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The antigens of *Mycoplasma hominis*

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Mycoplasma hominis is the only species of human origin, other than *M. pneumoniae*, to be implicated in human disease. Despite this, little is known of its antigenic structure. Indeed, only a few species of *Mycoplasma* have been examined in this respect, although it is already clear that the chemical structure of their antigens is far from uniform. A polysaccharide hapten is present in *M. mycoides* var. *mycoides* (Plackett & Buttery, 1958; Buttery & Plackett, 1960; Plackett, Buttery & Cottew, 1963), whereas a hapten of *M. pneumoniae* contains several active glycolipids (Plackett, Marmion, Shaw & Lemcke, 1969) and *M. laidlawii*, type B contains a glucolipid hapten (Plackett & Shaw, 1967). *M. pulmonis*, on the other hand, apparently has no lipid hapten but contains protein and polysaccharide antigens (Deeb & Kenny, 1967).

M. hominis is a potential pathogen causing inflammatory pelvic disease in women (Marmion, 1967) and generalized blood-stream infection following childbirth (Stokes, 1955; Tully & Smith, 1968), abortion (Tully *et al.* 1965; Harwick, Iuppa, Purcell & Fekety, 1967) or hysterectomy (Stokes, 1959). We have fractionated the antigens of *M. hominis* and determined their behaviour in a variety of serological tests.

MATERIALS AND METHODS

Strains

Strain SC4 was isolated from the urethra of a male patient. It was isolated on rabbit serum agar, so that antisera could be prepared after only a few subcultures. The strain was cloned twice from single colonies. Stocks of the fourth subculture were frozen at $-30°$ C., and lyophilized ampoules prepared from the sixth, so that cultures which had undergone fewer than ten subcultures could be used for fractionation. SC4 was identified as *M. hominis* by growth-inhibition tests on agar (Huijsmans-Evers & Ruys, 1956; Clyde, 1964) with antisera against authentic strains of *M. hominis*, *M. pneumoniae*, *M. orale*, type I, *M. fermentans* and *M. salivarium*.

Media and cultural conditions

Liquid medium consisted of Difco PPLO broth (without crystal violet) supplemented with Oxoid yeast extract (0·5 %, w/v), sodium deoxyribonucleate (0·002 %, w/v), penicillin (50 units/ml.) and human plasma (20 %, v/v). The plasma was citrated plasma from out-dated transfusion blood, treated by a modification of the method of Maizels (1944) to remove fibrin. Plasma was mixed with sterile kaolin

(160 g./l.), shaken thoroughly, allowed to stand for 24 hr. at 4° C. and shaken again. The kaolin was allowed to settle out at 4° C. for 5–6 days, and the plasma siphoned off, clarified and sterilized by Seitz filtration, and stored at 4° C. It was sometimes necessary to refilter the treated plasma before use owing to the precipitation of residual fibrin, but this did not affect the growth of the mycoplasma. Unlike media containing horse serum, this medium contained very little extraneous material that sedimented on centrifugation of mycoplasma cultures; in fact not more than 5 % of the dry weight of the cells.

For fractionation, *M. hominis* SC4 was grown for 18 hr. at 37° C. without shaking. For seeding, 1 vol. of a similar 18 hr. culture was added to each 1000 vol. of medium. Determinations of the optical density at 580 mμ and of the colony-forming units per ml. from counts on agar, showed that the logarithmic phase of growth ended at 16–18 hr. Cells were harvested at 15,000 *g* for 30 min. in an MSE 1800 centrifuge (or, in the case of a batch prepared at Porton, in a Sharples centrifuge) and washed in an appropriate buffer, first in 20 % of the original volume of culture and then in 10 %. At first, a 0·02 M phosphate buffer containing 0·01 M Mg^{2+} ion at pH 7·1 (Marmion, Plackett & Lemcke, 1967) was used to prevent loss of cell contents by spontaneous lysis. Later, however, it was found that the release of protein from the cells by alternate freezing and thawing was increased when no Mg^{2+} ion was present. Thereafter, for all disruption procedures, cells were washed in 0·15 M sodium chloride, or in sodium chloride/tris buffer (0·15 M sodium chloride, 0·05 M tris) at pH 7·4. Centrifuged deposits not used immediately were stored at − 30° C.

When material for the inoculation of rabbits was required, unheated rabbit serum (10–15 %, v/v) was substituted for human plasma in the medium. For growth-inhibition tests, Difco PPLO agar replaced the broth, and unheated horse serum (Burroughs Wellcome no. 3) the human serum or plasma, because with this medium the zones of inhibition were more distinct.

Disruption and fractionation of Mycoplasma hominis *by physical methods*

Osmotic lysis

The method of Razin (1963) was used, except that the suspensions were incubated at 37° C. for 30 min. not at room temperature.

Alternate freezing and thawing

Washed cells suspended in deionized water to approximately one-tenth of the original culture volume were frozen by alcohol and dry ice at − 25° C. or below and thawed at 37° C. After 10 freeze–thaw cycles, unbroken cells were removed by centrifugation at 8000 *g* for 30 min. Only a little more protein was released after ten more cycles.

Gas cavitation under nitrogen

Washed cells from 2·5 l. of culture were suspended in 30 ml. dilute sodium chloride/tris buffer (0·0075 M sodium chloride, 0·0025 M tris), equilibrated for

20 min. with stirring, under nitrogen (1600 lb./in.^{2}) in a modified bomb calorimeter, which was held in ice and water, and the pressure then released (Wallach & Ullrey, 1962). Suspensions were treated six times and centrifuged at 8000 *g* for 30 min. The resulting pellet was resuspended in the same volume of buffer and treated six times more before centrifugation at 8000 *g*. The 8000 *g* supernatants were pooled for further fractionation and the deposits of unbroken cells discarded.

Ultrasonic treatment

Washed cells from 2·5 l. of culture were suspended in 10 ml. dilute sodium chloride/tris buffer, and treated for 2 min. with a Branson S 75 Sonifier (Branson Instruments Inc., Stamford, Connecticut, U.S.A.) at 20 kc./sec. The suspension was held in ice and water throughout. After centrifugation at 8000 *g* for 30 min., the resuspended pellet was treated again, and the supernatants pooled.

