preparations prepared by centrifugation.

EXPERIMENTAL

Materials

Glucose-6-phosphate, NADPH and uridine diphosphoglucuronic acid (C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany). Acetylsalicylic acid, nicotinamide, nitrobenzoic acid, *o*-aminophenol (technical) and *N*-1-naphthylethylenediamine dihydrochloride (British Drug Houses Limited, Poole, Dorset). Riboflavin (Sigma Chemical Company, St. Louis, Mo.). Benzphetamine (Upjohn Company, Kalamazoo, Mich., U.S.A.). The *o*-aminophenol was purified by recrystallization from aqueous ethanol. Young adult male Wistar albino rats (250–300 g), Hartley albino guinea-pigs (350–400 g) and Dutch rabbits (1·5–2·0 kg), fasted for 24 h, were used. They were killed by decapitation after stunning.

Methods

Preparation of liver sample. The livers were removed immediately after death, rinsed in ice-chilled isotonic KCl and divided into one 20 g and three 10 g (±100 mg) portions. If several livers were required to yield 50 g, three portions of equal and one of double weight were removed from each liver to give a total of three 10 g and one 20 g samples. The three 10 g liver samples were each placed in 20 ml of ice-chilled isotonic KCl and cut into small pieces (*ca* 5 mm^3). All subsequent operations were at 2–4°.

Homogenization procedure. One 10 g sample (A) was homogenized in a Potter-Elvejheim (PE) glass homogenizer with a Teflon pestle (diam. 19 mm) having a 0·10–0·15 mm clearance. Three up and down strokes were used during a period of 60 s whilst the pestle speed increased from an initial 2500 rev/min to the pre-set speed of 4000 rev/min. The second 10 g sample (B) was homogenized by an Ultra-turrax (UT) homogenizer (Janke and Kunkel K.G., Stanfen i. Br., Germany) for two periods of 5 s (5 s pause) in a Potter-Elvejheim glass mortar which had an internal diameter 1·5 mm larger than the external diameter of the homogenizer tube. The third 10 g sample (C) was placed in a 100 ml vortex beaker and homogenized for six 10 s periods (5 s pauses) by an MSE homogenizer rotating at 12000 rev/min. The 20 g sample was forced through a nylon mesh (St. Martin's Bolting Cloth, 142 μm) and a 10 g sample (D) of the homogenate weighed. The volume of each homogenate was adjusted to 50 ml with isotonic KCl.

Subfractionation of microsomes. The relative centrifugal force used to separate the more dense homogenate material was investigated and homogenates (2 × 20 ml) were centrifuged at between 1000 and 12000 *g* for 20 min in a rotor SS34 of a Sorvall Superspeed RC2-B. The decanted supernatant (2 × 15 ml) was (i) centrifuged in an MSE Superspeed 40 at 140 000 *g* for 45 min, or (ii) adjusted to pH 5·0 by the gradual addition of 0·02M acetate buffer pH 4·0 and the precipitate collected by centrifugation in the Sorvall Superspeed at 6500 rev/min for 10 min, to give a microsomal pellet above a small glycogen pellet. The microsomes prepared as in (i) were washed by resuspending in isotonic KCl and recentrifuged. When microsomes were prepared by procedure (ii), the supernatant was divided into two equal parts before centrifugation, and one part was treated as in (i) whilst the other as in (ii). All microsomal

preparations were suspended in 0·05M phosphate buffer at 7·4, the final volume being adjusted to 15 ml.

Enzyme activities

Assay of esterase (EC.3.1.1.6). Activity of a 50-fold dilution of the microsomal preparations in 0·05M phosphate buffer pH 7·4 was assayed by the method of Howes & Hunter (1968) using 2×10^{-3}M acetylsalicylic acid as substrate. The change in absorbance at 295 nm was continuously monitored during the 30 min incubation period at 37° in a Gifford-Unicam model 2000 dual wavelength spectrophotometer.

Assay of UDP-transglucuronylase (EC.2.4.1.17). Activity was assayed essentially by the method of Storey & Dutton (1955). Reaction mixtures contained 1·5 μmol of UDPGA, 10 μmol of $MgCl_2$, 1·5 μmol *o*-aminophenol and 6 μmol of ascorbic acid in a total volume of 3·0 ml of 0·05M phosphate buffer pH 7·4. One ml of microsomal preparation was added to start the reaction, which was incubated at 37° for 30 min in a Mickle shaking incubator. The reaction was stopped by the addition of 1 ml of 7·5% trichloroacetic acid. Controls consisted of the above system in which the microsomal preparation was replaced by water or in which the trichloroacetic acid was added to the reaction mixture before the microsomal preparation. The precipitated protein was removed by centrifugation.

