Larger uncertainties, perhaps of the order of 0.1 Å, should be expected for the hydrogen atom positions.

Several structural features of the bilirubin bis anion do not differ substantially from those of the free acid in the crystal.\(^2\) Thus, the ridge tile shape is confirmed with a dihedral angle of 98° between the two oxypyrimethylen moieties which are both planar in a cisoid arrangement (syn-Z configuration). Values of bond lengths (Fig. 3) indicate an essentially double and single bond order for C4-C5 (and C15-C16) and C5-C6 (and C14-C15) respectively. Bond distances and bond angles are in good agreement with those determined in a single oxypyrimethylen compound.\(^2\) In the chloroform molecule and in the isopropylylammonium ion values of bond lengths and angles are also in the normal range.

The carbon-oxygen bond lengths in the carbonyl group are approximately equal, corresponding to a carboxylate form. The nature of lactim-lactam tautomerism in bilirubin is controversial.\(^3,^4\) In this study the values of carbon-nitrogen and carbon-oxygen bond lengths in the terminal rings, the experimental evidence in these rings of a hydrogen bond to the nitrogen atom, and the large value observed for the C4-C5-C6 bond angle—as a consequence of H···H repulsion in the oxypyrimethylen portion (see Fig. 3) allow the lactam form to be assigned.

**Fig. 3** The oxypyrimethylen moiety in the bilirubin-isopropyllamine-chloroform complex.

The hydrogen bond pattern is of particular relevance (see Fig. 2). In contrast to bilirubin,\(^5,^6\) in the present complex the conformation of the pigment molecule is stabilized by only two pairs of NH···O intramolecular bonds, each pair involving one carboxylate oxygen. Each of the remaining carboxylate oxygens is engaged not only in strong hydrogen bonds with two isopropylylammonium ions but also in a weak CH···O hydrogen bond with chloroform. The shortest H bond of the complex involves the lactam oxygen atom and the third hydrogen of the isopropylylammonium ion. Thus the structure of the di-isopropylylammonium bilirubinate might be viewed as a model for bilirubin-protein associations.

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**Protein transfer across microsomal membranes reassembled from separated membrane components**

The synthesis of secretory proteins is initiated on ribosomes in the cell cytoplasm and the first part of the polypeptide chain to appear is a short sequence termed the signal sequence. This signal is thought to direct the ribosomal complex to the endoplasmic reticulum (ER) membrane where the synthesis of the rest of the secretory protein is tightly coupled to its transfer across the membrane into the cisternal space. In all but one case, it is clear that the polypeptide chain is processed during transfer to remove the signal sequence. Little is known about the membrane proteins involved in conveying the growing polypeptide chain across the membrane. A classical approach to learning more about these proteins would be to dissect the microsomal membrane into inactive components that can be reassembled subsequently into a functional entity. This would allow the purification of components involved in protein transfer. Here we report the first such reassembly.

Messenger RNA coding for light-chain immunoglobulin (Ig) was translated in a wheat germ cell-free system in the presence of RNase-treated rough microsomes (RMa) from canine pancreas. The authentic Ig light chain (Li) was synthesised, together with a small amount of the light-chain precursor (p-Li) containing the signal peptide (Fig. 1, track 1). The authentic light chain was resistant to added proteases and its precursor was completely degraded (Fig. 1, track 2). Since added proteases degrade all but those proteins inside the vesicles, the transfer of the light-chain precursor into the vesicles is tightly coupled to the removal of the signal peptide. In the absence of membranes, no authentic light chain was synthesised (Fig. 1, track 3) and the precursor was completely degraded by added proteases (Fig. 1, track 4). The precursor synthesised in the presence of RMa membranes was < 10% of the total Ig Li and could be increased by limiting the amount of membrane, and hence the number of sites, available for protein transfer. In all the experiments described the total membrane surface area was adjusted (using the phospholipid content of the membrane) to the same level, which, for RMa membranes, was sufficient to transfer and process about 90% of the total Ig Li synthesised.

When RMa membranes were washed with 0.5 M KCl, the resulting RMa membranes had almost lost the capacity to transfer and process the Ig Li. Precursor was synthesised together with only a small amount of the authentic light chain (Fig. 1, track 5), and it was completely degraded by added proteases (Fig. 1, track 6). The small amount of authentic light...
The light chain was protected against digestion by protease and represented the residual transfer activity of the RM<sub>KK</sub> membranes. Protein transfer and processing could be fully restored to RM<sub>KK</sub> membranes by readdition of the salt extract (SE); the authentic light chain was synthesised together with some proteases (Fig. 1, track 7) but only the former was resistant to added proteases (Fig. 1, track 8). Treatment with high salt thus removes, reversibly, membrane components essential for protein transfer and processing.

