SHORT COMMUNICATIONS

BBA 43274

Assay of hydroxylation activity in immunoprecipitates

Hydroxylation of various lipid-soluble drugs, steroids and dyes is thought to be catalyzed by a NADPH-dependent electron transport system known to be present in liver microsomes[1–3] and possibly also in other organs[4]. The reaction has been termed mixed function oxidation by MASON[5], as one of the oxygen atoms of the O_2 molecule is incorporated into the reaction product, while the other oxygen atom is incorporated into water. The hydroxylating system is now generally believed to consist of at least two components: a flavoprotein, the NADPH–cytochrome *c* reductase and a cytochrome, the cytochrome P-450.

The liver microsomes also contain a second electron transport system, which uses NADH as electron donor and consists of the flavoprotein NADH–cytochrome *c*-reductase and cytochrome b_5. The biological function of this system is still unknown. There is evidence[6,7] that electrons can be transferred from one of the electron transport systems to the other, as NADH is capable of reducing cytochrome P-450 and NADPH cytochrome b_5 in liver microsomes. The reduction rates of these reactions are, however, much lower than those observed in the normal electron transport pathways.

In a recent publication[8] a method for revealing hydroxylation activity in immune precipitates was briefly described. New results obtained with an improved method will be reported in this paper.

The work was done with rat liver microsomes, separated into one rough (R) and two smooth types of membrane (Sa and Sb) as previously described[8]. Soluble protein antigens were extracted from the microsomal subfractions by treatment with 0.5 % Lubrol W (cetylpolyoxyethylene condensate, ICI, England) and 1 % sodium deoxycholate. The protein content of the antigen solutions was determined according to LOWRY *et al.*[9]. Protein concentration was 6–10 mg/ml in all experiments.

Antisera against each of the three membrane fractions were prepared in rabbits. Groups of 4 rabbits were used for each fraction. Each rabbit received 3–4 intramuscular injections, given at 3-week intervals, of 5–10 mg of protein, incorporated into complete Freund's adjuvant. Sera of rabbits injected with the same fraction were pooled. Prior to use, all antisera were exhaustively absorbed with lyophilized rat serum. The precipitates were developed after immunoelectrophoresis in agarose (electrophoretic separation for 5 h in 0.05 M barbiturate buffer, pH 8.6) and assayed for hydroxylating activity. The staining procedure for the hydroxylation reaction is a modification of the method of BOOTH AND BOYLAND[10], initially developed by one of us (C. P.). 100 mg β-acetamidonaphthalene was dissolved in 5–10 ml of ethylene glycol monoethyl ether. 0.2 M phosphate buffer (pH 7.5), containing 25 mg nicotinamide and 0.1–1.0 mM NADPH or NADH, was then added to give a final volume of 100 ml. The plates were incubated in this medium for 1 h at 37°. They were stained

for 1–2 h in a solution of 50 mg Garnet salt (Echtgranatsalz GBC, Chroma-Gesellschaft, Stuttgart, W. Germany) in 100 ml 0.2 M phosphate buffer (pH 7.5). The precipitates were stained red in positive reactions. A number of diazonium compounds, which differed in their coupling speed and energy at given pH were tested. Garnet GBC salt was chosen since it gave the best results.

Control experiments were performed by incubation of plates in complete medium but without the substrate. Additional controls to demonstrate hydroxylation activity in the Lubrol sodium deoxycholate-extracted microsomes were done in test tubes according to ORRENIUS *et al.*[11]. It was thereby shown that the extracted microsomes could hydroxylate aminopyrene, but to a lesser extent than the unextracted. The microsomal extracts also contained NADPH–cytochrome *c* reductase and cytochrome P-450, measured by the method of DALLNER[12,13]. The Lubrol–extracted Sa membranes showed the same NADPH–cytochrome *c* reductase activity as the untreated controls (approx. 300 μmoles cytochrome *c* reduced per min per mg protein). The cytochrome P-450 activity of the Lubrol–Sodium deoxycholate-treated membranes was, however, diminished to half that of the controls (0.035 $A_{450-500\ nm}$/mg protein).

In immunoelectrophoresis we obtained two red precipitates showing hydroxylation activity both in the R and the smooth Sa fractions (Fig. 1). No activity was seen in the Sb membranes. The two precipitates were, however, more strongly stained in the Sa than in the R membranes. Agarose strips containing electrophoretically separated proteins fractionated according to the method of LUNDKVIST AND PERLMAN[14] were assayed in parallel without antiserum. Strips corresponding to precipitates with hydroxylating atcivity contained the flavoprotein and cytochrome P-450 and were able to hydroxylate aminopyrene, while control strips were completely negative. These results constitute an independent confirmation of the specificity of the staining of the immune precipitates.

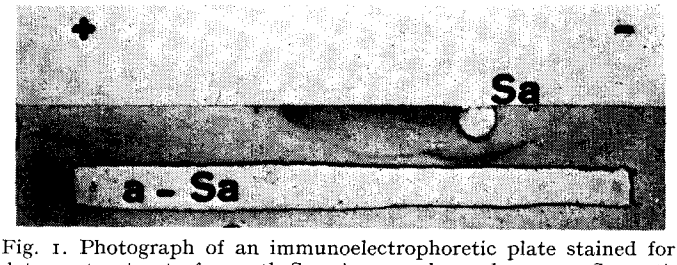

Fig. 1. Photograph of an immunoelectrophoretic plate stained for hydroxylation activity. Sa, detergent extract of smooth Sa microsomal membranes; a-Sa, antiserum against Sa membranes absorbed with lyophilized rat serum.