Assay of nitroreductase (EC.1.6.99.1). Activity was assayed in an atmosphere of N_2 at 37° by the procedure described by Umar & Mitchard (1968), using *p*-nitrobenzoic acid as substrate. *Assay of* N-*demethylating ability.* The assay system contained in a total volume of 5·0 ml: 0·05M phosphate buffer, pH 7·41, 20 μmol of NADPH, 4 μmol of semicarbazide (dissolved in 0·05M phosphate buffer pH 7·4 and neutralized with dilute ammonia to pH 7·4 before adding to incubate), 5 μmol of benzamphetamine, 2 ml of washed microsomes. Incubation was in an atmosphere of air at 37° for 60 min in a Mickle shaking incubator. The reaction was stopped by addition of 2 ml of $ZnSO_4$ and 2 ml of saturated solution of $Ba(OH)_2$, and the precipitate separated by centrifugation. The amount of formaldehyde semicarbazone produced was determined by the method of Nash (1953); 3 ml of Nash reagent being added to 3 ml of supernatant. Controls were prepared by adding the $ZnSO_4/Ba(OH)_2$ protein precipitant *before* the washed microsomes, or consisted of the above system minus the benzamphetamine.

Determination of protein. Microsomal protein concentrations were determined on samples diluted 20-fold by the method of Lowry, Rosebrough & others (1951).

In all assays involving spectrophotometry, a Unicam SP.800 (Unicam Instruments Ltd., Cambridge) was used.

Weight of freeze-dried material. To determine the amount of solid material in each microsomal preparation, samples (2 ml) of washed microsomes were freeze-dried in tared tubes on an Edwards freeze dryer, Model 10P (Edwards High Vacuum Ltd., Crawley).

RESULTS

These are presented from typical experiments in which values are compared only with others obtained during the same experiment from the same homogenate.

Influence of homogenization procedure on microsomal yield and enzyme activity. Table 1 shows the enzyme activities, protein concentrations and microsomal yields

Table 1. *Influence of technique used for homogenizing rabbit, rat and guinea-pig livers on protein yield and enzyme activity of microsomal preparations*

Method of homogenization	Esterase		Trans glucuronylase		Nitro-reductase		Demethylating activity		Protein mg/ml	Weight of freeze-dried material mg/ml
	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein	Total activity μmol/mg in 30 min	Specific activity μmol/mg protein	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein		
Rabbit liver										
Ultra-Turrax	21·8	2·4	0·39	0·043	0·43	0·048	1·65	0·18	9·0	23·3
Potter-Elvejheim	11·1	2·1	0·16	0·030	0·22	0·042	1·48	0·28	5·2	13·9
MSE homogenizer	18·8	2·0	0·40	0·043	0·42	0·045	1·23	0·13	9·2	25·0
Nylon mesh (142 μmesh) ..	9·4	2·1	0·15	0·033	0·15	0·033	1·27	0·28	4·5	16·1
Rat liver										
Ultra-Turrax	59·8	5·0	0·108	0·009	0·31	0·016	0·34	0·028	11·9	31·4
Potter-Elvejheim	49·1	5·5	0·052	0·006	0·10	0·011	0·57	0·065	8·8	26·5
MSE homogenizer	53·0	4·9	0·108	0·010	0·27	0·025	0·23	0·021	10·8	30·6
Nylon mesh (142 μmesh) ..	49·1	5·7	0·032	0·004	0·13	0·015	0·67	0·078	8·6	26·2
Guinea-pig liver										
Ultra-Turrax	70·9	6·2	0·64	0·056	0·19	0·016	0·22	0·019	11·4	23·9
Potter-Elvejheim	53·8	6·9	0·26	0·033	0·06	0·007	0·30	0·038	7·8	21·3
MSE homogenizer	59·8	5·5	0·92	0·085	0·17	0·015	0·20	0·020	10·8	24·7
Nylon mesh (142 μmesh) ..	53·4	6·5	0·50	0·061	0·03	0·003	0·30	0·036	8·2	20·2

(in terms of weight of freeze-dried material) of rabbit, rat and guinea-pig liver microsomal suspensions respectively, as determined by the procedures described in this paper. In all cases there was a close correlation between protein concentration and

