THE OPACITY FACTOR OF GROUP-A STREPTOCOCCI

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SUMMARY. Cell-bound opacity factor (OF) was extracted with sodium dodecyl sulphate (SDS) to yield stable extracts with titres of > 20000. The mol.-wt distributions of extracellular and SDS-extracted OF, determined by ultrafiltration or chromatography on Sepharose 4B, suggested that the high mol. wt (1 × 10⁶) of extracellular OF is due to aggregation, because cell-bound and extracellular OF in the presence of SDS had an average mol. wt of only 2 × 10⁵.

At least four apparent multiple-molecular forms (mol. wt 7-4–12-0 × 10⁴) of OF were detected by SDS polyacrylamide-gel electrophoresis. It seemed more probable that these were due to aggregation rather than the existence of different stable conformations. To explain the molecular-size distribution, the subunit would have to be as small as 1 × 10⁴ but this was supported by the finding that OF can be detected after passing through a dialysis membrane provided that its “substrate”, α₁-lipoprotein, is present on the other side. This raises the possibility that OF is associated with a carrier molecule.

The isoelectric-focusing profiles of OF were complex and differed markedly with the method used to prepare OF. Extracellular OF had a simple profile with an isoelectric point of 4-0, whereas Triton-extracted OF was the most complex and formed three peaks, the position of which varied depending on whether the detergent was present or absent during focusing runs.

INTRODUCTION

Although the importance of the opacity factor (OF) of group-A streptococci is well established (Widdowson, Maxted and Grant, 1970; Maxted and Widdowson, 1972; Maxted et al., 1973b; Widdowson, 1980), its physicochemical nature and its role in the serum-opacity reaction is little understood (Krumwiede, 1954; Rowen and Martin, 1963). It has been suggested that OF has a large mol. wt (Widdowson et al., 1971; Martinez et al., 1978), and its sensitivity to proteolytic enzymes indicates that it may be a protein (Hill and Wannamaker, 1968), but neither of these aspects has been fully investigated and the mol.-wt studies were done either on acid-extracted or extracellular OF in conditions that allowed aggregation. We have re-examined the effect of proteolytic enzymes and studied the mol.-wt distribution of extracellular and sodium...
dodecyl sulphate (SDS)-extracted cell-bound OF by several methods designed to keep aggregation to a minimum. Extracellular OF has a reported isoelectric point (pI) of 4.3 (Martinez et al., 1978). We have examined OF, extracted by several methods, to determine whether OF is composed of molecules with one or several pIs.

**Materials and Methods**

**Bacterial strains.** *Streptococcus pyogenes* strain R68/3116 which is M-positive, OF-positive (serotype T12, M22) was selected because it consistently gave high titres of OF in the culture supernate. An M-negative, OF-negative variant of this strain and an M-positive, OF-negative strain, B930/24 (serotype T3, M3) were used as controls in some experiments. The cultures were incubated overnight at 37°C in Todd Hewitt broth (Difco) supplemented with Neopeptone (Difco) 2% (w/v). The cells were sedimented by centrifugation at 1500 g for 20 min.

**Preparation of supernates.** The culture supernate was filtered through a Millipore membrane (pore size 0.45 μm—Millipore Ltd, Abbey Road, London NWlO 7SP) and 200-ml volumes of filtered supernate were concentrated tenfold in an ultrafiltration cell with either a PM10 Diaflo membrane (exclusion limit 1×10^4) or an XM300 membrane (exclusion limit 3×10^5). In some experiments, extracellular OF was precipitated by addition of ammonium sulphate to 60% saturation. After standing at 4°C for 24 h, the precipitate was collected by centrifugation, dissolved in distilled water and dialysed against distilled water for 48 h at 4°C.

**Extraction of cell-bound OF.** Cells from 500 ml of culture were washed three times with physiological saline. The washed cells were extracted with 10 ml of detergent solution either SDS 1% (w/v) or Triton X-100 0.5% (w/v) in water for 2 h at room temperature with mixing by end-over-end rotation, or 5 ml of 0.05 NaOH for 30 min at 56°C. The alkaline extract was cooled and neutralised with HCl. Cell debris was removed from the extracts by centrifugation and filtration through a Millipore filter (pore size 0.45 μm).

