Biochimica et Biophysica Acta, 291 (1973) 442–453

BBA 76115

MEMBRANE FRACTIONS FROM RAT HEPATOMA

III. IMMUNOCHEMICAL CHARACTERIZATION OF DETERGENT-SOLUBLE MEMBRANE PHOSPHATASES, ELECTRON TRANSPORT CHAINS AND CATALASE

MARIKA RAFTELL AND FRED BLOMBERG

The Wenner-Gren Institute for Experimental Biology, Department of Immunology, University of Stockholm, Stockholm (Sweden)

(Received July 3rd, 1972)

SUMMARY

1. A plasma membrane fraction (tP) and two submicrosomal fractions (rough tR, smooth tSa) were isolated from the 4-dimethylaminoazobenzene-induced rat hepatoma D23. Membrane extracts were tested in immunodiffusion and stained for various phosphatases, electron transport chains and catalase.

2. In the tP extract one 5′-nucleotidase (NMPase) active antigen, immunologically different from those present in liver, was found. tP also contained two of the nucleoside tri- and diphosphatase (NTPase and NDPase) active antigens present in liver plasma membranes. The uridine diphosphatase (UDPase) active antigen (U_1) characteristic for liver microsomes was only found in the tR fractions, while both tR and tSa contained an additional antigen with similar activity.

3. No hydroxylating capacity was found in any of the tumour fractions, not even after phenobarbital treatment, when 2-acetamidonaphthalene was used as substrate. However, some of the components of the liver electron transport chains, NADPH–cytochrome *c* reductase, NADH– and NADPH–neotetrazolium (NT)-reductase and cytochrome b_5 were present in the tR fraction. Furthermore, all tumour fractions contained one NADH–NT-reductase active antigen common to liver microsomes and plasma membranes.

4. One catalase active antigen was present in all liver and tumour fractions studied.

INTRODUCTION

Several antigens with different nucleoside phosphatase activities have earlier been found in liver plasma membranes and microsomes[1,2]. It was of interest to determine whether the different NDPase and NMPase activities found in hepatocellular membranes[3] were also present in the hepatoma D23 and if some new

Abbreviation: NT, neotetrazolium.

antigens of these types could be detected. Moreover, various physiological and pathological states of rapid hepatocellular growth, such as regenerating liver[4], neonatal liver[5] and hepatic tumours[6], are known to be deficient in microsomal drug metabolizing enzymes. Hepatic tumours, however, vary considerably in this respect. Thus, the well differentiated "minimal deviation" hepatomas[7,8] have retained the drug-metabolizing capacity present in normal liver, while the less differentiated "multiple deviation" hepatomas[9] have lost it. This study, therefore also comprises an immunochemical investigation of the drug metabolizing capacity of hepatoma D23.

MATERIAL AND METHODS

Membranes

Microsomes from rat liver were fractionated into rough membranes (R) and two different smooth membrane fractions (Sa and Sb) as described[2]. Corresponding fractions from the hepatoma D23 (tR, tSa and tSb) were isolated as described in the preceding paper[9]. Plasma membranes were prepared from liver (P) and the hepatoma D23 (tP) according to Emmelot *et al.*[10,11]. The fractions were washed in saline. Rat erythrocytes were isolated from heparinised blood, washed in phosphate-buffered saline, and broken up in distilled water. The ghosts were sedimented and repeatedly washed in saline until the supernatant was colourless.

Antisera

Antisera against the different membrane fractions, called a-R, a-Sa, a-P, a-tR, a-tSa and a-tP, respectively, were prepared. Antisera from 3–4 rabbits were pooled and exhaustively adsorbed with lyophilized rat serum[9]. The preparation of mono-specific antisera has been described earlier[12]. Membrane antigens were extracted with 1 % sodium deoxycholate and 0.5 % Lubrol W as previously reported[2,9]. The protein content of the extracts was determined according to Lowry *et al.*[13], with bovine serum albumin as standard, and adjusted to 8–10 mg protein per ml before analysis.

Immunodiffusion analyses were carried out as described earlier[1].

Characterization of immune precipitates

Histochemical staining reactions were used for enzymatic characterization of immune precipitates.

Nucleoside phosphatases

NTPase (nucleoside triphosphatase) and NDPase (nucleoside diphosphatase, EC 3.6.1.6) activities were assayed according to Wachstein and Meisel[14] with the substrates ATP, ADP or UDP as indicated in the text and figure legends. The same method was used to demonstrate NMPase (5′-nucleotidase, EC 3.1.3.5) but utilizing AMP or GMP as substrate.

Acid phosphatase (EC 3.1.3.1) and alkaline phosphatase (EC 3.1.3.1) were investigated according to Allen and Hunter[15] and Ozato and Okada[16].

Catalase (EC 1.11.1.6) was demonstrated with 5 % H_2O_2 according to Uriel[17].

