pacemakers are located in paired structures in bilaterally symmetric invertebrates, and the degree to which bilaterally paired oscillators are mutually coupled appears to be quite variable among species (*16, 17*). Hudson and Lickey (*18*) demonstrated that the two bilaterally symmetric circadian pacemakers in the eyes of *Aplysia* can become desynchronized from each other and free-run out of phase under constant lighting conditions. One interpretation of our data is that the bilaterally paired SCN might function as separate circadian oscillators in a manner similar to that of the paired oscillators of invertebrates. To extend this analogy further, the two SCN oscillators in the hamster might normally be coupled, but this coupling might be altered under certain environmental conditions (such as constant light), thus giving rise to the split condition.

Our results demonstrate the importance of the interaction of the SCN in the generation of circadian rhythms. One interpretation of this interaction consistent with the results is that each SCN has the capacity to serve as a circadian oscillator. Another possibility is that a set of interacting pacemakers may reside within each SCN, and the loss of the split rhythm may be a consequence of the total number of these oscillators destroyed; whether or not the destruction is unilateral may not be important. Experiments designed to sever the reciprocal neural connections between the SCN and partial bilateral SCN lesions in animals with split activity rhythms may clarify the role of the interaction between the SCN in the generation and maintenance of circadian rhythms.

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Spiroplasmas: Cultivation in Chemically Defined Medium

Abstract. *A chemically defined medium, CC-494, supports the cultivation in vitro of several spiroplasmas belonging to three distinct serogroups. Medium CC-494 supports the growth of flower spiroplasmas FS 23-6 and FS SR-3 and of honey bee spiroplasma HBS AS-576. The maximal populations of the two flower spiroplasmas and honey bee spiroplasma are comparable to those cultured in the undefined medium C-3G. The growth rate for all three spiroplasmas is slower in the defined medium.*

Spiroplasmas, which are organisms pathogenic to plants, insects, and several vertebrates, have been grown in media that, like those used for mycoplasma cultivation, are supplemented with one or more undefined constituents such as PPLO (pleuropneumo-like organismic) broth base, horse or fetal bovine serum, and yeast extract. These constituents are complex and difficult to replace with defined chemicals (*1*). Although completely defined media (*2*) have been developed for *Mycoplasma mycoides* and *Acholeplasma laidlawii*, no defined formulation has been reported for the culture of spiroplasmas. Knowing the chemical nature of the culture medium is important for determining the nutritional requirements, metabolic pathways, and biosynthetic capabilities of spiroplasmas and for characterizations of spiroplasma isolates. We now report the successful cultivation of spiroplasmas in a chemically defined medium.

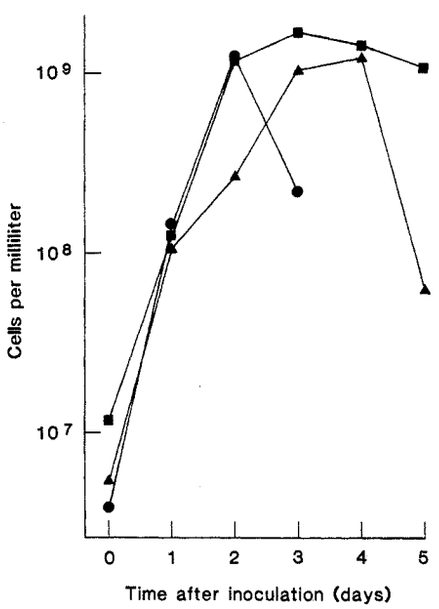

Fig. 1. Growth of FS 23-6 ●, FS SR-3 ▲, and HBS AS-576 (■) under optimal osmolarity.

Three strains of spiroplasmas, representing three of the six serogroups (*3, 4*), were used for the study. Flower spiroplasmas FS 23-6 (ATCC 29989) and FS SR-3 (ATCC 33095) were isolated from flowers of tulip trees in Maryland and Connecticut, respectively (*5*). Honey bee spiroplasma HBS AS-576 (ATCC 29416) was isolated from diseased bees (*6*). Routinely, the spiroplasmas were maintained in C-3G medium (*7*).