**Detection of OF.** Human plasma was used as a substrate for the reaction. Preliminary experiments showed that although the development of opacity is proportional to the amount of OF at low concentrations of OF, high concentrations of OF inhibited the development of opacity. However, the primary step in the reaction, the interaction of OF with x1-lipoprotein, was directly proportional to the OF concentration (Hallas, 1980). The simplest routine tests used for OF activity were the development of opacity in a slide or tube test. In the slide method, titres of crude OF preparations were determined by spotting a 2-mm loopful of doubling dilutions of the OF preparations on to plasma-agar slides by the method described by Maxted, Widdowson and Fraser (1973a). The dilution that gave a just-visible spot was taken as the OF titre of the preparation. The tube test was done by incubating 0.005-0.1 ml of column fractions with 0.5 ml of human plasma containing thiomersal 0.02% (w/v) for 16 h. After suitable dilution with saline in the range 0.5-2.0 ml the extinction at 475 nm (E475) was measured, in comparison with a control containing buffer in place of the OF sample. Because high OF titres depress the E475, each fraction was assayed at two levels to determine the one with greater OF activity.

**Treatment of OF with enzymes.** The effects of trypsin (Koch-Light Laboratories, Cohnbrook, Bucks SL3 0BZ), pepsin (Sigma London Chemical Co., Poole, Dorset BH17 7NH), lipase (Sigma), DNAase and RNAase (BDH Chemicals, Poole, Dorset BH12 4NN) on OF activity were measured. The reaction mixture for trypsin digestion contained 1 ml of a 1 in 15 dilution of concentrated extracellular OF, plus 1 ml of 0.02 m Tris-HCl buffer pH 7.8 and 0.025 ml of a solution of crystalline trypsin 10 μg/ml, specific activity 800 units/mg of protein. The reaction mixture was incubated in a water bath at 37°C and 0.1-ml samples were removed at intervals and titrated for OF activity by the slide method. The control tubes contained 0.025 ml of buffer in place of the trypsin solution. The reaction mixture for pepsin digestion which contained 1 ml of culture supernate (strain R68/3116) plus 1 ml of distilled water was acidified to pH 2.0 with 2N HCl and pepsin (0.025 ml of a 0.5 mg/ml solution, specific activity 3060 units/mg) was then added. The mixture was incubated at 37°C and 0.1-ml samples were removed at intervals and titrated for OF activity by the slide method. Serial dilutions were made in 0.1 m Tris-HCl buffer pH 8.0 to inactivate the pepsin. The reaction mixture for lipase treatment...
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...continued 0.1 ml of concentrated extracellular OF, 1.9 ml of 0.02 M phosphate buffer pH 7.8 and 0.025 ml of a 1 mg/ml solution of lipase, specific activity 28 560 units/mg, in distilled water. The lipase used was purified from pig pancreas (Sigma, product no. L2253) and contains protease activity 0.1–0.2 Sigma units/mg. The buffer used for the effect of ribonuclease on OF was 0.05 M acetate pH 5.5 and it was supplemented with 0.005 M MgSO4 for deoxyribonuclease. The proportions of the reaction mixtures were the same as described for lipase treatment.

**Gel filtration.** OF was separated on Sepharose 4B and Sephacryl S-300. Sepharose 4B (Pharmacia Ltd, Abbey Road, Hounslow, Middlesex TW3 1NE) was formed into a 1.5 × 30-cm column. Samples of 1.0 ml were applied and eluted with Veroval buffer pH 8.6; (ionic strength 0.03) and 1-ml fractions collected. The void volume was determined with Dextran Blue 2000 (Pharmacia) and the column was calibrated with thyroglobulin, catalase and apoferritin. Sephacryl S-300 gel was packed into a 1.6 × 70-cm column. A sample of 3 ml of SDS-extracted OF, concentrated tenfold by ultrafiltration with a PM 10 membrane, was applied and eluted with 0.05 M Tris-HCl containing SDS 1% (w/v). OF-positive fractions were pooled in a series of two fractions per pool and each pool was concentrated to 1 ml in a Minicon concentrator (B-15, Amicon Ltd, 2 Kingsway, Woking, Surrey GU21 1UR).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE).** The method of Weber and Osborn (1969) was used. The standard proteins, bovine serum albumin, catalase, egg albumin and chymotrypsinogen, were used to determine the relationships between the mol. wt and mobility of the proteins. The gels were stained with Coomassie Blue R250 (BDH) and the mobilities of the protein bands were calculated from the formula

\[
\frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}
\]