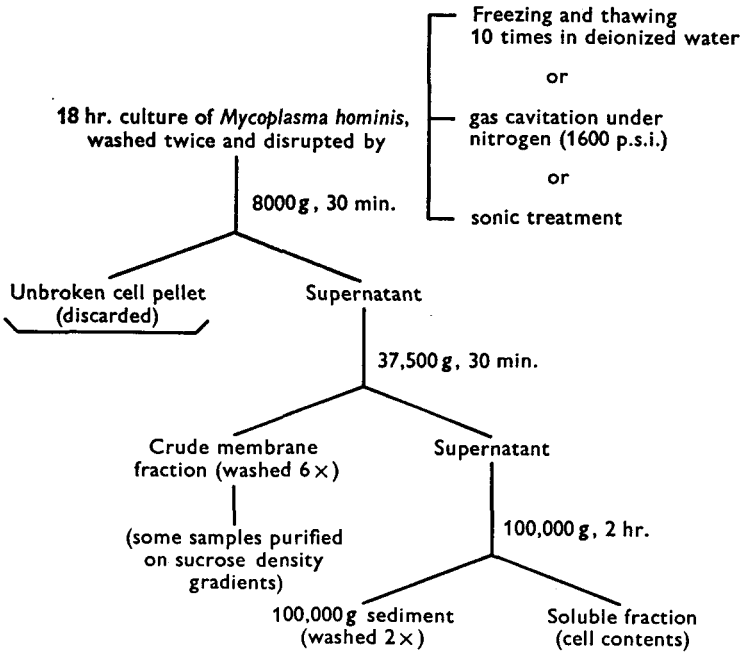

Fig. 1. Scheme for the disruption and fractionation of *M. hominis*.

Fractionation of disrupted cells

The 8000 *g* supernatants were centrifuged according to the scheme in Fig. 1. The crude membrane fraction gave a yellow-brown transparent pellet, which was washed six times in dilute sodium chloride/tris buffer. The soluble fraction (cell contents) was concentrated by pressure dialysis at 4° C. The material sedimenting at 100,000 *g* usually formed a small transparent pellet which was washed twice in buffer.

Purification of membrane fraction

Washed crude membrane fraction (0·4 ml. volumes containing 1–2 mg. of membrane protein) in dilute sodium chloride/tris buffer containing sucrose (10 %, w/w) was layered on top of a sucrose gradient prepared from buffered sucrose solutions containing 55, 50, 36 and 23 % (w/w) sucrose. Tubes were centrifuged at 20,000 rev./min. for 20 min. at 1° C. in a Spinco model L centrifuge on an SW 39 L rotor. When layered on top of a similar gradient, whole cell suspensions at the equivalent protein concentration passed to the bottom of the tube. Membrane preparations gave a discrete band approximately half-way up the tube and sometimes a small amount of sediment, presumably of unbroken cells, at the bottom. Fractions were collected by upward displacement on an ISCO Model 180 Density Gradient Fractionator (Instrumentation Specialities Co. Inc., Lincoln, Nebraska, U.S.A.) with a buffered sucrose solution (60 %, w/w) containing potassium hydrogen phthalate (0·15 %, w/v). The optical density at 245 mμ of the displaced material was plotted continuously on an ISCO Model UA 2 Analyser. The fractions containing the purified membrane were pooled, dialysed against buffer overnight at 4° C., and then centrifuged at 37,500 g for 40 min.

Lysis of membranes by detergents

Successive 0·1 ml. volumes of sodium lauryl sulphate, sodium deoxycholate, or Triton X-100 were added to 3 ml. amounts of a suspension of membranes at 0·5 mg. protein per ml. in dilute sodium chloride/tris buffer. The final concentration was 9 mg. detergent per mg. membrane protein. After each addition of detergent, the mixtures were incubated at 37° C. for 15 min. and the optical densities at 500 mμ determined in a Unicam SP 500 spectrophotometer. The readings were corrected for dilution by the detergents.

Protein estimations

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine serum albumin (puriss. grade, Koch–Light Laboratories, Colnbrook, Bucks.) as a standard.

Antisera

Since SC4 was isolated on rabbit serum agar, it was possible to raise antisera against whole cells from cultures which had been subcultured less than seven times and had never been in contact with the serum of other species. For subcutaneous (s.c.) inoculation, washed cells from 200 ml. of 2- to 3-day cultures were suspended in 2 ml. saline (0·15 M) and emulsified in 3 ml. of Drakeol–Arlacel adjuvant (Lemcke, 1965). Rabbits received two such doses at an interval of 13 days. Thirteen days after the second s.c. inoculation, 13 ml. of a washed saline suspension from 400 ml. of culture was given in six intravenous (i.v.) doses, (1, 1, 2, 2, 3 and 4 ml.), on alternate days. Rabbits were bled 5–7 days after the last inoculation. Sera were stored at −30° C. without preservative.

For antiserum to purified membranes, a suspension containing 3·5 mg. protein

was inoculated i.v. in six doses on alternate days. At first, an attempt was made to prepare antiserum to soluble fraction produced by gas cavitation, but the resulting antiserum reacted strongly in gel-diffusion with detergent-lysed membranes. Accordingly, freeze-thaw soluble fraction was used. The response to i.v. inoculation of this fraction was negligible even after the administration of 10 mg. protein, but good antiserum resulted when 5 mg. protein in Drakeol–Arlacel adjuvant was given in two s.c. inoculations separated by an interval of 13 days.

Serological tests

Complement fixation

The quantitative method of Fulton & Dumbell (1949) was adapted to the Microtiter System (Cooke Engineering Co., Arlington, Va.). In titrations with antiserum to whole cells, the amount of complement fixed by the fractions or extracts at a concentration of 0·5 mg. protein per ml. was compared with that fixed by whole cells at the same concentration. With lipid extracts and the aqueous phase of the phenol extract, comparisons were made with the antigens at 1·0 mg. dry weight per ml. Pre-immunization serum was included in all titrations. The logarithms of the units of complement fixed by each antigen were plotted against dilutions of antiserum, and the areas under the curve calculated. The 'area' of fixation by whole cells at 0·5 mg. protein per ml. or 1·0 mg. dry weight per ml. was recorded as 1·0 and the amount of complement fixed by fractions or extracts then expressed in terms of this unit fixation.

For comparing the complement-fixing activity of antisera against the purified membrane and soluble fractions, graded dilutions of a whole-cell antigen were titrated against graded dilutions of each antiserum. Since there was an antigen concentration at which all the antisera had a maximum serum titre, the titres recorded were those obtained at this optimal antigen concentration.

All sera were absorbed with packed sheep red blood cells to remove Forssman type antibody and, when anticomplementary, held with 1/10 guinea-pig serum overnight at 4° C. before inactivation at 56° C. for 30 min. Complement was obtained as guinea-pig serum, separated, pooled and stored in small quantities at −30° C.

