STUDIES ON PLASMA MEMBRANES
IV. THE ULTRASTRUCTURAL LOCALIZATION AND CONTENT OF SIALIC ACID IN PLASMA MEMBRANES ISOLATED FROM RAT LIVER AND HEPATOMA

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SUMMARY
Plasma membranes were isolated from rat liver and a transplanted rat hepatoma of the hepatocellular type. After glutaraldehyde fixation the membranes were treated with colloidal iron hydroxide (CIH) at pH 1.7, which was found to react specifically with the neuraminidase-sensitive sialic acid of the liver membranes. The CIH-reactive, neuraminidase-sensitive sialic acid, comprising 70% of the membrane-bound sialic acid, was exclusively located in the outer leaflet of the liver membranes as shown by the rather regular distribution of electron-dense CIH granules. This granular, asymmetric type of staining was also observed in the hepatoma membranes, which contained some 50% more sialic acid than did the liver membranes. In addition, the hepatoma membranes showed an intense and uniform staining by CIH of short segments of both membrane leaflets; the latter type of staining was but little impaired by neuraminidase pre-treatment. None of the junctional complexes of the liver membranes was stained by CIH. Tight junctions were very rarely observed in the hepatoma membrane preparations, and the desmosomes and intermediate junctions of these membranes not infrequently exhibited a loosened appearance exposing CIH-reactive neuraminidase-sensitive sialic acid at their opposite plates. This aspect could be induced in the desmosomes and intermediate junctions, but not in the tight junctions, by pre-treatment of the liver membranes with the chelating agent ethylenediaminetetra-acetate.

INTRODUCTION
In all cases studied so far sialic acid (N-acetylneuraminic acid) has been found to be a constituent of the plasma membrane of mammalian cells. In red-cell and Ehrlich ascites carcinoma-cell membranes, sialic acid is O-glycosidically linked to N-acetylgalactosamine, which in turn is bound to protein either directly or via other carbohydrate moieties (Cook, 1962; Okhuma & Ikemoto, 1966; Langley & Ambrose, 1964, 1967). Sialic acid bound to glycosphingolipid (ganglioside) has also been considered as a possible constituent of the plasma membrane, especially of nervous tissue cells (Bogoch, 1958; Woolley & Gommi, 1964; McIlwain, 1963, 1964; Johnston & Roots, 1965). Gangliosides have been isolated from red-cell stroma (Kuhn & Wiegandt, 1964; Klenk & Padberg, 1962).

By treatment of whole cells with the enzyme neuraminidase, which splits the O-glycosidic bond between sialic acid and glycoprotein or lipid, it has been found that
sialic acid may contribute to the negative surface charge of cells, as shown by their electrophoretic mobility (reviewed by Ambrose, 1966; see also Ambrose, 1965). The latter contribution is, however, highly variable according to cell type. Although roughly similar quantities of sialic acid may be released by neuraminidase from different cells, the negative surface charge of only certain cells, such as blood and ascites tumor cells, is decreased (and to a variable extent at that), whereas with other cells, including liver cells and cells from solid sarcomas, no decrease at all occurs (Cook, Heard & Seaman, 1961; Eylar, Madoff, Brody & Oncley, 1962; Cook, Seaman & Weiss, 1963; Simon-Reuss, Cook, Seaman & Heard, 1964; Wallach & DePerez Esandi, 1964; Ambrose, 1965). Thus, one has been led to suppose (Wallach & DePerez Esandi, 1964) that sialic acid might occupy various positions in the membrane with respect to the hydrodynamic plane of shear, differing not only among various cell surfaces but also from one site on the cell surface to another.

Staining techniques have recently been developed for the electron-microscopic identification of acid mucopolysaccharides using colloidal iron or thorium hydroxide at low pH (Mowry, 1963; Revel, 1964; Ito, 1965; Wetzel, Wetzel & Spicer, 1966). The specificity of the reactions was established by the results obtained after pre-treatment of tissue samples with certain enzymes; selectivity of the method was ascertained by methylation of tissue carboxyl and sulphate groups. Gasic & Berwick (1963) demonstrated with this method the presence of sialic acid in the surface of intact ascites tumour cells. However, because of the huge precipitate resulting from the formation of Prussian blue with ferrocyanide, the plasma membranes in the latter experiments completely disappeared under the electron-dense material, and the precise location of sialic acid in the membrane structure could not be determined. By omitting the precipitation step with ferrocyanide, it is possible to obtain a more precise localization of acidic, carbohydrate-containing material (Revel, 1964; Lovell, Clark & Curran, 1966).

