rats, some with and others without hormonal implants were also fed CD for 17 weeks. Total fat, "steroids," corticosteroids, and ascorbic acid were determined on adrenal glands, liver and tumor. Rats fed CD showed rise in fat, "steroid" and ascorbic acid values as early as 2 weeks and continued high through the experimental period. Adrenocorticosteroid values in intact CD fed rats were 30% higher at 17 weeks than those in control rats. Gonadectomy lowered in males and increased in females steroid values over those in comparable intact rats. Stilbesterol and testosterone implants respectively, in these rats, reversed this effect. It is suggested that high adrenal steroid values indicate increased secretion of adrenocortical hormones, which are necessary to promote the growth of altered cells to a recognizable tumor mass.

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Growth of *Mycoplasma gallisepticum* Strain J Without Serum.* (30969)

PAULINE G. LUND AND MARY S. SHORB
Poultry Science Department, University of Maryland, College Park

The nutritional requirements of the genus *Mycoplasma* are known to be complex(1). All parasitic strains have a protein requirement supplied by serum or serum fraction which is added to defined media(2,3,4). Growth of a saprophyte, *Mycoplasma laidlawii,* has been obtained in a defined medium upon addition of serum fraction, albumin(5, 6), or positively-charged tryptic peptides from ribonuclease(7).

This paper reports the development of media for the growth of the avian parasite, *Mycoplasma gallisepticum* strain J, which permit heavy growth without added serum fraction.

Methods and materials. All work referred to here was done with strain J, kindly given to us by Dr. H. M. DeVolt, Dept. of Veterinary Science, University of Maryland, Col-

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lege Park. For maintenance, Difco PPLO broth, without crystal violet, supplemented with 1.5% (v/v) Difco PPLO serum fraction, 0.3% maltose (w/v) and 0.05% thallium acetate (w/v) was used. Difco PPLO agar, with the serum fraction, maltose and thallium acetate supplements was used for plate counts. To avoid any carry-over of serum protein, the serum fraction supplement was replaced with TEM 4T-cholesterol, each at 10 mg/l. This will be referred to as PPLO TEM 4T-ch. broth. A stable inoculum culture was maintained in this as a 48-hour serial subculture, continuously incubated at 37°. Inoculation of 0.05 ml into 10 ml of experimental medium gave an initial count of 10^7 colony-forming units/ml. The period of incubation is given under *Results* for each set of experiments. Bellco borosilicate glass test tubes, 16 × 150 mm, covered with glass caps were used in all experiments. Before use, the tubes were washed thoroughly with Alconox detergent, rinsed thoroughly with distilled water, filled with distilled water, autoclaved, and dried.

The chemicals used were analytical grade except Hycase,† a vitamin-free, acid-hydrolyzed, salt-free product.

All media (10 ml per tube) were sterilized at 121° for 12 minutes. Coenzyme A and calcium pantothenate were sterilized by filtration and added aseptically to the sterilized assay tubes. Initial pH of all media before autoclaving was 7.8.

† Hycase was obtained from the Sheffield Chemical Co., Norwich, N. Y., amino acids, cholestrol, adenosine, cytidine, guanosine and thymidine from Nutritional Biochemicals Corp., Cleveland, Ohio. Calcium leucovorin (folinic acid) was a gift of Dr. John T. Litchfield, Lederle Laboratories, Pearl River, N. Y.; TEM 4T, a diacetyl tartaric acid ester of tallow monoglyceride, a gift of Dr. H. Birnbaum, Hachmeister Co., Pittsburgh, Pa., and Trypticase, a gift of Dr. Harriette D. Vera, Baltimore Biological Laboratory, Baltimore, Md. Coenzyme A (a product of P-L Biochemicals Inc., Milwaukee, Wisc.) was a gift of Dr. Brinton M. Miller of Merck and Co., Rahway, N. J., who also supplied vit. B_{12}, pantetheine, and calcium pantothenate. Pantethine was from Sigma Chemical Co., St. Louis, Missouri; DL-thioctic acid (α-lipoic acid) from Calbiochem; Los Angeles, Calif.

TABLE I. Growth of *M. gallisepticum* Strain J in Supplemented PPLO Broth.

Supplements to PPLO broth		
Na_2HPO_4, mg/tube	TEM 4T + cholesterol, 100 μg each per tube*	PPLO serum fraction 1%
	ml 0.02N NaOH	
0	3.5	4.3
10	4.3	5.4
20	4.2	6.4
30	4.0	6.4

* 10 ml medium per tube; 3-day incubation.

