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PREPARATION OF ANTISERA AGAINST CYTOCHROME b_5 AND NADPH–CYTOCHROME *c* REDUCTASE FROM RAT LIVER MICROSOMES

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SUMMARY

Antisera were prepared in rabbits against purified rat liver cytochrome b_5 and NADH–cytochrome *c* reductase, respectively. With the antiserum against cytochrome b_5, the cytochrome could be precipitated in immunoelectrophoresis from rough and smooth liver microsomal membranes, kidney microsomes and outer mitochondrial membranes. The cytochrome was precipitable from the rough membranes on the 2nd prenatal day, from the smooth membranes on the day of birth and from the kidney microsomes on the 2nd postnatal day.

The NADPH–cytochrome *c* reductase could be precipitated from the rough and smooth membranes and from kidney microsomes by the specific antiserum. It was precipitable from the rough and smooth membranes at birth and from kidney microsomes on the 2nd postnatal day. No NADPH–cytochrome *c* reductase was found in the mitochondrial membranes.

None of the two antisera contained inhibiting antibodies against the catalytic action of the enzyme against which it was directed.

INTRODUCTION

Liver microsomes are known to contain two electron-transport systems. One is linked to NADPH oxidation and generally believed to consist of at least two components: the NADPH–cytochrome P-450 reductase, which is closely related to the flavoprotein NADPH–cytochrome *c* reductase, and cytochrome P-450 (ref. 1). According to LU *et al.*[2,3] a third, heat stable component, is also involved in this system. It catalyzes the hydroxylation of a number of lipid soluble substances including steroids, drugs and various carcinogenic dyes[4]. The other electron transport system, which uses NADH as an electron donor, consists of NADH–cytochrome b_5 reductase and cytochrome b_5 (ref. 5). It has recently been shown to be involved in the oxidative desaturation of fatty acids catalyzed by liver microsomes[6].

Although both electron-transport systems are associated with microsomal membranes, the association seems not to be equally firm for the individual components. Thus, it has been shown by a number of authors[7–9] that treatment of liver microsomes with trypsin solubilizes NADPH–cytochrome *c* reductase and cytochrome b_5, but

leaves the NADH–cytochrome b_5 reductase and cytochrome P-450 in a membrane-bound state. From their studies on the effects of a number of proteolytic enzymes on smooth surfaced liver microsomes, ITO AND SATO[10] suggested that NADPH–cytochrome *c* reductase and cytochrome b_5 are located superficially in the microsomal membranes, while cytochrome P-450 and NADH–cytochrome b_5 reductase seem to be associated with the membranes in such a way that they are not readily attacked by proteolytic enzymes.

The present paper reports the preparation and properties of antisera produced in rabbits against purified cytochrome b_5 and NADPH–cytochrome *c* reductase.

MATERIALS AND METHODS

Cytochrome b_5 and NADPH–cytochrome *c* reductase were purified from liver microsomes—isolated according to the technique described by ERNSTER *et al.*[11]—of phenobarbital-treated Sprague–Dawley rats (80 mg phenobarbital per kg body weight injected intraperitoneally once daily for 3–5 days), as described by OMURA *et al.*[7] by trypsin digestion of KCl-washed microsomes. The supernatant was passed through a column of Sephadex G-100, whereby the two proteins were separated from each other. For further purification, the fractions were chromatographed on DEAE-cellulose columns. The recovery from 30 g rat liver of purified NADPH–cytochrome *c* reductase was about 1–2 mg and that of cytochrome b_5 about 0.5–1 mg. The concentration of cytochrome b_5 in the purified fractions was determined by measuring the absorbance at 424 nm of the $Na_2S_2O_4$-reduced sample[8]. The activity of the NADPH–cytochrome *c* reductase was determined by measuring the rate of reduction of cytochrome *c* as described by DALLNER[12]. The purification of the two enzymes was 100–150-fold.

Antisera against the enzymes were produced in rabbits. Four rabbits were used for each enzyme. Each rabbit received 4–5 intramuscular injections, given at 3 weeks' intervals, of 0.5–1 mg of protein incorporated into Freund's complete adjuvant. The antisera were tested in double diffusion plates and in immunoelectrophoresis against rough- and smooth-surfaced microsomal membranes and against the purified cytochrome b_5 and NADPH–cytochrome *c* reductase, respectively. Antisera giving identical reactions were pooled.