Isolated plasma membranes represent a more suitable material for studying the localization and function of sialic acid in membrane than do intact cells or thin tissue sections. Plasma membranes can be isolated from normal and neoplastic liver without being contaminated to any marked extent by other cellular organelles (Emmelot, Bos, Benedetti & Rümke, 1964; Emmelot & Benedetti, 1967). The isolated membranes allow the estimation of the total amount of sialic acid and of the neuraminidase-sensitive part. By definition, the latter determination is not complicated by the possible uptake of neuraminidase by whole cells and the subsequent release of intracellular sialic acid. Moreover, the extra- and intracellular sides of the plasma membranes isolated from solid tissues can easily be identified in the electron microscope on account of the presence of the junctional complexes between these membranes, which survive the isolation procedure. Since both sides of the plasma membranes can be exposed to similar experimental conditions in vitro, the ultrastructural localization of sialic acid in the isolated membranes can in principle be established. The results of such experiments on plasma membranes isolated from rat liver and transplants of the hepatocellular rat hepatoma 484 are reported in the present paper. A preliminary account of some of the results has been given (Benedetti & Emmelot, 1966).
MATERIALS AND METHODS

Livers were obtained from rats of the inbred strain R-Amsterdam. The hepatocellular rat hepatoma 484, originally induced in a female rat of this strain by 4-dimethylaminoazobenzene, was maintained by intraperitoneal transplantation in rats of the same strain.

Plasma membranes were isolated from rat liver, homogenized in 1 mM NaHCO₃ at pH 7.5, with or without addition of 2 mM CaCl₂, as described previously (Emmelot et al. 1964). When added, CaCl₂ was present in the homogenization medium only; all subsequent low-speed centrifugations were carried out in the dilute bicarbonate solution. The plasma membranes of the hepatoma transplants were always isolated from homogenates prepared in 1 mM bicarbonate containing 2 mM CaCl₂ for reasons previously described (Emmelot & Bos, 1966a).

The electron-microscopic experiments were carried out with freshly isolated membranes and membranes which had been pre-incubated: (a) in 50 mM sodium acetate buffer at pH 5.5 containing 0.15 M NaCl for 1 h at 37 °C, either with or without neuraminidase as indicated; (b) with 5 mM ethylenediaminetetra-acetate (EDTA), pH 6.0, for 30 min at 0 °C; (c) with EDTA as in (b), followed by neuraminidase as in (a); or (d) with 50 µg polylysine per mg membrane protein per ml physiological saline for 1 h at room temperature. In all cases except the last, the sialic acid content of the membranes and that of the supernatant following pre-incubation of the membranes was measured by the thiobarbituric acid method (Warren, 1963; see Table 1). The neuraminidase, obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A., was prepared from Clostridium perfringens. Two preparations were used routinely: neuraminidase preparation a (lot 33B-691, 1 mg of this preparation released 0.16 µmole neuraminic acid from N-acetylneuramin lactose at pH 5.1 per min at 37 °C), and neuraminidase preparation b (lot 86B-8510; 1 mg released 0.05 µmole neuraminic acid per min at 37 °C). Preparation a was used for most of the electron-microscopic experiments with liver membranes, 0.2 mg of the enzyme being added per 2.5-3.0 mg of membrane protein (Biuret) per ml medium (a) mentioned above. Preparation b (0.9 mg enzyme per 2.5-3.0 mg of membrane protein) was used in some of the experiments with liver membranes and all experiments with hepatoma membranes. In a few experiments (see Table 1) use was also made of neuraminidase preparations derived from Vibrio cholerae obtained from Koch–Light Laboratories, Colnbrook, England, and Serva Entwicklungslabor, Heidelberg, Germany; these preparations were incubated in the presence of 9 mM CaCl₂. The colloidal iron hydroxide (CIH) solution was prepared by adding 12 ml of a 32 % solution of FeCl₃ to 750 ml of boiling twice-distilled water; when used the solution had a pH of 1.7. The variously treated membranes were fixed with phosphate-buffered glutaraldehyde, pH 7.2, and carefully washed with the phosphate buffer. The pellet was twice resuspended in 5 % acetic acid (pH 2.5). The membrane pellet was then suspended in an aliquot of the CIH solution for 1 h at room temperature. For removal of nonspecific precipitate the membranes were subsequently washed 3 times with 5 % acetic acid. Finally the pellets were post-fixed in 1 % osmium tetroxide for 1 h and embedded.
RESULTS

Location of sialic acid in isolated plasma membranes

Liver membranes. The CIH solution which was used in the present experiments contained electron-dense particles with diameters ranging from 30 to 200 Å. Plasma membranes, isolated from liver homogenates prepared in bicarbonate medium with or without addition of CaCl₂, were fixed with glutaraldehyde and allowed to interact with the CIH solution, followed by postfixation and staining with uranyl acetate to give contrast to the membrane structure. Examination showed that the liver membranes were dotted with electron-dense granules similar in appearance to those present in the CIH solution (Figs. 1, 3). The attached granules were more or less uniformly distributed along the membranes and showed an average separation of about 200 Å. At places where the membranes had been cut tangentially, the granules were lying in the plane of the membrane. As shown by Fig. 2, a similar aspect was found for spread preparations of glutaraldehyde-fixed CIH-stained membranes which had not been subjected to the postfixation with osmium tetroxide, embedding and uranyl acetate staining.

