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[11,12]. Thus, the ridge tile shape is confirmed with a dihedral angle of 98° between the two oxodipyrromethene 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 oxodipyrromethene compound[24]. In the chloroform molecule and in the isopropylammonium ion values of bond lengths and angles are also in the normal range.

The carbon–oxygen bond lengths in the carboxyl group are approximately equal, corresponding to a carboxylate form. The nature of lactim–lactam tautomerism in bilirubin is controversial[7,25]. 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 bound 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 oxodipyrromethene portion (see Fig. 3) allow the lactam form to be assigned.

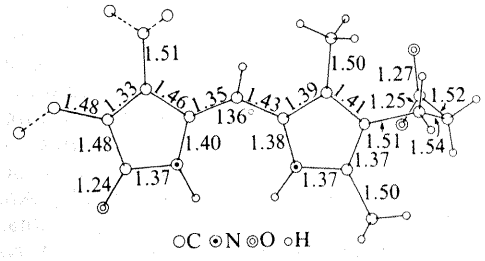

Fig. 3 The oxodipyrromethene moiety in the bilirubin–isopropylamine–chloroform complex.

The hydrogen bond pattern is of particular relevance (see Fig. 2). In contrast to bilirubin[11,12], in the present complex the conformation of the pigment molecule is stabilised 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 isopropylammonium 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 isopropylammonium ion. Thus the structure of the di-isopropylammonium bilirubinate might be viewed as a model for bilirubin–protein associations.

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1. Schmid, R. in *Jaundice* (eds Goresky, C. A. & Fischer, M. M.) (Plenum, New York, 1975).
2. Schmid, R. & McDonagh, A. F. *Ann. N. Y. Acad. Sci.* **244**, 533–552 (1975).
3. Bissel, D. M. *Gastroenterology* **69**, 519–538 (1975).
4. Kuenzle, C. C. in *The Hepatobiliary System* (ed. Taylor, W.) 309–335 (Plenum, New York, 1976).
5. Etter-Kjelsaas, H. & Kuenzle, C. C. *Biochim. biophys. Acta* **400**, 83–94 (1975).
6. Brodersen, R. & Theilgaard, J. *Scand. J. clin. Lab. Invest.* **24**, 395–398 (1969).
7. Kuenzle, C. C., Weibel, M. N., Pelloni, R. R. & Hemmerich, P. *Biochem. J.* **133**, 364–368 (1973).
8. Manitto, P., Severini-Ricca, G. & Monti, D. *Gazzetta Chim. Ital.* **104**, 633–637 (1974).
9. Knell, A. J., Hancock, F. & Hutchinson, D. W. in *Metabolism and Chemistry of Bilirubin and Related Tetrapyrrols* (eds Bakkan, A. F. & Fog, J.) 234–240 (Pediatric Research Institute, Oslo, 1975).
10. Manitto, P. & Monti, D. *J. chem. Soc. chem. Commun.* 122–123 (1976).
11. Bonnett, R., Davies, J. E. & Hursthouse, M. B. *Nature* **262**, 326–328 (1976).
12. Le Bas, G., Allegret, A. & de Rango, C. *4th European Crystallographic Meeting* Oxford (1977).
13. Billing, B. H. in *The Liver* (ed. Gall, A.) ch. 1 (Williams and Wilkins, Baltimore, 1973).
14. Kuenzle, C. C., Gitzelmann-Cumarasamy, N. & Wilson, K. J., *J. biol. Chem.* **251**, 801–807 (1976).
15. Kamisaka, J. K., Habig, W. H., Ketley, J. N., Arias, I. M. & Jakoby, W. B. *Eur. J. Biochem.* **60**, 155–161 (1975).
16. Kamisaka, J. K., Listowsky, I., Gatmaitan, Z. & Arias, I. M. *Biochemistry* **14**, 2175–2180 (1975).
17. Tipping, E., Ketterer, B. & Christodoulides, L., *Biochem. J.* **157**, 211–216 (1976).
18. Jacobsen, J. *Int. J. Peptide Protein Res.* **9**, 235–239 (1977).
19. Jacobsen, C. *Eur. J. Biochem.* **27**, 513–519 (1972).
20. Jacobsen, C. *Int. J. Peptide Protein Res.* **7**, 159–163 (1975).
21. McDonagh, A. F. & Assisi, F. *FEBS Lett.* **18**, 315–317 (1971).
22. Germain, G., Main, P. & Woolfson, M. M. *Acta crystallogr.* A **27**, 368–376 (1971).
23. Sheldrick, W. S. *J. C. S. Perkin II* 1457–1462 (1976).
24. Cullen, D. L. *et al. Tetrahedron* **33**, 477–483 (1977).
25. Severini-Ricca, G., Manitto, P., Monti, D. & Randall, E. V. *Gazzetta Chim. Ital.* **105**, 1273–1277 (1975).