The active components in the SE were characterised using a rapid, quantitative assay (see legend to Table 1) devised to facilitate their eventual purification. The assay determines the percentage of total protein synthesised that is resistant to added proteases because of transfer into vesicles. We used the same pancreatic mRNA in this assay since it codes mainly for secretory proteins and 40-55% of the protein synthesised is resistant to protease digestion. At least 80% of the pancreatic mRNA used in these experiments codes for secretory proteins (unpublished observations), so this level of resistance suggests that a fraction of the microsomal vesicles, containing newly-synthesised secretory proteins, are leaky to added proteases.

Fig. 1 Effect of KCl treatment of canine rough microsomes on the transfer and processing of light chain immunoglobulin. Rough microsomes (RM) from canine pancreas were prepared as described previously, and suspended to 1 mg membrane phospholipid ml<sup>-1</sup> in 20 mM HEPES pH 7.5 (at 20 °C), 110 mM potassium acetate, 3 mM magnesium acetate and 2 mM dithiothreitol (microsomal buffer). CaCl<sub>2</sub> was added to 1 mM and microsomal nuclease (Boehringer) to 10 μg ml<sup>-1</sup> followed by incubation at 20 °C for 15 min to remove endogenous mRNA. The nuclease was inhibited by chelating Ca<sup>2+</sup> with EGTA added to 2 mM, the membranes washed by pelleting through 0.5 M sucrose, 20 mM HEPES pH 7.5 at 60,000g for 20 min at 4 °C and resuspended in microsomal buffer containing 1 mM EGTA. Aliquots of these RM<sub>M</sub> membranes containing 50 μg of membrane phospholipid (90 μg protein) were used in each 100-μl assay. For the salt treatment, RM<sub>M</sub> membranes were suspended in ice-cold 0.5 M KCl, 20 mM HEPES pH 7.5 to 10 mg membrane phospholipid ml<sup>-1</sup> and pelleted as above. The pellet (RM<sub>KK</sub> membranes) was resuspended in microsomal buffer and 50 μg membrane phospholipid (80 μg protein) was used in each 100-μl assay. In a parallel experiment, the sucrose cushion was omitted and the mixture centrifuged at 100,000g for 1 h at 4 °C. The supernatant salt extract (SE) was retained and 8 μg of protein was used in every 100-μl assay. The RM<sub>M</sub>, RM<sub>KK</sub> and SE preparations were frozen in liquid nitrogen and stored at -80 °C in small aliquots. The assay for transfer and processing has been described in detail previously. Briefly, mRNA was isolated from rough microsomes (Fig. 1) and translated in a cell-free wheat germ system containing <sup>35</sup>S-methionine in the presence and absence of microsomal membranes. Aliquots of these RM<sub>M</sub> membranes containing 50 μg of membrane phospholipid were incubated at 37 °C for 1 h, samples were either immunoprecipitated with antibody to light chain, or treated with proteases to determine the nature of the light chain transferred into the microsomal vesicles. Aliquots from both treatments were analysed by SDS-PAGE. The upper band is the precursor light chain (P-Li) and the lower band, derived from the precursor by proteolytic removal of the signal peptide, is the authentic light chain (Li) of immunoglobulin that would be secreted in vivo.

The supernatant salt extract (SE) was retained and 8 μg of protein. More than 90% of SE protein added to RM<sub>KK</sub> membranes (50 μg membrane phospholipid per 100 μl assay). The supernatant salt extract (SE) was retained and 8 μg of protein. More than 90% of SE protein added to RM<sub>KK</sub> membranes (50 μg membrane phospholipid per 100 μl assay). The supernatant salt extract (SE) was retained and 8 μg of protein. More than 90% of SE protein added to RM<sub>KK</sub> membranes (50 μg membrane phospholipid per 100 μl assay). The supernatant salt extract (SE) was retained and 8 μg of protein. More than 90% of SE protein added to RM<sub>KK</sub> membranes (50 μg membrane phospholipid per 100 μl assay).
Table 1  Characterisation of the components in the salt extract that restore transfer activity to salt-washed microsomal membranes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Membrane or lipid</th>
<th>Treatment</th>
<th>Protease-resistant protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM₄ membranes</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>RM₆K membranes</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>RM₆K membranes</td>
<td>8 μg SE protein added at beginning of incubation</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>RM₆K membranes</td>
<td>8 μg SE protein added at end of incubation</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>RM₆K membranes</td>
<td>8 μg protein</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>RM₆K membranes</td>
<td>8 μg SE dialysed protein</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>RM₆K membranes</td>
<td>8 μg heat-treated SE protein</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>RM₆K membranes</td>
<td>8 μg trypsin-treated SE protein</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>Trypsin-treated RM₆K membranes</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Trypsin-treated RM₆K membranes</td>
<td>8 μg SE protein</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>Sonicated RM</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>Sonicated RM</td>
<td>8 μg SE protein</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>Sonicated egg phosphatidylcholine</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>Sonicated egg phosphatidylcholine</td>
<td>8 μg SE protein</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>RM₆K membranes</td>
<td>8 μg SE protein from SM membranes</td>
<td>13</td>
</tr>
<tr>
<td>16</td>
<td>SM membranes</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>17</td>
<td>SM membranes</td>
<td>8 μg SE protein</td>
<td>14</td>
</tr>
</tbody>
</table>