NAD(P)H–neotetrazolium reductase (E.C. 1.6.99.2) active immune precipitates were stained as reported by Raftell and Perlmann[18].

Hydroxylation activity in precipitates was developed as previously described[19]. Cytochrome P450, cytochrome b_5, NADPH– and NADH–cytochrome *c* reductases were determined in the test tube according to Dallner *et al.*[20].

RESULTS

Nucleoside phosphatases

NMPase, one of the marker enzymes of liver plasma membranes[11] has previously been identified by immune electrophoresis[21]. Its presence in the tumour was investigated, using AMP or GMP as substrates. The two stained precipitates found in the P/a-P combination[21] did not appear in the P/a-tP or tP/a-P combinations. However, tP/a-tP gave one very weakly stainable NMPase active precipitate (indicated by an arrow in Fig. 1). No NMPase activity was found in R, Sa, tR and tSa immunoprecipitates.

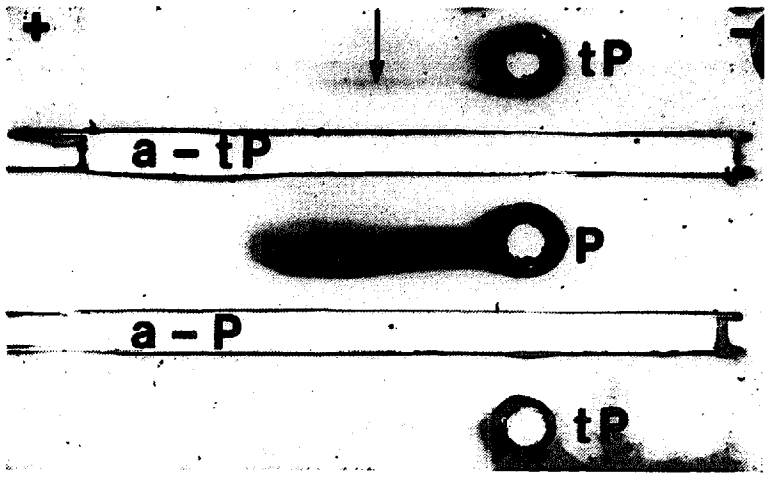

Fig. 1. Immunoelectrophoretic plate stained for NMPase activity with AMP as substrate. P and tP: detergent extracts of plasma membrane fractions from liver and hepatoma D23, respectively. a-P and a-tP: antisera against corresponding fractions; antisera absorbed with lyophilized rat serum. Arrow indicates a precipitate not found in the P fraction.

NDPase and NTPase activities were tested with ATP, ADP or UDP as substrates. The P/a-P reaction has earlier been reported[21] to give rise to 6 different precipitates, all hydrolyzing both NDP and NTP substrates. a-tP precipitated two of these antigens from P (X_3 and X_6) and two from tP (indicated by arrows in Fig. 2a). The latter two precipitates are hitherto unidentified, but double-diffusion experiments indicate that at least one of them is identical with a P/a-P precipitate.

ATP and ADP were neither hydrolysed by microsomal precipitates from normal liver nor from the hepatoma. The liver microsomes (both R and Sa) contain one UDPase active antigen (designated U_1)[1,12] not present in plasma membranes. This antigen was also precipitated from the tR fraction by a-R and faintly from R by a-tR (Fig. 2b). In contrast, no U_1 was found in tSa in corresponding tests. On the other hand, this fraction contained another UDPase active precipitate (Fig. 2c), which turned out to consist of 2 precipitates, as seen in double-diffusion tests according to Piazzi[22] (Fig. 3). One of the antigens was also present in Sa mem-