(Weber and Osborn, 1969). SDS-extracted OF and extracellular OF were analysed by SDS-PAGE and the mobilities of the protein-staining bands and OF-positive bands were compared. In some experiments, the OF-containing gels were also stained for glycoprotein by the method described by Segrest and Jackson (1972) and for lipoprotein with a saturated solution of Oil Red O (BDH) in 60% (v/v) ethanol for 18 h at 37°C. The pooled Sephacryl fractions of SDS-extracted OF were also analysed by SDS-PAGE. A sample of 5 μg of protein of each pool was applied to a polyacrylamide-gel rod and stained for protein after electrophoresis. The OF titres of the pools were equalised before application to separate gels for the analysis of the OF components.

**OF detection in polyacrylamide gels.** The gel rods were washed with distilled water and placed in a dish. A mixture prepared by addition of human plasma to an equal volume of Ion Agar (Oxoid) 2% (w/v) was poured onto the gels until they were covered. After the agar had solidified, the dish was incubated overnight at 37°C. The gels were examined and the positions of the zones of opacity recorded.

**Isoelectric focusing.** Sucrose-density gradient isoelectric focusing was performed in a 110-ml capacity LKB (8101) focusing column according to the manufacturer’s instructions. OF samples of 1.5–8 ml, dialysed against glycyne 1% (w/v) to remove electrolytes, were then added to the light solution before the density gradient was formed. The average focusing time was 48 h. Fractions of 2 ml were collected and the OF content was measured by the tube method after neutralisation of the fractions.

**Dialysis experiments.** One ml of each of concentrated extracellular OF, SDS-extracted OF, and the concentrated culture supernate of an M-negative, OF-negative serotype (B930/24) were placed in dialysis bags (Visking Tubing 8/32-in; Medicell International Ltd, 239 Liverpool Road, London W1 1LX) and dialysed against distilled water for 8 h at 4°C. The intact dialysis bags were then washed in water and transferred to separate test tubes containing 9 ml of human plasma with thiomersal 0.02% (w/v) as a preservative and incubated at 37°C. After incubation for 48 h, 1.5 ml of plasma was removed from each tube, diluted with 1.5 ml of saline and the E475 was measured in an SP600 spectrophotometer against a water blank. Each sample of plasma was separated by electrophoresis in a gel of agarose 1% (w/v) (Koch-Light) in Veroval buffer pH 8.6, ionic strength 0.03. The gel slides were stained for lipoprotein with Oil Red O (see SDS-PAGE methods) to confirm that the increase in opacity in plasma treated with OF-positive
preparations was due to the serum-opacity reaction and this was shown by a decrease in the mobility of the α-lipoprotein spot (Krumwiede, 1954). In another experiment, two 1-ml samples of SDS-extracted OF were placed in dialysis bags and dialysed against distilled water. One of the dialysis bags was transferred to 10 ml of human plasma and the second control bag was transferred to fresh distilled water. A "detergent control" containing 1 ml of SDS 1% (w/v) in the bag was suspended in 10 ml of human plasma. The tubes were incubated at 37°C. After 48 h the "test" dialysis bag was removed from the substrate and the optical density of the plasma substrate was determined with the plasma from the detergent control as a blank. The residual OF titre of the contents of the test and control bags was determined by the slide method.

RESULTS

Treatment of OF with enzymes

DNAase and RNAase had no effect on OF activity; there was no loss of OF activity after incubation for 1 h in the presence of 25 μg of either enzyme. OF was sensitive to the proteolytic enzymes trypsin and pepsin and also to lipase; it was most sensitive to trypsin. Fig. 1 shows the loss of OF activity with time for each enzyme.

