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MEMBRANE FRACTIONS FROM RAT HEPATOMA

I. ISOLATION AND CHARACTERIZATION

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SUMMARY

1. A transplanted hepatoma (D23) originally induced by 4-dimethylaminoazobenzene, was fractionated into three different microsomal subfractions (rough tR, smooth tSa and tSb) and a plasma membrane fraction (tP).

2. On electron micrographs the tR fraction consisted of vesicles mostly covered with ribosomes, while tSa, tSb and tP vesicles were of the smooth type.

3. The highest activity of the plasma membrane marker 5′-nucleotidase (NMPase) was found in tSb and tP. The activity in the tumour was, however, low in relation to liver. No significant activity of glucose-6-phosphatase or succinate–cytochrome *c* reductase were detected in any of the membrane fractions.

4. A 10-fold increase in adenosine-diphosphatase (ADPase) activity was found in the tSb fraction as compared to all other tumour and liver fractions. A similarly elevated activity in tSb was found with UDP and ATP as substrates. This activity was strongly inhibited by freezing.

INTRODUCTION

Chemically induced tumours in rat have been shown to possess new tumour-specific antigens. It has also been shown that such tumours lose some of the antigens typical for the tissue of origin[1,2]. Each tumour seems to have individually distinct tumour antigens and cross-reactions between different tumours are usually not seen. The causes of acquirement or depletion of antigens in chemically induced tumours are not known, but most tumours have long latency periods suggesting that immunoselective processes may be operating. It has, however, been demonstrated that different carcinogenic compounds induce tumours with varying strength of immunogenicity. Polycyclic hydrocarbons, *i.e.* 3-methylcholanthrene, give liver tumours which are more immunogenic than others induced by 2-acetylaminofluorene[3]. What effect the various carcinogenic compounds exert on the depletion of antigens in tumours they have induced, is not known.

Earlier work with immunological methods combined with specific staining for various enzyme activities, has given a relatively detailed picture of the antigen pattern of normal adult and early postnatal rat liver and of the membrane subfractions iso-

Abbreviation: DMAB, 4-dimethylaminoazobenzene.

lated from them[4–7]. This material provides the reference for the present studies on antigens of rat liver tumours. The aim of this study was to characterize different membrane fractions from an established hepatocellular carcinoma, isolated from rat liver after induction by 4-dimethylaminoazobenzene (DMAB) diet and further propagated by transplantation. In this paper the tumour and the membrane fractions isolated from it will be described. Quantitative enzyme assays and electron microscopy demonstrate that the isolated fractions consist of different types of membranes. An immunological characterization of the tumour fractions will be given in two companion papers.

MATERIAL AND METHODS

A transplanted rat liver tumour, originally induced by 4-dimethylaminoazobenzene (DMAB) in an inbred Wistar rat strain, was obtained from professor Baldwin's laboratory (Nottingham, England, 1971). The solid tumour was classified histologically[1] as a hepatocellular carcinoma. The hepatoma, designated D23, was isolated in 1964 and maintained by serial passages in syngenic Wistar rats. The tumour was, however, never transferred for more than 25 passages, after which, early generations, stored at −70 °C, were withdrawn for re-implantation. In our laboratory the hepatoma has only been transplanted 5 passages, after which material from the original batch (kept at −196 °C) was re-implanted. The hepatoma was transferred to rats by subcutanous injections of mechanically suspended tumour cells. After 2–3 weeks tumours measuring about 2–3 cm in diameter were usually found at the inoculation site. Only non-necrotic material was used in the preparation.

Fractionation of liver and tumour tissue

For preparation of microsomes the tumour tissue, freed from connective tissue and necrotic material was thoroughly minced and homogenized mechanically (ultraturrax, type TP 18/2) for 30 s at 20000 rev./min. For the isolation of plasma membranes, the tumour tissue was homogenized by 7–10 strokes in a Teflon–glass homogenizer. All preparations were made in an ice bath. The isolation of the microsomal subfractions "rough" (tR) and "smooth" (tSa and tSb) from the tumour was carried out as earlier described for normal liver[4]. The tR fraction was sedimented in the presence of Cs^+ and the tSa fraction in the presence of Mg^{2+}. The tSb fraction consisted of membranes sedimented neither by Cs^+ nor Mg^{2+}. Plasma membranes from the tumour (tP) and liver plasma membranes (P) were prepared according to the methods described by Emmelot *et al.*[8,9]. Tumour and liver homogenates were designated tH and H, respectively. "Rough" (R) and "smooth" (Sa and Sb) membranes from adult rat liver were prepared as earlier reported[4]. Isolated membrane fractions from either liver or tumour were washed twice with saline. For enzyme assays the fractions were lightly homogenized in distilled water and for extraction of detergent-soluble antigens in 0.25 % sucrose. In the latter case the fractions were treated with 0.5 % Lubrol W (cetylpolyoxyethylene condensate, ICI, England) and 1 % sodium deoxycholate for 1 h and then centrifuged at 105000 × *g* in 60 min. The protein content was measured according to Lowry *et al.*[10], with bovine serum albumin as standard. Antiserum against fetal serum proteins was prepared as earlier described [11] and was designated a-2RS. Immunoelectrophoretic analysis was carried out as earlier described[7].