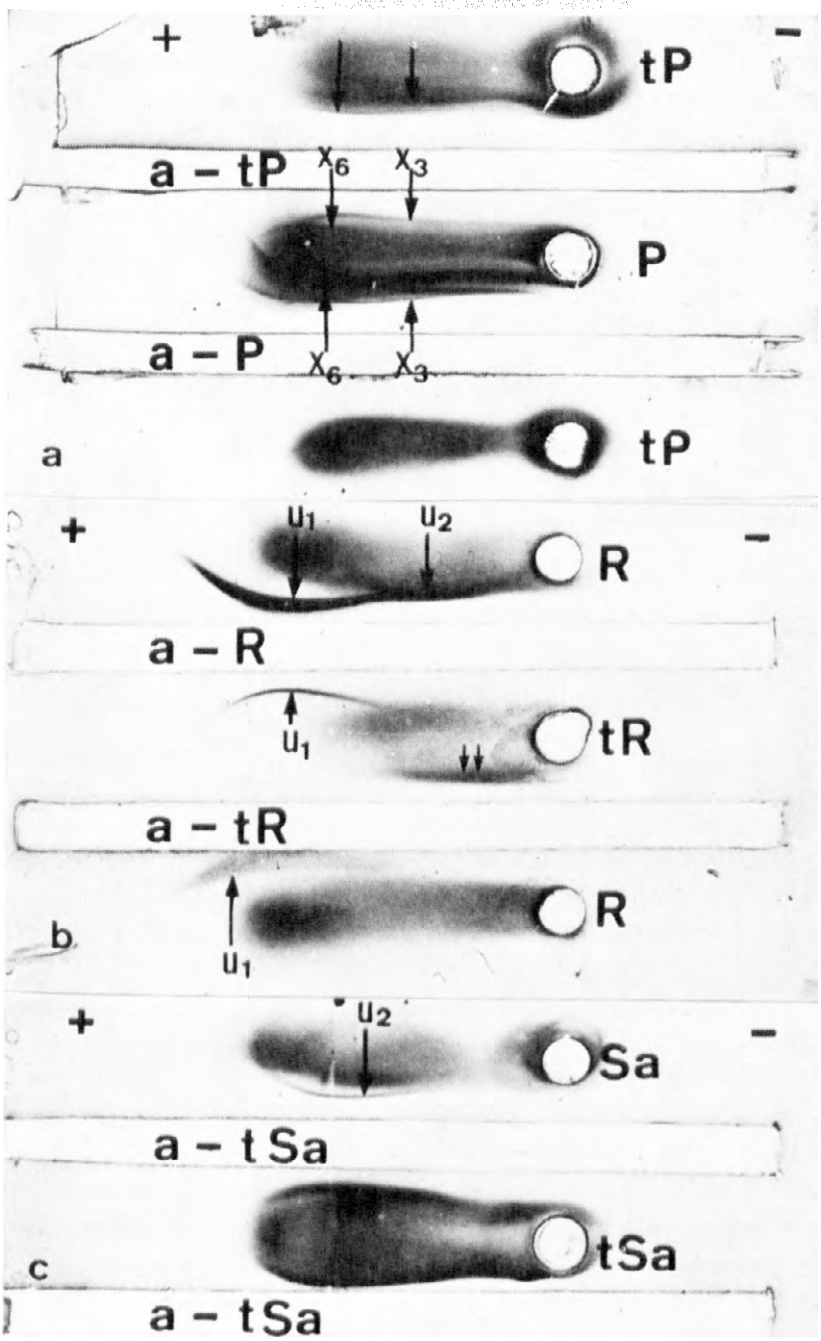

Fig. 2. UDPase staining of immune precipitates. R, tR, Sa and tSa: detergent extracts of liver and hepatoma rough and smooth microsomal membranes, respectively. a-R, a-tR, a-Sa and a-tSa, antisera prepared in rabbits against corresponding fractions, and absorbed with lyophilized rat serum. For other symbols see Fig. 1(a). At least two different precipitates (indicated by arrows) can be seen in the tP fraction, X_3 and X_6 correspond to an earlier classification of NTPase and NDPase active antigens in liver plasma membranes[21]. (b, c) Double arrows indicate a precipitate not found in the corresponding liver fractions, U_1 is an antigen specific for R and Sa fractions from liver, and also found in tR: U_2 is an antigen common to many fractions (*i.e.* R, Sa, Sb).

branes (designated U_2), while the other seemed to be "specific" for the tumour. This antigen was also precipitated in the tR/a-tR reaction (indicated by double arrows in Fig. 2b).

All antisera when tested in double diffusion, precipitated one additional

UDPase active antigen which was common to all fractions of both liver and tumour origin. This UDPase seemed not to be identical with any of the antigens described in the P fraction, as none of the microsomal antigens was able to hydrolyze ADP, ATP or, in the absence of Mg^{2+}, UDP[21]. Because of the complexity of the phosphatases it has thus far not been possible to further characterize this antigen. A test system with higher resolution is needed before a proper identification of all phosphatase active antigens can be carried out.

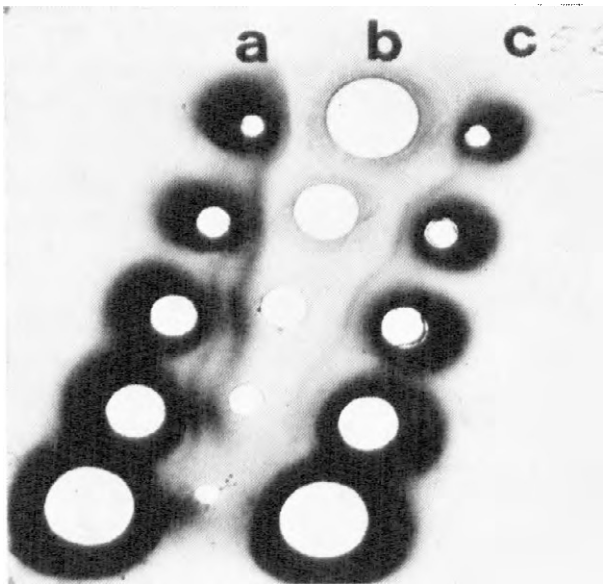

Fig. 3. Photograph of a Piazzi[22] test plate stained for UDPase activity. a and c: detergent extracts of smooth membranes from hepatoma D23 and liver (tSa and Sa), respectively. b: antiserum to smooth membranes from the hepatoma (a-tSa).