The isolated liver membranes, which at high magnification show a triple-layered membrane element (Benedetti & Emmelot, 1966, 1967; Emmelot & Benedetti, 1967), consist of long sheets of plasma membranes of various adjacent cells interconnected by means of junctional complexes (Emmelot, Benedetti & Rümké, 1964). The tight junction (zonula occludens), intermediate junction (zonula adhaerens) and desmosome (macula adhaerens) are present, and not infrequently these junctions are found (Emmelot & Benedetti, 1967; compare Farquhar & Palade, 1963, 1965) in a fixed sequence along the isolated liver membranes (Fig. 1). In the present experiments none of the various types of membrane junction was stained by CIH (Figs. 1, 3). Since the membrane junctions serve as markers for the identification of the intra- and extracellular side of the membranes, it was possible to ascertain (Fig. 3) that the iron granules were confined to the extracellular side of the membrane corresponding to the outer leaflet of the triple-layered membrane element. Thus, the target of the iron granules appears to be asymmetrically located in the membrane element.

Effect of neuraminidase. That the CIH staining revealed sialic acid was demonstrated by the almost complete absence of the electron-dense granules from liver membranes which had been stripped of their sialic acid by the action of neuraminidase prior to glutaraldehyde fixation and CIH staining (Fig. 4). The two neuraminidase preparations, a and b, which were used for these experiments, yielded identical results. Liver membranes which had been pre-incubated for 1 h at 37 °C in buffer (pH 5.5) without neuraminidase and subjected to CIH staining, served as controls in the neuraminidase
Sialic acid in plasma membranes

experiments. These preparations exhibited the same pattern of staining as that observed for the freshly isolated membranes.

Effects of polylysine. Since the CIH staining results from ionogenic interaction between the positively charged iron hydroxide complex and the negatively charged carboxyl groups of sialic acid, experiments were carried out in which any acidic receptors present in the membranes were blocked by the strongly basic polypeptide polylysine. Incubation of the membranes with polylysine in physiological saline at neutral pH prevented the subsequent association of the electron-dense particles of the CIH solution with the membranes (Fig. 5). After incubation with polylysine, the membranes showed a distorted and loosely clumped appearance due to the formation of ‘cross-bridges’ of filamentous material between individual sheets. The membrane thickness was irregularly increased, probably as a result of the adherence of polylysine to both sides of the membrane element.

Effect of ethylenediaminetetra-acetate. By pre-treating the isolated liver membranes for 30 min with EDTA (5 mM) at pH 6·0 and 0 °C, the otherwise ‘closed’ and CIH-unreactive desmosomes were converted to an ‘open’ form in which the intercellular plug was loosened and electron-dense granules were present at the extracellular sides of the two opposing plates of each junction after staining with CIH (Figs. 6, 8). A similar aspect was noted for the intermediate junction (Figs. 6, 8, 9). The electron-dense granules were not present in these junctions after CIH staining of membranes which had been pre-treated with EDTA and subsequently with neuraminidase. In contrast, the tight junction remained unreactive towards CIH following pre-incubation of the membranes with EDTA (Fig. 7). EDTA did not change the CIH staining of the plasma membranes proper.

Hepatoma membranes. The cells of the transplanted rat hepatoma 484 show a markedly decreased mutual contact, and bile spaces are virtually absent (Emmelot & Benedetti, 1967). Consequently the isolated plasma membranes of this hepatoma (homogenized in bicarbonate medium containing CaCl₂) showed in general less structural complexity and coherence, forming less extended sheets than did the liver membranes. In the hepatoma membranes tight junctions were very rarely observed, but desmosomes and intermediate junctions were present. Not infrequently the latter junctions seemed to be less well cemented together, the intracellular plugs being less dense (‘swollen’) than in the case of the liver-membrane junctions, and occasionally absent.