For electron microscopic studies the tumour material was fixed in 1 % OsO_4 and embedded in Epon[12]. The specimens were stained with uranyl[13] and lead[14]. Micrographs were taken in a Siemens Elmiskop I.

Enzyme assays

All enzyme assays were performed with membrane homogenates from tumour and liver fractions. Tests for NTPase (nucleoside triphosphatase, nucleoside triphosphate phosphohydrolase), NDPase (nucleoside diphosphatase, nucleoside diphosphate phosphohydrolase, EC 3.6.1.6) and NMPase (5'-nucleotidase, 5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) were performed in ATPase medium as described by Emmelot *et al.*[9]. The medium contained 100 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris buffer (pH 7.2) and the incubation volume was 1 ml. Up to 200 μg protein were incubated with 6 mM substrate (ATP, ADP, UDP or AMP) during 15 min at 37 °C. The reactions were stopped by the addition of 0.5 ml 15 % trichloroacetic acid. The samples were then centrifuged at 3000 $\times$ *g* for 30 min. Inorganic phosphate was determined by the method of Ames and Dubin[15].

Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed in 0.1 M Tris–maleate buffer (pH 6.6)[16] containing 10 mM glucose 6-phosphate. The analysis was then carried out as described above for NTPase. Succinate–cytochrome *c* reductase activity was determined according to Sottocasa *et al.*[17].

RESULTS

When sections of the hepatoma D23 were examined in the light microscope, the tumour cells showed unorganized growth in comparison with normal liver. The tumour cells were smaller than normal hepatocytes and differed in size within the tumour. The nuclei were of about the same size as in hepatocytes, but the cytoplasm was diminished. The electron micrographs showed poorly differentiated cells with small mitochondria and numerous free ribosomes. Cells with two nuclei were seen occasionally. Most cells contained a fairly well-developed endoplasmic reticulum with membranes of both rough and smooth type. Microvilli were seen on most of the exposed plasma membranes. No clearly visible desmosomes or junctions could be detected (Fig. 1).

Electron micrographs of the isolated tumour fractions indicated that different types of membranes were present in the preparations (Fig. 2). The tR fraction consisted of vesicles mostly covered with ribosomes, while the vesicles of the tSa and tSb fractions were of the smooth type. The tP fraction consisted of smooth membranes forming vesicles and sheets of various sizes.

In order to characterize the membrane fractions, a number of enzyme activities were assayed. The determinations were carried out on the third and fourth day after the start of the membrane preparations. NMPase and glucose-6-phosphatase were investigated primarily, as these enzymes are considered to be markers for plasma membranes and microsomes, respectively[11,19]. The glucose-6-phosphatase activity in our liver preparations was mostly found in the microsomes. The specific activity of glucose-6-phosphatase was about 30 times higher in microsomes (Sa) than in plasma membranes. In the tumour, however, this activity was hardly measurable in any of the fractions studied.