Incubations were carried out in the presence of [³⁵S]-methionine as described in the legend to Fig. 1 except that 4 μg of canine pancreatic mRNA was added to each 100-μl assay instead of light chain mRNA. Unless otherwise stated, membranes, lipids and the salt extract were added at the beginning of the assay and the SE was derived from RM membranes. At the end of the 1-h incubation at 25 °C, the samples were cooled in ice and three aliquots taken for assay: In the first, 10-μl aliquots were sampled directly on to filter paper to determine the total protein synthesised; in the second, a 20-μl aliquot was treated with 80 μl of 2 mg ml⁻¹ proteinase K (Merck) in 50 mM HEPES pH 7.5 and incubated at 25 °C for 10 min, before a 50-μl aliquot was sampled on to filter paper; in the third, a 20-μl aliquot was treated with 80 μl of 2 mg ml⁻¹ proteinase K in 50 mM HEPES pH 7.5, 15 mM deoxycholate and incubated as in the second. All filter papers were treated according to the method of Mann and Novelli.10 The proteinase K degrades nearly all of the proteins not inside the vesicle to such a small size that they do not precipitate on the filter paper when washed in 10% TCA. The background [³⁵S]-methionine added in the filter paper after proteinase K treatment in the presence of deoxycholate, was always <5% of the [³⁵S] in the total synthesised protein and was subtracted from the total counts and those obtained in the presence of proteinase K alone. These corrected counts were used to determine the percentage of protease-resistant protein.[³⁵S]Counts resistant to proteinase K/Total [³⁵S]counts × 100. In the absence of RM₆, RM₆K and SM membranes, the total [³⁵S] counts in the newly-synthesised proteins was typically 150,000 ± 20,000 c.p.m. In the presence of these membranes and irrespective of the various treatments, the total [³⁵S] counts were between 90,000 and 120,000 c.p.m. Smooth microsomes were prepared by the method of Adelman et al.13 and were extracted with 0.5 M KCI as for RM₄ membranes in Fig. 1. Dialysis of the SE was carried out against 2.000 vol 0.5 M KCI, 20 mM HEPES pH 7.5 at 4 °C for 1 h. Heat treatment was carried out on a 50-μl aliquot of SE (0.8 mg protein ml⁻¹) at 70 °C for 5 min. Trypsin treatment of RM₆K and SE was carried out by treating 50-μl aliquots with 1 μl of 1 mg ml⁻¹ trypsin and incubating at 25 °C for 20 min. Soya bean trypsin inhibitor (1 μl of 5 mg ml⁻¹) was then added and the samples used in the assay. Controls using a mixture of trypsin and soya bean trypsin inhibitor were shown to have no effect on any of the incubations. RM lipids were extracted from RM membranes using 20 vol CHCl₃-MeOH (2:1, v/v) and the organic phase removed under a stream of N₂ and then in vacuo. Egg phosphatidylcholine was purchased from Sigma. Lipids were suspended to 5 mg ml⁻¹ in 50 mM HEPES pH 7.5 by sonication for 5 × 2 min at <10 °C using a Branson sonicator with microtip set at 60 W. Phospholipids were assayed by the method of Bartlett14 and proteins by the method of Lowry et al.16.

In all cases the samples contained 50 μg phospholipids.

RM lipids or egg phosphatidylcholine (Table 1, experiments 9–14). The membrane proteins in the SE are not found in ER membranes devoid of attached ribosomes in vivo. Smooth microsomal (SM) membranes, substantially freed of RM membranes, were extracted with 0.5 M KCI and this salt extract did not confer transfer activity on RM₆K membranes (Table 1, compare experiments 2 and 15). The SM vesicles were themselves unable to transfer secretary proteins (Table 1, experiment 16) and this inability was not due simply to a lack of the membrane proteins present in the SE from rough microsomes. Addition of this SE to SM vesicles did not result in protein transfer (Table 1, experiment 17).

The membrane proteins isolated by treating RM membranes with high salt are needed for the transfer of secretory proteins across the ER membrane. They can restore transfer activity to inactive, but intact, salt-washed vesicles and are normally bound tightly to the membrane. They are thus proteins bound to the cytoplasmic side of the microsomal membrane and probably do not span it; they may, however, be bound to proteins that do span the bilayer. Their location would suggest that they are involved in the binding of the signal peptide and associated ribosomal complex to the membrane. Other membrane proteins are necessary for protein transfer but they are more likely to be involved in events subsequent to binding.

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