Growth inhibition on agar

Tests were made by the method of Clyde (1964), using preimmunization sera as controls.

Metabolic inhibition

The test designed by Purcell, Taylor-Robinson, Wong & Chanock (1966) for the inhibition of arginine metabolism by antibody was used, except that the medium contained human instead of horse serum. Preimmunization sera were included in all tests. For absorption of metabolism-inhibiting (MI) antibody, 0·1 ml. volumes of whole-cell antiserum were absorbed for 48 hr. at 4° C. by whole-cell suspensions, fractions or extracts containing 1 mg. protein, and precipitates were removed by centrifugation. With the chloroform–methanol extract, whole-

cell antiserum (0·1 ml.) was absorbed by a dispersion containing 1 mg. dry weight of lipid and compared with antiserum similarly absorbed with 1 mg. dry weight of whole cells. Control antiserum was held in the same way with buffer or suspending fluid.

Indirect haemagglutination

A suspension of washed sheep erythrocytes (4 %, v/v) was treated with an equal volume of tannic acid (Mallinckrodt, 1 in 20,000, w/v) in phosphate-buffered saline, pH 7·2 (PBS), for 30 min. at room temperature. After three washes, the tanned cell suspensions (2 %, v/v) were treated for 45 min. at room temperature with an equal volume of a sonicated suspension of SC4 in buffered saline. The sensitized erythrocytes were washed once in PBS and twice in PBS containing 1 % (v/v) normal rabbit serum (NRS diluent). For test, 0·1 ml. amounts of erythrocytes (1 %, v/v) in NRS diluent were mixed with an equal volume of antiserum in the same diluent. Preimmunization sera were also included in the test. The results were read after 3 and 18 hr. at room temperature. Since maximum titres were obtained when erythrocytes were sensitized with sonicated suspensions of SC4 at 0·125–0·063 mg. protein per ml., fractions were compared at the same concentrations. Membrane fractions were sonicated briefly to obtain a fine dispersion. All sera, including the normal rabbit serum used in the NRS diluent, were first absorbed with packed sheep erythrocytes. Absorption of indirect haemagglutinating (IHA) antibody by whole cells and fractions was carried out as described for the absorption of MI antibody.

Gel diffusion

The method described by Lemcke (1964) was used. Whole-cell lysates and fractions were used at 2 mg. protein per ml.

Immunoelectrophoresis

Slide cover glasses (8·2 cm × 8·2 cm) were covered with 7 ml. of Difco Noble agar (1 %, w/v) in barbitone buffer, pH 8·6 (Oxoid), at an ionic strength of 0·025 and containing sodium azide (0·1 %, w/v). Antigen wells were 2 mm. in diameter and electrophoresis was at 4° C. for 90 min. at 150 mV. in barbitone buffer, ionic strength 0·05. Antiserum was in troughs 1 mm. in width and precipitin arcs were allowed to develop at 4° C.

Chemical extractions

Chloroform–methanol

Washed whole cells were extracted as described for *M. pneumoniae* by Marmion *et al.* (1967). After evaporation of the solution to dryness at 35° C. *in vacuo*, the lipid was taken up in a little chloroform and stored at −10° C. For serological tests, the chloroform was evaporated by a stream of nitrogen and the lipid dispersed in veronal-buffered saline, pH 7·2 (CFT diluent, Oxoid), by shaking with glass beads or by sonic treatment for 2 min. at 4° C. The dry weight of the lipid in the dispersion was determined and serological activity was recorded in terms of dry weight.

Aqueous acetone

Washed whole cells were extracted with aqueous acetone (80 %, v/v) on a magnetic stirrer for 10 min. at room temperature. The insoluble residues, deposited at 8000 *g*, were re-extracted in the same way. The supernatants were pooled and filtered through glass wool. Acetone was removed by evaporation *in vacuo* and finally with a stream of nitrogen. The lipid was dispersed in the remaining water by sonic treatment for 1 min. at 4° C. and the dry weight determined. The residue remaining after lipid extraction was washed, resuspended in CFT diluent and the complement-fixing activity determined at 1 mg. dry weight per ml.

Phenol

Two methods were used: (i) Cells previously extracted with chloroform–methanol were washed, suspended in water and extracted with warm aqueous phenol as described by Marmion *et al.* (1967). After removal of the phenol by dialysis, the aqueous phase was lyophilized, and dissolved in CFT diluent for serological tests. (ii) Cells suspended in water were shaken vigorously with an equal volume of aqueous phenol (90 %, w/w) for 1 hr. at 4° C. (Gierer & Schramm, 1956; Westphal, Luderitz & Bister, 1952). After separation, the aqueous and phenolic layers were dialysed against water to remove the phenol. The clear aqueous phase was lyophilized. The insoluble material which separated from the phenolic phase as the phenol was removed was also lyophilized; it was partly soluble in sodium lauryl sulphate, sodium deoxycholate and dilute sodium hydroxide, and all three solutions gave identical precipitin lines in gel-diffusion. Accordingly, the rest of the material was extracted at 37° C. for 30 min. with 0·06 N sodium hydroxide, clarified by centrifugation for 10 min. at 3000 *g* and dialysed against 0·001 M tris-HCl buffer, pH 7·4 at 4° C. for 24 hr.

Potassium hydroxide

The method of Warnaar *et al.* (1965) for extracting an antigen from vaccinia virus was used on a suspension of *M. hominis* containing 2 mg. protein per ml.

RESULTS

Disruption of cells

The cell materials absorbing at 260 mμ, released by osmotic lysis, increased as the salt concentration decreased from 0·256 M to 0·022 or 0·014 M and thereafter decreased as the concentration decreased to 0·006 M (Fig. 2). In this respect, *M. hominis* behaved differently from several species examined by Razin (1963), who found that maximum lysis of *M. laidlawii* and *M. bovigenitalium* occurred at the lowest salt concentration tested (0·006 M) and of *M. mycoides* var. *capri*, at the highest concentration (0·256 M). These differences may reflect some fundamental species differences in the composition or structure of the membranes. Nevertheless, even at the tonicities which allowed the maximum lysis of *M. hominis*, the amount of materials absorbing at 260 mμ was only twice that released in the 0·256 M suspensions. Similarly, the optical density at 500 mμ was reduced by less

than one-third. Osmotic lysis was, therefore, unlikely to give satisfactory yields of fractions. Of the three other methods, sonic treatment released most protein (67–88 %) from the cells, compared with 45–65 % by gas cavitation and 31–52 % by freezing and thawing.