The activity of the plasma membrane marker, NMPase, tested with AMP as

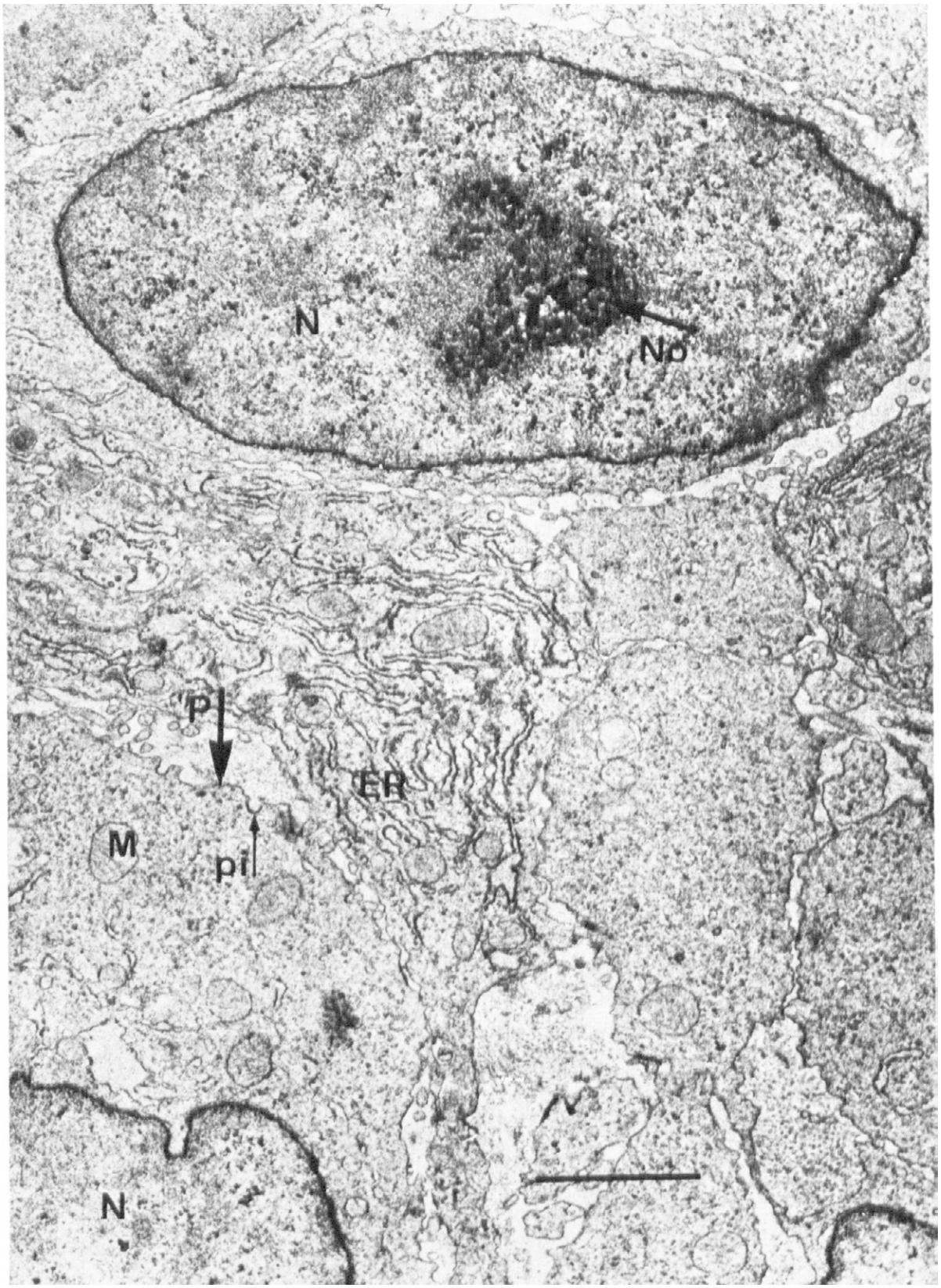

Fig. 1. Electron micrograph of a section from hepatoma D23. Poorly differentiated cells with little cytoplasm and polymorphic nuclei (N) and prominent nucleoli (No). Small mitochondria (M), free ribosomes and endoplasmic reticulum (ER), predominantly of the rough variety, can be seen. The plasma membranes appear mostly as microvilli and signs of pinocytotic activity is observed (pi). Plasma membrane criteria such as desmosomes and clearly visible junctions are absent. The bar equals 2 μm.