Rough and smooth microsomal membranes were isolated from rat liver microsomes as previously described[13]. Soluble protein antigens were extracted from the microsomal subfractions and from rat kidney microsomes—isolated by the same method as described for liver microsomes[11]—by treatment with 0.5 % Lubrol W (acetylpolyoxyethylene condensate, ICI, England) and 1 % sodium deoxycholate. Inner and outer mitochondrial membranes from rat liver were prepared according to SOTTOCASA *et al.*[14]. Proteins were solubilized from the mitochondrial membranes by sonication in a Branson Sonifier at 0° of 3.5-ml aliquots for 4 × 15 sec at 5A. The protein content of the antigen solutions was determined according to LOWRY *et al.*[15]. Protein concentration was adjusted to 6–10 mg/ml for the rough and smooth microsomal membranes, kidney microsomes and for the inner and outer membranes of liver mitochondria, and 0.5–1 mg/ml for the purified enzymes in all experiments. Immunoelectrophoresis was carried out in agarose gel. The electrophoretic separation was run for 5 h in 0.05 M barbiturate buffer, pH 8.6.

RESULTS

Properties of anti-cytochrome b_5 serum

Only one precipitate was obtained when purified cytochrome b_5 was reacted with its antiserum (anti-cytochrome b_5). In some experiments, however, it was found that this precipitate consisted of a double line, as can be seen in Fig. 1. Thus, it most probably contained two antigens which were immunologically somewhat different, but had the same electrophoretic mobility. A corresponding precipitate was also developed between anti-cytochrome b_5 and the extracts of the rough and smooth microsomal fractions (Fig. 2). That this precipitate really corresponded to the cytochrome b_5–anti-cytochrome b_5 complex was ascertained by demonstrating spectral properties characteristic of cytochrome b_5 in the precipitate. Agarose strips containing electrophoretically separated proteins prepared according to LUNDKVIST AND PERLMANN[16] were assayed in the absence of antiserum. Strips corresponding to membranes 5–7 in Fig. 2 showed the presence of cytochrome b_5 while strips corresponding to 1–4 were completely negative in this respect. No cross reactions were seen between purified NADPH–cytochrome *c* reductase and the anti-cytochrome b_5 serum.

The appearance of cytochrome b_5 during liver development was also studied. With the anti-cytochrome b_5 serum it could be precipitated from 2 days prenatal rough microsomal membranes, but only from the smooth membranes of the newborn.

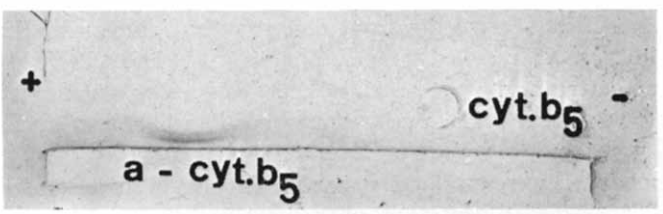

Fig. 1. Photograph of immunoelectrophoretic plate stained for protein. a-cyt. b_5, antiserum against purified rat liver cytochrome b_5; cyt. b_5, purified rat liver cytochrome b_5.

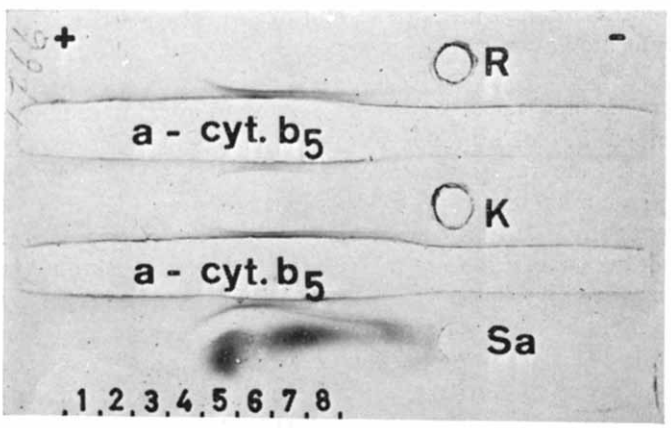

Fig. 2. Photograph of immunoelectrophoretic plate stained for protein. R, Sa, K are detergent extracts of rough and smooth microsomal membranes from adult rat liver microsomes and rat kidney microsomes, respectively. For abbreviations see Fig. 1. 1–8 indicate agarose strips cut out of electrophoretic plates. (For further explanations see the text.)

This difference in appearance of the cytochrome in the two types of membranes is in line with the findings of DALLNER *et al.*[17], who showed that during development many microsomal enzymes appear first in the rough and subsequently in the smooth membranes. However, they were not able to demonstrate the presence of cytochrome b_5 in the total liver microsomal fraction until the 1st postnatal day, and then the level was very low.