Pepsin is active only at low pH and therefore the enzyme activity would be inhibited in the sample that was removed for titration of OF activity by making the serial dilutions in Tris buffer pH 8.0. Human serum contains a trypsin inhibitor and the action of trypsin was also presumably inhibited on contact with the substrate. It is not possible to inactivate the lipase enzyme and therefore traces of enzyme were present in the OF dilutions that were applied to the OF slide. The titre of OF decreased with increasing incubation time for all three enzymes, which indicates that loss of OF activity is not due to the action of the enzyme on the OF substrate, a serum lipoprotein. Although OF activity was destroyed by the lipase enzyme, the preparation used is not entirely free from protease and the loss of OF activity could be attributed to the presence of a protease in the preparation.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Exclusion limit (mol. wt)</th>
<th>SDS-extracted OF</th>
<th>extracellular OF from cells grown in “whole broth”*</th>
<th>“ultra filtrate broth”†</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM300</td>
<td>$3\times10^5$</td>
<td>48.0</td>
<td>2.5</td>
<td>22.0</td>
</tr>
<tr>
<td>XM100</td>
<td>$1\times10^5$</td>
<td>10.0</td>
<td>1.3</td>
<td>4.0</td>
</tr>
<tr>
<td>XM50</td>
<td>$5\times10^4$</td>
<td>Nil</td>
<td>Nil</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* "Whole broth" contained Todd Hewitt Broth (Difco) 3% (w/v) + Neopeptone (Difco) 2% (w/v).
† "Ultra filtrate broth" was "whole broth" concentrated tenfold in an ultrafiltration cell (Model 202, Amicon) with an XM300 Diaflo membrane (exclusion limit $3 \times 10^5$). The ultrafiltrate was distributed into 50-ml portions and sterilised by autoclaving.
Fig. 1.—Effect of enzymes on OF activity. — = Enzyme-treated OF activity; O = control (no enzyme). (a) Trypsin-treated OF; (b) pepsin-treated OF; (c) lipase-treated OF.
Membranes of different pore size were used in an ultrafiltration cell to estimate the mol. wt of OF in extracellular and SDS-extracted preparations. OF preparations were concentrated tenfold in membranes of decreasing pore size until OF was no longer recoverable in the filtrate. Only 2.5% of the OF activity in the extracellular preparation was able to pass through the XM300 membrane, suggesting that the bulk of the material had a mol. wt greater than $3.0 \times 10^5$. In contrast, only just over half of the SDS-extracted OF was retained by the XM300 membrane (table I). When the strain was grown in broth from which the larger-mol.-wt components had been removed by ultrafiltration through an XM300 membrane, only 22% of the extracellular OF was excluded by the XM300 membrane in comparison with 2.5% from "whole broth". These results suggest the aggregation of OF with large-mol.-wt components of broth.

Characterisation of OF by gel filtration

Preliminary experiments showed that Sepharose 4B (fractionation range $6 \times 10^4$–$2 \times 10^7$) was the most suitable gel for investigating the mol. wt-dispersion of OF because extracellular OF was excluded completely from Sephadex G200 (fractionation range $5 \times 10^3$–$2.5 \times 10^5$) and greatly retarded on Sepharose 2B (fractionation range $7 \times 10^4$–$4 \times 10^7$). Fig. 2 shows the mol. wt-distribution of extracellular OF and SDS-extracted OF on Sepharose 4B. Extracellular OF was more heterogeneous in size than SDS-extracted OF which was confined to fewer fractions. Calibration of the column with standard proteins suggested an average mol. wt for extracellular OF of $1 \times 10^6$ and that of SDS-extracted OF of $2 \times 10^5$. The apparently high mol. wt of extracellular OF might be due to aggregation and did not occur in detergent-extracted OF in which SDS was present. Extracellular OF, pretreated with SDS, was separated on Sepharose 4B in the presence of detergent. Treatment of extracellular OF with SDS effectively reduced the apparent molecular size of OF to that obtained for SDS-extracted OF. In all the preparations we studied, OF could be separated from the bulk of the protein in crude preparations by means of an appropriate gel-filtration system. Fig. 3 shows the separation of SDS-extracted OF, concentrated by ultrafiltration, on Sephacryl S-300. The protein peak was separated from the OF peak and fractions 17 and 18 had high OF titres but very little protein.