The CIH staining of the isolated hepatoma membranes yielded also the granular, asymmetric type of staining (Figs. 10, 14) described above for the liver membranes. The spacing of the CIH granules in the hepatoma membrane outer leaflets was about the same as that of the liver membranes, but the size and form of the granules attached to the hepatoma membranes was sometimes more uneven than in the case of the liver membranes. In addition to this type of staining, another aspect consisting of an intense and uniform staining by CIH of both the outer and inner leaflets of short segments of the hepatoma membranes was obtained (Figs. 10, 11). This uniform, symmetric type of staining was never observed in fresh or EDTA-treated liver membranes. The granular, asymmetric and uniform, symmetric types of staining were present in the same hepatoma membrane sheets (Fig. 10). Treatment of the hepatoma
membranes with neuraminidase (preparation b) had a differential effect on the two types of CIH staining. It markedly reduced the granular-asymmetric type of staining, as in the case of the liver membranes, but had little effect on the short segments of the hepatoma membranes, which became uniformly stained in both leaflets (Figs. 15, 16). The intensity of the latter type of staining was but slightly reduced by neuraminidase pre-treatment as compared with control preparations subjected to buffer without enzyme.

The difference in morphological integrity of the desmosomes and intermediate junctions between untreated liver and hepatoma membranes, and the resemblance between the 'open' hepatoma-membrane junctions and those of liver membranes subjected to EDTA, were in general also expressed by the CIH-stainability of the various junctions. Whereas none of the membrane junctions of the fresh liver membranes was stained, not infrequently the desmosomes and intermediate junctions of the fresh hepatoma membranes were reactive towards CIH. As seen in Figs 12 and 13, the latter junctions, like those of liver membranes subjected to pre-treatment with EDTA, exhibited internal rows of electron-dense granules which were sensitive to (that is, absent after) neuraminidase pre-treatment. The rather regularly spaced location of the iron granules on the opposite plates of the open junctions represents a continuation of the similar aspect noted for the membrane element not involved in the junctional complexes, and reveals the presence of sialic acid in these junctions.

**Sialic acid content of liver and hepatoma membranes**

The sialic acid contents of rat-liver and rat-hepatoma membranes following hydrolysis for 1 h in 0.1 N H₂SO₄ at 80 °C are listed in Table 1. Extension of the hydrolysis for another hour increased the yield of sialic acid from the liver membranes by 17 %. At least 95 % of the sialic acid could be recovered in the protein fraction of the liver membranes and no evidence for the presence of gangliosides in these membranes has been obtained (R. P. van Hoeven & P. Emmelot, unpublished observations).* The isolated liver and hepatoma membranes contain a certain proportion (18—33 %) of protein that is readily soluble in physiological saline (Emmelot et al. 1964; Emmelot & Benedetti, 1967). It has been shown that at least part of these proteins are derived from the cytoplasm (Emmelot & Bos, 1966a; Benedetti & Emmelot, 1967; Emmelot & Benedetti, 1967). However, all the sialic acid of the liver membranes was present in the saline-insoluble membrane portion (Emmelot et al. 1964). Thus, if it is assumed that on average 25 % of the total membrane protein is of non-membrane origin, the sialic acid content of the ‘chemically pure’ plasma membranes is 25 % higher than that listed in Table 1.

Table 1 shows that the sialic acid content of the rat-hepatoma membranes per unit weight of protein was some 50 % higher than that of the rat-liver membranes; the same increase is obtained when the data are expressed per µmole of phospholipid-P, since the protein:phospholipid-P ratios of the 2 types of membrane have been found to be similar (Emmelot & Benedetti, 1967). Trypsin (0.025 %, 1 h at 37 °C in

* Recently traces of what appear to be gangliosides have been detected by thin-layer chromatography.
phosphate buffer at pH 8.5) released 40–50% of the sialic acid from rat-liver and hepatoma membranes as sialopeptide, as shown after hydrolysis of the membrane supernatant and the residual membranes.

The sialic acid content of the various plasma membranes has been determined in both freshly isolated membranes and membranes pre-incubated for 1 h at pH 5.5 and 37 °C in the acetate buffer used in the neuraminidase experiments. Pre-incubation of the membranes with this buffer (without neuraminidase) did not release the membrane sialic acid. Neither has pre-treatment of the rat-liver membranes with EDTA, under the conditions used for the electron-microscopic experiments, been found to decrease the sialic acid (or hexosamine) content of the membranes. As shown in Table 1, 70% of the sialic acid was released from rat-liver membranes by neuraminidase preparation a. When used in a similar concentration of 0.2 mg enzyme per 2.5–3.0 mg of membrane protein, neuraminidase preparation b released less than 50% of the sialic acid. However, 0.9 mg of neuraminidase preparation b per 2.5–3.0 mg of membrane protein released 65–70% of the sialic acid from the rat-liver membranes. This amount was identical to that released by preparation a and by