The extent of growth was measured by the viable count technique as described by Butler and Knight(8) or the titration of the acid formed by the organisms during growth. All titration results are reported as the ml of 0.02 N NaOH required per 10 ml of incubated, inoculated medium to reach the pH of the sterile uninoculated medium, usually 7.6 as measured by a glass electrode.

Results. The Difco PPLO serum fraction in the PPLO broth supplied the required cholesterol and fatty acids. Since TEM 4T and cholesterol were effective lipid supplements for *Trichomonas gallinae*(9) this mixture was tested as a replacement for the PPLO serum fraction. Some growth of *M. gallisepticum* was obtained with 100 μg of each/tube (Table I, column 2). Because the pH of this medium dropped rapidly with growth, dibasic sodium phosphate was added as a buffer. There was an increase in acid production with TEM 4T-cholesterol plus 10-20 mg of buffer/tube; higher levels delayed growth somewhat. As greater acid production was observed with serum fraction plus buffer (Table I, column 3), serum was obviously supplying additional stimulants.

To correlate growth with acid titration, plate counts of colony-forming units were made. A rapid increase in counts, from 10^7 to 10^9 units/ml, occurs within 24 hours on the crude PPLO TEM 4T-ch. broth. The numbers remain stable until the 4th day, then rapidly fall. Acid titration increased a little more slowly, becoming maximum after 3 days' incubation. Counts and titration, however, will vary with different lots of both PPLO broth and serum fraction. These results are similar to those observed with *M.*

TABLE II. Basal Media.

	A	B
	g/l	g/l
Hycase	10.0	10.0 *
L-cystine	.03	.03*
DL-Threonine	.1	.1 *
Trypticase		.3–5.0
DL-Tryptophane	.03	.03
Cysteine HCl	.03	.03
Maltose	5.0	5.0
Trist	6.0	6.0
Adenosine	.01	.01
Guanosine	.01	.01
Cytidine	.01	.01
Thymidine	.01	.01
$MgSO_4 \cdot 7H_2O$	.2	.2
NaCl	6.8	6.8
$(NH_4)_2SO_4$	6.0	6.0
Na_2HPO_4	.1	.1
KCl	.4	.4
	mg/l	mg/l
B_{12}		.01
Biotin		.01
Pantetheine or pantethine		.5
Pyridoxamine phosphate	.5	.5
Lipoic acid	.48	.48
Choline chloride	16.	16.
Razin and Cohen Vitamin mix(6)		
TEM 4T	10.	10.
Cholesterol	10.	10.

* Medium C was like medium B, with Razin and Cohen's amino acid mix(6) replacing Hycase, L-cystine and DL-threonine. Glutamic acid at 100 mg/l replaced glutamine in the amino acid mix(6). Inositol was added at 5.75 mg/l.

† Tris; 2-amino-2 hydroxymethyl-1,3-propanediol.

laidlawii(5). Titration has been used in all later experiments.

Preliminary experiments showed that addition of amino acid mixes and a vitamin mix to PPLO broth resulted in better growth; therefore, experiments with a more defined medium were attempted. A combination of the media of Razin and Knight(5) and Razin and Cohen(6) was tried. Some additions and modifications were made: Hycase was substituted for Bacto Casamino acids, cysteine HCl and threonine were added; thymidine was added to the other nucleosides and the amounts of all nucleosides were reduced. Pyridoxamine phosphate and lipoic acid (dl-α-thioctic acid) were included. Maltose was substituted as the sugar at a reduced level, serum fraction or albumin was omitted and TEM 4T and cholesterol added. The amounts of the above ingredients are shown in medium A, Table II. With these changes, only minimum growth was obtained (1.5 ml titration). When Trypticase was added at 20 mg/tube, growth increased to 3 ml titration.

Razin and Cohen(6) had found ammonium sulfate, and tris (2-amino-2 hydroxymethyl-1,3-propanediol) as a buffer, stimulated the growth of *M. laidlawii*. Therefore, various levels of these compounds were tested to determine the amount needed for optimum growth of *M. gallisepticum*. Fig. 1 shows that *M. gallisepticum* requires, at 5 days' incubation, 60 mg/tube of both tris and ammonium sulphate, when Trypticase is at 20 mg/tube. With these amounts of tris and ammonium sulphate, and Trypticase increased to 50 mg/tube, a titration of 6.4 ml can be obtained at 2 days' incubation. This is the same titration value as found with serum plus sodium phosphate (Table I), after 3 days' incubation.

