REQUIREMENT OF A SOLUBLE PROTEIN FOR MAXIMAL ACTIVITY OF THE MONO-OXIDASE SYSTEM OF HEPATIC MICROSOMES

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SUMMARY. Hepatic microsomes were shown to possess only about 1/3 to 1/2 of the aminopyrine and ethylmorphine (EM) N-demethylase activities of the 9000 x g supernatant fraction from which they were derived. Activity was restored with the 105,000 x g supernatant fraction (SF). The factor in SF is heat labile, precipitated by ammonium sulfate or acetone, none-dializable, and resists sedimentation at 170,000 x g for 4 hr. SF elevated the K_m for EM N-demethylation. These observations suggest that the factor in SF is a macromolecule which functions as an unknown component of the mono-xidase system, as an activator of the system, or by removing an inhibitor of the system.

INTRODUCTION. When supplemented with TPNH and Mg^{++}, hepatic microsomes are generally considered to possess as much activity of the mono-oxidase system responsible for the oxidation of xenobiotics as that found in the 9000 x g supernatant fraction from which the microsomes are obtained. For example, on numerous occasions throughout the years we have compared the ethyl-morphine N-demethylase activities of 9000 x g supernatant fractions with those of microsomes and found them to be approximately equal. However, more recently we have observed that microsomes possessed about two-thirds to one half the activity of the 9000 x g supernatant fraction and that the 105,000 x g supernatant fraction largely restored activity. This communication is a preliminary report describing the nature of the accessory factor contained in the 105,000 x g supernatant fraction (SF).

MATERIAL AND METHODS. Microsomes and SF were prepared from livers of Simonson or Holtzman, male rats (250-260 g) perfused *in situ* with ice cold isotonic KCl solution as follows. A 50% homogenate of the livers in isotonic KCL solution was made using 15 strokes of a loose fitting Dounce homogenizer. The 9000 x g supernatant fraction of the homogenate was

centrifuged at 105,000 g_{max} for 60 min. The supernatant fraction (SF) was removed with a syringe. Typical SF and microsomal fractions from one gram of wet liver contained about 25 and 14 mg of protein/ml, respectively. SF was diluted with isotonic KCl solution to contain 20 mg of protein/ml when aminopyrine and ethylmorphine N-demethylase activities were measured and 6 mg of protein/ml when aniline hydroxylase activity was determined. Formaldehyde formed by the N-demethylation reactions was determined by the method of Nash (1); p-aminophenol formed by the aniline hydroxylase reaction was measured by the method of Imai et al. (2).

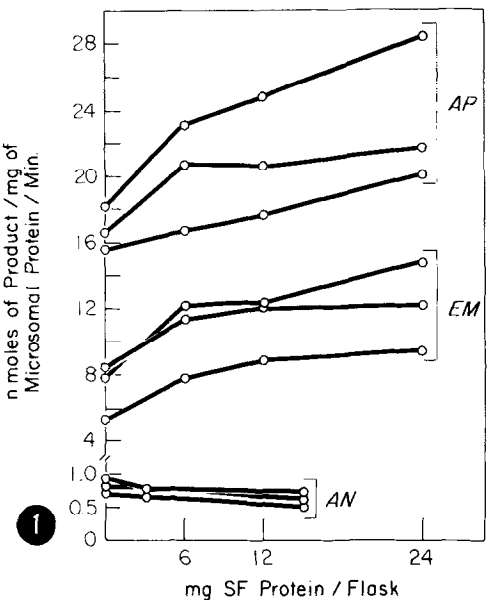
Acetone powder was made from SF (3), dried overnight in vacuo over sulfuric acid at 4°C and resuspended in isotonic KCl solution or in 0.1M phosphate buffer, pH 7.5. SF (200 mg of protein in 10 ml) was dialyzed against one litter of 0.1M phosphate buffer, pH 7.5, overnight at 4°C.

Cytochrome P-450 and cytochrome b_5 were determined by the method of Omuar and Sate (4), while NADPH-cytochrome c reductase was determined by the method of Lu et al. (5).