From the 2nd postnatal day and onwards cytochrome b_5 could also be precipitated from extracts of kidney microsomes. The precipitate obtained between adult kidney microsomes and the anti-b_5 serum can be seen in Fig. 2.

In a series of experiments, extracts of inner and outer mitochondrial membranes, prepared from rat liver, were reacted with the anti-cytochrome b_5 serum. The outer membranes gave one precipitate, as can be seen in Fig. 3, whereas no such precipitate was observed with the inner membrane preparation.

The anti-cytochrome b_5 serum did not contain antibodies which inhibited the catalytic action of the cytochrome in the NADH–cytochrome *c* reductase reaction. Neither the total antiserum nor the γ-fraction, when added in concentrations of 0.5–10 mg/ml, caused a significant inhibition of the rate of NADH–cytochrome *c* reduction in the liver microsomes. 0.55 μmole cytochrome *c* was reduced per min per mg protein. The same value was obtained in the control system, where the γ-fraction of unspecific antiserum (anti-rough membranes) was used.

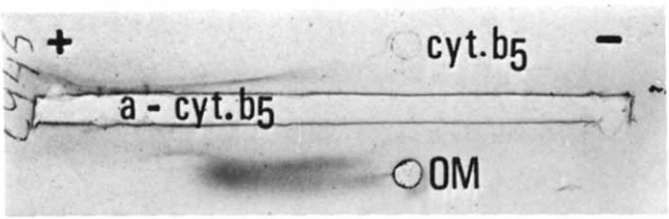

Fig. 3. Photograph of immunoelectrophoretic plate. OM, outer membranes of rat liver mitochondria. For other abbreviations see Fig. 1. The large spot on the OM side represents protein denatured during electrophoresis and is not an immune precipitate. It could not be washed out of the agarose by the washing procedures used in this study.

Properties of anti-NADPH–cytochrome c reductase serum

When the antiserum against NADPH–cytochrome *c* reductase (anti-cyt. *c* red.) was reacted with the purified reductase, one precipitate was obtained in immunoelectrophoresis. However, when this antiserum was reacted against extracts of rough or smooth microsomal membranes, it gave several precipitation lines (see Fig. 4). These precipitates were tested for various enzyme activities, such as esterase, acid phosphatase, neotetrazolium reductase and UDPase, by histochemical staining procedures developed for agarplates[13]. It was demonstrated that the NADPH–cytochrome *c* reductase preparation injected into rabbits was contaminated with esterase and UDPase active antigens. The concentration of these impurities was low in the purified reductase preparation, as none of these enzyme activities was detected in the NADPH–cytochrome *c* reductase – anti-cyt. *c* red. precipitate. They were, however, present in a sufficiently high concentration to induce antibody production in rabbits. None of the above mentioned enzyme activities was detected in the precipitate

obtained between the rough and smooth microsomes and anti-cytochrome b_5, indicating that the cytochrome b_5 preparation was fairly pure.

Agarose strips corresponding to the different precipitates developed between smooth microsomal membranes and the anti-cyt. *c* red. preparation (Fig. 4) were tested for NADPH–cytochrome *c* reductase activity. It was shown that strips corresponding to the reductase–anti-cyt. *c* red. precipitate (indicated by arrow in Fig. 4) catalyzed the reduction of cytochrome *c*, while all the other strips were negative. In order to produce a more specific antiserum, the precipitate corresponding to the reductase active strips was cut out of a number of agarose plates, emulsified in Freund's complete adjuvant and injected into rabbits. Each rabbit, which received the total protein corresponding to 8–10 precipitates, was given 4 injections at 3 weeks' intervals. When this new antiserum was reacted in immunoelectrophoresis against the purified reductase and rough or smooth microsomal membranes, only one visible precipitate

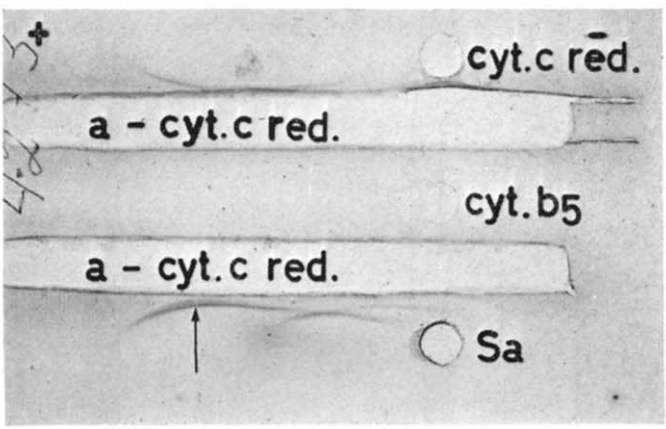

Fig. 4. Photograph of immunoelectrophoretic plate. Cyt. *c* red., NADPH–cytochrome *c* reductase purified from rat liver; a-cyt. *c* red., antiserum against the purified NADPH–cytochrome *c* reductase. For other abbreviations see Fig. 2. Arrow indicates the cytochrome *c* reductase specific precipitate. (For further explanations see the text.)