Medium A was not supplemented with pantothenic acid, biotin, or B_{12}. Since fatty acids are required for growth without serum and because coenzyme A is involved in fatty acid synthesis, the pantothenic requirement was investigated. Since pantetheine is the most active vitamin form used by some bacteria(10), it was tested in basal medium A with 50 mg of Trypticase/tube.

Fig. 2 (chart A) shows the increase in counts of colony-forming units/ml obtained when *M. gallisepticum* was grown with and without 5 μg pantetheine/tube and (chart B) the increase in titration which resulted under these same growth conditions.

With the demonstration of a pantetheine response, the requirement for Trypticase was reinvestigated. Poor growth was observed without pantetheine (Fig. 3, curve A) until levels of 50-100 mg of Trypticase were reached. With pantetheine (Fig. 3, curve B), partial growth occurs with 1 to 10 mg of Trypticase and very good growth with 50 mg/tube. Biotin had been found necessary for growth of *Mycoplasma* by other workers (2,3,7). When biotin and vit B_{12} were added to the medium supplemented with pantetheine, this enhanced growth still further (Fig. 3, curve C). Biotin alone (tests not shown) was responsible for most of this growth and may indicate an absolute requirement for it. Some growth was observed with 1-2 mg of Trypticase and very good growth

with only 10 mg of Trypticase per tube. The composition of the medium producing this growth is shown in Table II, Medium B. This medium with Trypticase at 3 mg/tube has permitted growth of strain J through 100 serial subcultures.

The Razin and Cohen(6) amino acid mix was next substituted for Hycase. Glutamic acid was substituted for glutamine as preliminary work indicated growth with glutamic acid was the same as with glutamine, whether filter sterilized or autoclaved. Inositol was added as trials showed it improved growth slightly. The composition of this medium is shown in Table II, Medium C. Fig. IV illustrates the Trypticase response on basal Medium C, with B_{12}, biotin and pantetheine omitted, curve A; curve B shows growth with these vitamins added, after 48 hours' incubation, while curve C shows slightly improved growth after 72 hours. The level of growth in the amino acid basal medium is lower than