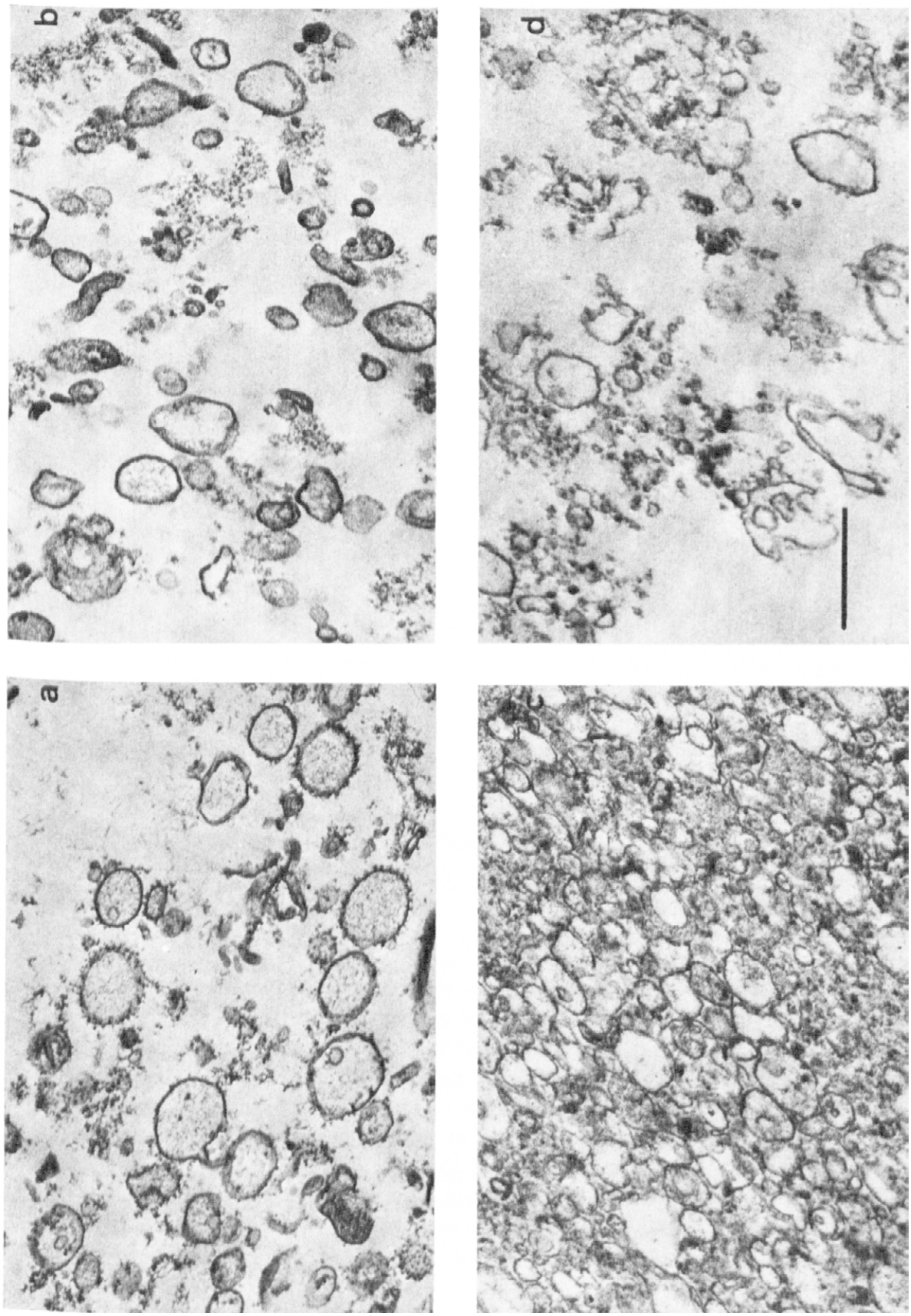

Fig. 2. Electron micrographs of different membrane fractions a ... tR, mostly consisting of ribosome-coated vesicles. b, c, d = tSa, tSb and tP, respectively. All these fractions consist of smooth microsomes. The bar corresponds to 0.5 μm.

substrate, is illustrated in Fig. 3. As expected, this marker was highly concentrated in the P fraction, while liver microsomes were considerably less active. The activities of these membranes were, however, somewhat elevated in comparison with liver homogenate.