A defined medium was developed for the spiroplasma cultivation; CC-494 supports the growth of FS 23-6, FS SR-3, and HBS AS-576 (Table 1). The basal medium was prepared by mixing stock solutions (*8*) of the different fractions in Hepes. The lipid portion and bovine serum albumin (BSA) were prepared separately (*9*) and added to the basal medium in the ratio 1:4 (by volume). The *p*H of the basal medium and lipid-BSA portion was adjusted to 7.5 before mixing. The completed medium was then filter-sterilized (pore diameter, 0.45 μm), and 2.5-ml portions were dispensed into test tubes. Inoculum (30 μl) of each spiroplasma from a culture in the log phase of growth was added to each tube containing CC-494 medium, and the cultures were incubated at 31° ± 1°C. Subse-

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quent serial subcultures were made by transferring 30 μl of the log-phase culture into 2.5 ml of fresh medium for at least ten passages to avoid carry-over of unknown factors from the original undefined medium. To date, FS 23-6 has been subcultured for more than 100 passages, and FS SR-3 and HBS AS-576 have been subcultured for 70 passages in CC-494.

Seven different osmolarities ranging from 420 to 750 mos*M* were prepared for the defined medium by the addition of various concentrations of sodium chloride. Thirty microliters of spiroplasma culture at log phase was inoculated into each of seven tubes, each tube containing a medium of different osmotic properties. Each set of spiroplasma cultures was duplicated. The osmolarity required for optimal growth of each of the three spiroplasmas, based on the measurement of maximal cell populations (*10*) was determined to be 420 mos*M* for FS 23-6, 570 mos*M* for HBS AS-576, and 620 mos*M* for FS SR-3. Under optimal osmolarity, the maximum growth (in cells per milliliter) was 1.96×10^9 for FS 23-6, 1.63×10^9 for FS SR-3, and 3.29×10^9 for HBS AS-576 (Fig. 1). The growth rates of the three spiroplasmas in the defined medium were slower than those in undefined media; the population of FS 23-6 reached its peak in 2 days, of FS SR-3 in 4 days, and of HBS AS-576 in 3 days in our medium, as compared to 1 day for each in the undefined medium C-3G (*11*). The maximal populations of the two flower spiroplasmas and the honey bee spiroplasma in CC-494 were comparable to those attained in C-3G (*11*).

Bovine serum albumin, a protein containing 582 sequenced amino acids, used in our medium (*12*) probably serves as the transport vehicle for long-chain fatty acids such as palmitic and oleic acids, which are poorly soluble in aqueous solution. The formation of a noncovalent complex with BSA reportedly allows appreciable quantities of these free fatty acids to dissolve in the medium. We do not know whether the BSA in our medium is metabolized.

Table 1. Composition of CC-494.

