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ENHANCEMENT OF RAT LIVER MICROSOMAL OXIDASES BY MAGNESIUM AND A HEAT-STABLE FACTOR FROM THE SOLUBLE FRACTION*

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Abstract—Aldrin epoxidation and ethylmorphine *N*-demethylation by rat liver microsomes were increased approximately 2-fold when the microsomal incubates were supplemented with Mg^{++} or the microsomal supernatant. Enhancement was maximal at an Mg^{++} concentration of 5 mM and the effect was not diminished in the presence of oxalate, citrate, or fluoride. The Mg^{++} effect was not due to a stimulation of NADPH generation. The enhancing factor in the soluble fraction was destroyed by extraction with 2-butanol but not with acetone, was heat stable (boiling water bath for 10 min), and could be partially purified by fractionation on a gel filtration column. This factor eluted from the column simultaneously with protein, endogenous Mg^{++}, and endogenous glucose 6-phosphate dehydrogenous activity. Although Mg^{++} and the heat-stable soluble factor possessed some properties in common, there was considerable evidence that the two enhancers acted independently.

THE SOLUBLE fraction of liver homogenates is often included as a component of microsomal drug metabolizing systems, either by addition as the postmicrosomal fraction or as a part of the postmitochondrial supernatant. The property usually valued in this fraction is its content of the enzyme G 6-P dehydrogenase, although a few reports mention other advantages. The hydroxylation of bile acids[1] was stimulated by the addition of the soluble fraction of rat liver homogenate to a microsomal system. A soluble factor prepared from liver microsomes is reported[2] to stimulate the oxidation of fatty acids by liver microsomes and a heat-stable factor in rat, dog and guinea pig liver microsomes was found to stimulate the deamination of amphetamine by microsomes prepared from rabbit liver.[3]

Microsomal enzymes have been activated by metal ions, especially magnesium. Addition of Mg^{++} to a liver homogenate increased the demethylation of 4-dimethylaminoazobenzene by 25 per cent[4] and this ion was required in the hydroxylation of steroids by beef adrenal homogenates.[5] LaDu *et al.*[6] assumed that the Mg effect was due to its stimulation of the NADPH generating system. A heat-stable factor in a 'heavy microsome fraction' which stimulated the side-chain oxidation of hexabarbitol was related to the magnesium content of this fraction.[7] NADPH oxidase activity was tripled by the addition of Mg or Ca^{++} to a rat liver microsome system.[8] Other workers reported only slight[3, 6] or no increase in enzyme activity[9, 10] when magnesium was added to their systems.

We have observed an enhancement of the epoxidation of the insecticide aldrin by rat

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liver microsomes supplemented with either rat or trout liver soluble fraction.[11] An acetone powder of a rat liver homogenate was also active in this respect. In a further study of this enhancement by the soluble fraction we also considered the possibility that magnesium was responsible for the increased microsomal activity. We have found that both factors stimulate epoxidation of aldrin and the *N*-demethylation of ethylmorphine. The soluble factor is heat stable and can be partially separated from the postmicrosomal supernatant by gel filtration. The enhancement by the two factors is additive.

MATERIALS AND METHODS

The special chemicals used in this study and their sources were as follows: NADP, NADPH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase from General Biochemicals; Sepharose 4B, Pharmacia Fine Chemicals, Inc.; and Na isocitrate-isocitric dehydrogenase, Sigma Chemicals.

Isolation of rat liver microsomes. Livers were removed from male, white rats, 3–5 months old, while under ether anesthesia. In most experiments, the liver was thoroughly perfused with cold isotonic KCl prior to excision. A 10% liver homogenate was then prepared by homogenizing the livers in 1·15% KCl with a Virtis 45 homogenizer operated at full speed for 30 sec. Microsomes were isolated from the homogenate by differential centrifugation.[12] When washing was required, the microsomes were suspended in cold isotonic KCl at one-half volume of the original homogenate and recentrifuged at 50,000 rpm for 1 hr. After thorough draining, the tubes containing the wet microsomal pellets were stored at −10° and used, without loss of activity, during a 3-week period.

Treatment of the soluble fraction. After it was determined that heating in a boiling water bath for 10 min coagulated part of the protein in the soluble fraction without reducing its enhancing activity, this treatment was applied in most of the experiments. Lyophilization also provided a means of concentrating and storing the soluble fraction without loss of enhancement activity. This treatment produced a fraction which will be referred to as the HLSF. It was prepared by placing 40 ml of the soluble fraction in a boiling water bath for 10 min, centrifuging the coagulated protein, passing the supernatant through a glass-wool filter and lyophilizing the filtrate. The resulting dry powder was stored in a vacuum dessicator and before use was reconstituted to its original volume. When stored in this manner, enhancing activity was retained at least 2 months.

