Biochemical Analysis of Membranes

Edited by
A.H. Maddy

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Preface

In natural membranes biochemists have, perhaps for the first time, attempted the analysis of a dynamic supramolecular structure. This study requires the application of the most sophisticated physico-chemical techniques, yet the interpretation of the data obtained by these techniques is ultimately limited by our knowledge of the molecular components of membranes, and the provision of information on these building units falls largely within the province of the biochemist. It is hoped that this book will assist biochemists, and biologists without a formal biochemical training, involved in this quest in two ways; firstly, by providing practical guidance on the most important current techniques, and secondly by providing sufficient theoretical background to these techniques to enable workers to modify the details to fit their own precise requirements.

The book falls into two sections, the first of which deals with methods for the isolation of various types of membrane, (the absence of a chapter on the erythrocyte membrane is not due to an oversight but due to the fact that several excellent modern reviews on this subject already exist, e.g. G. Schwach and M. Passow (1973), *Molec. Cell. Biochem.*, 2, 197; thus making a further one superfluous), whilst the second is devoted to biochemical methods for the study of the isolated membranes. In this latter section the first four chapters relate to the isolation and chemical analysis of the membrane proteins, lipids and carbohydrates, while later chapters are concerned with the spatial distribution of lipids and proteins within membranes, and a review of the application of modern immunological methods to membrane study. Each chapter ends with a special section devoted to experimental procedures.

August 1975 A. H. Maddy

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Preface

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SECTION ONE

1 Techniques for the manipulation of mycoplasma membranes

SHMUEL RAZIN and SHLOMO ROTTEM

1.1 Introduction
1.2 Cultivation of mycoplasmas
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1.1 Introduction

Mycoplasmas are minute prokaryotic micro-organisms, varying in shape from spherical structures with diameters of 0.3 to 1.0 μm to slender branched filaments of uniform diameter (0.2–0.3 μm) ranging in length from a few μm to 150 μm. Hence, the name *Mycoplasma*, fungus form (Fig. 1.1). Electron microscopy of thin sections of mycoplasmas reveals an extremely simple ultrastructure, supporting the idea that these may be the simplest and most primitive organisms capable of autonomous growth. The mycoplasma cell is built of only three organelles: the cell membrane, the ribosomes, and the prokaryotic nucleoid (Fig. 1.2). Unlike all other prokaryotes, mycoplasmas have no cell walls or intracellular membranous structures. The absence of a cell wall in mycoplasmas is a characteristic of outstanding importance, to which the mycoplasmas owe many of their peculiarities, for example, their morphological instability, osmotic sensitivity, tendency to penetrate and grow in depth of solid media, resistance to antibiotics which interfere with cell wall synthesis, susceptibility to lysis by detergents, alcohols, specific antibody and complement etc. [1,2].

The fact that mycoplasma cells contain only one membrane type, the plasma membrane, constitutes one of their most useful properties for membrane studies since it is certain that the plasma membrane, once isolated, is uncontaminated with other membrane types. This is also true for erythrocytes. However, working