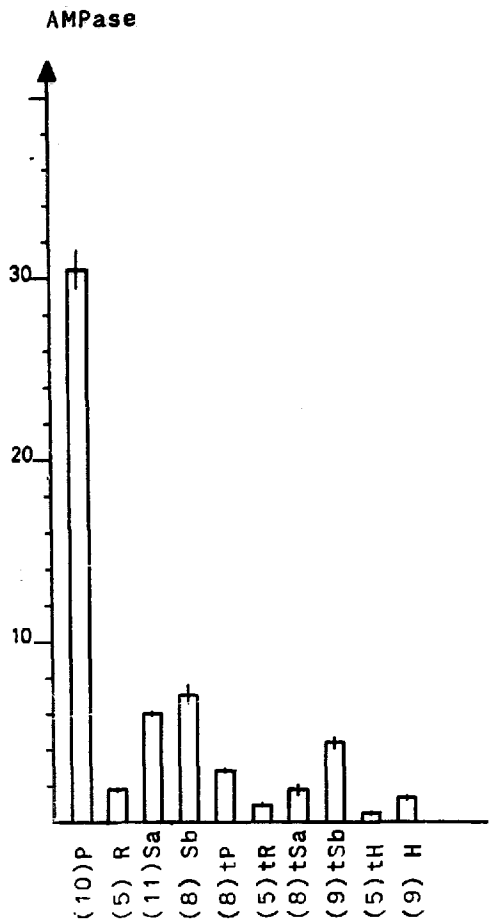

Fig. 3. AMPase activity (± S.E.) in the different membrane fractions from liver and hepatoma D23, respectively. H = homogenate, P = plasma membranes, R, Sa and Sb = different microsomal fractions. Corresponding fractions from the tumour are marked with t. Activities are given in μmoles P_i/mg protein per h. Three fractionations were made. Numbers of determinations are given in parentheses.

In the tumour homogenate, NMPase activity was low, reflecting decreased activities in all fractions studied. The highest activity was found in tSb, but also tP was more active than tR and tSa. At present there is no proper explanation for the relatively high NMPase in the tSb fraction. One obvious possibility may be that some plasma membrane component has been lost from the tumour plasma membranes and instead has become concentrated in tSb, or that the subcellular distribution of NMPase in hepatoma differs from that in rat liver.

Succinate–cytochrome *c* reductase, used as a marker for mitochondrial inner membranes[17] was also investigated in order to establish to what extent the tP fraction was contaminated with mitochondria. Electron micrographs showed only minor mitochondrial contamination, as clearly visible mitochondria were only seen occasionally. This was supported by the enzyme determinations, giving no measurable activities.

Two different substrates, ADP and UDP, respectively, were used for NDPase determinations, as liver microsomes had been shown to hydrolyze UDP selectively (refs. 20, 21). As shown in Fig. 4a the highest UDPase activity was found in R, while the other microsomal fractions and also P gave moderate activities.