Column fractionation of the soluble fraction. A glass column, 2·5 cm × 35 cm, coated with 1% dimethyldichlorosilane in benzene (from Bio-Rad Laboratories), was partially filled with Tris buffer (0·001 M, pH 8·0). Sepharose 4B gel was mixed with a small volume of the same buffer and added to the column. The gel was allowed to settle and the buffer allowed to drain at a flow rate of 3 ml in 5 min. Additional gel was added until the packed column was 27 cm in length. The column was then washed with three volumes of buffer. The sample to be fractionated, 4 to 6 ml of an 8- or 10-fold concentrate of the original soluble fraction, was introduced and eluted at a flow rate of 3 ml per 5 min with Tris buffer, pH 8·0, in a linear gradient between 0·001 M and 0·10 M. Sixty 3-ml fractions were usually collected.

Protein and magnesium determinations. The protein concentration of microsomal suspensions and the soluble fraction preparations were determined by the method of Lowry *et al.*[13] This method, or the spectrophotometric method (absorbance at 280

mμ), was used to measure the protein content of fractions collected from the Sepharose columns.

The Mg^{++} content of the various preparations was determined by direct aspiration in a Jarrell–Ash atomic absorption spectrophotometer.

Enzyme assays. Aldrin epoxidase was assayed in a system containing 50 m μmoles aldrin in 0·10 ml 2-methoxyethanol, microsomes equivalent to 0·5 to 1·5 mg microsomal protein (unless otherwise stated), an NADPH generating system and sufficient Tris buffer at pH 8·0 for a total volume of 6 ml. Incubation was at 37° in a shaking water bath. The following NADPH sources were used, depending on the nature of the experiments: (a) G 6-P (18 μmoles), NADP (1·8 μmoles), and G 6-P dehydrogenase (3·3 units); (b) isocitrate (48 μmoles), NADP (1·98 μmoles), and isocitric dehydrogenase (2·16 units); (c) 0·5 to 1·0 ml of one of the soluble fraction preparations of a Sepharose column eluate, G 6-P (18 μmoles), and NADP (1·8 μmoles); and (d) chemically reduced NADPH (2·0 μmoles). System (a) was the usual source.

In enhancement studies, the added fraction, or magnesium chloride, was introduced shortly before the microsome suspension. The reaction was terminated after 15 min, except in time course studies, with a 3:1 mixture of hexane:2-propanol and extracted and analyzed for dieldrin as described previously.[12]

Ethylmorphine *N*-demethylase was assayed in a reaction mixture consisting of 3·6 μmoles ethylmorphine HCl, NADPH generating system (a) (described above), 5 to 7 mg of microsomal protein and Tris buffer (pH 7·4) to a total volume of 6 ml. Mg^{++} (5 mM) or soluble fraction was added to the tubes before the microsomal suspension. Tubes with the complete system minus substrate were used as controls. Incubation was at 37° for 10 min.[14] The reaction was stopped by adding 2 ml of a 20% $ZnSO_4$ solution. The amount of formaldehyde formed was estimated by the modified method of Nash,[15] correcting for formaldehyde formed in the control flasks.

Spectrophotometric assays. The reduction of NADP in systems containing G 6-P dehydrogenase from yeast or the rat liver soluble fraction was determined by the rate of increase in absorption at 340 mμ using a Cary model 11 spectrophotometer. The system contained NADP (0·3 mM), G 6-P (3 mM), and yeast G 6-P dehydrogenase (3·3 units) or a soluble fraction preparation (1·0 ml).

The spectral changes due to interactions between microsomal cytochromes and the substrate[16] were also investigated. The cuvettes contained microsomes (2 mg protein per ml) and either aldrin (18·3 or 36·6 μM in 0·05 ml 2-methoxyethanol) or ethylmorphine (0·30 or 0·60 mM), was equilibrated at room temperature for 5 min. They were scanned against a reference solution containing the same amount of microsomes (and 2-methoxyethanol in the case of aldrin). These spectra were compared with those of similar mixtures containing magnesium (5 or 10 mM) or soluble fraction. The microsomal cytochrome P-450-CO complex was estimated spectrophotometrically.[11]

Polyacrylamide gel electrophoresis. Polyacrylamide gel columns, 5 mm × 10 cm, were prepared according to Davis.[17] Acetone powder and HLSF or fractions collected from the Sepharose column, all containing approximately 0·3 mg protein, were electrophoresed.