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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[1]. 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[2,3]. 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 (RM_R) from canine pancreas[5]. 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 RM_R 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 RM_R membranes, was sufficient to transfer and process about 90% of the total Ig Li synthesised.

When RM_R membranes were washed with 0.5 M KCl, the resulting RM_{RK} 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

chain was protected against digestion by protease and represented the residual transfer activity of the RM_{RK} membranes. Protein transfer and processing could be fully restored to RM_{RK} membranes by readdition of the salt extract (SE); the authentic light chain was synthesised together with some precursor (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 canine 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

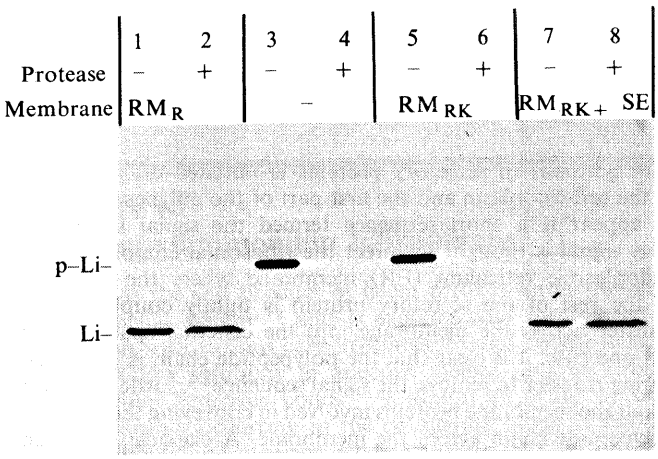

Fig. 1 Effect of KCl treatment of canine rough microsomes on the transfer and processing of light chain immunoglobulin. Rough microsomes (RM) from canine pancreas were prepared as described previously[1], and suspended to 1 mg membrane phospholipid ml^{-1} in 20 mM HEPES *p*H 7.5 (at 20 °C), 110 mM potassium acetate, 3 mM magnesium acetate and 2 mM dithiothreitol (microsomal buffer). $CaCl_2$ was added to 1 mM and micrococcal nuclease (Boehringer) to 10 μg ml^{-1} followed by incubation at 20 °C for 15 min to remove endogenous mRNA. The nuclease was inhibited by chelating Ca^{2+} with EGTA added to 2 mM, the membranes washed by pelleting through 0.5 M sucrose, 20 mM HEPES *p*H 7.5 at 60,000*g* for 20 min at 4 °C and resuspended in microsomal buffer containing 1 mM EGTA. Aliquots of these RM_R membranes containing 50 μg of membrane phospholipid (90 μg protein) were used in each 100-μl assay. For the salt treatment, RM_R membranes were suspended in ice-cold 0.5 M KCl, 20 mM HEPES *p*H 7.5 to 10 mg membrane phospholipid ml^{-1} and pelleted as above. The pellet (RM_{RK} 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 100,000*g* 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_R, RM_{RK} 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[1]. Briefly, mRNA was isolated from a MOPC 41 tumour secreting Ig light chain, and translated in a cell-free wheat germ system containing ^{35}S-methionine in the presence and absence of microsomal membranes. After incubation at 25 °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*.