RESULTS. Fig. 1 shows data from representative experiments illustrating the effects of SF on aminopyrine and ethylmorphine N-demethylation. At the highest level of SF addition, rates of aminopyrine N-demethylation were increased by 23 to 36% and those of ethylmorphine, by 45 to 86%. At the lowest level of SF addition, respective increases were 6 to 24 and 35 to 45%. Aniline hydroxylation was not stimulated by SF.

The effect of SF on the synergistic effect of DPNH on TPNH-dependent ethylmorphine N-demethylation (6) was studied using the additions of SF given in Fig. 1. DPNH (1 mM) produced no increase in HCHO formation in the presence of SF above that observed in the absence of SF (about 35%) and SF did not replace DPNH as a DPNH synergist.

Fig. 2 illustrates the effect of SF on the apparent kinetic constants for ethylmorphine N-demethylation. SF is seen to increase the apparent K_m as well as the apparent V_{max}.



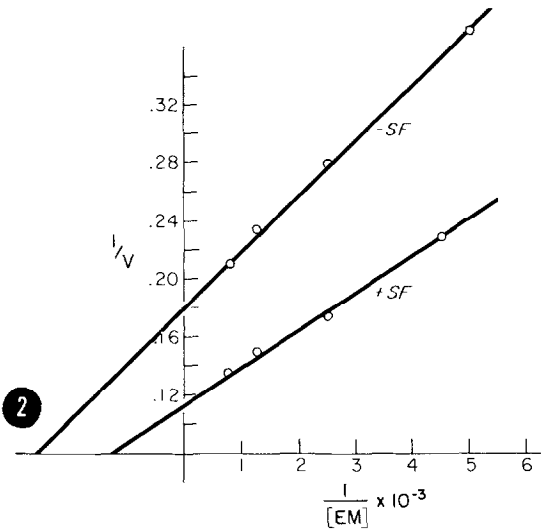


Figure 1.

Effects of supernatant factor (SF) on rates of metabolism of aminopyrine (AP), aniline (An) and ethylmorphine (EM). When AP and EM were substrates, the incubation medium consisted of the following (μmoles/6 ml): TPN^+ (2), glucose-6-PO_4 (20), glucose-6-PO_4 dehydrogenase (2 units), $MgCl_2$16 H_2O (10), phosphate buffer (200), semicarbazide (37.5), AP (25) or EM (10), 2 mg of microsomal protein and indicated amounts of SF. When An was the substrate, the incubation medium consisted of the following (μmoles/3 ml): TPN^+ (1), isocitric acid (10), isocitrate dehydrogenase (17 units), $MgCl_2$.6 H_2O (15), phosphate buffer (50), aniline (0.6), 6 mg of microsomal protein and indicated amounts of SF. Substrate concentrations were saturating and reaction rates were linear throughout the incubation periods of 5, 15 and 20 min for AP, EM and An, respectively. Incubations were conducted with shaking 120 oscillations/min in air at 37°C.

Figure 2.

Effects of supernatant factor (SF) on the apparent kinetic constants for ethylmorphine (EM) N-demethylation. Experimental conditions are given in Fig. 1. Each flask contained 6 mg of SF protein and 2 mg of microsomal protein. The apparent Km and V_{max} values with and without SF are 0.77 and 0.38 mM and 9.43 and 5.55 nmoles of HCHO formed/mg of microsomal protein/min, respectively. N = 3 for each point.

Experiments summarized in Table 1 strongly suggest that the accessory factor is a macromolecule. It is non-dializable and precipitated by acetone and ammonium sulfate. Placing SF in a boiling water bath for 10 min completely destroyed activity. Frozen SF stored for 3 wks. at -10°C lost little of its activity.

Table 1. Effects of dialysis, acetone precipitation and ammonium sulfate precipitation on the activity of the supernatant factor (SF).

Addition	HCHO formed (nmoles/mg microsomal protein/min)	Increase (%)
None	11.4	--
SF	18.0	58
Dialized SF	16.1	42
Acetone powder		
in phosphate buffer	18.7	65
in isotonic KCl	18.3	61
$(NH_4)_2SO_4$ ppt.	15.4	35

Assay conditions were the same as those given in Fig. 1 except that Holtzman rather than Simonson rats were used. All additions were made at the level of 6 mg of protein/flask. Each flask contained 2 mg of microsomal protein. Values represent the means of 2 or 3 experiments.