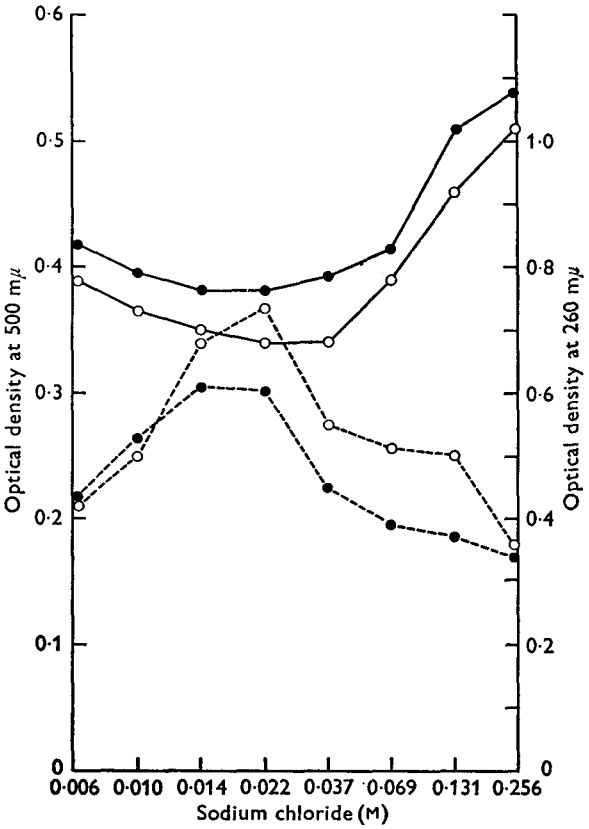

Fig. 2. Lysis of an 18 hr. culture of *M. hominis* SC4 in salt solutions of decreasing tonicity. —, Optical density at 500 mμ; ---, optical density at 260 mμ. Expt. 1. (●); Expt. 2. (○). The concentrations of sodium chloride include the amount of salt added with the suspension.

Fractions obtained by physical disruption

Serology

The complement-fixing activities of fractions from typical experiments with SC4 disrupted by freezing and thawing, gas cavitation and sonic treatment are shown in Table 1. All the fractions fixed complement with antiserum to whole cells, and the corresponding fractions produced by the three methods differed little.

The results of metabolic inhibition, using antisera absorbed with whole cells and fractions from five different experiments, are summarized in Table 2. Crude and purified membrane fractions absorbed MI antibody even more strongly than whole cells at the same protein concentration. Soluble fractions absorbed at least 32 times less effectively than membranes, but the 100,000 *g* sediments were nearly as active as the membranes.

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Both crude and purified membranes were highly active in sensitizing erythrocytes to agglutination by antisera against whole cells or purified membranes (Table 3). The membranes were nearly as active as sonicated suspensions of whole cells at the same protein concentration, whereas the soluble fractions were much less so.

Table 1. *Complement fixation, with antiserum to whole cells, of fractions of* Mycoplasma hominis *SC*4 *containing* 0·5 *mg. protein per ml., expressed as fractions of the amount of complement fixed by whole cells*

Method of cell disruption	Whole cells	Crude membrane	Soluble fraction	100,000*g* sediment
Freezing and thawing	1·00	1·05	0·92	NT
Gas cavitation	1·00	0·86	1·00	NT
Sonic treatment	1·00	0·85	1·00	0·78

Table 2. *Absorption of metabolism-inhibiting (MI) antibody from antiserum to whole cells (titre* 1280–2560) *by fractions of* Mycoplasma hominis *SC*4

	Factor by which titre decreased after absorption by				
		Membrane			
Method of cell disruption	Whole cells	Crude	Purified	Soluble fraction	100,000*g* sediment
Gas cavitation (Expt. 9)	128	256	NT	4	32
Gas cavitation (Expt. 11)	128	> 256	NT	4	NT*
Sonic treatment (Expt. 1)	128	> 256	NT	8	128
Sonic treatment (Expt. 2)	128	> 128	NT	2	> 256
Sonic treatment (Expt. 12)	128	128	128	2	NT

Titre = highest dilution of the serum inhibiting a colour change of 0·5 pH units.
NT = not tested.
* Insufficient to test.

Table 3. *Agglutination, by antisera to whole cells and to purified membrane, of tanned erythrocytes sensitized with sonicated cells or fractions of* Mycoplasma hominis *SC*4 *at* 0·125 *mg. protein per ml.*

	Titres	
Erythrocytes sensitized with	Antiserum to whole cells	Antiserum to purified membrane
Sonicated cells	5120	2560–5120
Crude membrane	5120	2560
Purified membrane	1280–2560	640–1280
Soluble fraction (gas cavitation)	20–40	10–20
Soluble fraction (freeze–thaw)	160	NT

NT = not tested.

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Both crude and purified membranes strongly absorbed IHA antibody from antiserum to whole cells (Table 4). A soluble fraction produced by freezing and thawing absorbed very little, but one produced by gas cavitation decreased the titre 128-fold, probably because of the presence of membrane components, since a similar fraction produced by gas cavitation stimulated the formation of antibody to membrane antigens (see above).

Table 4. *Absorption of indirect haemagglutinating antibody by whole cells or fractions of* Mycoplasma hominis *SC*4

(Sera titrated against tanned erythrocytes sensitized with a sonicated suspension of SC4 at 0·125 mg. protein per ml.)

Antiserum to whole cells absorbed with	Titre of haemagglutinating antibody
Nil (control)	2560
Whole cells	< 10
Crude membrane	10
Purified membrane	20
Soluble fraction (freeze–thaw)	1280
Soluble fraction (gas cavitation)	10–20

With antiserum to whole cells, soluble fractions, whether produced by freezing and thawing, gas cavitation or sonic treatment, gave a characteristic pattern in gel-diffusion or immunoelectrophoresis (Pl. 1, fig. 1*a*, *b* and Pl. 2, fig. 1*a*, *b*). In the gel-diffusion results, the precipitin lines were numbered 1–6, starting at the antiserum well (Pl. 1, fig. 1*b*). The lines 4 and 5 were heaviest and sometimes appeared as a single broad band; line 6 was heavy, and sometimes double. In contrast, membrane fractions were inactive (Pl. 2, fig. 1*a*) unless first lysed with detergents such as sodium lauryl sulphate, sodium deoxycholate, Triton X-100 or Brij 58, or with dilute sodium hydroxide (0·1–0·05 M). Fractions treated with the first three detergents gave the most precipitin lines. According to electron micrographs, Triton X-100 produced the greatest degree of membrane breakdown, although the reduction in optical density at 500 mμ was greatest with sodium deoxycholate and sodium lauryl sulphate (Fig. 3). Moreover, some batches of sodium lauryl sulphate and sodium deoxycholate gave diffuse, non-specific zones of precipitation which were not entirely absent with serum absorbed three times with detergent. Thus, Triton X-100 seems the detergent of choice for lysing membranes for gel-diffusion tests. The intensity and resolution of the precipitin lines was best when 5 mg. per mg. of membrane protein was used.