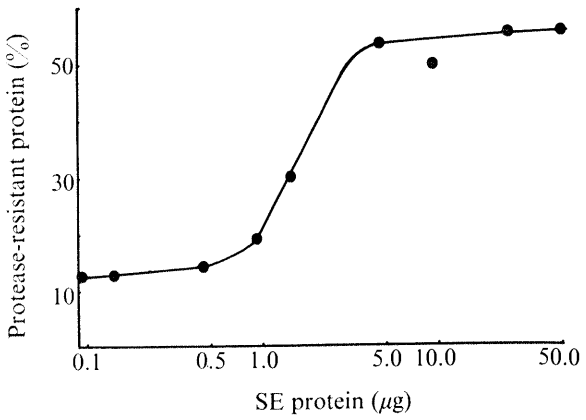

Fig. 2 Titration of the salt-washed microsomal membranes with the salt-extracted membrane proteins. Incubations were carried out as described in Fig. 1 and the quantitative assays as described in Table 1. The salt extract was concentrated by ultrafiltration using a Minicon with a B15 membrane (molecular weight cut-off = 15,000) and varying amounts were added to RM_{RK} membranes (50 μg membrane phospholipid per 100 μl assay).

(compare ref. 6). The percentage of leaky vesicles in any membrane preparation, however, was unaffected by the membrane treatments we used. RM_R membranes containing radiolabelled secretory proteins were subjected to these various treatments and 40–50% of the proteins synthesised were found to be resistant to added proteases. Membrane disruption is therefore not the cause of some of the results presented here.

RM_R membranes protected 39% of the total proteins synthesised and salt washing reduced this to 12%. Approximately half of the protection afforded by RM_{RK} membranes to pancreatic secretory proteins can be attributed to residual transfer activity (compare with Fig. 1, tracks 5,6 for the Ig Li); the other half is a feature of the assay and represents newly-synthesised proteins associated with membrane vesicles so as to allow only partial digestion by added proteases. This small amount of protein did not vary significantly from experiment to experiment and did not affect interpretation of the results. Readdition of SE to RM_{RK} membranes at the beginning of the incubation restored the original level of protection, whereas readdition at the end had no effect. In the absence of any other membranes, SE alone was unable to protect the secretory proteins (Table 1, experiments 1–5).

Proteins are the active components in the SE. Dialysis to remove small molecules did not impair its ability to restore transfer activity to RM_{RK} membranes, whereas heat or trypsin treatment destroyed it (Table 1, experiments 6–8). RNA does not seem to be an active component because the extract was derived from RNase-treated membranes. No phospholipids were detected in the extract.

The proteins which restore transfer activity to RM_{RK} vesicles are bound tightly to the membrane. By varying the amount of SE added to RM_{RK} membranes it was possible to increase the protection from 12 to 55% at saturating concentrations (Fig. 2). We estimated that 3.4 μg of SE protein had to be added to RM_{RK} membranes (containing 50μg phospholipid) to saturate the membrane sites that rebind the SE proteins. From RM_R membranes (containing 50 μg phospholipid) we obtained 3.1 μg of SE protein. More than 90% of SE protein added to RM_{RK} membranes must, therefore, have been rebound. These data also indicate that RM_R membranes, as isolated, are nearly saturated with SE proteins.

The membrane proteins extracted with high salt are not solely responsible for protein transfer across microsomal membranes because the SE will not restore transfer activity to RM_{RK} membranes that have been trypsin treated, nor will it confer transfer activity on sonicated liposomes derived from

Table 1 Characterisation of the components in the salt extract that restore transfer activity to salt-washed microsomal membranes