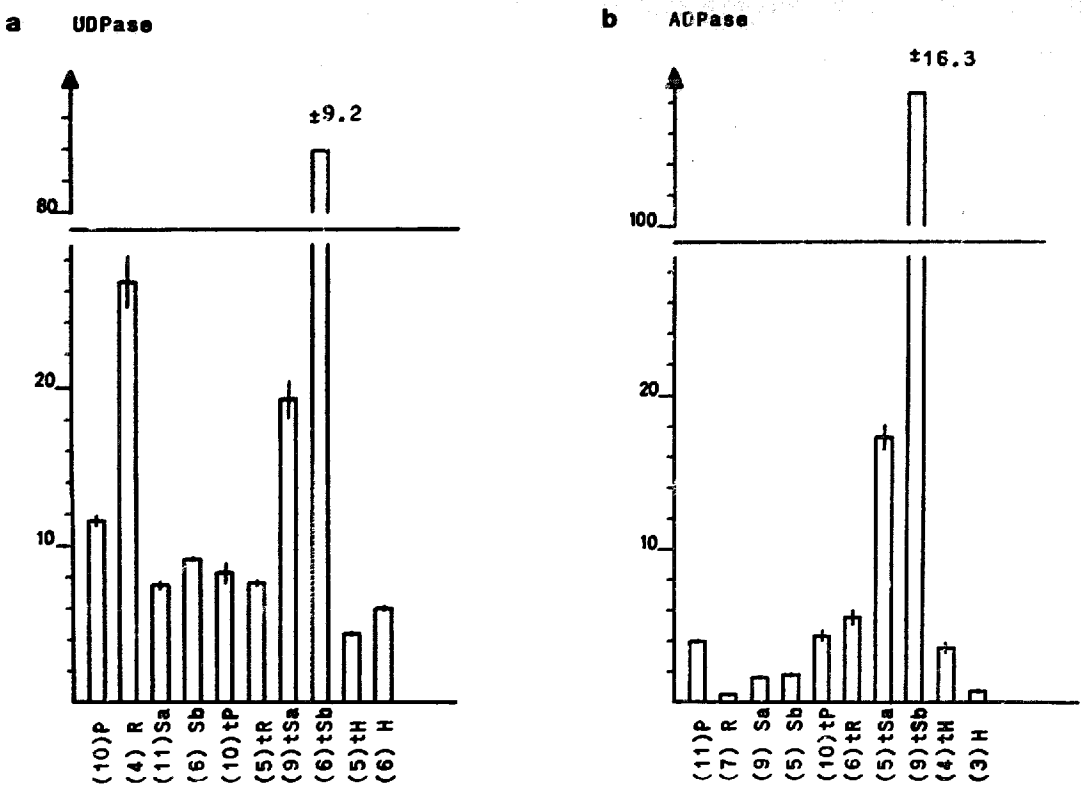

Fig. 4. NDPase determinations (± S.E.) with UDP and ADP as substrate (Figs a and b, respectively). For further explanations see legend to Fig. 3. Activities are given in μmoles P_i/mg protein per h.

Tumour homogenate showed a somewhat decreased activity in relation to liver homogenate. The pronounced UDPase activity of the R fraction was not recovered in tR. Instead tSb showed a strongly elevated activity; a 10-fold increase was found as compared to tR and tP. The relatively high activity found in tSa probably depends on a certain admixture of components present also in tSb.

In agreement with the substrate specificity of liver microsomes, the lowest ADPase activity was found in the R fraction (Fig. 4b). ADPase was about 2-fold enriched in P in comparison to Sa and Sb. The P and tP activities were almost equal, while ADPase of tumour microsomes was strongly increased, especially in tSb.

When the various tumour fractions were tested for Mg^{2+}-ATPase, a similar distribution as for ADPase was found, *i.e.* very high tSb activity. In order to distinguish different enzyme species with similar activities, an experiment was performed, where the membranes were kept frozen (−20 °C) for three days (day 3–7). The results are shown in Fig. 5. This procedure decreased the activities in all fractions with the exception of UDPase in liver microsomes and tP, where the activities were elevated. The largest decrease with both ADP or UDP used as substrate was seen in tSb. On the basis of these results it may be suggested that one enzyme or a group of enzymes is responsible for the extreme NDPase and NTPase activities found in tSb.