Buffer (g/liter)	
Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)	11.25
Inorganic salts (g/liter)	
$CaCl_2$	0.46
KCl	1.15
$MgSO_4 \cdot 7H_2O$	2.18
NaCl	2.1 (FS 23-6) 8.4 (FS SR-3) 6.3 (HBS AS-576)
$NaHCO_3$	1.20
$NaH_2PO_4 \cdot H_2O$	0.06
Na_2HPO_4	0.07
KH_2PO_4	0.03
Keto acids (g/liter)	
α-Ketoglutaric acid–monosodium salt	0.30
Pyruvic acid–sodium salt	0.30
Nucleosides and nucleotide (mg/liter)	
Adenosine	36.0
Guanosine	36.0
Cytidine	36.0
Thymidine	36.0
Uridine	36.0
2′-Deoxyadenosine	36.0
2′-Deoxyguanosine	36.0
2′-Deoxycytidine	36.0
5-Methyl-deoxycytidine	1.8
Uridine-5′-triphosphate	3.6
Carbohydrates (mg/liter)	
Glucose	8.0×10^3
D-(−)-Ribose	4.5
2′-Deoxy-D-ribose	4.5
Amino acids (mg/liter)	
L-Alanine	270
L-Arginine	270
L-Aspartic acid	210
L-Asparagine	270
L-Cysteine	210
L-Glutamic acid	450
L-Glutamine	630
Amino acids (mg/liter)	
Glycine	135
L-Histidine	225
L-Isoleucine	90
L-Leucine	90
L-Lysine	900
L-Methionine	450
L-Phenylalanine	90
L-Proline	900
L-Serine	135
L-Threonine	180
L-Tryptophan	54
L-Tyrosine	270
L-Valine	180
Vitamins (mg/liter)	
Ascorbic acid	45.0
Biotin	0.009
Calcium pantothenate	0.009
Choline chloride	0.45
Folic acid	0.009
i-Inositol	0.045
Niacin	0.023
Niacinamide	0.023
p-Aminobenzoic acid	0.045
Pyridoxine HCl	0.023
Pyridoxal HCl	0.023
Riboflavin	0.009
Thiamine HCl	0.009
Lipids (mg/liter)	
Palmitic acid	12.30
Oleic acid	9.20
Cholesterol	18.50
Tween 40 (ml/liter)	0.095
Tween 80 (ml/liter)	0.095
Bovine serum albumin (g/liter)	12.0
Others (mg/liter)	
NADP	3.60
FAD	1.80
Glutathione (reduced)	9.00
Cocarboxylase	0.90
Coenzyme A	1.13
Sodium acetate $\cdot 3H_2O$	75.00
Sodium glucuronate	3.75
Phenol red	15.00

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8. Stock solutions (percent): Hepes, 15; $CaCl_2$, 1.9; KCl, 4.8; $MgSO_4 \cdot 7H_2O$, 9.7; NaCl, 17.5; $NaHCO_3$, 5; $NaH_2PO_4 \cdot H_2O$, 3.8; Na_2HPO_4, 0.9; KH_2PO_4, 1.1; α-ketoglutaric acid, 4; pyruvic acid, 4; cysteine, 2; aspartic acid, 1; tyrosine, 0.5; all other amino acids, 3; adenosine, 1.2; guanosine, 1.2; cytidine, 1.2; thymidine, 1.2; uridine, 1.2; deoxyadenosine, 1.2; deoxyguanosine, 1.2; deoxycytidine, 1.2; 5-methyldeoxycytidine, 0.06; uridine-5-triphosphate, 0.12; glucose, 20; D-(−)-ribose, 0.6; deoxy-D-ribose, 0.6; ascorbic acid, 6; biotin (10×), 0.012; calcium pantothenate (10×), 0.012; choline chloride, 0.06; folic acid (10×), 0.12; *i*-inositol (10×), 0.06; niacin (10×), 0.03; niacinamide (10×), 0.03; aminobenzoic acid (10×), 0.06; pyridozal HCl (10×), 0.03; pyridoxine-HCl (10×), 0.03; riboflavin (10×), 0.012; thiamine-HCl (10×), 0.12; nicotinamide-adenine dinucleotide phosphate, 0.12; flavin-adenine dinucleotide, 0.12; glutathione (reduced form), 1.2; cocarboxylase, 0.12; coenzyme A, 0.15; $NaOOCCH_3 \cdot 3H_2O$, 10, sodium glucuronate, 0.5; phenol red, 0.2.
9. For lipid-defined medium (CC-494), 40 mg of palmitic acid, 30 mg of oleic acid, and 60 mg of cholesterol were added to a bottle containing 2 ml of 100 percent ethanol, 0.3 ml of Tween 40, and 0.3 ml of Tween 80. An adequate amount of the above stock solution was added to an adequate volume of 6 percent BSA solution (in water).
10. Two tubes containing 2.5 ml each of fresh defined medium were inoculated with 30 μl of log-phase culture of the test spiroplasmas. Two 5-μl portions of culture sample were pipetted from each tube, and cell number was calculated from counts of ten random fields from each slide (*7*).
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