been well studied in the cat retina. Ganglion cells exhibit morphological exuberance beginning roughly at E50, a time when synaptogenesis within the inner plexiform layer has just commenced (*17*), and reach their peak complexity at about the time of eye opening (1 week postnatal). Then there is a decline to nearly adult levels by about 1 month after birth, which suggests that visual experience may play a role in the loss of morphological exuberance. The period of decline also coincides with the time during which retinal ganglion cells are capable of rearranging their dendrites in response to the destruction of their neighbors (*18*). Thus, these transient processes may represent morphological correlates of the competition for presynaptic inputs that Perry and Linden (*18*) have suggested underlies the phenomenon of dendritic remodeling. Retinal ganglion cells could communicate through these processes with each other directly or via other cell types, thereby providing a morphological basis for local synaptic interactions that may play a role in the establishment of retinal ganglion cell form and function.

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7. It is possible to confuse ganglion cells with glial cells or displaced amacrine cells early in development. However, neither of these cell types can be labeled by the injection of microspheres into retinorecipient targets, either in adults or in fetal animals. In addition, amacrine cells were never labeled in postnatal animals that had been injected with microspheres when of fetal age—thus suggesting that amacrine cells never project an axon centrally (*8*).
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A Defined Medium for a Fastidious Spiroplasma

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A defined medium (H-1) was developed for cultivation of the suckling mouse cataract agent, *Spiroplasma mirum*, a fastidious member of the class Mollicutes that causes cataracts and chronic brain infection in inoculated neonate mice. The H-1 medium was used to show the importance of sphingomyelin as a growth factor for the culture of the spiroplasma in vitro. The growth of *Spiroplasma mirum* and the pathology it induces in sphingomyelin-rich tissues in vivo may be related to this dependency.

THE SUCKLING MOUSE CATARACT agent (SMCA) was isolated in 1961 from a pooled extract of rabbit ticks, *Haemaphysalis leporispalustris*, collected from a cottontail rabbit (*1*). Thought to be a slow virus prior to 1973 (*1*) and a mycoplasma-like organism thereafter (*2, 3*), SMCA was shown in 1976 to be a spiroplasma (*4*) (Mollicutes, Spiroplasmataceae) (*5*), and was eventually named *Spiroplasma mirum* (*6*).

Primary isolates of *S. mirum* were originally obtained by inoculating embryonated chicken egg yolk sacs with tick extracts; the cultured organisms killed the eggs in 4 to 10 days (*1*). High titers of the agent were demonstrated in the eyes and brains of intracerebrally inoculated newborn mice; cataracts, retinitis, and chronic brain infections resulted. The severity of symptoms varied and appeared to depend on the *S. mirum* strain used (*1, 7*). In 1971, *S. mirum* was cultivated in rabbit lens organ culture (*8*) and in 1984, in rabbit lens cell culture (*9*). Recently, Kotani *et al.* showed that *S. mirum* could infect and transform mammalian cells in vitro; malignant tumors were observed in mice inoculated with the transformed cells (*10*).

Although initial attempts to cultivate SMCA in conventional liquid or solid media were unsuccessful, Tully *et al.* (*11*) succeeded in 1977 in cultivating *S. mirum* from the allantoic fluid of embryonated chicken eggs in a newly developed medium (SP-4). This medium, based on a defined cell culture medium [CMRL 1066 (*12*)], also contained complex undefined additives such as peptones, yeast hydrolysate, fresh yeast extract, and serum. Strain SMCA growing in SP-4 medium reached titers of 10^8 to 10^9 infectious units per milliliter, as determined by end point titrations after 5 to 7 days of incubation. The growth rate of *S. mirum*, very slow in comparison to most spiroplasma species, suggested that *S. mirum* and similar spiroplasmas possess specialized nutritive requirements.

The SP-4 formulation was subsequently found to be an excellent medium for other spiroplasmas, particularly those associated with ticks (*13*), but it also proved to be useful for isolation and cultivation of certain slow-growing nonhelical mollicutes such as *Mycoplasma pneumoniae* (*14*) and *M. genitalium* (*15*). Once this unanticipated growth enhancement of human mollicutes had been discovered, a search began for factors in the SP-4 medium that were responsible for growth enhancement.

Initially, we analyzed the effect of deletion

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of SP-4 medium components on the growth of strain SMCA. Amino acids, and to a lesser extent peptones, were found to be particularly important for growth of the spiroplasma. However, a high background level of undefined components made it difficult to assess the importance of single factors. The study of growth enhancement could best be pursued with a defined medium, but the extremely fastidious nature of strain SMCA appeared to rule out the development of such a medium. Nevertheless, the eventual development of defined media for cultivation of certain fast-growing spiroplasmas (*16*) suggested that a defined medium could be developed for the fastidious *S. mirum*. We report here the development of such a defined formulation, medium H-1 (*17, 18*) (Table 1), containing the component sphingomyelin.