RESULTS

The soluble fraction of male rat liver homogenate, added to the standard microsomal

aldrin epoxidase system, increased dieldrin production by 50–75 per cent. There was no epoxidase activity by soluble fraction alone. This increase, or enhancement, was not affected by washing the microsomal pellet with KCl, Tris buffer or EDTA. An acetone powder prepared from the soluble fraction of a 10% liver homogenate contained 29% of its protein as determined by the method of Lowry *et al.*[13] Although there was some loss of enhancement (based on the weight of liver represented), the specific activity of the acetone powder (per cent increase in epoxidation per mg protein) increased about 2-fold. The specific activity of the enhancement factor in the HLSF (defined in methods) was two to six times higher than that given by the soluble fraction or the acetone powder.

The acetone powder of the soluble fraction was reconstituted to its original volume (9 ml buffer per gram liver equivalent) and dialysed against 0·001 M Tris buffer, pH 8·0, for 16 hr without loss of epoxidase stimulating activity. However, when this fraction was extracted with 2-butanol the aqueous phase was devoid of enhancing activity and this activity could not be recovered from the 2-butanol fraction after removal of the solvent.

Column fractionation. When a 10-fold concentrate of the lyophilised and heated soluble fraction was fractionated on a Sepharose 4B column, as described earlier, a major protein peak occurred in the 120- to 150-ml fraction. This fraction contained nearly all of the epoxidase enhancing activity of the preparation, giving a maximum increase of 90 per cent (curve C, Fig. 1).

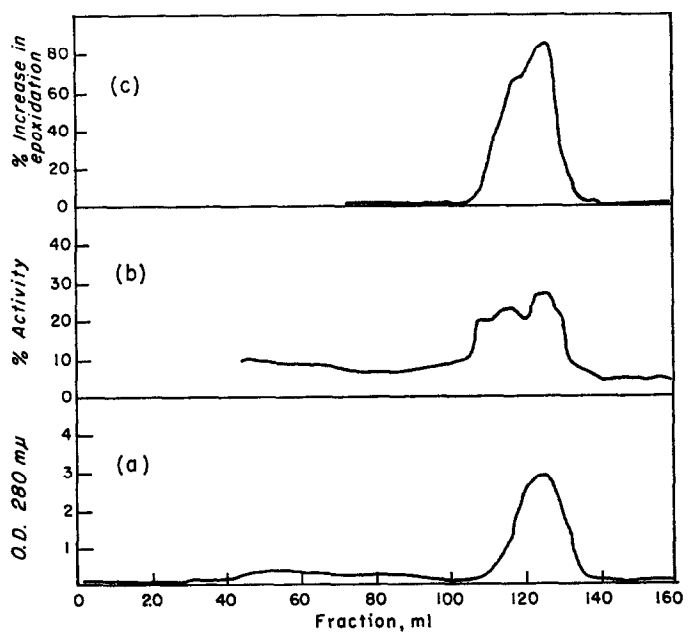

FIG. 1. Fractionation of a lyophilised, heated, soluble fraction on a Sepharose 4B column. (a) Protein determined by optical density at 280 mμ. (b) Epoxidase activity supported by endogenous G 6-P dehydrogenase of column fractions. Determined by substituting 1 ml of column eluate for yeast G 6-P dehydrogenase in the standard incubation mixture. Expressed as per cent of epoxidase activity of the standard system. (c) Per cent increase in epoxidase activity with column fractions (1·0 ml) added to the standard incubation mixture.

The polyacrylamide gel electrophoretograms indicated that some purification was achieved as the soluble fraction was heated, freeze-dried and subjected to Sepharose column fractionation. Of the seven protein bands in the acetone powder, at least two were eliminated by heating, the remainder having R_f values of 0·4, 0·47, 0·57, 0·62 and 0·72. Upon Sepharose column fractionation, represented in Fig. 2, only the 0·4, 0·47 and 0·57 bands remained in the 120- to 125-ml fractions.