One protein with acid phosphatase activity, seemingly unique for the tumour, was precipitated from tR and tSa by their homologous antisera (Fig. 4). tP/a-tP gave a similar active precipitate. The antigens were not detected in normal liver membranes with any antisera. Alkaline phosphatase activity has been reported[23] to increase in other tumours of hepatoma type, but we were unable to detect any immunoprecipitates with this activity, in either tumour or in normal liver membranes.

Electron transport enzymes

In earlier work, it was shown that two distinct immune precipitates originating from R and Sa membranes could carry out hydroxylation of 2-acetamidonaphthalene to 2-acetamide-6-naphthol[19]. When tumour precipitates were assayed for hydroxylating activity, no staining appeared in any combinations of antigen and antisera, indicating that the hepatoma D23 could not carry out this reaction. The absence of the hydroxylating capacity was also ascertained by test tube assay of tumour microsomes for cytochrome P450, known to be a part of the hydroxylating system of liver microsomes[24-26]. No cytochrome P450 was found in the tumour microsomes.

We also tested for NADPH–cytochrome *c* reductase, the other known component of the hydroxylating system. The assays were performed both as a test tube reaction and by immunoelectrophoresis with a mono-specific antiserum as earlier described[12]. One weak but clearly visible precipitate was obtained on reacting

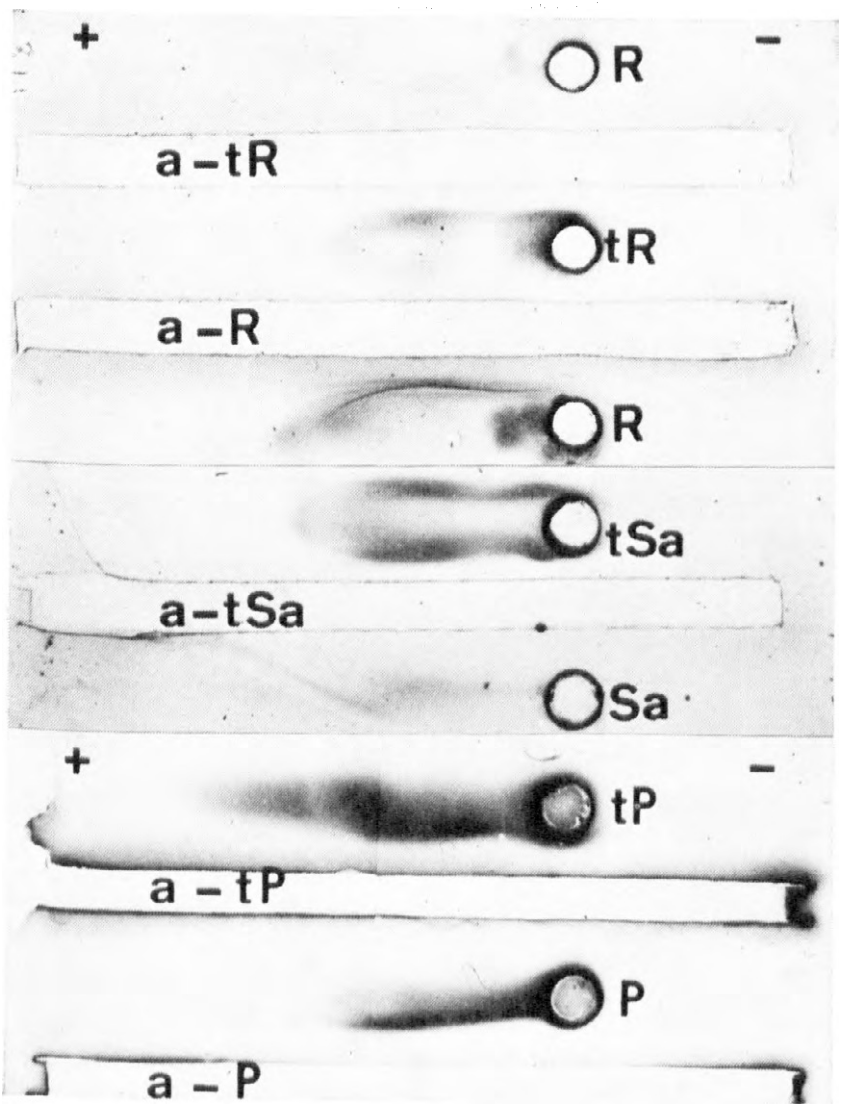

Fig. 4. Acid phosphatase staining of immune precipitates. For symbols see Figs 1 and 2.

tR with anti-NADPH–cytochrome *c* reductase. In the test tube, tR also gave a weak but significant activity (0.70 μmole cytochrome *c* reduced per mg protein per h as compared with 5.68 for R membranes). tSa gave a faint activity in the test tube, but no visible precipitate with anti-NADPH–cytochrome *c* reductase. No activity was found in tP or P membranes.