was obtained (see Fig. 5). On testing this precipitate for the presence of contaminating enzyme activities, it was found that all but one esterase active antigen had been eliminated. This antigen had exactly the same electrophoretic mobility as the NADPH–cytochrome *c* reductase and therefore could not be removed by cutting out

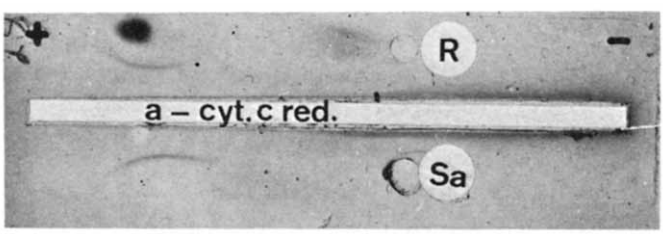

Fig. 5. Photograph of immunoelectrophoretic plate. For abbreviations see Fig. 2. a-cyt. *c* red., antiserum against NADPH–cytochrome *c* reductase prepared by injecting precipitates with reductase activity, emulsified in Freund's complete adjuvant into rabbits. For explanation of the intense spot on the R side, see legend to Fig. 3.

the precipitates for injection into rabbits. Moreover, the esterase active precipitate was not visible until after staining.

No cross reactions were obtained between purified cytochrome b_5 and the anti-cyt. *c* red. serum (Fig. 4).

Using this new and more specific anti-cyt. *c* red. serum, the NADPH–cytochrome *c* reductase could be precipitated from extracts of rough and smooth liver microsomes of newborn animals. This is compatible with the results of DALLNER *et al.*[17], who demonstrated the presence of a weak NADPH–cytochrome *c* reductase activity in rat liver microsomes from the 3rd prenatal day. Furthermore, a NADPH–cytochrome *c* reductase active antigen was also found to be present in rat kidney microsomes from the 2nd postnatal day. On the other hand, no NADPH–cytochrome *c* reductase was precipitated from the extracts of the outer and inner membrane preparations of liver mitochondria.

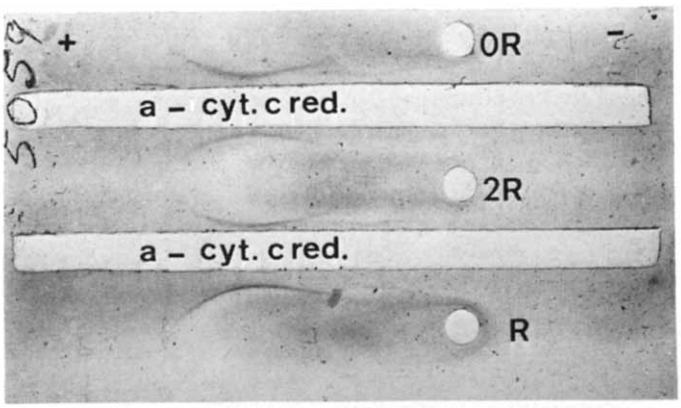

Fig. 6. Photograph of immunoelectrophoretic plate. oR, 2R and R, detergent extracts of rough membranes from newborn, 2-day and adult rat liver, respectively. For other abbreviations see Fig. 5.

The anti-cyt. *c* red. serum contained no blocking antibodies against the enzymatically active site of the enzyme, as it did not inhibit the reduction of cytochrome *c* catalyzed by the purified NADPH–cytochrome *c* reductase preparation. 0.9 μmole cytochrome *c* was reduced per min per mg protein both in the presence of unspecific γ-globulin (anti-albumin) and the γ-globulin precipitated from anti-cyt. *c* red. serum. Concentrations of added γ-globulin between 0.5 and 10 mg/ml were investigated and found to be without effect.