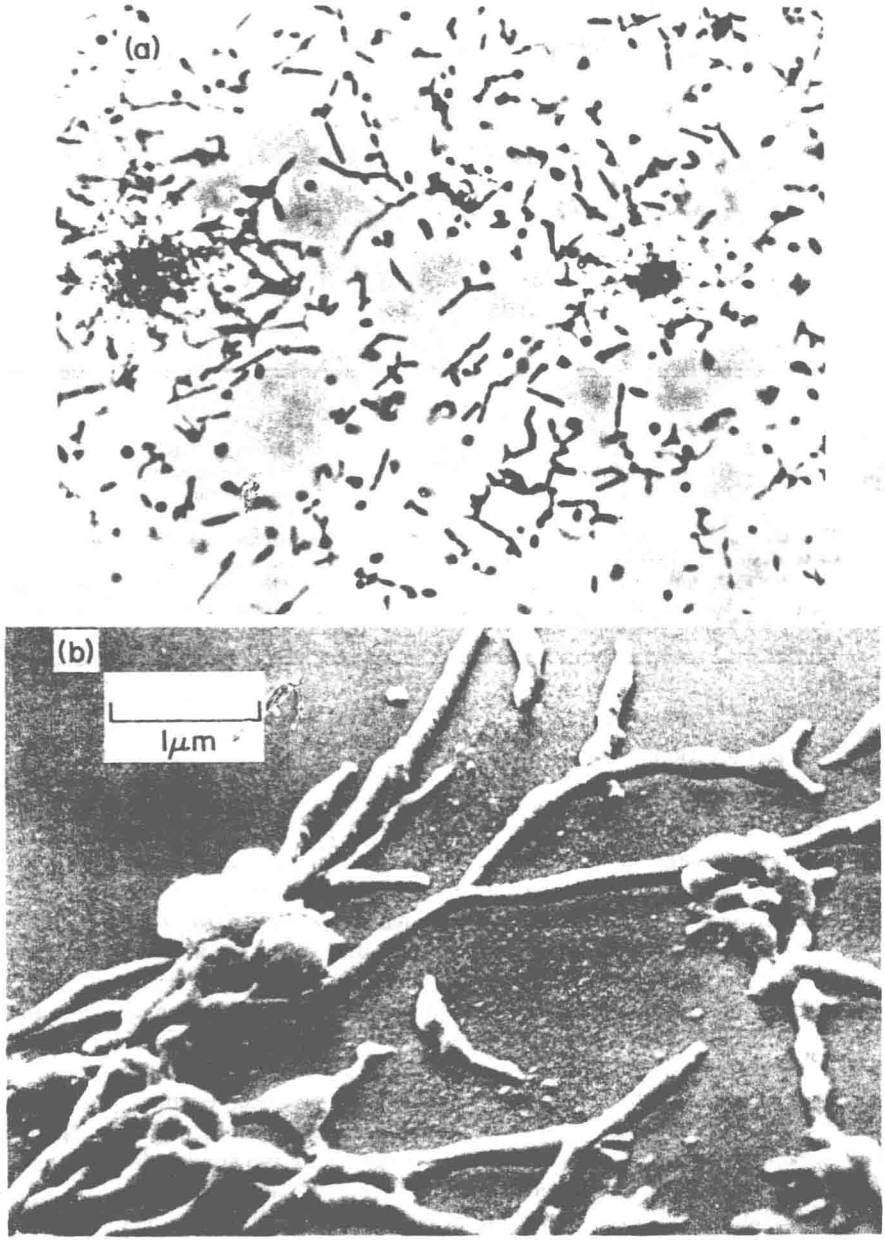

Fig. 1.1 (a) Phase-contrast micrograph of a 24 hour broth culture of *Acholeplasma laidlawii* (x1250). From Razin, S., Cosenza, B. J. and Tourtellotte, M. E. (1967), *Ann. N.Y. Acad. Sci.*, **143**, 66. (b) Scanning electron micrograph of a 6 day culture of *Mycoplasma pneumoniae*. From Biberfeld, G. and Biberfeld, P. (1970), *J. Bact.*, **102**, 855. The micrographs show the characteristic morphological elements that can be seen in mycoplasma cultures. These include branched filaments, chains of cocci and single spherical cells.

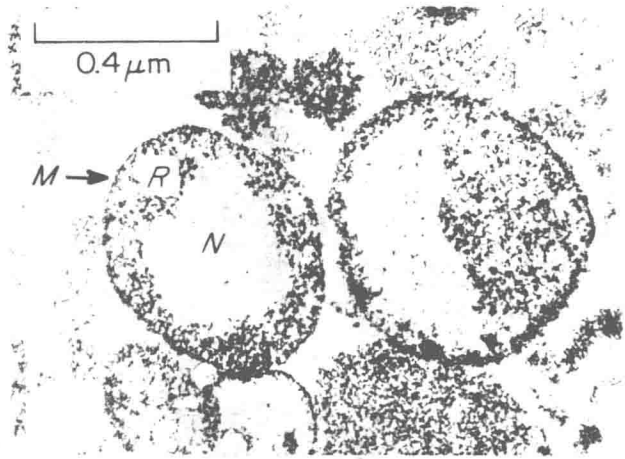

Fig. 1.2 Thin sections of *Mycoplasma hominis* cells showing the trilaminar shape of the plasma membrane (*M*), ribosomes (*R*), and the fibrillar nucleoid region (*N*). From Anderson, D. R. and Barile, M. F. (1965), *J. Bact.*, **90**, 180.