To further establish if these results depended on a true loss of the capacity of the tumours to produce some of the components of the hydroxylation chain, phenobarbital was injected into tumour-bearing animals (80 mg phenobarbital per kg body weight injected intraperitoneally once daily for 3–5 days). Several authors[27,28] have reported that agents like 3-methylcholanthrene or phenobarbital stimulate the hydroxylation reaction in some tumours. No increase in drug metabolising capacity, tested on immunodiffusion plates, was found in the hepatoma D23 after phenobarbital administration, suggesting that essential components of the chain were truly missing.

When the capacity of tR, tSa and tP to reduce cytochrome *c* with NADH as electron donor was measured, tR was the only tumour fraction showing any

activity in the test tube assay (1.6 μmoles cytochrome *c* reduced per mg protein per h). All liver fractions gave positive reactions (R giving 71.1 and P 7.22).

We then investigated the presence of cytochrome b_5 both in the test tube and by immunodiffusion with monospecific antisera[12], since the reduction of cytochrome *c* with NADH, according to the proposed scheme of Dallner *et al.*[20], proceeds by a flavoprotein and cytochrome b_5. The monospecific antisera did not precipitate cytochrome b_5 from any of the tumour fractions. However, tR showed a low but significant activity in the test tube reaction (0.020 $A_{424-410\,nm}$ unit per mg protein; the corresponding value for R was 0.055 unit). The other tumour fractions were negative.

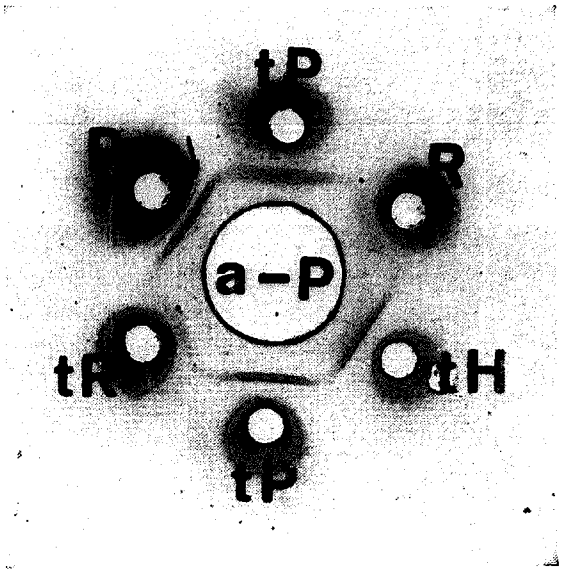

Fig. 5. A diffusion plate stained for NADH–NT-reductase activity. For symbols see Figs 1 and 2. tH: detergent extract of tumour homogenate. Note the line of identity obtained with all extracts tested. Precipitate indicated by arrow is not stained.

Immune precipitates were tested for NADH- and NADPH–neotetrazolium (NT)-reductase activity, as NT can function as an electron acceptor for the two electron transport chains present in liver microsomes[20]. One of the two NT-reductase active antigens present in liver microsomes[18] was also present in liver plasma membranes and in all tumour fractions studied. An identity reaction was obtained between P, tP and tR membranes, when reacted with a-P (Fig. 5), a-tP and a-tR. a-tR and a-tSa could also precipitate the antigen from corresponding tumour and liver fractions. This NT-reductase was inhibited by 1 mM *p*-chloromercuribenzoate (PCMB) but not by 1 mM rotenone. The significance of the finding that one of the NT-reductase active antigens present in liver microsomes is lost in the tumour microsomes, while the other, which is shared with liver plasma membranes, is retained, is at present unclear.

Catalase

Catalase activity was strongly diminished in the microsomal subfractions of the tumour, as judged from the decreased reactivity in tR and tSa precipitates. The weak precipitation reaction of tSa with a-tSa and a-Sa is shown in Figs 6a