<table>
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<tr>
<th>Membrane source</th>
<th>Homogenization medium</th>
<th>m×moles sialic acid/mg protein</th>
<th>Preparation used</th>
<th>Sialic acid released (%)</th>
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<tr>
<td>Rat liver</td>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>33 ±2 (26–36; 10)</td>
<td>a</td>
<td>60 ±3 (6)</td>
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<td></td>
<td></td>
<td>32 ±1 (30–33; 3)</td>
<td>b</td>
<td>68 ±3 (3)</td>
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<td>32</td>
<td>K-L</td>
<td>68</td>
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<td></td>
<td></td>
<td>33</td>
<td>S</td>
<td>67 [48]</td>
</tr>
<tr>
<td>Rat hepatoma 484</td>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt; + CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>30 ±2 (26–32; 5)</td>
<td>a</td>
<td>72 ±1 (3)</td>
</tr>
<tr>
<td></td>
<td>NaHCO&lt;sub&gt;3&lt;/sub&gt; + CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>44 ±1 (41–48; 4)</td>
<td>a</td>
<td>50, 56 (2)</td>
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<tr>
<td></td>
<td></td>
<td>47 ±3 (42–51; 3)</td>
<td>b</td>
<td>75 ±3 (3)</td>
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Table 1. Content of total and neuraminidase-sensitive sialic acid of plasma membranes isolated from rat liver and hepatoma

Total (and neuraminidase-insensitive) sialic acid was measured after 1 h hydrolysis of the membranes in 0.1 N H<sub>2</sub>SO<sub>4</sub> at 80 °C using the thiobarbituric acid procedure. Neuraminidase-sensitive sialic acid was measured by the latter method as free sialic acid, following incubation of membranes for 1 h at 37 °C with neuraminidase preparation a (0.2 mg enzyme/2.5–3.0 mg membrane protein), preparation b (0.9 mg enzyme/2.5–3.0 mg membrane protein; figure in square brackets shows the percentage of sialic acid released by a suboptimal amount of 0.2 mg of this enzyme), a preparation from Koch–Light (abbreviated as K–L; 0.2 ml preparation/2.4 mg membrane protein), or a preparation from Serva (abbreviated as S; 0.4 ml preparation/2.4 mg membrane protein and, in square brackets, the percentage of sialic acid released by 0.25 ml of this preparation) per ml medium (see Materials and Methods). In the experiments with the latter two preparations 9 mM CaCl<sub>2</sub> was present in the medium. After incubation the membranes were spun down at 1500 g for 15 min and washed once with twice-distilled water. Sialic acid was measured in the combined supernatants and in the hydrolysed membranes. Listed in the Table are the mean values with standard deviation and, in round brackets, the range of values and/or number of experiments.
optimal amounts of two other commercially obtained neuraminidase preparations (see Table 1) which had been derived from *V. cholerae* and which, in contrast to the enzyme preparations *a* and *b* originating from *Clostridium perfringens*, needed to be activated by addition of calcium ions. At least as much sialic acid was released from the hepatoma as from the liver membranes with neuraminidase preparation *b*; two earlier experiments (Emmelot & Benedetti, 1967) with preparation *a* had yielded lower values.

For each type of membrane preparation and neuraminidase used, the sum of the free sialic acid released by enzymic action and that retained in the membrane pellet has been found to be equal to the sialic acid content of the membranes not treated with enzyme. These results showed that, regardless of the neuraminidase sample and its calcium requirement, a maximum of about 70% of the sialic acid could be split off from the rat-liver membranes. Thus the question of the nature of the neuraminidase-insensitive part of the sialic acid arises. Any sialic acid not released by enzyme action must either involve a chemical linkage that is not broken by neuraminidase (such forms are known for gangliosides; Svennerholm, 1963a; Kuhn & Wiegandt, 1963), or be spatially inaccessible to the enzyme. Since the electron-microscopic observations had shown that pre-treatment of the liver membranes with EDTA exposed the otherwise ‘masked’ sialic acid in the desmosomes and intermediate junctions, some experiments were carried out in which the effect of EDTA pre-treatment on the amount of sialic acid subsequently released from the membranes by neuraminidase was studied. This type of experiment was feasible because of the calcium-ion independence of the *Clostridium* neuraminidase. However, pre-treatment of liver membranes with EDTA did not increase the amount of neuraminidase-sensitive sialic acid released from the membranes (controls 70% of the sialic acid released; after EDTA, 63% and 68% released in two experiments). This result may suggest that, although the sialic acid in the desmosomes and intermediate junctions of fresh membranes is not available for staining with CIH, it may still be released by neuraminidase. The type of binding and the location of the neuraminidase-insensitive sialic acid are as yet unknown.