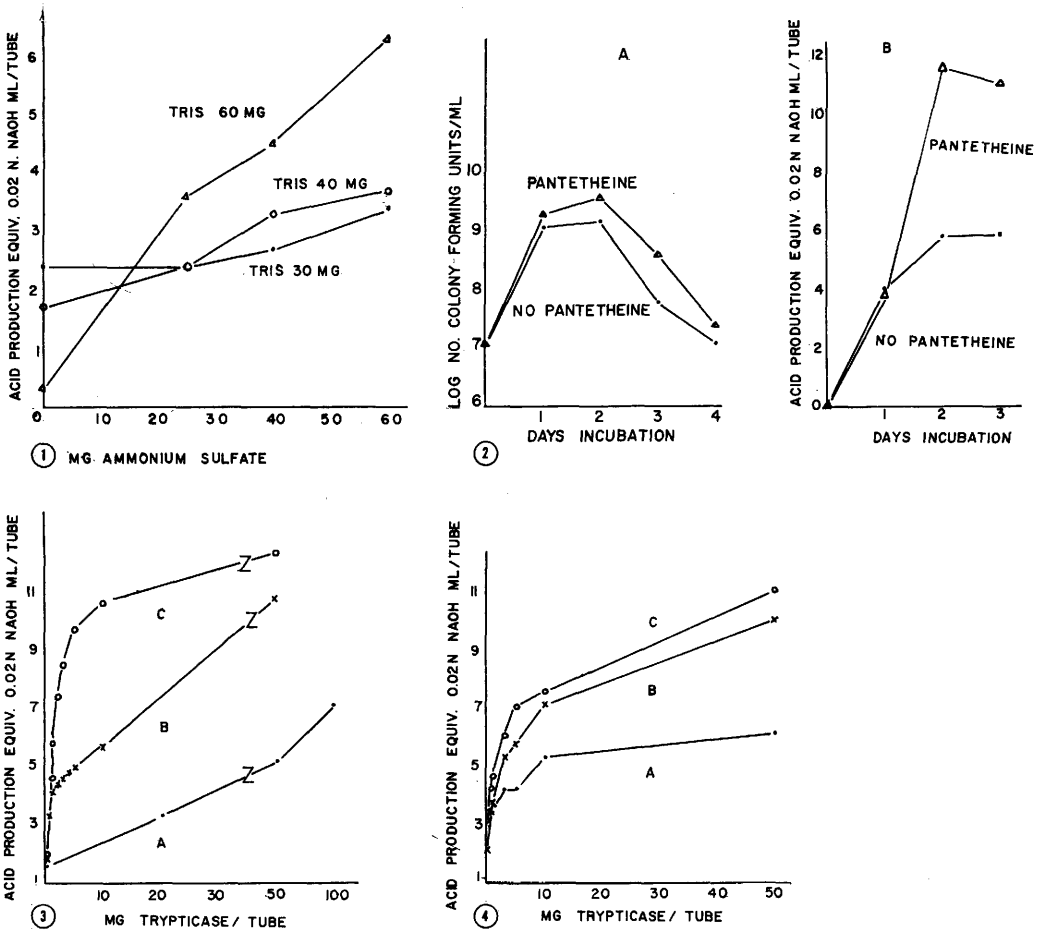

FIG. 1. Effect of ammonium sulfate and tris buffer on growth of *M. gallisepticum* strain J with Trypticase at 20 mg/tube, 5 days' incubation.

FIG. 2. *M. gallisepticum* strain J grown in Hycase basal medium A with 50 mg/tube Trypticase. A) Counts of colony-forming units/ml of *M. gallisepticum*, △——△ with 5 μg pantetheine/tube; ●——● without pantetheine B) Acid production, △——△ with pantetheine at 5 μg/tube ●——● without pantetheine.

FIG. 3. Growth of *M. gallisepticum* strain J in basal medium A in response to Trypticase. Curve A, no additions to basal; curve B, 5 μg pantetheine/tube; curve C, 5 μg pantetheine plus 0.1 μg each of biotin and B_{12}/tube, 2 days' incubation.

FIG. 4. Growth of *M. gallisepticum* strain J in medium C in response to Trypticase. Curve A, no additions to basal medium; curve B, 5 μg pantetheine/tube plus 0.1 μg each of biotin and B_{12}/tube. Curve A and B, 2 days' incubation; Curve C, same as curve B but 3 days' incubation.

TABLE III. Growth of *M. gallisepticum* Strain J on 3 Media, with Various Members of the Pantothenic Acid Group, all at 2 Days' Incubation.

μg of compound/tube	Medium A		Medium B			Medium C†			
						Pantethine		Coenzyme A	
	Calcium pantothenate	Pantetheine	Calcium pantothenate	Panthethine	Coenzyme A	Filtered vitamins*	Autoclaved vitamins*	Filtered vitamins*	Autoclaved vitamins*
	(ml 0.02N NaOH)								
0	5.4	3.8	6.4	6.4	6.4	5.1	5.2	5.1	5.2
.01		4.0							
.1		4.5				7.3	7.4		
1.0		11.1	6.1	10.6	9.0	11.4	10.9	7.9	7.4
3.0				10.6		10.9	10.6		
5.0	5.5	11.5	6.4	8.7	9.4	10.0	10.5	11.0	11.7
10.0			7.0	9.1	11.4	10.3	10.9	10.3	10.1
15.0	5.5	11.3							
20.0			7.3	8.3	11.3	10.3	10.6	11.4	11.5
25.0	5.7	11.3							
50.0			8.0		10.6			7.9	10.5
100.0			7.0						

Trypticase used at 50 mg in all experiments.

* Razin and Cohen's vitamin mix plus vitamin B_{12} were filter-sterilized or autoclaved in the medium, as indicated in Table. Pantethine and pantetheine were autoclaved in the medium while calcium pantothenate and coenzyme A were filter-sterilized.

† Hycase ash, 50 mg/l added to Medium C, unpublished work.

that obtained when Hycase furnishes the amino acids, especially with 1-10 mg of Trypticase (Fig. 3, curve C).