Characterisation of OF by isoelectric focusing

OF extracted by various methods was subjected to focusing to determine whether OF was composed of molecules with one or several isoelectric points (pI). Focusing of extracellular OF, concentrated by ammonium sulphate fractionation to a titre of 64, formed a simple profile with a single, well defined OF peak at pH 4.2 (fig. 4a). However, focusing of extracellular OF concentrated by ultrafiltration (OF titre 5120) showed that although there was a peak of OF activity at about pH 4, OF was also detected in the anode after neutralisation of acid and some activity was also present in most of the other fractions (fig. 4b). Alkali-extracted, cell-bound OF formed a broad peak of activity over the pH range 5.2–6.5 and a minor peak at pH 4 (fig. 4c). Triton-extracted OF was focused in the presence and absence of detergent and both
profiles produced three peaks of OF activity (fig. 4 c and d). The position of the first peak was unchanged whether or not detergent was present during focusing, but that of the second peak was 4.0–5.0 in the presence of and 5.0–5.7 in the absence of detergent. Although the focusing profiles varied considerably according to the method of extraction, the profiles of OF extracted by the same method were identical in duplicate experiments.

**Characterisation of OF by SDS-PAGE**

The most consistent feature of OF is the development of several bands of
OF-activity after separation by SDS-PAGE and exposure of gels to human plasma. Three distinct bands of activity could be demonstrated in concentrated extracellular OF with sample volumes ranging from 0·025 to 0·125 ml (OF titre 5120). With sample volumes of 0·01 to 0·05 ml (OF titre 40960) of SDS-extracted OF, run on 10 replicate gels, four OF bands were always present and a fifth band was occasionally present. However, when the sample size was effectively increased by the application of fivefold concentrate of SDS-extracted OF, one extra band of smaller mol. wt and another of high mol. wt were seen. The average mobilities (and 95% confidence limits) of OF bands in the SDS extract calculated from 10 replicate gels on unconcentrated

<table>
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<tr>
<th>Preparation in bag</th>
<th>Optical density (E_{475}) of plasma after 48 h</th>
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<tr>
<td>Concentrated extracellular OF</td>
<td>0·60</td>
</tr>
<tr>
<td>SDS-extracted OF</td>
<td>0·96</td>
</tr>
<tr>
<td>Concentrated supernate: OF-negative variant</td>
<td>0·38</td>
</tr>
<tr>
<td>Concentrated supernate: OF-negative serotype</td>
<td>0·36</td>
</tr>
</tbody>
</table>
Preparations were: $0.277 \pm 5\%$, $0.295 \pm 5\%$, $0.399 \pm 5.6\%$, $0.382 \pm 5\%$ and $0.439 \pm 2.5\%$. The average mol. wts corresponding to these mobilities were $12.0 \times 10^4$, $11.8 \times 10^4$, $10.3 \times 10^4$, $9.6 \times 10^4$ and $8.3 \times 10^4$ indicated by the standard proteins described (Methods). The average mol. wts of extracellular OF bands were $9.8 \times 10^4$, $8.8 \times 10^4$ and $7.8 \times 10^4$. Although the multiple bands of OF on SDS gels appear to be a consistent feature of OF irrespective of the method of extraction, they may be artefacts. This possibility is suggested by the results obtained for the analysis of pooled, concentrated Sephacryl S-300 fractions of SDS-extracted OF by SDS-PAGE (see fig. 5). Analysis of these preparations by SDS-PAGE showed that some separation of protein had been achieved by gel filtration on Sephacryl S-300 as the higher-mol.-wt protein bands were concentrated in pool 1. In contrast to the protein bands, the positions of the OF bands were similar in the different pools,
suggeasing that very little separation of OF had apparently taken place on Sephacryl S-300 chromatography. The same multiple OF bands were visible in most of the gel pools, except that pool 4 showed some enrichment of the minor low-mol.-wt component.