Compared to defined media reported for fast-growing spiroplasmas (*16*), medium H-1 contains lower concentrations of inorganic salts and higher concentrations of amino acids, nucleic acid precursors, and cofactors. Since spiroplasmas are defective in their fatty acid biosynthesis (*19*), saturated (palmitic) and unsaturated (oleic) fatty acids were added. To satisfy the sterol requirement (*19, 20*), we added cholesterol. We incorporated sphingomyelin (*21*) because recent observations indicated its importance for certain members of the class Mollicutes. For example, sphingomyelin was found to be preferentially incorporated into the cell membranes of several spiroplasma species (*22*). In membranes, sphingomyelin may act as a stabilizing factor, preserving cellular integrity (*21*). In media, it may interact with cholesterol to form sphingomyelin-cholesterol vesicles that serve as preferred cholesterol donors (*23*).

The SMCA strain of *S. mirum* used for initial cultivation had been passaged 57 times (1:10) in SP-4 medium. To facilitate adaptation to the completely defined medium, this culture was first transferred 33 times in the H-1 basal medium (*17*) plus 20% fetal bovine serum (FBS) [with no serum substitute (*18*)], and then by three passages in basal medium plus 10% FBS and 10% serum substitute. We have subsequently passaged the SMCA strain 60 times (1:10) in H-1 medium. The identity of the cultured organisms was confirmed serologically by the spiroplasma deformation test (*24*) at various intervals, including the 60th passage.

Growth titers of SMCA (*25*) in H-1 medium were originally low; however, after 25 passages in this medium at 30°C, the agent achieved titers of 7×10^7 organisms per milliliter of culture in 3 days, with an exponential doubling time of 21 hours (Fig. 1). Similarly, after 45 passages, titers were 2×10^8 organisms per milliliter with a doubling time of 7 hours. Titers of 5×10^8 organisms per milliliter were obtained at the

Table 1. Composition of H-1 medium.

Buffer (mg/liter)	
Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)	16,000
Inorganic salts (mg/liter)	
$CaCl_2 \cdot 2H_2O$	95
KCl	180
KH_2PO_4	285
$MgSO_4 \cdot 7H_2O$	95
NaCl	2,350
$NaH_2PO_4 \cdot H_2O$	285
Organic acids (mg/liter)	
α-Ketoglutaric acid (Na)	290
Pyruvic acid (Na)	190
Oxalacetic acid (Na)	190
Amino acids (mg/liter)	
L-Alanine	380
β-Alanine	95
L-Arginine · HCl	1,900
L-Asparagine	550
L-Aspartic acid	950
L-Cysteine · HCl	550
L-Glutamic acid	1,400
L-Glutamine	1,700
Glycine	380
L-Histidine	760
L-Hydroxyproline	450
L-Isoleucine	450
L-Leucine	900
L-Lysine	380
L-Methionine	380
L-Phenylalanine	950
L-Proline	950
L-Serine	380
L-Threonine	200
L-Tryptophan	380
L-Tyrosine	380
L-Valine	380
Nucleic acid precursors (mg/liter)	
Adenosine	45
Cytidine	45
Guanosine	45
Inosine	45
Thymidine	45
Uridine	45
2'-Deoxyadenosine	45
2'-Deoxycytidine	45
Nucleic acid precursors (mg/liter)	
2'-Deoxyguanosine	45
5-Methyldeoxycytidine	2.0
Inosine monophosphate	2.0
Uridine 5'-triphosphate	4.5
D-(−)-Ribose	9.0
2'-Deoxy-D-ribose	9.0
Cofactors (mg/liter)	
Cocarboxylase	4.5
Coenzyme A	4.5
Flavin-adenine dinucleotide	4.5
Nicotinamide adenine dinucleotide	4.5
Nicotinamide adenine dinucleotide phosphate	4.5
Vitamins and reducing agents (mg/liter)	
Ascorbic acid	30
Biotin	0.45
Calcium pantothenate	0.85
Choline chloride	0.65
Folic acid	0.45
Glutathione	25
i-Inositol	0.65
Niacin	0.45
Niacinamide	0.45
p-Aminobenzoic acid	0.45
Pyridoxal · HCl	0.45
Pyridoxine · HCl	0.85
Riboflavin	1.30
Thiamine	0.45
Carbohydrates (mg/liter)	
Fructose	900
Glucose	4,800
Sodium glucuronate	2.0
Sucrose	1,900
Trehalose	950
Xylose	100
Lipids	
Cholesterol (mg/liter)	20
Oleic acid (mg/liter)	10
Palmitic acid (mg/liter)	10
Sphingomyelin (mg/liter)	20
Tween 40 (ml/liter)	0.1
Tween 80 (ml/liter)	0.1
Proteins (mg/liter)	
BSA, essentially fatty acid–free	11,750
Other components	
Penicillin G (10^5 U/ml)	10
Phenol red (0.2%) (ml/liter)	10
*p*H 7.35; 440 mOs*M*	