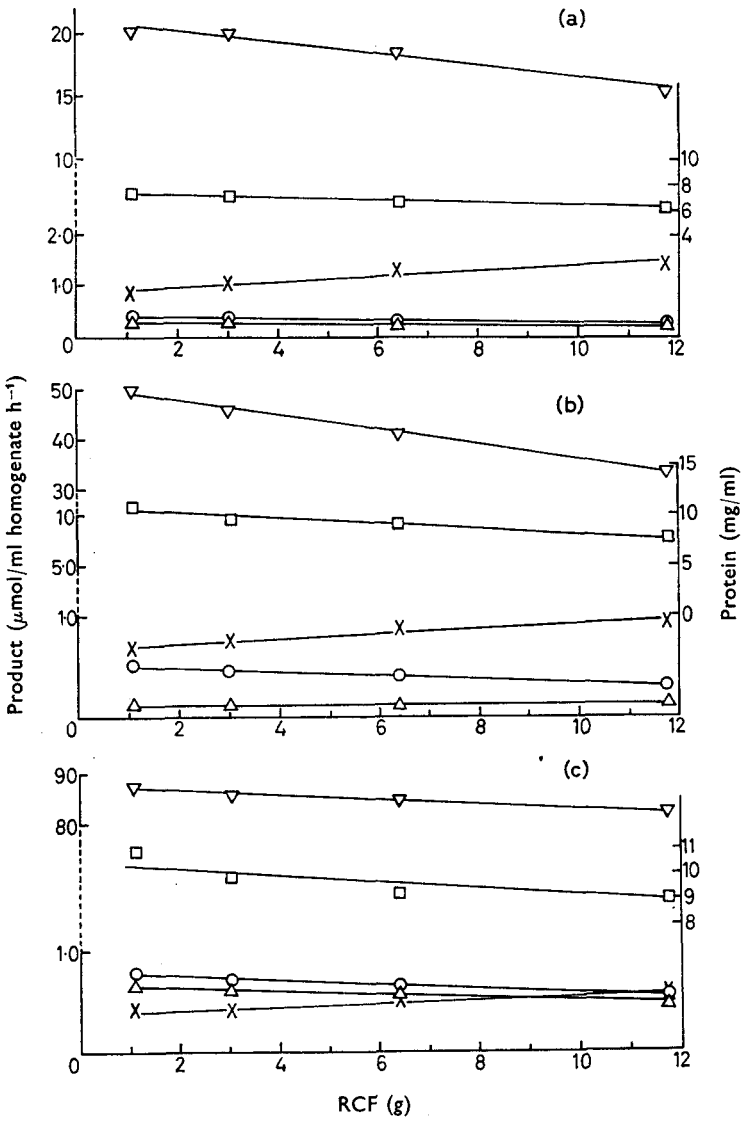

FIG. 1. Influence of the relative centrifugal force which was used to fractionate microsomes from rabbit (a), rat (b) and guinea-pig (c) livers on microsomal protein yield □—□ and microsomal esterase ▽—▽, transglucuronylase △—△,* nitro-reductase ○—○ and *N*-demethylating X—X activities. Experimental details are given in the text.

* Transglucuronylase activity expressed as μmol product/ml in 30 min.

microsomal yield, but there was a significant difference between values obtained for samples prepared with the UT or MSE homogenizers and those prepared by the PE and nylon mesh techniques. Although there were differences in microsomal yield and protein concentrations of samples prepared from different species, the UT and

MSE homogenizers always gave homogenates from which microsomal fractions having similar values of total enzyme activity were obtained and which were consistently higher than values obtained from samples prepared by the other procedures. In all three species the specific activity of *N*-demethylation (i.e. enzyme activity/mg protein) was much higher (2–3-fold), and the specific activity of esterase slightly higher, in samples prepared by the PE or nylon mesh techniques.

Influence of the relative centrifugal force (RCF), used to fractionate the homogenate, on microsomal yield and enzyme activity. Fig. 1 illustrates the effect of RCF, which was used to separate the more dense subcellular materials, on microsomal yield as measured by protein concentration and enzyme activities of rabbit, rat and guinea-pig preparations. The initial homogenate was prepared by the UT. Except for *N*-demethylating ability there was a relation between decrease in enzyme activity and decrease in microsomal yield. Interestingly, the activity of the enzyme responsible for *N*-demethylation increased as the microsomal content (as measured by the protein concentration of the preparation) decreased.

