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Mg^{+2}, a requirement for some microsomal drug oxidations

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AFTER differential ultracentrifugation we have observed two distinct rabbit liver microsomal fractions which could be separated, owing to differences in density, color, and gross appearance. This report presents preliminary data on the nature and relationship of these fractions to some microsomal drug oxidations known to require $NADPH_2$ and oxygen.[1]

Livers from female albino rabbits were homogenized at 0°–4° with 2 volumes of 0·15 M KCl in a Waring Blendor at top speed for 15 sec or in a Potter–Elvehjem homogenizer for 2 min. The rest of the procedure was also carried out at the same temperature. Nuclei, mitochondria, and cell debris were removed by centrifugation at 9,000 *g* for 20 min in the Spinco model L ultracentrifuge. The supernatant, containing microsomes and soluble fraction, was then centrifuged at 105,000 *g* for 1 hr. The pellet was found to consist of a lower, and hence heavier, microsomal fraction (HMS) which was homogeneous, colorless, and translucent, and an upper, lighter microsomal fraction (LMS) which was reddish-brown and opaque. The 105,000 -*g* supernatant (soluble fraction) was removed and the LMS gently aspirated into a pipette with the aid of a suction bulb. The two particulate fractions were then individually washed two or three times, further separating the LMS and HMS from each other, and then reconstituted with fresh 0·15 M KCl so that 1 ml of each finally represented 2 g of the original liver. The HMS appeared opalescent and milky white; the LMS was opaque and reddish-tan to brown in color.

The latter could be stored at 4° and used for about 6 days, after which a marked loss in drug-oxidizing activity was noted. Dialysis of 10 ml of LMS against 1 liter of 0·01 M Tris buffer, pH 8·6, for 3 hr at 4° (with three changes of medium) produced no loss in the drug-oxidizing activity of this fraction. The rate of oxidation was greater in the presence of 0·01 M Tris buffer at pH 8·2 than in 0·1 M phosphate buffer at pH 7·4.

Table 1 indicates that both the LMS and HMS were necessary for the side-chain oxidation of 74–94 mμmoles/mg protein/hr of hexobarbital in the presence of $NADPH_2$. The 0–8 mμmoles barbiturate oxidized by the LMS or HMS alone is within the limits of experimental error in the procedure. If the LMS was first exposed to a boiling water bath for 10 min and then added to a reaction mixture containing HMS, only 14 mμmoles of drug was oxidized. However, the HMS treated similarly proved to be heat stable, since 72 mμmoles barbiturate was oxidized by the combination of LMS+boiled HMS. Furthermore, the addition of an equivalent amount of an ashed preparation of HMS to the LMS in two experiments resulted in the oxidation of 96 mμmoles substrate. This was similar to the 94 mμmoles of drug metabolized in the presence of unaltered HMS. Finally, Table 1 shows that the HMS could be completely replaced by 5×10^{-3} M Mg^{+2} or by a $NADPH_2$-generating system which contained the same amount of Mg^{+2}. No combination of components or the $NADPH_2$-generating system without Mg^{+2} was able to replace the HMS, indicating that Mg^{+2} was the active component in this system. That $NADPH_2$ was also necessary was apparent from the fact that it could not be replaced by NADP in the presence of LMS+HMS or LMS+Mg^{+2}. NADP, when included as part of the $NADPH_2$-generating system containing Mg^{+2} for the optimal production of $NADPH_2$, was associated with the oxidation of barbiturate. However, under these conditions both Mg^{+2} and $NADPH_2$ were then available to the hexobarbital-oxidizing enzyme system. Other metals including Fe^{+3}, Fe^{+2}, Cu^{+2}, Cu^{+1}, Mn^{+2}, Mo^{+6}, and Ca^{+2} could not replace Mg^{+2} in this system. The HMS produced no additional stimulation in the presence of 10^{-2} M Mg^{+2} as the chloride or the sulfate salt.

Some LMS preparations were found to oxidize hexobarbital to a limited extent in the absence of HMS or Mg^{+2}. This effect could be abolished, however, by EDTA. The addition of excess (10^{-2} M) Mg^{+2} to such a reaction mixture then produced a marked oxidation of substrate, further indicating the reliance of this system on a metal ion. Preliminary evidence also suggested similar requirements for the sulfoxidation of chlorpromazine.

The drug-oxidizing enzymes were localized in the LMS fraction which contained all of the micromal protein,[3] but no Mg^{+2} or glycogen, unlike the glycogen-bearing microsomal drug-oxidizing subfractions of Fouts.[4] Significantly, the HMS fraction, although devoid of protein, was found to contain Mg^{+2} (determined by emission spectroscopy on one preparation) and glycogen.[5] The amount of HMS fraction present in a given preparation appeared to vary with the state of nutrition of the animal, being diminished or absent in an animal starved for more than 12 hr.

TABLE 1. EFFECTS OF MICROSOMAL FRACTIONS ON HEXOBARBITAL OXIDATION

The reaction mixtures contained 1·0 μmole hexobarbital, 2·0 μmoles $NADPH_2$, and buffer to the final concentrations and pH stated below; 0·2 ml LMS and 1·0 ml HMS or an equivalent volume of ashed HMS or 25 μmoles $MgCl_2$ were added as noted. The $NADPH_2$-generating system, when present, consisted of 0·5 unit glucose-6-phosphate dehydrogenase or soluble fraction from 1 g liver, 25 μmoles glucose-6-phosphate, 100 μmoles nicotinamide, 25 μmoles $MgCl_2$, and 0·5 μmole $NADPH_2$ or NADP. The total volume was 5·0 ml. When indicated, separate components were placed in a boiling water bath for 10 min prior to being added to the rest of the reaction mixture. Incubations were carried out for 30 min in air at 37°. The disappearance of substrate was measured to follow the metabolism of hexobarbital[2] and was linear for the period studied. Values in the table are averages. Figures in parentheses refer to number of preparations assayed.

Components	Hexobarbital oxidized (mμmoles/mg protein/hr)	
	0·1 M phosphate buffer pH 7·4	0·01 M Tris buffer pH 8·2
HMS	8 (6)	0 (3)
LMS	8 (6)	4 (6)
LMS + HMS	74 (5)	94 (3)
Boiled LMS + HMS	14 (3)	
LMS + boiled HMS	72 (3)	
LMS + ashed HMS		96* (2)
LMS + $MgCl_2$	78 (6)	111 (8)
LMS + $NADPH_2$-generating system	73 (4)	

* Two assays were performed on one ashed preparation.

Finally, the possibility exists that the HMS fraction (which can be replaced by Mg^{+2} in our system) is similar to Axelrod's heat-stable rat liver microsomal factor, which was found to stimulate the deamination of amphetamine by rabbit liver microsomes.[6]

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REFERENCES

1. B. B. BRODIE, J. R. GILLETTE and B. N. LADU, *Ann. Rev. Biochem.* **27**, 427 (1958).
2. J. R. COOPER and B. B. BRODIE, *J. Pharmacol. exp. Ther.* **114**, 409 (1955).
3. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
4. J. R. FOUTS, *Biochem. biophys. Res. Commun.* **6**, 373 (1961).
5. C. A. GOOD, H. KRAMER and M. SOMOGYI, *J. biol. Chem.* **100**, 485 (1933).
6. J. AXELROD, *J. biol. Chem.* **214**, 753 (1955).