The gel-diffusion pattern of detergent-lysed membranes with whole cell antisera was quite distinct from that of soluble fractions (Pl. 1, fig. 2). The gel-diffusion pattern of the soluble fraction was not affected by the addition of sodium lauryl sulphate or Triton X-100 at the concentration used to lyse membranes and whole cells. Whole cells lysed with detergents gave lines characteristic of both fractions (Pl. 1, fig. 2). The lines given by detergent-lysed membranes developed close together and were more difficult to resolve than those given by soluble fractions,

but where the resolution was good, at least three lines were detectable (Pl. 1, figs. 2, 3). Immunoelectrophoresis of membrane lysed with the anionic detergent, sodium lauryl sulphate, gave a strong arc, which was probably complex, toward the anode (Pl. 1, fig. 5*b*). With the non-ionic detergent Triton X-100, crude membranes gave a strong arc and purified membranes a double arc near the origin (Pl. 1, fig. 5*a*). In addition, both crude and purified membrane preparations gave an arc at the cathode end (Pl. 1, fig. 5*a*). When membranes are lysed with sodium lauryl sulphate the active components migrating toward the anode are probably complexes of detergent and membrane protein which migrate according to the charge on the detergent ion. Membranes purified on sucrose density gradients gave the same gel-diffusion pattern as crude membrane fractions (Pl. 1, fig. 3). However, in immunoelectrophoresis crude, but not purified membranes, gave a faint, diffuse arc in the position of the main arc of the soluble fraction (Pl. 1, fig. 5*a*).

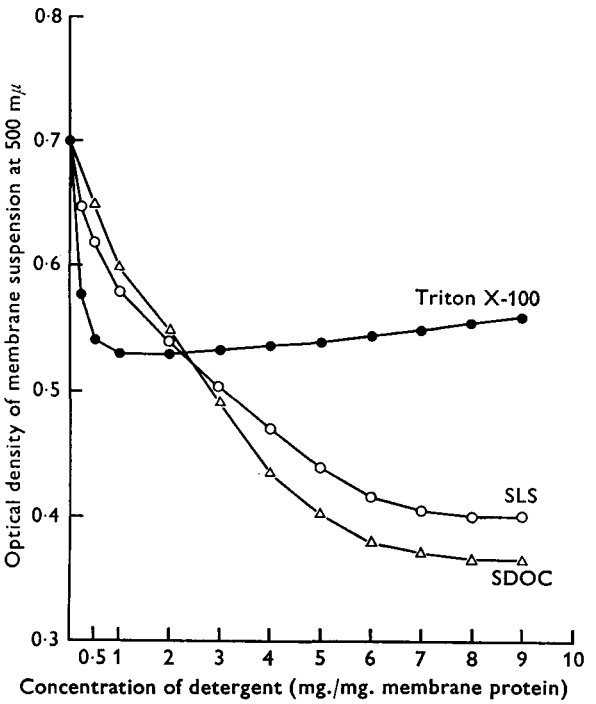

Fig. 3. Decrease in optical density at 500 mμ of *M. hominis* SC4 membrane suspensions with increasing concentrations of sodium lauryl sulphate, sodium deoxycholate and Triton X-100.

Soluble fractions from some batches of sonically treated cells proved to be contaminated by membrane components. Besides the pattern characteristic of soluble fractions, there were one or two additional bands near the antigen well, which linked up with some given by detergent-lysed membrane (Pl. 1, fig. 4). Sonic treatment evidently rendered some membrane components unsedimentable at 100,000 *g*, and is therefore not suitable for preparing soluble fraction free of membrane components.

Some of the resuspended 100,000 *g* sediments gave lines characteristic of the soluble fraction, in particular, line 6. However, when lysed with detergents, the deposits invariably gave strong lines characteristic of the membrane fraction. Repeated washing of one preparation removed the line 6 component, leaving only the membrane components to be revealed by detergent lysis. The material sedimenting at 100,000 *g* therefore consists largely of membranes, and this undoubtedly accounts for its activity in absorbing MI antibody (Table 2). Moreover, since 100,000 *g* sediments contain no reactive components different from those in the membrane and soluble fractions, the 37,500 *g* centrifugation stage of the fractionation process can be omitted, sedimenting the membranes at 100,000 *g* and increasing the number of washes.

Table 5. *Serological reactivity of antisera produced by inoculation of membranes* (3·5 *mg. protein*) *or soluble fraction* (5 *mg. protein*) *of* Mycoplasma hominis *SC4 into rabbits*

(The activity of a typical antiserum to whole cells is included for comparison.)

Antiserum to	Zone of growth-inhibition (mm.)	Titre			No. of precipitin lines with	
		Metabolic inhibition	Indirect haemagglutination	Complement-fixation*	Detergent-lysed membrane	Soluble fraction
Purified membrane	3–5	160	2560–5120	160	3	0
Soluble fraction (freeze–thaw)	0	< 10	160	1280–2560	2†	5
Whole cells	4–6	2560–5120	5120	2560	3	6

* Antigen = whole cell suspension of SC4. † Very faint.

Immunogenicity

Of the antisera against purified membranes and the soluble freeze–thaw fraction, only that against the membranes had growth- and metabolism-inhibiting activities (Table 5). In the indirect haemagglutination test, the membrane antiserum had a titre of 2560–5120, whereas that of the antiserum to the soluble fraction was only 160 (Table 5). In gel-diffusion tests, the membrane antiserum reacted only with detergent-lysed membrane and not with soluble fractions, but antiserum to the soluble fraction gave two faint lines with both crude and purified membranes (Table 5). It is highly probable, therefore, that the IHA activity of this serum was due to small amounts of antibody to membrane antigens. The absence of MI antibody and the small amounts of IHA antibody in the antiserum to the soluble fraction was not due to a general failure of the immune response in the rabbit; the complement-fixing titre of this antiserum was higher than that of the membrane antiserum (Table 5). Preimmunization sera gave no reaction in growth-inhibition and gel-diffusion, and had titres of < 10 in the other tests.