with mycoplasmas has the added advantage that the mycoplasma cell is fully capable of growth and reproduction. This can be exploited to introduce variations in membrane composition in ways not at all possible with erythrocyte membranes. The minute mycoplasmas, which are all parasites, lack many of the biosynthetic pathways found in the more familiar bacteria, such as *E. coli*, and therefore need complex growth media to provide the numerous essential nutrients. These include membrane lipid components or their precursors. This dependence on an external supply of lipids provides a powerful tool for the introduction of controlled changes in membrane lipids, so that the effects of specific lipid components on membrane structure and function can be analysed as will be described in detail in a later section. For extensive recent reviews on the physiology of mycoplasmas and their membrane biochemistry the reader is referred to Maniloff and Morowitz [3] and to Razin [2,4].

The number of established species of mycoplasmas has been increasing most markedly during the past few years. Some mention should, therefore, be made of the taxonomy and nomenclature of these organisms. All the mycoplasmas are members of the class Mollicutes (soft-skin organisms) established in 1967 to include all the prokaryotes without cell walls. This class so far contains only one order, Mycoplasmatales, and two families, *Mycoplasmataceae* and *Acholeplasmataceae*. The *Mycoplasmataceae* is divided into two genera: *Mycoplasma*, which includes the cholesterol-requiring species, and *Ureaplasma*, which are also capable of hydrolysing urea (T-mycoplasmas), while the *Acholeplasmataceae*, which do not require cholesterol for growth, contains so far a single genus, *Acholeplasma* [5–7]. The trivial term 'mycoplasmas' is generally used to include all the organisms classified in both families of the order

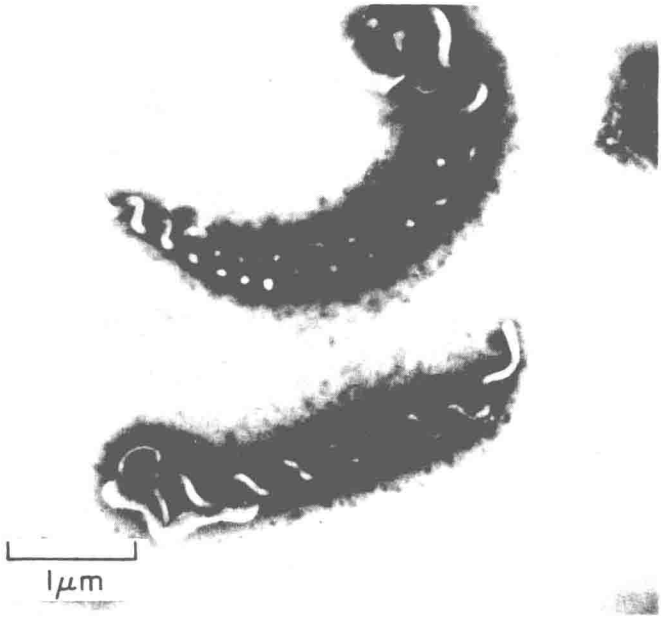

Fig. 1.3 A negatively-stained 2 day culture of *Spiroplasma citri* showing the characteristic helical filaments. From Cole, R. M., Tully, J. G., Popkin, T. J. and Bové, J. M. (1973), *J. Bact.*, **115**, 367.

Mycoplasmatales. A group of organisms resembling the mycoplasmas in most characteristics, but differing in that they have a helical shape (Fig. 1.3), has recently been described and cultured from diseased plants and insects and named *Spiroplasma* [8,9]. Another group of micro-organisms which seem to be less closely related to mycoplasmas though they, too, lack a cell wall are thermophilic organisms isolated from coal refuse piles [10]. These organisms grow best at 59°C and at pH 2.0 and so were named *Thermoplasma acidophilum*. Their remarkable stability to lysis by osmotic shock, by detergents, and by heat [11] suggests an unusual membrane structure. Recent analysis of their membrane components indeed showed some unusual features such as the presence of long-chain isopranols instead of fatty acids [12], a remarkably low number of free amino and carboxyl groups in membrane proteins [13] and 'highly rigid' lipid regions [14].