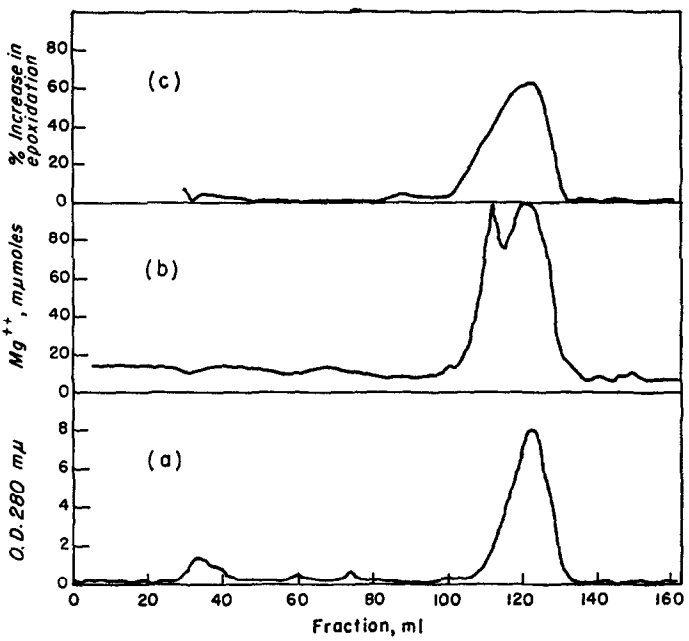

FIG. 2. Fractionation of HLSF containing 60 μmoles of added $MgCl_2$ on a Sepharose 4B column (a) Protein determined by absorbance at 280 mμ. (b) Mg^{++} content of fractions. (c) Per cent increase in epoxidase activity with column fractions (1·0 ml) added to the standard incubation mixture.

Role of NADPH generation. The untreated soluble fraction, its lyophilised powder, and its acetone powder, contained G 6-P dehydrogenase in sufficient quantity to at least partially replace the yeast G 6-P dehydrogenase used in our NADPH generating system. Of greater interest was the fact that the endogenous G 6-P dehydrogenase activity eluted from the Sepharose column in the same fractions as the protein and the epoxidase enhancing factor. The actual amount of the enzyme present in this fraction was not measured, but it was sufficient, in combination with the epoxidase enhancing factor, to achieve normal levels of epoxidation without added G 6-P dehydrogenase. As shown in Fig. 1, this endogenous G 6-P dehydrogenase activity was largely destroyed by heating (curve b), while the enhancement factor, as previously noted, was not affected.

Role of Mg^{++}. During the experiments with the NADPH generating system the usual G 6-P dehydrogenase system was replaced with the isocitrate–isocitric dehydrogenase system. This resulted in an even greater aldrin epoxidase activity, more

than 100 per cent increase, than had been seen previously. This increased activity, occurring without the addition of the soluble fraction, was found to be due to the presence of $MgCl_2$ in the isocitrate dehydrogenase system. Deletion of the magnesium from this system in a standard incubation mixture returned the aldrin epoxidase activity to its normal level and the addition of 30 μmoles (5 mM) of $MgCl_2$ to an incubate containing the G 6-P dehydrogenase NADPH system more than doubled its epoxidase activity. When chemically reduced NADP replaced the NADPH generating system, the augmentation was even greater (Table 1).

TABLE 1. EFFECT OF THE NADPH SYSTEM ON THE ENHANCEMENT OF ALDRIN EPOXIDATION BY THE SOLUBLE FRACTION AND BY Mg^{++}

NADPH generating system	mμmoles Dieldrin/mg protein		
	Microsomes alone	Microsomes plus sol. fract.	Microsomes in 5 mM Mg^{++}
Isocitrate–isocitric dehydrogenase	4·63	7·27	9·10
G 6-P–G 6-P dehydrogenase	4·26	7·70	9·03
NADPH	4·08	8·36	11·40

Magnesium concentration in the aldrin epoxidase system is optimal at approximately 5 mM. Table 1 shows that at this level aldrin epoxidase is stimulated to a greater extent by Mg^{++} than by the soluble factor. The HLSF differed from Mg^{++} in its effect on microsomal aldrin epoxidase from young (5 weeks) male rats, causing a 50 per cent increase in enzyme activity while Mg had no effect. Neither factor stimulated microsomal epoxidase activity in preparations from adult (3 months) female rats.

Metal binding agents such as isocitrate (8 mM), oxalate (5 mM), or fluoride (5 mM), had little or no effect on the stimulation of epoxidase activity by the HLSF or magnesium ion (Fig. 3). It can be seen that the magnesium stimulation was greater than that of the HLSF. When both factors were present, the amount of stimulation was additive.