Extracts of whole cells by chemical methods

On the average, 22·2% of the dry weight of the original cells was recovered in chloroform–methanol extracts, which contained 0·9% protein. In the aqueous acetone extract, 16·5% of the dry weight of the cells was recovered, and 3·2% of this was protein. Neither extract had much complement-fixing activity compared with whole cells (Table 6), confirming the results of Kenny (1967) with chloroform–methanol extracts of *M. hominis*. Chloroform–methanol extracts, dispersed with glass beads or by sonic treatment, were equally active. The low reactivity of the chloroform–methanol extracts with homologous serum is apparently specific, in that they did not react with antisera to *Mycoplasma orale* type 1, *M. salivarium* and *M. arthritidis*. This is contrary to Kenny's observation that a chloroform–methanol extract of *M. hominis* cross-reacted with antisera to other mycoplasma species. Kenny attributed the cross-reactions to lipids adsorbed by the mycoplasmas from the calf or horse serum in the medium. The reaction of the lipid extracts with homologous antiserum is unlikely to be due to an antigen of the Forssman type (Provost, Perreau & Queval, 1964), since all antisera were absorbed by sheep erythrocytes before titration.

Table 6. *Serological activity of extracts of* Mycoplasma hominis *SC* 4 *with antiserum to whole cells of SC* 4

Suspension or extract under test	Complement-fixing activity	No. of precipitin lines (gel-diffusion)	Titre in metabolic inhibition test after absorption by suspension or extract*
Whole-cell suspension	1·00	NT	20
Chloroform–methanol	0·15	1	1280–2560
Aqueous acetone	0·12	1†	NT
Residue after aqueous acetone extraction	0·57	NT	NT
Aqueous phenol (at 68° C.)–aqueous phase	0·00	0	NT
Aqueous phenol (at 4° C.)–aqueous phase	0·00	0	NT
Aqueous phenol (at 4° C.)–alkaline extract of phenolic phase	0·68	4	160
Potassium hydroxide	0·81	4	80

* Titre of unabsorbed serum in metabolic inhibition test = 2560.
† Very faint. NT = not tested.

The chloroform–methanol extract did not absorb MI antibody (Table 6). In gel-diffusion tests with whole cell antisera, it gave a single, rather diffuse line, but this did not link up with any of the lines given by the soluble fraction or detergent-lysed membranes. The aqueous acetone extracts gave only a faint reaction in gel-diffusion.

The aqueous phases after warm or cold aqueous phenol extraction contained, respectively, 4·1 and 15·8% of the dry weight of the cells extracted and gave a

positive Molisch reaction for carbohydrate. However, neither of the extracts was active in complement-fixation or gel-diffusion tests (Table 6).

The solid from the phenolic phase after cold aqueous phenol extraction, represented 31 % of the dry weight of the cells extracted and contained 81 % protein. An extract of the solid with dilute sodium hydroxide contained 27 % of this protein and was appreciably active in complement fixation and in absorbing MI antibody (Table 6). In gel-diffusion, the extract contained at least three components in common with detergent-lysed membranes, and one component corresponding to line 1 of the soluble fraction (Pl. 2, fig. 2*a*, *b*).

The soluble material extracted from whole cells with dilute potassium hydroxide contained, on the average, 11·6 % of the original cell protein. Like the phenolic extract, it was active in complement fixation, gel-diffusion and the absorption of MI antibody (Table 6). It contained two gel-diffusion components (Pl. 2, fig. 3*a*, *b*) in common with detergent-lysed membranes, one of which was also shared with the phenolic extract (Pl. 2, fig. 2). Components corresponding to lines 1 and 3 of the soluble fraction were also present, but it is clear that the serologically reactive components in the phenolic and potassium hydroxide extracts were derived mainly from the membrane. It is noteworthy that none of the major precipitating components of the soluble fraction, i.e. those which are associated with lines 4, 5 and 6, was present in any of the extracts.

DISCUSSION

The diversity of antigenic structure within the genus *Mycoplasma* is emphasized by our results with *M. hominis*. Whereas a major lipid hapten can be extracted from *M. pneumoniae* with chloroform–methanol or acetone (Kenny & Grayston, 1965; Marmion *et al.* 1967; Soběslavský, Prescott, James & Chanock, 1966, 1967), comparable extracts of *M. hominis* have little serological activity and clearly do not contain any major hapten or antigen. Nor is there any serologically reactive polysaccharide comparable to that in *M. mycoides* var. *mycoides*; the aqueous phases from phenol extractions of *M. hominis* were consistently inactive, whereas similar extracts of *M. mycoides* contain the galactofuranose polysaccharide hapten (Plackett *et al.* 1963). In contrast, components of *M. hominis* active in complement-fixation, metabolic inhibition and gel-diffusion tests behave as proteins in that they pass into the phenolic phase during aqueous phenol extraction and can be extracted from the cells with dilute alkali. Thus, *M. hominis*, the only species that does not ferment carbohydrates and whose antigens have been studied, has antigenic determinants which are different from those of the carbohydrate-fermenting species. What is known of *Mycoplasma* species antigens suggests that the mycoplasmas comprise a heterogeneous group, a supposition compatible with the pronounced differences in the DNA base ratios within this genus (Neimark, 1967; McGee, Rogul & Wittler, 1967).

It was possible by gel-diffusion and immunoelectrophoresis to distinguish the antigens characteristic of the membrane of *M. hominis* from those of the soluble cell contents, and to identify them in any fraction or extract. Moreover, the anti-

gens which stimulate the production of growth-inhibiting and MI antibody are in the membrane rather than in the soluble fraction, judging from the ability of membrane fractions to stimulate MI antibody formation and to absorb MI antibody from antiserum to whole cells. The association of MI antibody with membrane components is also suggested by the ability of the phenolic and potassium hydroxide extracts to absorb MI antibody, since both contained mainly membrane components. It seems that IHA activity also is associated with the membrane. Since all three methods used for disruption resulted in some contamination of the soluble fractions with membrane, the low level of activity of soluble fractions in inducing or absorbing IHA antibody and in sensitizing erythrocytes for agglutination, is probably only due to membrane fragments.

The antigens associated with metabolic inhibition and indirect haemagglutination are apparently in the membrane of other mycoplasmas. Thus, Williams & Taylor-Robinson (1967) found that MI antibody was induced by membrane fractions, but not by the cell contents, of *M. gallisepticum* and *M. pneumoniae*. Moreover, in *M. pneumoniae* the lipid hapten, which is undoubtedly derived from the membrane, is active in blocking growth-inhibiting, MI and IHA antibody (Soběslavský *et al.* 1966, 1967; Lemcke, Plackett, Shaw & Marmion, 1968).