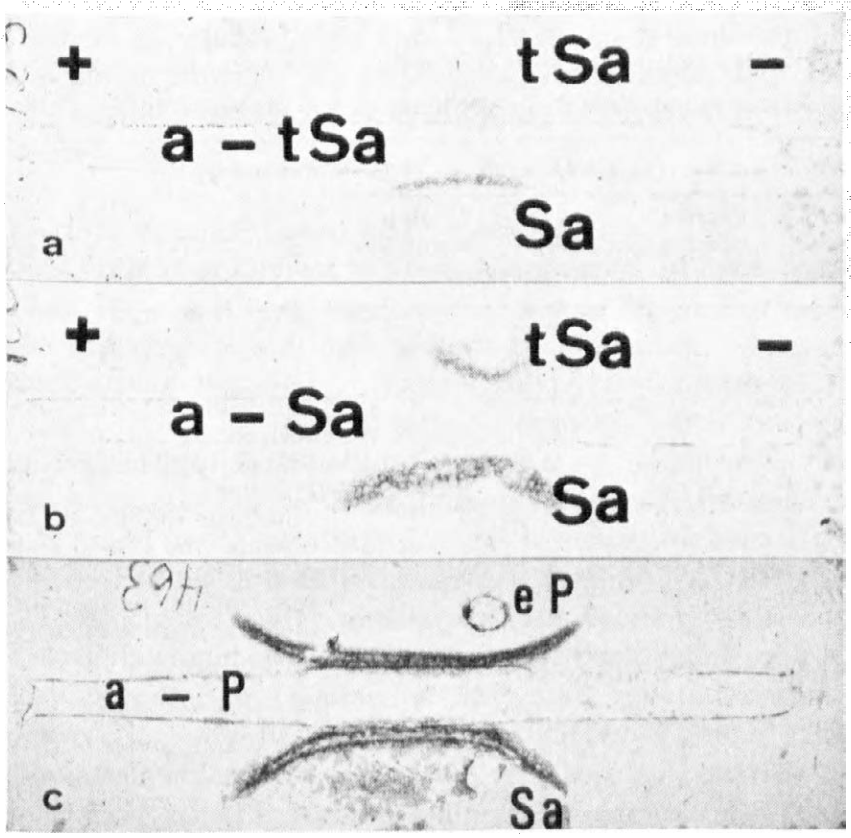

Fig. 6. Immunoelectrophoretic plates showing catalase activity. eP: detergent extracts of rat erythrocyte ghosts. For further explanations of symbols see Fig. 1.

and b, respectively. The tP/a-tP precipitate on the other hand, showed the same reactivity as the corresponding normal counterpart. It should also be mentioned in this connection that membranes from erythrocytes contain the same catalase-active antigen as liver membranes, since it can be precipitated from detergent extracts of washed erythrocyte ghosts with anti-liver membrane sera (a-P (Fig. 6c) and a-Sa). However, the catalase reaction of liver probably does not depend on contamination from erythrocytes, as membranes from perfused liver give an identical reaction with those from non-perfused liver. No differences in reactivity between precipitates developed with membrane fractions from normal liver and those from liver of tumour-bearing animals were observed.

DISCUSSION

A summary of the results is presented in Table I. As pointed out in the preceding paper it is not known whether the antigenic changes obtained depend on loss of antigens from the hepatocyte during dedifferentiation or if the hepatoma is derived from liver cells already lacking these antigens.

Nucleoside phosphatases

In accordance with the findings that plasma membrane ATPase activity is lower in hepatoma D23 than in liver[3], only two precipitates with NTPase and NDPase activity were detected in the plasma membranes of the tumour. At least one was immunologically identical with one of the six antigens seen in normal liver

TABLE I

PRECIPITATES WITH ENZYME ACTIVITIES IN DIFFERENT FRACTIONS OF LIVER AND HEPATOMA D23

One UDPase common to all fractions is not identified and therefore not presented in the Table.

	Liver		*Hepatoma*		*Remarks*
	Microsomes (R–Sa)	*Plasma membrane (P)*	*Microsomes (tR–tSa)*	*Plasma membranes (tP)*	
Nucleoside phosphatases					
NMPase	—	2	—	1	1 tP antigen immunologically different from liver antigen
NDPase–NTPase	—	6	—	2	1 tP antigen identical with P antigen
UDPase*	3		3–2		1 antigen specific for the tumour, the others common with liver
Acid phosphatase	3	3	1	1	the tumour antigen not identical with any of the liver antigens
Hydroxylating activity	2	—	—	—	
NADH(NADPH)–NT–reductase	2	1	1	1	1 antigen present in all fractions, the other specific for liver microsomes
Catalase	1	1	1	1	very weak in hepatoma microsomes

* Not hydrolysing ATP or ADP.

plasma membranes. The relationship of the NTPase-active precipitates found here to the various types of NTPase activities (*e.g.* Mg^{2+}- and (Na^+–K^+)-dependent ATPases) distinguished in the literature[29,30] is not known at present. Monospecific antisera prepared against the indivudual antigens will be a valuable tool for establishing their physiological significance.

The NMPase active antigens in liver plasma membranes and hepatoma seemed to be immunologically non-identical, thus implying possible different biological functions. The significance of this observation is unknown.