Endogenous Mg^{++}. The level of endogenous Mg^{++} in several microsomal and soluble fraction preparations was determined by direct aspiration with the atomic absorption spectrometer. The concentration found in several microsomal suspensions varied only slightly, although the specific activity of the aldrin epoxidase in these same preparations varied considerably (Table 2). The amount of magnesium present was much lower than that found to stimulate epoxidation when added exogenously. Similarly, the HLSF contained approximately 0·04 μmole of Mg^{++} per ml, the amount usually added to the microsome system in enhancement studies. Approximately two-thirds of the magnesium was lost from the soluble fraction by dialysis, but this did not affect the enhancing activity.

Comparison of Mg^{++} and the HLSF as activators. Activation of the epoxidase system when the two factors were added to increasing levels of microsomes is shown in Fig. 4. As before, the magnesium effect is greater than that of the heat-stable factor. When the data are plotted to show the increase in epoxidation, it is seen that the optimum level of microsomal protein is approximately the same for both factors.

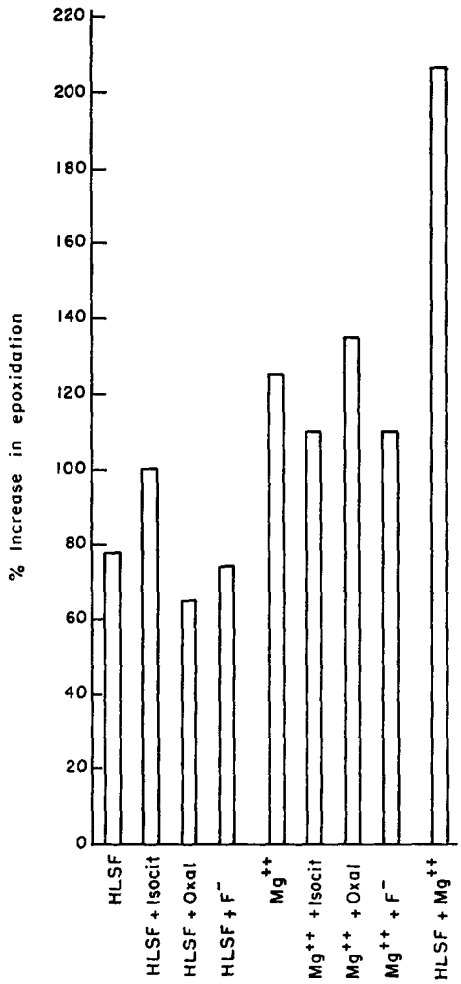

FIG. 3. Effect of isocitrate (8 mM), oxalate (5 mM), and fluoride (5 mM) on enhancement of microsomal aldrin epoxidation by the HLSF (1·0 ml) and by magnesium (5 mM). Also shown, the additive effect of HLSF and Mg^{++}.

TABLE 2. MICROSOMAL ALDRIN EPOXIDASE ACTIVITY COMPARED TO ENDOGENOUS MG^{++} CONCENTRATION

Experiment No.	Aldrin epoxidase, specific activity*	$(Mg^{++}) \times 10^{-4}$ M, microsomes†
52	4·86±0·13	0·74
57	8·07±0·08	0·98
59	20·60±0·20	0·87
60‡	0·63±0·05	1·40

* Specific activity = mμmoles dieldrin/mg microsomal protein in 15 min.
† Stock suspension used in incubation.
‡ Female rat liver microsome.

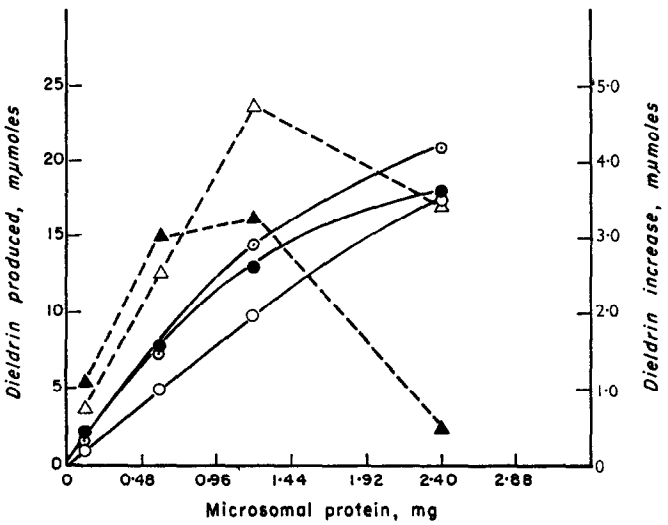

FIG. 4. Relationship between microsome level and magnesium or HLSF stimulation of microsomal aldrin epoxidation. ○, Standard incubation mixture; ⊙, plus 5 mM magnesium; ●, plus 1·0 ml HLSF; △, increase in dieldrin formation due to Mg^{++}; ▲, increase in dieldrin formation due to HLSF.