1.2 Cultivation of mycoplasmas

1.2.1 Growth media

Mycoplasmas are usually cultivated in complex media containing serum or serum components, heart infusion, peptone and yeast extract. Only a few species have

so far been cultivated in defined or semi-defined media [15]. Most of the *Mycoplasma* species and all the *Acholeplasma* species can be grown in the modified Edward medium [16] consisting (per litre) of:

Bacto-heart infusion broth, 13 g	K_2HPO_4, 2.4 g
Bacto-peptone, 5 g	Sodium deoxyribonucleate, 0.02 g
Bacto-yeast extract, 7 g	Bacto-PPLO serum fraction, 20 ml
Glucose, 5 g	Penicillin G, 300 000 units
NaCl, 2.5 g	Thallium acetate, 0.25 g.

A basal medium containing the heart infusion broth, peptone, yeast extract, sodium deoxyribonucleate and NaCl is prepared and its pH is adjusted to 8.0. The basal medium is autoclaved and may be kept at room temperature. Stock solutions of glucose (500 mg ml^{-1}, w/v), K_2HPO_4 (240 mg ml^{-1}, w/v) and thallium acetate (25 mg ml^{-1}, w/v) are prepared, autoclaved and kept at 4°C. For compounding the complete medium, 10 ml of each of the glucose, K_2HPO_4 and thallium acetate solutions, 20 ml of the PPLO serum fraction and a solution containing 300 000 units of penicillin G are added to each litre of the basal medium. The complete medium should be stored in the cold and used within a period of 1–2 weeks. Commercial lots of PPLO serum fraction may differ in their growth-promoting activity [17]. For growth of most of the laboratory-adapted strains, 3–5% (v/v) of horse serum can replace the more expensive and often less effective PPLO serum fraction.

To cultivate the more fastidious mycoplasmas, in particular for primary isolation, the PPLO serum fraction and the Bacto-yeast extract are replaced by 20% (v/v) horse serum and 10% (v/v) yeast extract prepared from dry yeast according to the procedure of Hayflick [18] and commercially available from several sources (cf. Microbiological Associates, Bethesda, Md.). The addition of 20 mM L-arginine to the medium greatly improves the growth of the non-fermentative mycoplasmas that possess the arginine dihydrolase pathway [19]. Growth of *Ureaplasma* (T-mycoplasmas) is most markedly improved by the addition of 0.05% urea to the medium [20]. *Spiroplasma citri* will grow in the modified Edward medium supplemented with sorbitol (7%, w/v), tryptone (Difco) (1%, w/v), sucrose (1%, w/v), fructose (0.1%, w/v), and glucose (0.1%, w/v) according to Saglio *et al.* [8]. The concentration of horse serum in the culture medium may be reduced to 5% (v/v) without greatly reducing the growth of laboratory-adapted *S. citri* strains (Razin, unpublished observation).

Since mycoplasmas incorporate exogenous fatty acids into their polar lipids, the membrane phospholipids and glycolipids of mycoplasmas can be specifically labelled by adding a radioactive fatty acid to the growth medium. Effective labelling of membrane lipids of all the mycoplasmas tested so far has been achieved by adding 25–50 μCi of ^{3}H-oleic acid or ^{3}H-palmitic acid per litre of the growth medium. Membrane proteins may by labelled by adding 100–200 μCi of ^{14}C-L-phenylalanine to the medium [21].

1.2.2 Growth conditions

Although most mycoplasma species are facultative anaerobes, they grow better aerobically. However, primary isolation of many strains requires an atmosphere of 95% N_2 and 5% CO_2. The optimal pH for mycoplasma growth varies between 6.0 and 8.5. The fermentative strains, such as *A. laidlawii*, usually produce higher yields when the pH of the medium is adjusted to 8.0–8.5, while the arginine-hydrolysing strains, such as *M. hominis*, and the urea-splitting *Ureaplasma* strains produce higher yields when the initial pH of the medium is adjusted to 6.5. These strains will stop growing and eventually lyse once the pH of the growth medium reaches 8.0–8.5 due to the ammonia liberated from the arginine or urea hydrolysed [7,22].