The presence of the UDPase activity antigen U_1, specific for liver microsomes[2], in rough but not smooth fraction of the hepatoma points to differences between these two fractions. The physiological significance of UDPase activity is not clear, but Ernster and Jones[31] have proposed that the UDPase of liver microsomes may have a function in removing UDP, formed in the various uridine nucleotide-linked conjugation reactions taking place in the membranes. The correlation of U_1 with conjugation reactions has not been investigated in either the liver or in the hepatoma. It may, however, be mentioned that the ability to stain U_1 in liver microsomes increases after phenobarbital treatment[32], suggesting involvement in drug metabolism. No corresponding increase in activity has been seen with rough tumour membranes from phenobarbital-treated hosts. It is possible that antigen U_1 is actually composed of several enzyme species which all show UDPase activity, but which have different functions in the liver membranes. Our earlier findings in polyacrylamide-electro-

phoresis experiments[18] of two UDPase active bands running close together towards the anode support this hypothesis. The tumour may then have become depleted of some molecular species, possibly those involved in drug metabolism, while others are retained.

Electron transport chains and hydroxylation

It was of interest to study the presence of hydroxylation capacity in the hepatoma D23, as it had been reported in the literature that some hepatomas have lost the capacity to hydroxylate various compounds, while others, being mostly of the minimal deviation type, have retained this property[27,28]. Moreover, it has been demonstrated by Hart *et al.*[6] and others[27,28] that pretreatment of tumour-bearing animals with phenobarbital and methylcholanthrene induces drug-metabolizing enzyme activities in several liver tumours. Although no hydroxylation activity was found in the hepatoma D23 microsomes, not even after phenobarbital treatment of the host, it was interesting to note that NADPH–cytochrome *c* reductase and NT-reductase activity remained in the tumour, while cytochrome P450 had been lost. This would suggest that the first components of the electron transport pathway proposed by Dallner *et al.*[20] and by Hildebrandt and Estabrook[33] among others, *i.e.* the flavoprotein, NADPH–cytochrome *c* reductase and component x, may interact with other, not yet identified, electron-dependent reactions. These may have a higher survival value to hepatoma cells, than the more specific hydroxylation reaction. This would explain the retention of a part of the NADPH-dependent system in the tumour.

In contrast to the NADPH-dependent system the entire NADH-dependent electron transport system, consisting of a flavoprotein, a component x and cytochrome b_5, was present in the tumour (tR fraction). The individual components in the system were demonstrated to be present in test tube reactions.

The presence of NADH-cytochrome *c* reductase activity in liver plasma membrane has previously been described by Emmelot and Benedetti[34]. They pointed out that it is unlikely that the activity seen in liver plasma membranes would entirely depend upon microsomal contamination. In case of contamination, one would have expected higher values for the other electron transport chain enzymes than those which were actually found.

The function of the NADH-dependent electron transport chain has, to our knowledge, not yet been completely established. Hildebrandt and Estabrook[33] have recently suggested that this system can interact with the hydroxylation reaction in liver microsomes through cytochrome b_5 reacting with reduced cytochrome P450. Oshino *et al.*[35] have proposed that the NADH-electron transport chain through cytochrome b_5 participates in fatty acid desaturation. The first pathway probably does not function in the hepatoma, as no hydroxylation activity has been found in it. The second alternative has not been investigated in hepatoma D23.

Catalase

In our studies we found only one catalase active precipitate in immunoelectrophoresis, when liver or tumour fractions were reacted with their homologous antisera. Hence we did not detect the heterogeneity of catalase reported by others[36–38].

It should, however, be pointed out that this study has been carried out on membrane fractions, while most of the work on catalase heterogeneity was done with isolated and purified catalase enzyme preparations. Davidova *et al.*[37] have suggested the existence of two forms of catalase in rat liver and hepatomas, one soluble and one lipid-bound. Our results coincide with their work in that some catalase appears in the supernatant[1],, while considerable activities stay bound to the membranes, but can be released by detergents. The catalase appearing in the supernatant was precipitated by all anti-liver membrane sera, giving[2]an identical reaction with the bound form. Thus, immunologically these two forms seem to be identical.

The liver of tumour-bearing animals gave exactly the same reaction with anti-liver sera as normal liver, showing no decrease in the ability to stain catalase of the immune precipitate. This is at first sight in contrast to the findings of others[37,38] but may depend on quantitative differences not measurable with our methods.

In our experimental system the catalase in hepatoma D23 was decreased in the microsomal fractions, but not in the plasma membranes, in comparison with normal liver. No explanation is at present available for this difference. It was interesting to find that erythrocyte and liver membranes had one catalase active antigen in common. This has earlier also been reported by Nishimura *et al.*[39], who studied purified catalase from rat liver and erythrocytes with anti-catalase sera and found what seemed to be one antigen common to both sources.

ACKNOWLEDGEMENTS

The authors want to thank Professor Peter Perlmann, head of the department, for stimulating discussions and advice during the course of the work. The excellent technical assistance of Miss I. Olsson is gratefully acknowledged. This work was supported by grant No. 113-K72-06XB from the Swedish Cancer Society.