**Correlation of protein-staining bands with OF-positive bands on SDS-PAGE**

The average mobilities of the five major OF bands of SDS-extracted OF were compared in 10 experiments with the average mobilities of protein bands in the OF region. The average mobility of OF bands 1 and 3 coincided with protein bands. OF band 2 had no corresponding protein band. OF bands 4 and 5 showed some
correlation with protein bands although this was less consistent than for bands 1 and 3. Analysis of pooled fractions of SDS-extracted OF separated by Sephacryl S-300 chromatography showed that pool 1 is rich in protein bands which corresponded to the region of OF. These protein bands were either absent in other pools or existed at such lower concentrations that they were not detectable when 5 μg of protein was applied to the gel. If these protein bands were associated with OF they should be at least as strong in pools 2 and 3, because these had the highest OF titres.

**Correlation of OF with lipoprotein and glycoprotein bands**

Staining of a 0.05-ml sample of a fivefold concentrate of SDS-extracted OF separated by SDS-PAGE failed to detect any glycoprotein or lipoprotein-staining bands in the preparation.

**Dialysis experiments**

Gel-filtration and ultrafiltration studies showed that OF appeared to be a large molecule. On the other hand, SDS-PAGE experiments with OF previously separated by gel filtration suggested that OF may exist in several forms, some of which are of high mol. wt. It seemed that OF might be a very small molecule and variation in its properties may be due to combination with different carrier molecules. Experiments were therefore devised in which OF was partitioned from the plasma substrate by dialysis tubing (exclusion limit approximately $5 \times 10^3 - 2 \times 10^4$) to determine whether there was any transfer of OF molecules through the membrane to the surrounding substrate. Table II shows the results of an experiment in which SDS-extracted OF, extracellular OF, and concentrated culture supernates of an OF-negative variant of the test strain and of an M-positive, OF-negative strain, were dialysed against human plasma. The optical densities of the plasma samples incubated with the OF-negative preparations were approximately equal to each other and were much lower than samples containing OF-positive preparations. Plasma incubated with SDS-extracted OF had a higher optical density than the extracellular OF preparation; this is consistent with the finding that the SDS extract has a higher OF titre (40 960) than the concentrated extracellular OF preparation (5120). These results indicate that OF may be a very small molecule because it appears to be able to move through the dialysis-bag membrane into the surrounding substrate. Each sample of plasma was separated by electrophoresis and stained with Oil Red O. Plasma treated with an OF-positive preparation showed a reduction in intensity of the α1-lipoprotein spot, which confirms that the increase in plasma opacity was due to the serum opacity reaction. Table III

**Table III**

Dialysis of SDS-extracted OF against human plasma

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Optical density (E475) of plasma</th>
<th>OF titre in bag after dialysis</th>
<th>Percentage of control titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-extracted OF</td>
<td>1.24</td>
<td>2560</td>
<td>12.5</td>
</tr>
<tr>
<td>OF control</td>
<td>...</td>
<td>20480</td>
<td>...</td>
</tr>
<tr>
<td>SDS control</td>
<td>0.4</td>
<td>...</td>
<td>...</td>
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</table>
shows the results from another experiment in which SDS-extracted OF was dialysed against human plasma for 48 h and the OF titre of the contents of the bag were compared with an OF control that had been incubated in distilled water. The OF titre of the test sample was only 12.5% of the control titre. The optical density of the substrate incubated with SDS-extracted OF was 1.24 in comparison with 0.4 for plasma incubated with SDS solution 1.0% w/v (no OF) as a control.

DISCUSSION

The production of OF by strains of some group-A streptococci is known to be a useful taxonomic marker, particularly in the identification of difficult M types (Maxted et al., 1973b; Fraser et al., 1977). It has a close association with M antigen (Widdowson et al., 1970; Widdowson et al., 1971) and may affect the pathogenicity and virulence of strains by virtue of its influence on the antigenicity of other streptococcal products (Goeder, 1961; Top and Wannamaker, 1968; Widdowson et al., 1970; Widdowson et al., 1974; Pinney and Widdowson, 1977). The aim of this study has been to elucidate the physicochemical nature of OF in terms of mol. wt and PI and to discover whether it is a protein as had been suggested by previous observations that it was destroyed by some proteolytic enzymes.