It is apparent from our fractionation studies that the cells of *M. hominis* are more difficult to disrupt than those of *M. laidlawii*, which lyse in solutions of low tonicity and from which membranes can be prepared by osmotic shock. Kim, Clyde & Denny (1966), using the criterion of cell viability, also found that a strain of *M. hominis* was relatively resistant to osmotic lysis, sonic treatment and alternate cycles of freezing and thawing. Of the three methods used to disrupt cells, sonic treatment released the most protein from the cells and gave the highest yield of membrane fraction, but gel-diffusion tests showed that the soluble fractions produced by this method were frequently contaminated with membrane components that did not sediment at 100,000 ***g***. It is of interest in this connexion that Pollack, Razin, Pollack & Cleverdon (1965) found membrane-associated enzymes in the 'soluble' fraction of sonically treated cells of *M. laidlawii*. Nevertheless, although sonic disruption is unsuitable for preparing soluble fractions, it seems to be the only practical method for preparing membranes in sufficient quantities for antigenic analysis. Soluble fractions produced by gas cavitation under nitrogen were also contaminated with membranes, although in amounts too small to be demonstrable in gel-diffusion tests or by immunoelectrophoresis. Freezing and thawing proved to be the best method for producing soluble fractions, although even they were not entirely free from membrane antigens.

It is, however, towards the isolation and characterization of the membrane antigens that current work is being directed, since they rather than soluble fractions are associated with metabolic inhibition and indirect haemagglutination. The corresponding antibodies may be of greater significance than complement-fixing antibodies in the diagnosis of naturally occurring human infections with *M. hominis*. Thus, Taylor-Robinson *et al.* (1965) found that indirect haemagglutination was more sensitive than complement fixation for detecting antibody to an oral strain of *M. hominis* in the serum of patients of all ages. Jones & Sequeira

(1966), who measured the complement-fixing and MI antibodies to *M. hominis* in about 3000 sera, concluded that MI antibody, although occurring less frequently than complement-fixing antibody, is probably produced only in response to severe infection. The characterization of the antigens associated with metabolic inhibition and indirect haemagglutination should help to elucidate the significance of these antibodies in human infection with *M. hominis*.

SUMMARY

A genital strain of *Mycoplasma hominis* was fractionated by differential centrifugation after disruption of the cells by alternate cycles of freezing and thawing, by gas cavitation under nitrogen, or by ultrasonic treatment.

Antigens of the cell membrane were distinct from those in the soluble cell contents, judging from metabolic inhibition (MI), indirect haemagglutination (IHA), gel-diffusion and immunoelectrophoresis tests, and by the antibody response in rabbits inoculated with these fractions. The antigens which gave rise to MI and IHA antibody were located in the cell membrane.

Extraction of whole cells of *M. hominis* by various chemical methods suggested that the active components were protein in nature and that there was no lipid hapten, as in *M. pneumoniae*, or polysaccharide, as in *M. mycoides* var. *mycoides*.

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EXPLANATION OF PLATES

PLATES 1–2

Gel-diffusion and immunoelectrophoresis reactions of fractions and extracts of *M. hominis* SC4 with antiserum to whole cells of SC4. Antiserum in centre wells in gel-diffusion tests.

PLATE 1

Fig. 1 (*a*). Precipitin patterns given by soluble fractions produced by freezing and thawing (Sf), by gas cavitation (Sg) and by sonic treatment (Ss). (*b*) System of numbering precipitin lines given by soluble fractions with antiserum to whole cells.

Fig. 2. Precipitin patterns of soluble fraction (Sg), detergent-lysed membrane (Mt) and detergent-lysed cells (Ct). Detergent was Triton X-100, 5 mg. per mg. of membrane or cell protein.

Fig. 3. Precipitin patterns of crude membrane fraction (Mt), membrane purified on a sucrose density gradient (Mp) and cells (Ct) after treatment with Triton X-100 (5 mg. per mg. of membrane or cell protein).

Fig. 4. Gel-diffusion reactions showing presence of membrane components in soluble fractions produced by sonic treatment of *M. hominis* SC4. Soluble fractions (Ss), crude membranes lysed with sodium lauryl sulphate, 5 mg. per mg. membrane protein (Ms). Arrow indicates common component.

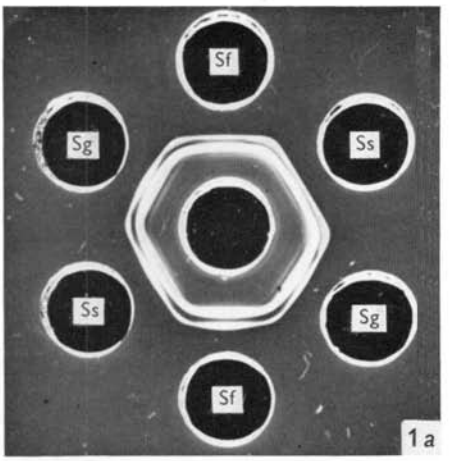
Fig. 5 (*a*) and (*b*). Immunoelectrophoresis of membranes lysed with detergents. (*a*) Purified membranes (Mp) and crude membrane (Mt) lysed with Triton X-100. (*b*) Crude membranes lysed with sodium lauryl sulphate (Ms), soluble fraction included as a control (Sg). Both detergents at 5 mg. per mg. membrane protein. Endosmosis (drift toward cathode) of components in soluble fraction stronger than in Pl. 2, fig. 1.

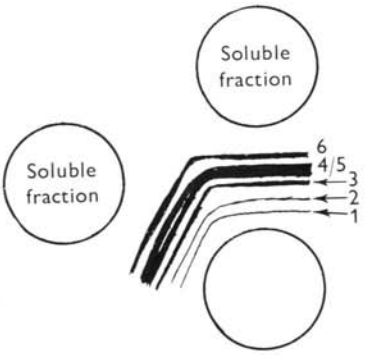
PLATE 2

Fig. 1 (*a*) and (*b*). Immunoelectrophoresis of soluble fractions produced by gas cavitation (Sg) and by sonic treatment (Ss), and of membrane not treated with detergent (M).

Fig. 2 (*a*) and (*b*). Comparison of precipitin lines given by phenolic extract (P, derived from phenolic phase after cold, aqueous phenol treatment) with those given by soluble fraction (Sg) and membrane lysed with 5 mg. sodium lauryl sulphate per mg. membrane protein (Ms). Potassium hydroxide extract (K) included for comparison.