DISCUSSION

Although both the enzymes studied were purified 100–150-fold as compared to the liver microsomes, only the cytochrome b_5 preparation was reasonably pure already after the initial purification procedure. Most often single precipitates were formed when the antiserum produced in rabbits against the purified cytochrome b_5 preparation was reacted with either the purified preparation or extracts of rough and smooth liver microsomes. In some experiments, however, this precipitate consisted of a double line, suggesting that the antiserum contained antibodies against two anti-

genic components. Since it was not possible to cut out the two lines and assay them separately for the presence of cytochrome b_5, it could not be decided whether the two precipitation lines were due to an impurity in the cytochrome b_5 preparation or possibly to the presence of two forms of cytochrome b_5 in the microsomal membrane. The latter alternative should be considered in the light of the observation of SARGENT AND VADLAMUDI[18] who isolated two similar components of cytochrome b_5 from rat liver microsomes. Slight differences in amino acid composition were reported for the two forms which could account for the immunological variation seen in our work. The two antigenic components were not only seen with the purified cytochrome b_5 preparations, but also with detergent extracts of liver and kidney microsomes, indicating that tryptic action was not the direct cause of the appearance of two components.

In contrast to the findings with cytochrome b_5, the NADPH–cytochrome *c* reductase preparation obtained after the initial purification procedure was contaminated with several impurities as demonstrated by the appearance of multiple precipitates when the rabbit antiserum, was reacted with microsomal extracts. Some of the precipitates exhibited enzymatic activities other than NADPH–cytochrome *c* reductase. This difficulty could, however, be largely overcome by cutting out the precipitates with NADPH–cytochrome *c* reductase activity from the agarose plates and using them to produce new antibodies. When the new antiserum so produced was reacted in immunoelectrophoresis against the NADPH–cytochrome *c* reductase preparation or detergent extracts of microsomal membranes, only one precipitate was formed which, however, also displayed a very weak esterase activity. This antigen could not be removed by cutting out the precipitate for injection into rabbits since it had exactly the same electrophoretic mobility as the NADPH–cytochrome *c* reductase.

The antisera produced against the liver microsomal enzymes also reacted with extracts of kidney microsomes suggesting the presence of NADPH–cytochrome *c* reductase and cytochrome b_5 with identical antigenic properties in both tissues. Like liver microsomes, kidney microsomes contain a NADPH-linked monooxygenase system, which, however, preferentially catalyzes the ω-oxidation of fatty acids, and the NADPH–cytochrome *c* reductase is involved in the reduction of cytochrome P-450 in the ω-oxidation reaction[19]. The identical antigenic properties of liver and kidney NADPH–cytochrome *c* reductases are of interest since differences in substrate specificity between the cytochrome P-450's of the two tissues may possibly turn out to be due to structural differences in the cytochrome.

Unlike the NADPH–cytochrome *c* reductase, cytochrome b_5 is a constituent also of the outer mitochondrial membrane (*cf.* ref. 20). Our finding that cytochrome b_5 of the outer membrane is precipitated when reacted with antisera produced against the microsomal cytochrome, suggests common antigenic properties of the two cytochromes. Furthermore, since TAKESUE AND OMURA have recently reported the inhibition of the rotenone-insensitive NADH–cytochrome *c* reductase of the outer mitochondrial membrane by an antibody prepared against microsomal NADH–cytochrome b_5 reductase[21], it now seems that there exist great similarities between the individual components of the microsomal NADH–cytochrome *c* reductase system and that present in the outer mitochondrial membrane. Observed differences between the properties of microsomal and outer mitochondrial cytochrome b_5 (*cf.* ref. 20) may thus be due to differences in the microenvironment of the cytochrome in the two organelles.

The immunological technique used in the present work proved very useful for demonstrating the presence of even minor amounts of the two enzymes. This was most obvious in the study of the appearance of the enzymes in the microsomal membranes of the developing rat liver. In agreement with the results of DALLNER *et al.*[17] both enzymes appeared first in the rough microsomes. Using this fraction, it was possible to demonstrate the presence of cytochrome b_5 already two days before birth, when spectrophotometric assays were difficult due to the very low concentration of the cytochrome present.

One of the aims of the present work was to obtain antibodies against the two enzymes which could inhibit their catalytic activities. KURIYAMA *et al.*[22] have obtained such antibodies in rabbits against purified rat liver NADPH–cytochrome *c* reductase. In this work, precipitating antibodies were obtained which, however, did not inhibit the catalytic activities of the two enzymes. A reason for this difference may well be that whereas they injected 2–5 mg of purified enzyme in Freund's complete adjuvant into each rabbit, we have injected only 0.5–1 mg at a time. This may mean that on injecting larger amounts of purified enzyme, antibodies are produced against more determinants on the enzyme protein. Some of these determinants may be close to the enzymatically active site and their reaction with antibody may have inhibitory effects on the enzymatic activity of the antigen[23].

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