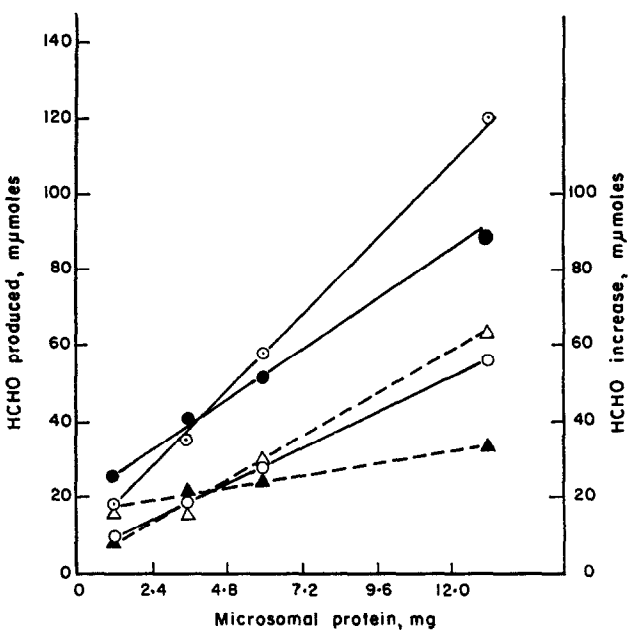

FIG. 5. Relationship between microsome level and magnesium or HLSF stimulation of microsomal ethylmorphine *N*-demethylation. ○, Standard incubation system; ⊙, plus 5 mM magnesium; ●, plus 1·0 ml of HLSF; △, increase in formaldehyde formation due to Mg^{++}; ▲, increase in formaldehyde formation due to HLSF.

Ethylmorphine *N*-demethylation was also stimulated by magnesium ion and the HLSF. The stoichiometry of the enhancement was somewhat different than with epoxidation, however, as is seen in Fig. 5. Stimulation was almost linear throughout the range of enzyme assayed and the increase in *N*-demethylase activity also continues to rise. As with epoxidase, the HLSF caused smaller increases than magnesium ion. When added to microsomes prepared from livers of young (5 week), male rats, however, the HLSF caused a greater stimulation of *N*-demethylation (94% increase) than Mg^{++} (44% increase).

Another difference in the magnesium and HLSF effects was seen in the kinetics of the reaction. It is evident from Fig. 6 that the HLSF, although less stimulatory, appears to stabilize the reaction to a greater extent than magnesium.

The effect of the two activators on aldrin epoxidase and ethylmorphine demethylase activity was also shown by K_m and V_{max} measurements (Table 3). The K_m values were

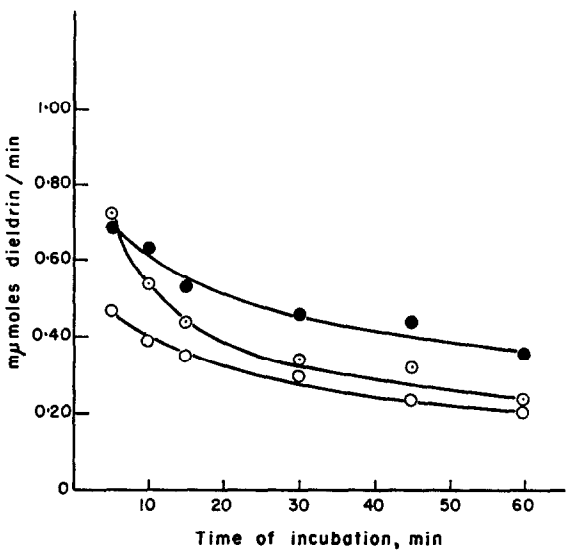

FIG. 6. Effect of Mg^{++} and HLSF on the rate of microsomal aldrin epoxidation. ○, Standard reaction mixture; ⊙, plus 5 mM Mg^{++}; ●, plus 1·0 ml HLSF.