**DISCUSSION**

There is a good deal of evidence that a glycoprotein or polysaccharide-rich layer, which may contain acidic components, exists on the outside of the plasma membranes of many, if not all, cell types (Brandt, 1962; Gasic & Berwick, 1963; Fawcett, 1965; Ito, 1965; Rambourg, Neutra & Leblond, 1966; Lovell et al. 1966). This extraneous coat, which has been termed ‘glycocalyx’ by Bennett (1963), may vary in composition, form and resistance to lytic agents, according to cell type. The carbohydrate-rich ‘cell coat’ of rat liver has recently been demonstrated by Rambourg et al. (1966) during a survey of some fifty rat tissues. The present observations confirm and extend this concept in respect to the glycoprotein-bound sialic acid of the plasma membranes isolated from rat liver. However, it cannot be excluded that material of the carbohydrate-rich coat of the liver cells, not resistant to the hypotonic conditions of isolating and the handling of the membranes, may have been lost in our experiments.
Liver plasma membranes

According to our results, CIH at pH 1.7 specifically stained the neuraminidase-sensitive sialic acid of the isolated rat-liver plasma membranes. This conclusion is valid on the reasonable assumption (Emmelot & Bos, 1966b) that the two neuraminidase preparations used in these experiments were not contaminated by enzymes which might split from the membranes other potential CIH receptors bearing negative charge at low pH. The CIH staining was performed on glutaraldehyde-fixed membranes but it is very unlikely that glutaraldehyde blocked negatively charged groups of the membranes. Furthermore, it was shown that the post-fixation with OsO₄ and the uranyl acetate staining did not affect the pattern of interaction of CIH with the membranes. The specificity of the CIH staining therefore indicates that among the acidic groups of the liver membranes only the carboxyl group of sialic acid is dissociated at low pH and capable of interacting with the positively charged iron complex. The neuraminidase-sensitive sialic acid of free liver cells (Granner, Fuhrmann & Ruhennstroth-Bauer, 1964; Baba, Ishii, Arai & Aoki, 1966) and of isolated liver plasma membranes (R. P. van Hoeven & P. Emmelot, unpublished results) consists predominantly of N-acetylneuraminic acid, and the latter compound is a relatively strong acid (pKa = 2.6; Svennerholm, 1963b). It follows that other acidic groups which are dissociated at low pH (such as carbohydrate sulphate), are absent from the liver membranes or inaccessible to CIH, and that no acid groups capable of dissociation at pH 1.7 are unmasked by the enzymic removal of sialic acid. The latter hypothesis (without stating the acidity of the unmasked group) has been advanced previously (Wallach & DePerez Esandi, 1964) as one of the possibilities that might explain the apparent failure of neuraminidase pre-treatment to decrease the negative surface charge of (liver) cells as measured by electrophoretic mobility (Doljanski & Eisenberg, 1965). Another explanation for this phenomenon could be that sialic acid is located more than 10 Å below the hydrodynamic plane of shear of these plasma membranes. For obvious reasons the CIH staining procedure does not allow one to check this possibility. However, it could be shown by the present experiments that the neuraminidase-sensitive sialic acid of the liver membranes is asymmetrically located in the membrane element, being present exclusively in the membrane outer leaflet in a rather regular spacing. Since the sialic acid is bound to membrane protein, it appears most likely that the CIH-reactive, neuraminidase-sensitive sialic acid, which represents 70% of the sialic acid present in the liver membranes, is O-glycosidically linked to glycoprotein of the membrane outer leaflet. Since pre-treatment with EDTA does not change the granular, asymmetric staining of the plasma-membrane element by CIH, whereas previous experiments have shown (Benedetti & Emmelot, 1966, 1967; Emmelot & Benedetti, 1967) that EDTA may change the triple-layered appearance of the plasma membrane element to a globular one, it follows that marked rearrangement of the sialic acid-containing glycoprotein of the outer leaflet by EDTA either does not occur or lies below the sensitivity of the CIH-staining method.

This location of sialic acid in the membrane outer leaflet furnishes another demon-
stration of the asymmetric architecture of the plasma membrane element. Previous instances of this kind, obtained with intact and isolated plasma membranes of various cells, include the differential effect of fixing and staining agents on the membrane outer and inner leaflets (Farquhar & Palade, 1963; Sjöstrand, 1963; Doggenweiler & Frenk, 1965), the asymmetric distribution of the sodium-pump reactions (Whittam & Ager, 1964; Kaye, Cole & Donn, 1965), ionogenic groups (Wallach, Kamat & Gail, 1966) and surface antigens (Emmelot & Benedetti, 1967; Benedetti & Emmelot, 1967). The asymmetry of the plasma membrane most certainly has some functional significance. This may apply especially to the case of sialic acid, since the plasma membrane appears to contain the highest amount of sialic acid per mg membrane protein (Table 1), as compared with the other liver-cell organelles (Li, Li & Shetlar, 1965; Patterson & Touster, 1962). Although sialic acid has been invoked in a number of plasma-membrane processes of cells other than those from liver (Cohen, 1963; Woolley & Gommi, 1964; Glick & Githens, 1965; Glick, Goldberg & Pardee, 1966), its function in the liver plasma membrane is not clearly established. The previous observation by Emmelot & Bos (1966) that neuraminidase (preparation a) abolished the K⁺-activated p-nitrophenylphosphatase of liver plasma membranes, which might be involved in K⁺-ion transport, could not be confirmed when in recent experiments neuraminidase preparation b was used. Moreover, the problem of the relation between the location and function of sialic acid is a complex one, in view of the varieties that have been found in (i) the contribution of sialic acid to the negative surface charge of different cells, and (ii) the location of sialic acid, as shown by the present experiments with the hepatoma plasma membranes.