Experiment	Membrane or lipid*	Treatment	Protease-resistant protein %
1	RM_R membranes	—	39
2	RM_{RK} membranes	—	12
3	RM_{RK} membranes	8 μg SE protein added at beginning of incubation	43
4	RM_{RK} membranes	8 μg SE protein added at end of incubation	4
5	—	8 μg SE protein	1
6	RM_{RK} membranes	8 μg SE dialysed SE protein	42
7	RM_{RK} membranes	8 μg heat-treated SE protein	11
8	RM_{RK} membranes	8 μg trypsin-treated SE protein	11
9	Trypsin-treated RM_{RK} membranes	—	5
10	Trypsin-treated RM_{RK} membranes	8 μg SE protein	5
11	Sonicated RM lipids	—	1
12	Sonicated RM lipids	8 μg SE protein	2
13	Sonicated egg phosphatidylcholine	—	3
14	Sonicated egg phosphatidylcholine	8 μg SE protein	3
15	RM_{RK} membranes	8 μg SE protein from SM membranes	13
16	SM membranes	—	11
17	SM membranes	8 μg SE protein	14

Incubations were carried out in the presence of ^{35}S-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 aliquot was 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 *p*H 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 *p*H 7.5, 15 mM deoxycholate and incubated as in the second. All filter papers were treated according to the method of Mans and Novelli[8]. 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 soaked in 10% TCA. The background ^{35}S-methionine bound to the filter paper after proteinase K treatment in the presence of deoxycholate, was always ≤5% of the ^{35}S in the total synthesised protein and was substracted 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 [(^{35}S Counts resistant to proteinase K/Total ^{35}S counts) ×100]. In the absence of RM_R, RM_{RK} and SM membranes, the total ^{35}S 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 ^{35}S counts were between 90,000 and 120,000 c.p.m. Smooth microsomes were prepared by the method of Adelman *et al.*[7] and were extracted with 0.5 M KCl as for RM_R membranes in Fig. 1. Dialysis of the SE was carried out against 2,000 vol 0.5 M KCl, 20 mM HEPES *p*H 7.5 at 4 °C for 15 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 RM_{RK} and SE were carried out by treating 50-μl aliquots with 1 μl of 1 mg ml^{-1} trypsin and incubating at 25 °C for 20 min. Soybean trypsin inhibitor (1 μl of 5 mg ml^{-1}) was then added and the samples used directly in the assay. Controls using a mixture of trypsin and soybean trypsin inhibitor were shown to have no effect on any of the incubations. RM lipids were extracted from RM membranes using 20 vol $CHCl_3$–MeOH (2:1, v/v) and the organic phase removed under a stream of N_2 and then *in vacuo*. Egg phosphatidylcholine was purchased from Sigma. Lipids were suspended to 5 mg ml^{-1} in 50 mM HEPES *p*H 7.5 by sonication for 5×2 min at ≤10 °C using a Branson sonifier with microtip set at 40 W. Phospholipids were assayed by the method of Bartlett[9] and proteins by the method of Lowry *et al.*[10].

* 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 KCl and this salt extract did not confer transfer activity on RM_{RK} membranes (Table 1, compare experiments 2 and 15). The SM vesicles were themselves unable to transfer secretory 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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1. Blobel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 835–851, 852–867 (1975).
2. Campbell, P. N. & Blobel, G. *FEBS Lett.* **72**, 215–226 (1976).
3. Palmiter, R. D., Thibodeau, S. N., Gagon, J. & Walsh, K. A. *11th FEBS Mtg, Copenhagen, 1977* (Pergamon, London, 1977).
4. Kreibich, G., Pereyra, B. & Sabatini, D. D. *Abstr. Tenth Int. Congr. Biochem., Hamburg*, 297 (1976).
5. Dobberstein, B. & Blobel, G. *Biochem. biophys. Res. Commun.* **74**, 1675–1782 (1977).
6. Sabatini, D. D. & Blobel, G. *J. Cell Biol.* **45**, 146–157 (1970).
7. Adelman, M. R., Blobel, G. & Sabatini, D. D. *J. Cell Biol.* **56**, 191–205 (1973).
8. Mans, R. J. & Novelli, G. D. *Archs. Biochem. Biophys.* **94**, 48–53 (1961).
9. Bartlett, G. R. *J. biol. Chem.* **234**, 466–471 (1959).
10. Lowry, O. H., Rosebrough, N. J., Farr, R. J. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).