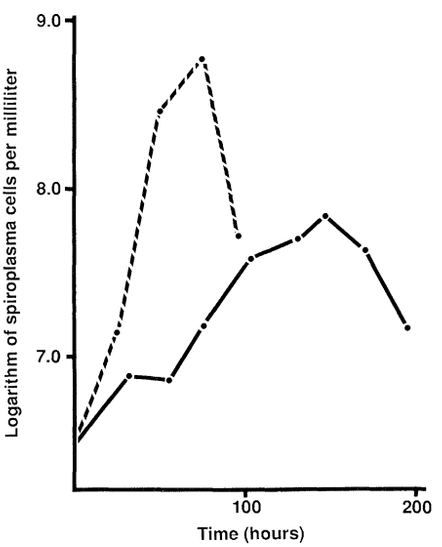

Fig. 1. Growth of the 25th passage of H-1 medium–adapted *S. mirum* strain SMCA in H-1 medium (solid line) and SP-4 medium–adapted SMCA in SP-4 medium (dashed line), at 30°C. Titers were determined by standard microscopic enumeration methods (*25*).

60th passage. The cultured organisms retained their characteristic helical morphology and motility in H-1 medium. Under the same growth conditions, SMCA reached titers of 6×10^8 organisms per milliliter in SP-4 medium, with an exponential doubling time of 6 hours. At 37°C, strain SMCA achieved titers in complete H-1 medium that were comparable to those at 30°C. Adaptation of strain SMCA to H-1 medium without sphingomyelin was very slow. Even after 60 passages in this medium variation, the spiroplasma reached titers of no more than 5×10^6 organisms per milliliter in 5 days at 30°C. At 37°C, spiroplasmas in the absence of sphingomyelin became essentially spherical within 24 hours, showed no evidence of multiplication, and were not viable (that is, they did not multiply when transferred to SP-4 medium).

Growth rates of spiroplasmas in conventional media vary enormously. Although some of the fastest growing spiroplasmas have been cultivated in defined media, slow-growing spiroplasmas had remained noncultivable in defined formulations (*26*). Prior to this study, *S. mirum* had been thought of as one of the slowest growing spiroplasmas, and it had been feared that its biochemical pathways and their pathogenic consequences could be studied only in undefined medium. Medium H-1 and the important growth factor sphingomyelin provide new opportunities for studying the metabolic basis for pathology induced by *S. mirum* in vertebrates. The critical importance of sphingomyelin for in vitro cultivation of strain SMCA at the mammalian body temperature of 37°C, the association of sphingomyelin with plasma membranes, particularly of neural and hepatic tissues (*27*), and the multiplication and persistence of *S. mirum* in the brain, retina, and liver of inoculated mice (*1, 7, 8*) suggest that sphingomyelin dependency should be investigated as a possible factor determining *S. mirum* tissue tropism and pathogenesis in mammals.

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18. Preparation of serum substitute. To 2.0 ml of warm (30°C) 100% ethanol, add (while stirring): 5 mg of palmitic acid, 5 mg of oleic acid, 10 mg of cholesterol, 10 mg of sphingomyelin, 0.05 ml of Tween 40, and 0.05 ml of Tween 80. While stirring, add 0.4 ml of this preparation dropwise to 19.6 ml of 6% BSA (essentially fatty acid–free, No. A-7511, Sigma), and adjust to *p*H 7.4 with 1*N* NaOH. Sterilize by filtration (0.22-μm pore diameter) and store at 26°C.
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Identification of a Family of Muscarinic Acetylcholine Receptor Genes

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Complementary DNAs for three different muscarinic acetylcholine receptors were isolated from a rat cerebral cortex library, and the cloned receptors were expressed in mammalian cells. Analysis of human and rat genomic clones indicates that there are at least four functional muscarinic receptor genes and that these genes lack introns in the coding sequence. This gene family provides a new basis for evaluating the diversity of muscarinic mechanisms in the nervous system.

MUSCARINIC RECEPTORS MEDIATE many of the actions of acetylcholine in the central and peripheral nervous systems (*1*). Two pharmacologically distinct classes of such receptors, M1 and M2, have been defined on the basis of their affinities, high and low, respectively, for the antagonist pirenzepine (*2*). However, a variety of heterogeneous properties of muscarinic receptors have not always correlated well with this classification, which suggests that there may be other classes. It has been unclear whether the apparently different receptors represent modifications of a single receptor or whether they are different proteins. Recently a porcine brain muscarinic receptor complementary DNA (cDNA) was cloned and expressed in *Xenopus* oocytes to produce functional receptors (*3*). The presence of related messenger RNA (mRNA) in tissues rich in M1 receptors and its absence in tissues rich in M2 receptors suggested that there is more than one muscarinic acetylcholine receptor gene. More recently a related porcine cardiac cDNA sequence with the tissue distribution expected of M2 receptors was reported (*4*). Although no expression data were presented to verify that it is a muscarinic receptor, its identity has since been confirmed by expression of the porcine gene (*5*) and the corresponding human gene in mammalian cells.

Muscarinic receptors are members of a large class of neurotransmitter, hormone, and light receptors which act through binding to and activation of guanosine 5′-tri-

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