REFERENCES

1 F. Blomberg and P. Perlmann, *Exp. Cell Res.*, 66 (1971) 104.
2 U. Lundkvist and P. Perlmann, *Immunology*, 13 (1967) 179.
3 M. Raftell and F. Blomberg, *Biochim. Biophys. Acta*, 291 (1973) 421.
4 J. R. Fouts, R. L. Dixon and R. W. Shultice, *Biochem. Pharmacol.*, 7 (1961) 265.
5 J. R. Fouts and R. H. Adamson, *Science*, 129 (1959) 897.
6 L. G. Hart, R. H. Adamson, H. P. Morris and J. R. Fouts, *J. Pharmacol. Exp. Ther.*, 149 (1965) 7.
7 A. H. Conney and J. J. Burns, in G. Weber, *Advance in Enzyme Regulation*, Vol. 1, Pergamon Press, Oxford, 1963 p. 189.
8 R. H. Adamson and J. R. Fouts, *Cancer Res.*, 21 (1961) 667.
9 F. Blomberg and M. Raftell, *Biochim. Biophys. Acta*, 291 (1973) 442.
10 P. Emmelot, C. J. Bos, E. L. Benedetti and P. H. Rümke, *Biochim. Biophys. Acta*, 90 (1964) 126.
11 P. Emmelot and C. J. Bos, *Biochim. Biophys. Acta*, 121 (1966) 434.
12 M. Raftell and S. Orrenius, *Biochim. Biophys. Acta*, 233 (1971) 358.
13 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 M. Wachstein and E. Meisel, *Am. J. Clin. Pathol.*, 27 (1957) 13.
15 J. M. Allen and R. J. Hunter, *J. Histochem. Cytochem.*, 8 (1960) 50.
16 K. Ozato and T. S. Okada, *Nature*, 197 (1963) 1310.
17 J. Uriel, *Ann. N.Y. Acad. Sci.*, 103 (1963) 956.
18 M. Raftell and P. Perlmann, *Exp. Cell Res.*, 57 (1969) 119.

19 M. Raftell and C. Powell, *Biochim. Biophys. Acta*, 216 (1970) 428.
20 G. Dallner, P. Siekevitz and G. E. Palade, *J. Cell Biol.*, 30 (1966) 97.
21 F. Blomberg and P. Perlmann, *Biochim. Biophys. Acta*, 233 (1971) 53.
22 S. E. Piazzi, *Anal. Biochem.*, 27 (1969) 281.
23 P. Emmelot and C. J. Bos, *Int. J. Cancer*, 4 (1969) 705.
24 L. Ernster and S. Orrenius, *Fed. Proc.*, 24 (1965) 1190.
25 G. C. Mueller and J. A. Miller, *J. Biol. Chem.*, 202 (1953) 579.
26 M. L. Das, S. Orrenius and L. Ernster, *Eur. J. Biochem.*, 4 (1968) 519.
27 F. D. Lotlikar, *Biochem. J.*, 118 (1970) 513.
28 L. A. Rogers, H. P. Morris and J. R. Fouts, *J. Pharmacol. Exp. Ther.*, 157 (1967) 227.
29 P. Emmelot and C. J. Bos, *Biochim. Biophys. Acta*, 120 (1966) 369.
30 D. F. H. Wallach and V. B. Kamat, in E. F. Neufeld and V. Ginsburg, *Methods in Enzymology*, Vol. 8, Academic Press, New York, 1966, p. 164.
31 L. Ernster and L. C. Jones, *J. Cell Biol.*, 15 (1962) 563.
32 U. Lundkvist and P. Perlmann, in H. Peeters, *Protides of the Biological Fluids, Proc. Colloq., Bruges*, 1967, Vol. 15, Elsevier Publ. Co., Amsterdam, 1968, p. 347.
33 A. Hildebrandt and R. W. Estabrook, *Arch. Biochem. Biophys.*, 143 (1971) 66.
34 P. Emmelot and E. L. Benedetti, in H. Peeters, *Protides of the Biological Fluids, Proc. Colloq., Bruges*, 1967, Elsevier Publ. Co., Amsterdam, 1968, p. 315.
35 N. Oshino, Y. Imai and R. Sato, *J. Biochem.*, 69 (1971) 155.
36 L. A. Holmes and C. D. Masters, *Arch. Biochem. Biophys.*, 109 (1965) 196.
37 S. Y. A. Davidova, V. S. Shapot and G. A. Drozdova, *Biochim. Biophys. Acta*, 220 (1970) 206.
38 T. Higashi, K. Kashiwagi and K. Warabioka, *Gan*, 59 (1968) 46.
39 E. T. Nishimura, S. N. Carson and T. Y. Kobara, *Arch. Biochem. Biophys.*, 108 (1964) 452.