Previous workers have suggested that OF is of large mol. wt, although the information on the size distribution of OF was incomplete in these studies because the choice of gel was limited. We have shown that Sepharose 4B is a suitable gel for use in the study of the mol.-wt distribution of extracellular OF and that the average mol. wt of extracellular OF, determined by ultrafiltration and gel filtration, was $1 \times 10^6$. However, extracellular OF was heterogeneous in size and formed a broad peak over many fractions. This phenomenon was also observed by Martinez et al. (1978), on Sepharose 6B. SDS-PAGE is the most accurate method used to investigate the size of OF, and the mol.-wt distribution of SDS-treated extracellular OF examined by SDS-PAGE was 7.4 to $9.8 \times 10^4$. The mol. wt of SDS-extracted OF estimated by ultrafiltration and gel filtration was approximately $2 \times 10^5$; and analysis by SDS-PAGE showed that the size distribution ranged from $8.4 \times 10^4$ to $12.0 \times 10^4$. SDS-PAGE analysis of several different OF preparations suggested that OF may be composed of multiple-molecular forms. Although aggregation of OF was minimal in these experiments, the existence of more than one stable conformation of OF is far from proven, and the multiple bands are more likely to be due to aggregation of smaller molecules. This hypothesis is also supported by the fact that prior separation of OF by gel filtration on Sephacryl S-300 had no effect on the multiple patterns of OF obtained in SDS-PAGE, suggesting that similar aggregates reform after separation. The size of the subunit would have to be as small as $1 \times 10^4$ to explain the size distribution of the multiple bands obtained.

We have shown that in all the preparations studied, OF may be separated from the bulk of the protein in crude preparations by gel filtration; in addition, some OF-positive fractions did not contain detectable protein. Similarly, analysis of SDS-extracted OF by SDS-PAGE showed that not all the OF-positive bands corresponded with protein-staining bands in duplicate gels, even when a protein stain with a high sensitivity was used; Coomassie Blue can detect as little as 0.5 μg of protein/cm of gel (Fazekas de St Groth, Webster and Datyner, 1963). Also, analysis
of pooled, concentrated Sephacryl S-300 fractions showed that protein bands could not be detected in the region of the OF bands derived from pool 2 although the sample had an OF titre of $1.3 \times 10^7$ and the OF bands gave a strongly positive result. It is difficult to explain the non-staining nature of the OF-positive band, although they may be protein with a very high specific activity.

Previous observations on the sensitivity of OF to proteolytic enzymes had not been reported in any detail. We re-examined the effect of enzymes on OF and showed that the loss of OF activity in the presence of trypsin or pepsin is time-dependent, which strongly suggests that the enzymes are acting on OF itself, or possibly on the OF carrier molecule and not on the lipoprotein substrate. We have also established that OF is not destroyed, or at least is inactivated at a slower rate, in control conditions in the absence of proteolytic enzymes; this confirms that the conditions themselves are not responsible for the loss of OF activity. Our results suggest that OF is probably a protein and possibly a lipoprotein because its activity is also destroyed by lipase. We cannot, however, rule out the possibility that the enzymes destroy a carrier molecule rather than OF itself. The variable characteristics of OF were its heterogeneity in mol. wt and in isoelectric-focusing profile and they seemed to depend upon the method of extraction. A possible explanation is that OF is a very small molecule which can associate with various carrier molecules in different extracts. This is known to be so for streptolysin S, which is a small peptide of $2.8 \times 10^3$ mol. wt that is reactive only in combination with various carrier molecules (Ginsburg, 1970). Dialysis experiments, designed to test this hypothesis for OF showed that, provided $\alpha_1$-lipoprotein was on the other side of the dialysis bag, the majority of the OF activity in an SDS extract was released from its hypothetical carrier and was able to move through the dialysis membrane, presumably because $\alpha_1$-lipoprotein was the preferred carrier molecule. This evidence suggests that although OF in various preparations may have an apparent mol. wt as large as $1 \times 10^5$ or $1 \times 10^6$, it may have a true mol. wt of less than $1 \times 10^4$. More experiments are necessary with potential carrier molecules including M protein, to determine whether the properties of OF depend on the nature of the carrier substance.

REFERENCES