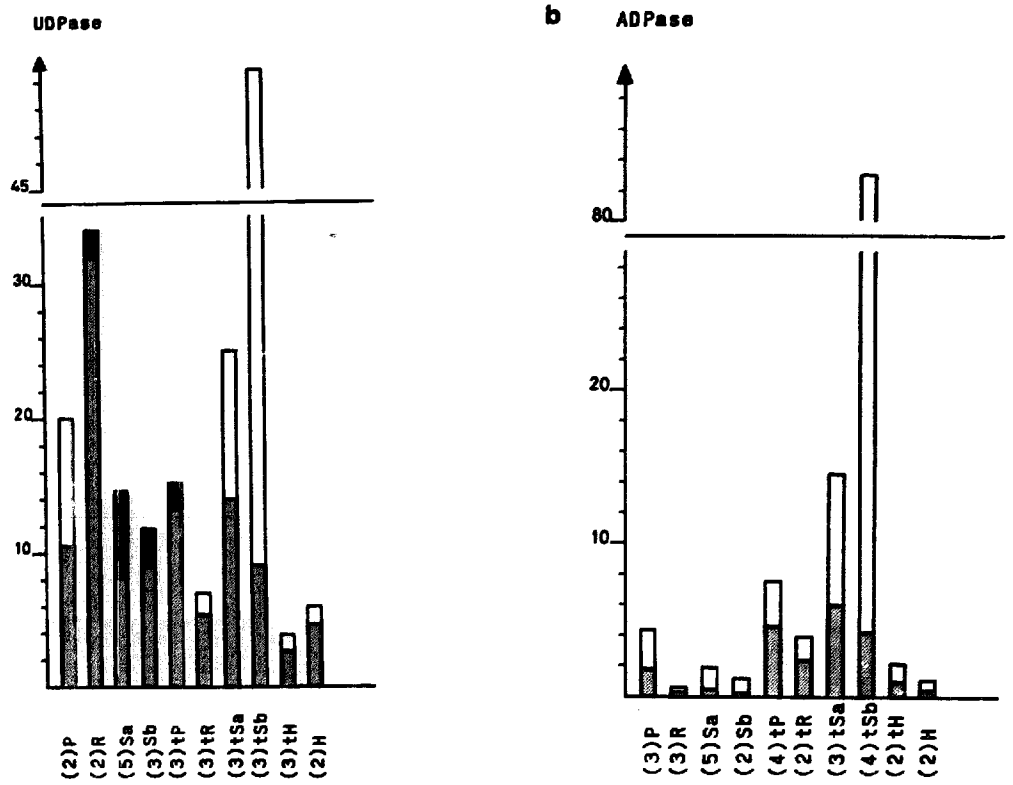

Fig. 5. NDPase activity (UDPase and ADPase) before and after freezing of the fractions (three days at −20 °C). Activities before freezing given in white stippling, decreased activities after freezing in shaded stippling and increased activities after freezing in black stippling. See further legend to Fig. 3. Numbers in parentheses give numbers of determinations. Activities are given in μmoles P_i/mg protein per h.

Fetal serum proteins

With an antiserum against serum from 2-day-old rats (a-2RS) absorbed with lyophilized adult rat serum powder, a fetal serum protein was found in the sera from animals bearing hepatoma D23. In immunoelectrophoresis the antigen was precipitated in the α_2 region. Serum from newborn animals contained two additional fetal serum proteins[11] which, however, could not be precipitated from the sera of tumour-bearing animals.

In order to study the site of origin of the fetal serum protein, detergent extracts of the hepatoma or the liver of tumour-bearing rats were reacted with a-2RS in immunodiffusion tests. However, neither from homogenates nor from microsomal fractions of these tissues could precipitable antigens be extracted. This probably indicates that the immunodiffusion methods were not sensitive enough to detect the small quantities of antigen present in the tissue producing it. It should be pointed out that the liver was perfused with ice-cold saline prior to preparation in order to remove all free serum. A more sensitive method like the radioimmunoassay for α-fetoprotein, described by Rouslahti and Seppälä[22], or immunofluorescence microscopy, would probably be more appropriate to detect the antigen.

DISCUSSION

The present paper deals with the isolation of different membrane fractions from a solid hepatocellular carinoma (D23). The technique used for preparation of plasma

membranes was that described by Emmelot *et al.*[8] for a solid rat hepatoma (484), while the isolation procedure for microsomal fractions has earlier only been used for rat liver.

The hepatoma fractions all had characteristic enzyme profiles indicating that membranes of different origin were obtained. The plasma membrane marker NMPase was strongly decreased in the tumour plasma membrane fraction. Emmelot and Bos[23] also found decreased NMPase activity in plasma membranes from hepatoma 484, although not as pronounced as in this study. It seems unlikely that a certain membrane compartment containing the NMPase was selectively lost during fractionation, since a marked decrease in activity was also found in the tumour homogenate.

Glucose-6-phosphatase used as marker for hepatocyte microsomes, was also strongly decreased in the hepatoma and could therefore not be used as marker for tumour microsomes. This is in line with findings of Emmelot and Bos[23].

Both liver and tumour plasma membranes contained significant amounts of ADPase activity in contrast to the results of Emmelot *et al.*[9,18]. The very strong ADPase activity found in tSb was reflected both in tSa and tH. When UDP was used as substrate instead of ADP, a similar distribution of activity was found in all tumour fractions. The freezing experiments demonstrated that such treatment inhibited most of the ADPase and UDPase activities in tSb, about half to the activities in tSa, while tR was only little affected. In tP and liver microsomes ADPase was decreased, but UDPase increased.

These results show that the hepatoma and liver plasma membranes were distinct from their microsomal counterparts, thus indicating a good separation. The similarity of tP and liver microsomes in regard to UDPase activity can at present not be explained.

The data presented here suggest that different enzyme species may be in part responsible for the NDPase activities in plasma membranes and microsomes from hepatoma and liver, respectively. This is in line with the immunological data, presented in the following papers[24,25]. Unfortunately, the yield of tSb was very low (30 g liver gave 5–10 mg Sb, while approx. 2 mg tSb was obtained from the same amount of hepatoma) and it has therefore not been possible to study this fraction immunologically in any detail. However, in preliminary experiments, in which tSb extracts were reacted with antisera against all the other fractions, no NDPase active precipitates appeared. This suggests that the NDPase which dominates in the tSb fraction, is not identical with any of the antibody-precipitable NDPases, detected in the other fractions[25]. An antiserum against tSb, presently in preparation, may be helpful for further characterization of the freeze-labile NDPase described in this paper.

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