To further establish the soluble nature of the factor, SF was centrifuged at 174,000 x g for 4 hr. A pellet was obtained which contained about one-third of the protein of SF and 18% of its stimulatory activity; 70% of the activity was recovered in the supernatant fraction.

On the basis of protein content, SF contained about 6, 10 and 3 percent, respectively, of the cytochrome P-450, cytochrome b_5 and TPNH-cytochrome c reductase found in microsomes. SF possessed only 2% of the ethylmorphine N-demethylase activity of microsomes. Addition of ten times the amount of TPNH-cytochrome c reductase found in microsomes in the form of a solubilized preparation of the reductase (7) increased ethylmorphine N-demethylation by only 20%.

The possibility was considered that albumin, which is present in large amounts in hepatic microsomes, might produce some non-specific stimulatory effect. Rat albumin (Sigma fraction V, 3 mg/flask) had no effect on the rate of ethylmorphine N-demethylation.

Phenobarbital administration (40 mg/kg/day for 3 days) did not increase the activity of SF.

DISCUSSION. The stimulatory effect of a macromolecular component of SF on the hepatic microsomal mono-oxidase system might be produced in several

ways: 1) SF may supply a previously unrecognized component of the system. The hepatic system is known to be composed only of TPNH-cytochrome c reductase (TPNH-cytochrome P-450 reductase) and cytochrome P-450, although in reconstituted systems, a heat-stable lipid factor activates the system (8). However, because the redox potentials of the known components of the system are unfavorable for the transfer of electrons (9) and because other mono-oxidase systems involving cytochrome P-450 are known to require components in addition to P-450 hemoprotein and nucleotide reductase, at least one additional component for the hepatic system has been anticipated. A likely prospect is a non-heme iron protein which would serve as a counterpart to adrenodoxin in the adrenal system (10,11) or to putidaredoxin in the cytochrome $P\text{-}450_{cam}$ system (12). 2) SF may supply a factor that stimulates the system without functioning as an electron transfer component. For example, the factor might combine with cytochrome P-450 or components in its environment and facilitate the transfer of electrons in the manner that type I substrates are thought to stimulate activity of the system (13, 14). 3) SF may supply a factor capable of removing an inhibitory endogenous type I compound from cytochrome P-450. Endogenous type I compounds which may interfere with the metabolism of exogenous type I substrates have been postulated from spectral data and other considerations (15,16).

The kinetic studies (Fig. 2) do not permit a selection of the most probable of these mechanisms. If mechanisn 3) was in force, the plots in Fig. 2 could represent uncompetitive inhibition (17), where the supplemented microsomes were freed of the inhibitor. The SF induced shift in K_m could also be explained by mechanism 1), in which case, the hypothetical electron transfer component in SF would be rate-limiting in thy absence of SF and its addition would create a new rate-limitation by another component of the system. A change in rate-limitation imposed by an SF induced shift in rates of acceptance of first and second electrons by cytochrome P-450-substrate complex (6), could also explain the kinetics illustrated in Fig. 2.

Finally, this shift in kinetic constants upon addition of SF could occur if more than one mono-oxidase system was involved in the N-demethylation of ethylmorphine and one of these was stimulated preferentially or made complete by the addition of SF.

The factor in SF is heat labile and therefore distinguishable from the heat stable factor described by Terriere and Chan (18), and the heat stable lipid factor of Lu et al. (8). Also, the factor in SF seems to resemble the heat labile factor described by Van Dyke and Wineman (19), which stimulated the microsomal dechlorination of 1,1,2-trichhloroethane, although the authors expressed some doubt that the reaction was cytochrome P-450 dependent. Washing removed 60% of the dechlorinase activity from their microsomes; repeated washing did not decrease ethylmorphine N-demethylase activity of our microsomes.

The question as to why the stimulatory effect of SF was not observed in earlier studies is worth pondering. If we visualize the SF factor as having a relatively loose association with the microsomal membrane, subtle changes in the membrane dictated by age, hormonal and nutritional status, environmental conditions, etc. might determine how firmly the factor remains bound to the membrane while the microsomes are being processed.

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