Hepatoma plasma membranes

The two conclusions which have been drawn from the observations on the CIH staining of the liver membranes, namely, that CIH does interact specifically with the neuraminidase-sensitive sialic acid, and that this sialic acid is exclusively present in the membrane outer leaflet, cannot be applied without restriction to the hepatoma membranes. These two conclusions are valid only for the granular, asymmetric type of CIH staining that the hepatoma membranes share with the liver membranes. The uniform symmetric type of staining that is limited to the hepatoma membranes seems to be due only in small part to neuraminidase-sensitive sialic acid, and there is not sufficient evidence to establish whether the neuraminidase-insensitive portion of the staining reflects interaction of CIH (i) with sialic acid that is poorly accessible or resistant to neuraminidase, and/or (2) with other acidic groups which are dissociated at low pH. However that may be, the results do point to an important difference in the chemical structure of the rat-hepatoma and rat-liver membranes; the former membranes contain also some 50 % more sialic acid than do the latter. Many other examples of diversity between the rat-liver and hepatoma membranes, observed in the course of our investigations, have recently been summarized (Emmelot & Benedetti, 1967), but in the present connexion the difference between the two types of membranes in respect to the reactivity of their desmosomal and intermediate junctions towards CIH merits attention.
Sialic acid in plasma membranes

Plasma membrane junctions

In the hepatoma membranes CIH-reactive sialic acid sensitive to neuraminidase could be observed on the opposite plates of desmosomes and intermediate junctions, while the intercellular plugs of the junctions frequently had a loosened appearance. This aspect of the junctions was not observed in liver membranes but could be induced by removing divalent cations (very probably Ca\(^{2+}\)) through pre-incubation of the membranes with EDTA. The open and CIH-reactive aspect of the hepatoma-membrane junctions was exhibited in spite of the fact that calcium ions had been present in the medium used for homogenization of the hepatoma tissue. These observations point to an irreversible change or at least to a greater lability on the part of many of the junctional complexes of the hepatoma as compared with those of the liver membranes. To what extent the in vitro handling of the membranes may have contributed to the manifestation of this difference cannot be stated. However, since the hepatoma tissue showed a decreased cell contact in situ (Emmelot et al. 1964; Emmelot & Benedetti, 1967) and tight junctions were virtually absent, the open aspect of the other junctional complexes may reflect a tendency of these tumour cells to escape from mutual contact. Intermediate junctions and desmosomes are generally considered to act as mere attachment devices between cells (Farquhar & Palade, 1965), whereas the tight junctions, by being highly permeable, may allow free exchange of molecules between the cells (Loewenstein, 1966; Penn, 1966). The virtual absence of tight junctions between the hepatoma plasma membranes may indicate that intercellular diffusion is lost in this kind of tumour, and this has actually been found to be the case (Loewenstein, 1966; Loewenstein & Kanno, 1966).

Chelating agents such as EDTA are capable of dissociating single cells from solid tissues, including the liver. The structural uncoupling of the desmosomes and intermediate junctions by EDTA, described in the present paper for isolated liver membranes, confirms a similar effect observed by Sedar & Forte (1964) for the oxytomic cells of frog gastric mucosa. The internal rows of CIH granules located on the opposite plates of the open junctions of EDTA-treated liver membranes represent a continuation of the similar aspect of the membrane outer leaflet at places other than those involved in the junctional complexes. This implies that when an intermediary or desmosomal junction between opposite liver membranes is formed in situ, the sialic acid becomes masked but remains present. In the intact junction the sialic acid carboxyl groups may be either spatially or chemically masked, and thus inaccessible to CIH. In the first case the voluminous CIH 'crystals' would not be capable of penetrating a junction cemented together by calcium ions. Secondly, the sialic acid carboxyl groups might themselves be firmly bound by way of calcium ions to other anionogenic groups (perhaps extending from the intercellular plug); in this case sialic acid would be involved in the mechanism that keeps the junction intact. In view of the lack of CIH staining of the EDTA plus neuraminidase-treated junctions, the partner anionogenic groups participating in this mechanism must be less acidic than are the sialic acid carboxyls, being not dissociated at pH 1-7. It cannot, however, be excluded that a soluble compound of acidic character may participate in this bonding.
Although the sialic acid carboxyl is masked in the intact junctions, sialo-glycoprotein might still be demonstrable; Rambourg & LeBlond (1967) have recently observed periodic acid/silver methenamine-positive material in the desmosomes of epithelial cells.