TABLE 3. EFFECTS OF HLSF AND MG^{++} ON KINETICS OF MICROSOMAL ALDRIN EPOXIDASE AND ETHYLMORPHINE DEMETHYLASE REACTIONS

Reaction mixture	Aldrin epoxidation* $K_m \times 10^{-6}$ M	V_{max}†	Ethylmorphine demethylation* $K_m \times 10^{-3}$ M	V_{max}‡
Microsomes	3·68	12·0	0·20	6·67
Microsome plus 5 mM Mg^{++}	3·60	16·4	0·20	16·67
Microsome plus HLSF	3·20	15·5	0·20	8·33

* Based on three replications, with Lineweaver–Burk points located by computer, method of least square.

† mμmoles Dieldrin per mg microsomal protein in 15 min.

‡ mμmoles HCHO per mg microsomal protein in 10 min.

unchanged but there was a marked increase in V_{max} values, especially in the case of magnesium and *N*-demethylation of ethylmorphine.

Spectrophotometric assay. Neither Mg^{++} nor HLSF affected the level of the cytochrome P-450-CO complex in the microsomes. Spectral interaction of microsomes with either aldrin or ethylmorphine gave a trough at 425 mμ and a peak at 390 mμ. Neither the intensity nor the positions of these spectra were affected by Mg^{++} or the HLSF.

DISCUSSION

The heat-stable microsomal activating factor we have described is located in the postmicrosomal fraction of liver homogenate and thus might be presumed to originate in the soluble fraction of the cell. In this respect it differs from the factors described by Axelrod[3] and by Lu and Coon[2] who prepared their activating fraction from microsomes. The factor we report is not removed by acetone extraction (although it is lost on extraction with 2-butanol) and is eluted from the Sepharose column with a protein containing fraction. This also indicates a difference from the enhancing factor described by Lu and Coon.[2] The factor which stimulates bile acid hydroxylation[1] was prepared from the soluble fraction of rat liver homogenates and may not differ from the factor we report. Judging from its ability to stimulate both aldrin epoxidase and ethylmorphine *N*-demethylase, this factor may be assumed to have a rather general effect on microsomal oxidizing systems.

A substance in the soluble fraction which prevented respiratory decline in mitochondria–microsome mixtures[18] was thought to counteract inhibitors in the microsomes, perhaps by protecting sensitive enzymes. A protective action by the soluble fraction has also been suggested in studies of fish microsomal systems.[19] There is evidence in our report (Fig. 6) of a protective or stabilizing action by the HLSF. If this is the case, it might be presumed that the action is nonspecific, perhaps requiring soluble protein.

There is some reason to believe that the enhancement is due to a specific component of the microsomal electron transport system which has been separated from the particulate fraction during isolation. We have observed, for example, that microsomal preparations unusually high in epoxidase or *N*-demethylase activity are enhanced much less than those of lower activity. Thus, the 'accidents' of the isolation procedures may dictate whether a key component remains attached to the microsomal membrane or is lost to the soluble fraction. This concept is supported by the results of Voight *et al.*[1] who observed that the 6β hydroxylase activity of microsomes prepared in 1·0 M phosphate buffer was stimulated by the soluble fraction. However, if the microsomes were prepared in 0·01 M phosphate buffer the enzyme was more active and was not stimulated by the soluble fraction.

The soluble fraction of liver homogenates provides an endogenous source of glucose 6-phosphate dehydrogenase required in the generation of NADPH.[20–24] Improved generation of NADPH resulting from the use of this fraction might explain our results. This seems unlikely, however, in view of the heat lability of endogenous G 6-P dehydrogenase (Fig. 1, curve b) and the results presented in Table 1 which show that enhancement occurs with added soluble fraction regardless of the source of NADPH.

The results in Table 1 also rule out the possibility that the Mg^{++} activation seen is

due to improvement in NADPH generation. Otherwise dieldrin production, when chemically reduced NADP was used, would have been lower than when generating systems were used.

There is some evidence that the heat-stable factor and Mg^{++} are associated in some way, e.g. a magnesium–protein complex. Both stimulate aldrin epoxidase and ethylmorphine *N*-demethylase to a similar extent, their effects on the kinetics of the reactions are similar (Table 3) and there is evidence (Fig. 2) that the active fraction from the Sepharose column contains Mg^{++}. This magnesium must be complexed in some manner since inorganic magnesium failed to elute from the column. This bound magnesium is not necessarily associated with the heat-stable factor, however, as polyacrylamide gel electrophoresis of the active fraction eluting from the Sepharose column showed that at least three protein components were present.