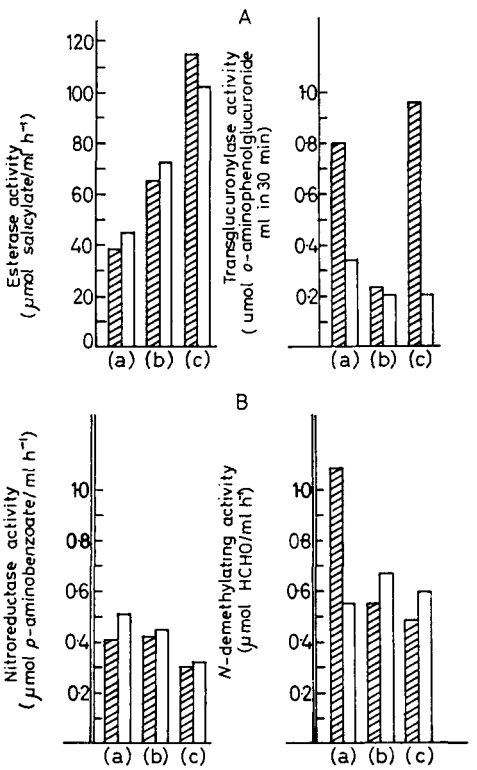

FIG. 2. Effect of precipitating microsomes obtained from rabbit (a), rat (b) and guinea-pig (c) livers at pH 5·0 on esterase and transglucuronylase (A), and nitro-reductase and *N*-demethylating activities (B) (open columns), compared with the activities of these enzymes in microsomes obtained by centrifugation at 140 000 *g* (hatched columns).

Preparation of microsomal fractions by iso-electric precipitation at pH 5·0. Fig. 2 shows the effect on microsomal enzyme activities when microsomes obtained from rabbit, rat and guinea-pig were prepared by iso-electric precipitation. Esterase and nitro-reductase activities were similar in each species to the activities of a microsomal

fraction obtained by centrifugation at 140 000 *g*. Transglucuronylase activity was much reduced in both rabbit and guinea-pig (about 2 and 5-fold respectively) and the *N*-demethylating activity of the rabbit preparation was reduced (about 2-fold).

DISCUSSION

In the present study, liver microsomal enzyme activities have been shown to be influenced both by the procedure used to prepare the initial tissue breis and by the RCF used to subfractionate the homogenate.

As the microsomal preparations were suspensions of insoluble washed subcellular particles in a buffer, it was assumed that the microsomal content was equivalent to the protein concentration or weight of freeze-dried material obtained for each preparation. Table 1 shows that homogenization by UT or MSE homogenizers produced microsomal preparations which had high microsomal content and high enzymic activity related to protein concentration or weight of freeze-dried product.

Microsomal preparations isolated from homogenates prepared by the use of violent procedures (i.e. UT or MSE homogenizers) did not appear to contain a high proportion of inactive protein, because specific activities calculated with respect to the protein concentration for esterase, transglucuronylase and nitroreductase were similar to, or higher than, the corresponding values obtained for comparable preparations prepared from homogenates obtained by the PE or nylon mesh techniques (Table 1). These latter techniques which utilize shearing forces to disrupt tissues, yielded microsomal preparations in which the *N*-demethylating activity was higher, whether calculated as total or specific activity, than activities measured in preparations obtained from UT or MSE homogenates. There would therefore appear to be a high proportion of inactive (devoid of *N*-demethylating activity) material in microsomal fractions obtained from homogenates prepared by the use of violent mechanical techniques.

When the RCF used to subfractionate microsomes was varied, the enzymic activities were again shown to parallel protein concentrations. However, in all three species, ability to demethylate increased as protein concentration (and presumably microsome content) decreased. It is also significant that a high demethylating activity was obtained in preparations isolated from homogenates obtained by the PE or nylon mesh techniques, preparations that also had a low protein concentration, and it would therefore appear probable that a membrane-associated protein component was inhibiting this process. This possibility is at present being investigated.

McLaren (1957) emphasized that extremes of pH do not kill living cells, and suggested that a wide variation in pH occurs in the micro environment of the cell. Leone & Redstone (1962) established that the pH of lowest solubility of cellular components was between 3·5 and 6·5, which supports the view of Morton (1954) that the particulate fraction precipitated at pH 5·0 during his studies on alkaline phosphatase was the microsomal fraction. The microsomal fraction obtained during this investigation by precipitation at pH 5·0 gave a preparation having similar enzymic activity to the microsomal preparations obtained by centrifugation, and, with the exception of transglucuronylase, the enzymes studied were stable at this pH; only rabbit liver microsomes showed an impaired ability to demethylate. Both rabbit and guinea-pig transglucuronylase activities were much decreased but although rat microsomal transglucuronylase is very unstable its activity was unaffected by the change in pH.

The present investigation demonstrates that microsomes retaining a high level of the enzymic activities involved in drug metabolism can be prepared by precipitation at pH 5·0 as an alternative to high speed centrifugation.

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