Preliminary Notes

Solubilization of aromatic hydroxylase system of liver microsomes and requirement of lipid-like factor

In attempts to elucidate the enzymic mechanisms involved in the hydroxylation of foreign aromatic compounds by liver microsomes[1], it was desirable to obtain the hydroxylase system in a soluble form. We have, therefore, tested a variety of methods and succeeded in solubilizing the aniline-hydroxylating activity of liver microsomes with the aid of heat-treated venom of *Trimeresurus flavoviridis*. Since this venom, like those of other snakes, is known to contain heat-stable phospholipase A[2], it is likely that this enzyme is the principal agent responsible for the solubilization. Digestion of microsomes with the untreated venom, on the other hand, resulted in a complete inactivation of the enzyme system probably owing to the action of powerful proteases present in the venom[3]. The other methods tested including treatments with pancreatic lipase, bile salts, several synthetic detergents, digitonin, *n*-butanol, and sonic oscillation all either caused inactivation or brought about insufficient solubilization of the hydroxylase system.

The microsomes used were prepared from rabbit liver and suspended in 1.15% KCl according to MITOMA *et al.*[4]. They were usually kept at 4° for about 20 h prior to digestion, since it was noted that microsomes thus aged are somewhat more readily solubilized than fresh preparations. The heat-treated venom was prepared by heating a crude venom solution (1% in 0.05 *M* Tris, pH 7.4) for 8 min in a boiling-water bath and then removing the resulting precipitate by centrifugation. The aniline-hydroxylating activity was measured as in the previous report[1] using glucose 6-phosphate, glucose 6-phosphate dehydrogenase and TPN as a TPNH-generating system.

A mixture containing 11 ml of microsomal suspension (equivalent to 20 g liver), 4 ml 0.5 *M* Tris, pH 9.0, and 1 ml of the heat-treated venom was incubated at 4° for 16 to 18 h. The digest was then adjusted to pH 7.5 (no measurable solubilization takes place at this pH), diluted to 60 ml, and subjected to centrifugation at 105,000 × *g* for 60 min. More than 90% of the hydroxylase activity and approximately 60% of microsomal protein were recovered in the supernatant thus obtained. Spectrophotometric investigations further showed that both cytochrome b_5 and DPNH–cytochrome b_5 reductase had also been solubilized during the digestion.

The 105,000 × *g* supernatant could be dialysed against 0.002 *M* Tris buffer (pH 7.5) for more than 3 h without appreciable loss of activity. It was further possible to lyophilize the dialysed preparation to give a reddish powder, the activity of which was quite stable when stored in the cold in a desiccator.

In a previous communication[1] it was shown that a lipid-like component indispensable for the hydroxylase activity can be extracted from microsomes with acetone–methanol–ether and that the activity of microsomes thus treated can be

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

restored by the addition of the component extracted in the solvent mixture. It was thus of interest to see whether the solubilized preparation behaves in the same way. Such experiments seemed especially desirable in view of our experiences that the extraction experiments with intact or acetone-dried microsomes were sometimes (notably in winter) not quite reproducible for reasons still to be elucidated.

Solvent extraction of the solubilized system was therefore conducted as follows: 100 mg of the lyophilized powder obtained above was suspended in 50 ml of cold acetone–methanol–ether (7:1:2, v/v/v) and homogenized for 15 min at — 10 to — 15°. The homogenate was filtered and the residue reextracted with another 50-ml portion of the cold solvent. The residue after second extraction was dried over conc. H_2SO_4 in a vacuum desiccator, whereas the combined extracts were mixed with a small amount of water and concentrated *in vacuo* to give about 1 ml of an aqueous lipid emulsion.

TABLE I

LIPID-LIKE FACTOR REQUIREMENT IN SOLUBILIZED ANILINE HYDROXYLASE SYSTEM

The reaction mixture contained 0.1 *M* Tris-acetate buffer (pH 8.5), 4 μmoles aniline, 8 μmoles nicotinamide, 0.12 μmole TPN, 2.5 μmoles $MgCl_2$, 7.5 μmoles glucose 6-phosphate, 0.64 unit glucose 6-phosphate dehydrogenase, 0.35 ml solubilized enzyme system, and indicated amount of lipid emulsion (obtained as described in the text). The total volume was 1.0 ml. The mixture was aerobically shaken for 60 min at 37°. Hydroxylation was followed by measuring *p*-aminophenol formation as described previously[1].

Solubilized enzyme system		*Lipid emulsion (ml)*	*p-Aminophenol formed (mμmole)*	*Spec. activity (mμmole p-aminophenol/mg protein)*
Treatment	*Protein (mg)*			
Solvent-extracted	2.17	0	7.3	3.4
Solvent-extracted	2.17	0.05	27.7	12.8
Solvent-extracted	2.17	0.10	39.4	18.2
Untreated	2.26	0	40.3	17.9

It was thus found, as can be seen from Table I, that the aniline-hydroxylating activity of the lyophilized powder was greatly diminished by the extraction, but could be fully restored by the addition of the lipid emulsion.

These experiments, so far invariably reproducible, again indicate an important role played by a lipid-like factor in the microsomal hydroxylation. Furthermore, they are of special significance in that these results can be obtained even with those microsomes which fail to give an active lipid-like factor on direct extraction and thus provide a more convenient means for the study of the factor.

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