Other evidence indicates that the two factors are different. The heat-stable factor can be dialyzed so as to lose more than two-thirds of its endogenous Mg^{++} without any loss of enhancing activity. The concentration of endogenous Mg^{++} in the soluble fraction is less than 1 per cent of that required for maximum activation. The two factors also have different effects on the rate of the epoxidase reaction, Fig. 6. The HLSF maintained the rate of dieldrin production 50 to 80 per cent above that of microsomes alone throughout the 60-min incubation, while Mg^{++} was much less effective, particularly when the incubation was prolonged.

Still other differences seen in our study of the Mg^{++} and soluble fraction enhancing effect include the lack of effect by Mg^{++}, but not HLSF on microsomal epoxidase from immature male rat livers. Also, there is a difference in the specific activity of the two factors in their stimulation of epoxidase (Fig. 4), and *N*-demethylase (Fig. 5). The additive enhancement effects (Fig. 3) when both Mg^{++} and the heat-stable factor are present in an epoxidase system also show that the two agents are different.

Evidently the stimulatory action of Mg^{++} involves rather strong binding to microsomal membranes since agents such as oxalate, isocitrate, and fluoride[25, 26] have no effect when present in the incubation mixtures (Fig. 3). Previous workers[8, 27] have shown that divalent cations such as Mg^{++} and Ca^{++} are bound to microsomal membranes which act as cation exchangers. If structural changes result from this binding of Mg^{++}, they cause a change in the rate of the oxidative reactions rather than in the affinity of the enzymes for their substrates. The K_m values are unaffected by either Mg^{++} or HLSF while the V_{max} values are increased.

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REFERENCES

1. W. Voigt, P. J. Thomas and S. L. Hsia, *J. biol. Chem.* **243**, 3493 (1968).
2. A. Y. H. Lu and M. J. Coon, *J. biol. Chem.* **243**, 1331 (1968).
3. J. Axelrod, *J. biol. Chem.* **214**, 753 (1955).
4. G. C. Mueller and J. A. Miller, *J. biol. Chem.* **176**, 535 (1948).
5. M. Hayano and R. I. Dorfman, *J. biol. Chem.* **201**, 175 (1953).
6. B. N. LaDu, L. Gaudette, N. Trousof and B. B. Brodie, *J. biol. Chem.* **214**, 741 (1955).
7. R. H. Trivus and M. A. Spirtes, *Biochem. Pharmac.* **13**, 1679 (1964).
8. H. V. Bogdanska, L. Kranch and B. C. Johnson, *Archs Biochem. Biophys.* **109**, 248 (1965).
9. P. Mazel, J. Simanis and A. Kerza-Kwiatecki, *Biochim. biophys. Acta* **114**, (1), 72 (1966).
10. C. C. Irving, *Cancer Res.* **26**, 1390 (1966).

11. T. M. CHAN, J. W. GILLETT and L. C. TERRIERE, *Comp. Biochem. Physiol.* **20**, 731 (1967).
12. J. W. GILLETT, T. M. CHAN and L. C. TERRIERE, *J. Agric. Fd Chem.* **14**, 540 (1966).
13. O. LOWRY, N. ROSEBROUGH, A. FARR and R. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
14. J. A. CASTRO and J. W. GILLETTE, *Biochem. biophys. Res. Commun.* **28**, 426 (1967).
15. J. COCHIN and J. AXELROD, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
16. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3**, 113 (1967).
17. B. J. DAVIS, *Ann. N. Y. Acad. Sci.* **121**, art. 2, 404 (1964).
18. J. A. GROVE, R. M. JOHNSON and J. H. CLINE, *J. biol. Chem.* **241**, 5564 (1966).
19. D. R. BUHLER and M. E. RASMUSSON, *Comp. Biochem. Physiol.* **25**, 223 (1968).
20. G. P. QUINN, J. AXELROD and B. B. BRODIE, *Biochem. Pharmac.* **1**, 152 (1958).
21. L. LEADBEATER and D. R. DAVIES, *Biochem. Pharmac.* **13**, 1607 (1964).
22. J. V. DINGELL, P. D. JOINER and L. HURWITZ, *Biochem. Pharmac.* **15**, 971 (1966).
23. L. G. HART and J. R. FOUTS, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **249**, 486 (1965).
24. L. E. GAUDETTE and B. B. BRODIE, *Biochem. Pharmac.* **2**, 89 (1959).
25. R. C. NORDLIE and A. W. GEHRING, *Biochim. biophys. Acta* **77**, 100 (1963).
26. R. C. NORDLIE and P. T. JOHNS, *Biochemistry, N. Y.* **7**, 1473 (1968).
27. A. P. CARVALHO, H. SANUI and N. PACE, *J. Cell. Biol.* **62**, 311 (1963).