Fig. 3 (*a*) and (*b*). Comparison of precipitin lines given by potassium hydroxide extract (K) with those given by soluble fraction (Sg) and membrane lysed with 5 mg. sodium lauryl sulphate per mg. membrane protein (Ms).





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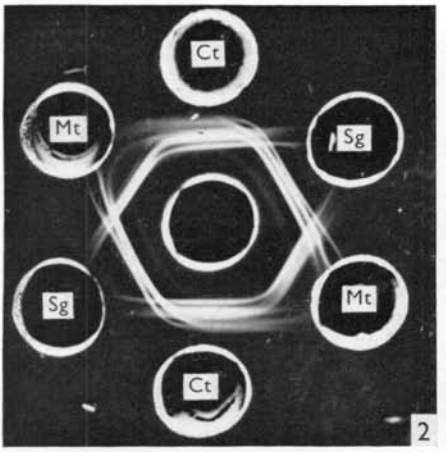

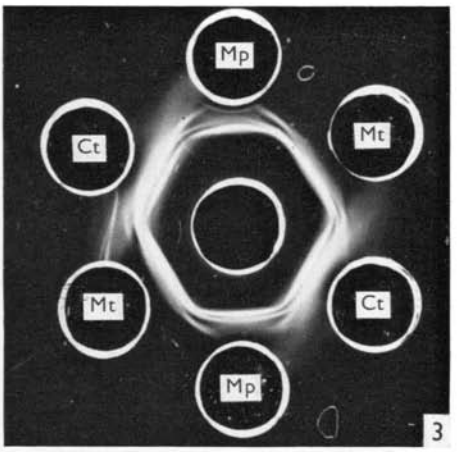

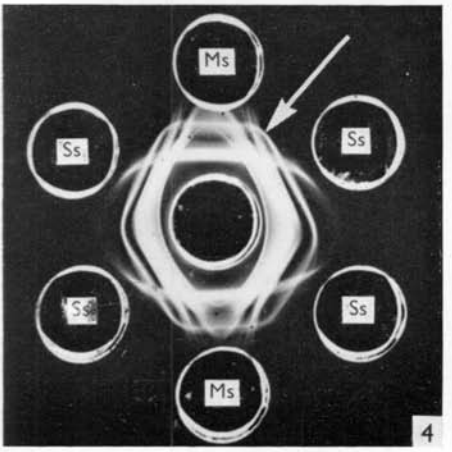

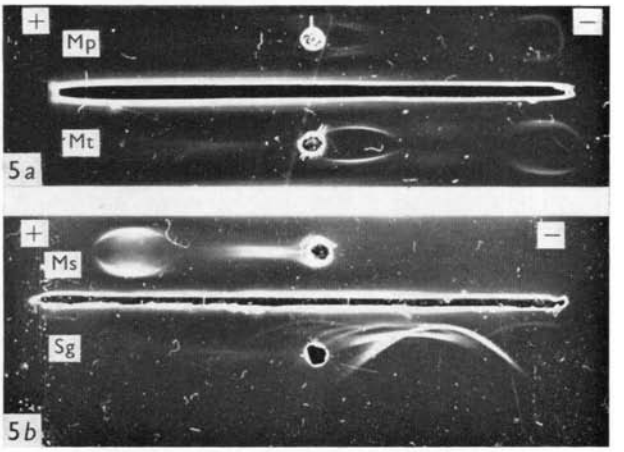

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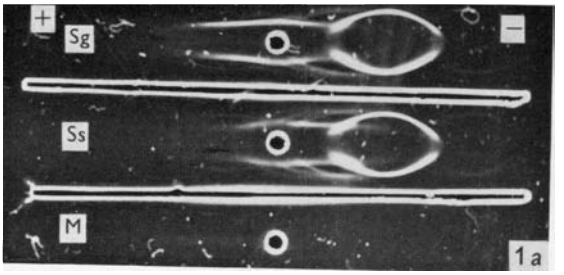
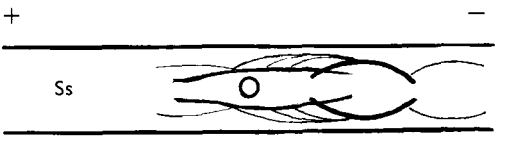
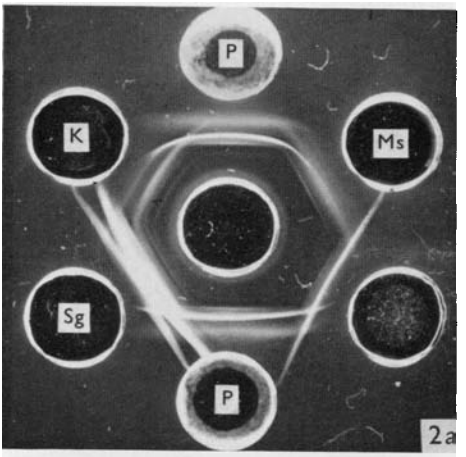
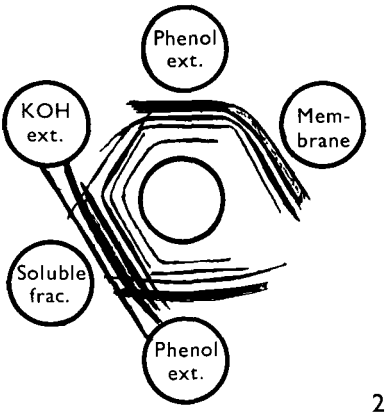


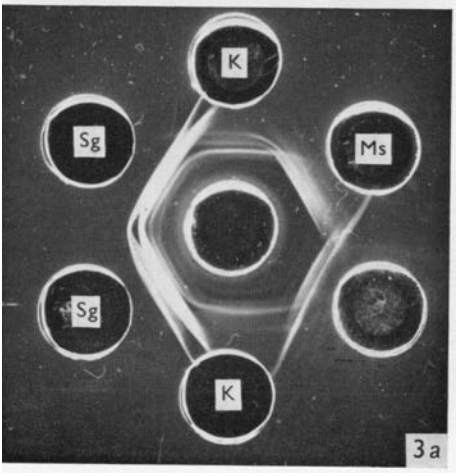


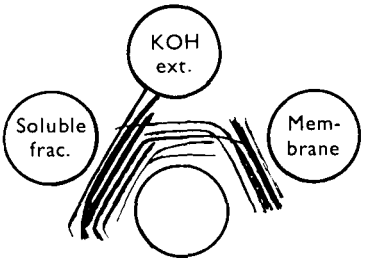
1*b*





2*b*





3*b*

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