Growth of most laboratory-adapted mycoplasmas can be followed by pH changes in the medium and may be assessed by turbidity measurements at 640 nm [23]. With the fast-growing mycoplasmas, yields of up to 100–150 mg cell protein per litre of medium may be expected. For successful membrane isolation the organisms must be harvested before the end of the logarithmic phase of growth, when turbidity is still on the rise. *A. laidlawii* cultures may reach this point after 16 to 24 hours of incubation, depending on medium batch and inoculum size. The turbidity of the culture at this point is about 0.4 at 640 nm and the pH of the medium drops from the initial value of about 8.5 to about 7.5.

1.2.3 Criteria for culture purity

Since the media used for growing mycoplasmas are very rich, even a few contaminating bacteria may overgrow the slower-growing mycoplasmas. The incorporation of high concentrations of penicillin and thallium acetate into the mycoplasma media usually prevents growth of air-borne contaminants but may fail to prevent the growth of contaminants originating from water, in particular pseudomonads. A rapid and simple check for bacterial contamination can be made by examining a drop of the culture by phase-contrast microscopy. The contaminating bacteria can easily be distinguished from mycoplasmas by their much larger dimensions, different shape, and frequently by their motility, as is the case with pseudomonads. A Gram-stained preparation might also be useful for detecting contamination. Mycoplasmas are Gram-negative and stain rather poorly.

On the whole, it is relatively easy to detect the contamination of a mycoplasma culture by other bacteria, yeasts or fungi. A heavier and coarser turbidity are hints of possible contamination, which should be verified by phase-contrast microscopy. It is much more difficult to detect cross-contamination of a mycoplasma culture with another mycoplasma species, an unfortunate event which may occur in laboratories working on different mycoplasma species simultaneously. In this case turbidity changes, phase

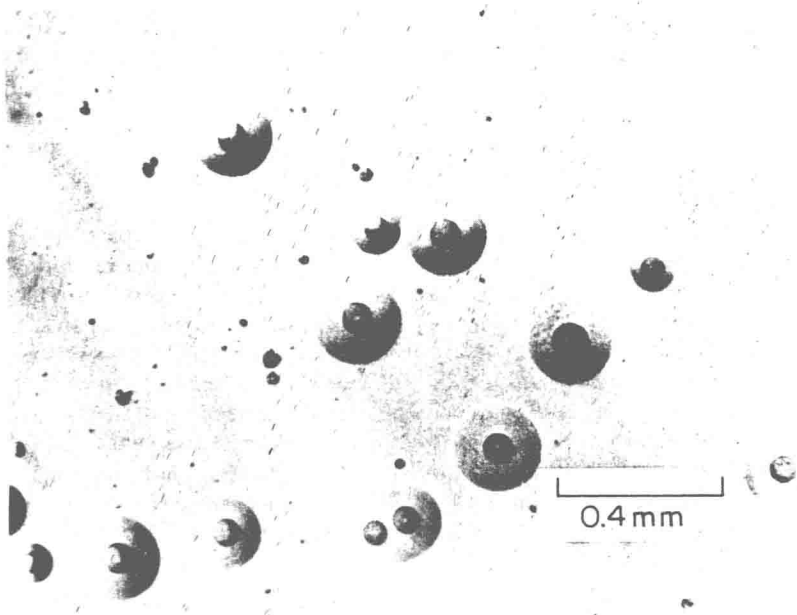

Fig. 1.4 A 4 day culture of *Acholeplasma laidlawii* on solid medium showing the characteristic 'fried-egg' colonies. From Razin, S. and Oliver, O. (1961), *J. gen. Microbiol*, **24**, 225.

contrast microscopy of culture and even colony morphology have little or no value. The different mycoplasmas have an essentially similar colony shape resembling a fried egg (Fig. 1.4). The colony consists of a central region embedded in the agar and a peripheral zone spread on the agar surface [24]. The best, and frequently the only, way to distinguish a mixed mycoplasma culture is by use of specific antisera. Antisera conjugated with fluorescein *iso*thiocyanate may be used for the direct identification of mycoplasma colonies on the plates [25]. Isolated colonies from the suspect culture may be cloned [26] and their identity verified by the growth inhibition test using standard antisera [27]. Reference antisera and seed are available from the FAO/WHO International Reference Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, DK 8000 Aarhus C, Denmark, or from Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

1.2.4 Storage of mycoplasma cultures

Some of the mycoplasma broth cultures may be kept in the cold for at least several weeks without total loss of viability. However, it is a routine practice in