In the earlier study[8], diazotized sulphanilic acid was used as diazonium salt to give the coloured product on the immunoprecipitates. The specific orange staining of the precipitates was very waek and the yellow background stains relatively strong. In extracts of Sa membranes, 5 precipitates appeared to be stained, but it was difficult to distinguish between specifically stained precipitates and some strong lines to which yellow stain was adsorbed nonspecifically. The improved method used here demonstrated conclusively that only two precipitates exhibited hydroxylation activity.

In these experiments either NADH or NADPH functioned as electron donors for hydroxylation of 2-acetamidonapthalene to 2-acetamido-6-naphthol. Obviously, the period of incubation was too long to distinguish between the two electron donors. Varying their concentrations from 1 to 0.001 mM revealed no differences in their capacities to give hydroxylation staining. The findings suggest that NADH–cytochrome *c* reductase also is present in the precipitates, as ORRENIUS *et al.*[15] have reported that the cross-reactions between the two electron transport chains are not due to lack of specificity of the flavoproteins but rather to an interaction in the flavin–cytochrome region.

As pointed out above, at least two components, the NADPH–cytochrome *c* reductase and cytochrome P-450, are needed to obtain a hydroxylation reaction. This suggest that the different components of the electron transport chains are not separated from each other by the methods of extraction used here and are precipitated together by antibodies. The antibodies are probably directed against determinants other than the enzymatically active sites. Our findings also indicate that the two electron transport systems may be situated close together in the microsomal membranes as both NADH- and NADPH–cytochrome *c* reductase seem to be present in the same precipitates. At the present time there is no explanation for the appearance of two immunologically distinct precipitates with hydroxylation activity. The results can not be explained by random adsorption of enzymically active complexes to inactive precipitates, as only 2 of more than 20 different precipitates (see Fig. 2) in the plates show this activity.

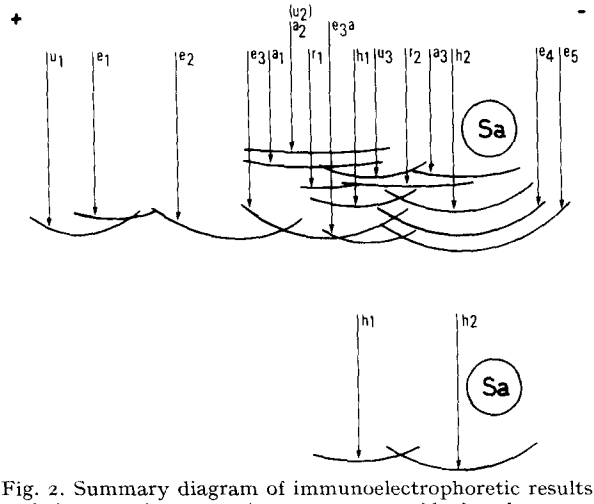

Fig. 2. Summary diagram of immunoelectrophoretic results with extract of Sa membranes tested with its homologous antiserum. a_1–a_3, acid phosphatase active antigens; e_1–e_5, esterase active antigens; h_1 and h_2, antigens with hydroxylation activity; r_1 and r_2 precipitates showing NADPH- and NADH–neotetrazolium reductase activity; u_1–u_3, UDPase active antigens.

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Application of the voltage-clamp technique for measuring the quantum efficiency of light-induced potential changes in *Nitella translucens*

It has been shown[1] that in algal cells, like *Nitella translucens*, the negative potential across the two cellular membranes and the cell wall reversibly changes upon illumination, and that these changes are caused by photosynthetic energy conversion in the two pigment systems of the chloroplast-localized photosynthetic apparatus. It has been suggested[1] that the potential changes primarily are caused by changes in the cytoplasmic proton concentration due to electron transport coupled uptake of protons by the chloroplast in the cytoplasm.

This communication reports on experiments with *Nitella translucens*, energized by monochromatic light, under conditions at which the membrane potential is continuously clamped at the potential in the dark. This voltage-clamp technique, widely applied in research on changes in ionic conductance underlying action potentials in excitable membranes of various organized structures of living organisms (*cf.* refs. 2–5), enables the measurement of the efficiency of light quanta for changing the membrane potential under controlled steady-state light conditions.

Nitella translucens was collected from a fresh water pool in the Netherlands. Cells were bathed in artificial pond water, containing 1.0 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$ and were kept growing at 15° in a conditioned growth room under weak light in a light–dark regime of 12h each. For the experiments, young fresh grown cells about 5–8 cm in length and 0.5 –0.8 mm in diameter were used. Measurements were carried out at room temperature. Fig. 1 shows a schematic diagram of the voltage-clamp arrangement. An increase in membrane potential (ΔV) brought about by actinic illumination causes a current (I) which flows in the feed-back