No sialic acid could be demonstrated by the CIH staining method in the tight junctions, and since Rambourg & LeBlond (1967) have shown that periodic acid/silver methenamine-reactive carbohydrate is absent from the tight junction in epithelium, it may tentatively be concluded that sialic acid is also missing; hence it might follow that the carbohydrates are removed from the membrane outer leaflets when the latter fuse to form a tight junction. Apart from the suggested absence of carbohydrate, the intermediate (fusion) line of the tight junction has recently been found (Benedetti & Emmelot, 1967) to differ from the free plasma membrane outer leaflet by its lack of preservation after fixation by glutaraldehyde only. Perhaps this particular molecular and structural organization of the intermediate line of the tight junction relates to the much higher permeability of the latter as compared with that of the plasma membrane proper.

In agreement with the results of Sedar & Forte (1964) on frog gastric mucosa, EDTA in the present experiments with isolated liver plasma membranes did not change the morphological continuity of the tight junction, which among the various types of junctions appears to be the one most resistant to mechanical strain (Farquhar & Palade, 1963, 1965). However, preliminary experiments (Benedetti & Emmelot, 1967) have revealed ultrastructural changes in the intermediate line of the tight junction after pre-treatment with EDTA, which might (i) be related to a decreased mechanical stability of the tight junction (and thus explain the dissociative effect of EDTA on liver tissue), and (ii) be related to the irreversible ‘uncoupling’ of the intercellular diffusion brought about by EDTA (Loewenstein, 1966).

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REFERENCES


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Fig. 1. Isolated rat-liver plasma membranes stained with colloidal iron hydroxide (CIH). Low-magnification micrograph showing the electron-dense granules attached to the membranes, but not to the junctional complexes (indicated by brackets).

Fig. 2. Isolated rat-liver plasma membranes fixed with glutaraldehyde, stained with CIH, and spread on carbon-coated grids. Electron-dense granules varying in diameter between 30 and 200 Å are scattered on the membrane sheet.

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Fig. 3. Isolated rat-liver plasma membranes stained with CIH. The electron-dense granules are restricted to the outer leaflet of the membranes (inset). Junctional complexes (brackets) are not stained.

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Fig. 4. Isolated rat-liver plasma membranes pre-treated with neuraminidase and stained with CIH. Electron-dense particles are absent.

Fig. 5. Isolated rat-liver plasma membranes pre-treated with polylysine and stained with CIH. The electron-dense granulation is practically absent. The membrane sheets are folded and clumped, and filamentous material is attached to the membrane element (arrows). Note the swelling of some areas of the tight junction (brackets).

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Fig. 6. Isolated rat-liver plasma membranes pretreated with EDTA and stained with CIH. The electron-dense granules are located in the outer leaflet of the membranes, including now also those participating in the desmosomal and intermediate junctions.

Fig. 7. A high-magnification micrograph showing that the tight junction is not stained (see also Fig. 6, top left).
Fig. 8. Isolated rat-liver plasma membranes treated with EDTA and stained with CIH. The electron-dense granules are located on the outer leaflet of the membrane element and are now also present in the 'open' desmosomes and intermediate junctions (brackets).

Fig. 9. An intermediate junction is shown after staining with uranyl acetate, without the use of colloidal iron hydroxide; note the decreased density of the intracellular electron-opaque material as a result of the EDTA treatment.
Figs. 10, 11. Isolated rat-hepatoma plasma membranes stained with CIH. Note the presence of the granular, asymmetric type of staining and the intense and uniform staining of both the outer and inner leaflet of certain membrane segments.

Figs. 12, 13. Isolated hepatoma membranes stained with CIH. Electron-dense granules are found scattered on the plasma membranes making up the intermediate junction. The granules in the latter situation are apparently smaller than those lining the membrane outer leaflet not involved in the junctional complex.
Fig. 14. Isolated rat-hepatoma plasma membranes stained with CIH. The electron-dense granules are regularly located at the outer leaflet of the membrane element. The desmosome is not stained.

Fig. 15. Isolated hepatoma membranes pre-incubated with neuraminidase and stained with CIH. The granular, asymmetric type of staining is significantly reduced.

Fig. 16. Segment of the hepatoma membrane element that is still stained by CIH in both leaflets after neuraminidase pre-treatment.
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