Three members of the pantothenic acid group were tested (Table III) using basal media A, B, and modified C (footnote, Table III). Those tests showing the same titration in the control tube were run on the same day. Calcium pantothenate had low activity, while Coenzyme A, pantetheine and panthethine were much more active. Panthenol was inactive on medium A. Pantetheine might be the most active form if tested on the more complete B medium. It was tested only on A medium. High titration values in all control tubes indicates that 50 mg of Trypticase contains one of the pantothenic acid group.

The activity of pantethine and coenzyme A were not affected by the treatment of the other vitamins, *i.e.*, filter sterilization or autoclaving, nor was pantetheine or pantethine affected by autoclaving. The exact requirements for the pantothenic acid group, as well as biotin and B_{12}, will have to be determined when Trypticase is finally eliminated from the medium.

Discussion. M. gallisepticum strain J does not need a native serum protein for growth as required by the saprophytic *M. laidlawii*(5, 6), and by the pathogenic *M. mycoides*(3) and *M. gallisepticum* strain 293(4). However, the Trypticase required by strain J may be fulfilling a need for nonspecific peptides as reported by Tourtellotte *et al*(7) for *M. laidlawii.*

The ability of *M. gallisepticum* to utilize pantethine and pantetheine demonstrates that these vitamins can be used in the synthesis of coenzyme A. Coenzyme A is probably involved in the formation of the multiple complex lipids present in *Mycoplasma*(11,12,13). The levels of coenzyme A used by *M. gallisepticum* strain J correspond closely to that (1 mg/l) used by Tourtellotte *et al*(7) for *M. laidlawii.*

The lower plane of growth obtained with amino acid medium indicates that Hycase may supply some trace substance needed for growth with low levels of Trypticase. Although Razin and Cohen(6) found glutamine essential for *M. laidlawii,* glutamic acid can be substituted for glutamine in the amino acid medium C for *M. gallisepticum* strain J. As the medium for *M. gallisepticum* is further defined, the requirements for known nutrients may change.

Summary. Mycoplasma gallisepticum strain J has been grown for over 100 serial subcultures in a medium in which the only nondefined components were Trypticase, Hycase

and TEM 4T. Hycase can be replaced by an amino acid mixture, with a slightly lower plane of growth. An interchangeable requirement for pantethine, pantetheine or coenzyme A was demonstrated. Calcium pantothenate had very low activity and panthenol was inactive. Biotin and vitamin B_{12} stimulated acid production during growth.

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The Electroencephalogram in Experimental Allergic Encephalomyelitis in the Lewis Rat. (30970)

BRUCE L. BAXTER AND MARVIN E. ROSENTHALE (Introduced by Richard F. Tislow)
(With the technical assistance of Rosemary Scerni, Rosalie Buonato and Louis Datko)
Pharmacological Evaluation Section, Wyeth Laboratories, Inc., Radnor, Pa.

Experimental allergic encephalomyelitis (EAE) is a distinct, reproducible disease that can be induced in a variety of animal species by injection of nervous tissue incorporated with Freund's adjuvant(1). Although considerable variability has been noted, electroencephalographic (EEG) abnormalities and convulsions have been reported to occur in all of the species so examined: monkey(2), rabbit(3), and guinea pig(4). In this communication we will report the results of a study on the effects of EAE on the EEG of the Lewis rat.

Methods. Electrodes were chronically implanted in 11 male Lewis strain rats (Microbiological Associates, Bethesda, Md., weighing 150-200 g) under sodium pentobarbital anesthesia (35 mg/kg). The electrodes consisted of stainless steel screws driven into the skull over the frontal, temporal and occipital cortex unilaterally on the left side. The screws were wired to a Winchester miniature socket and encased in acrylic cement. Bipolar EEG recordings were obtained using a Grass model III-D electroencephalograph. Recordings were obtained with the animals unrestrained in a 30 by 10 by 10 cm metal box to which the rats had previously been acclimated. A control record was obtained from each rat on day 1. On day 2, a 40% w/v homogenate of isologous spinal cord in distilled water containing 0.5% phenol was emulsified with an equal volume of Freund's complete adjuvant (4 mg/ml killed tubercle bacilli). A single dose of 0.05 ml of the encephalitogenic emulsion was then injected into the right hind foot pad(5). Recordings were obtained on days 4, 6, 7, 8, 11, 12, 13, 14, 15, 17, 19 and 20 after injection of emulsion. This included the period before, during, and after the paralysis observed in EAE. All animals were examined and weighed on the days when EEG records were obtained.

Results. Animals continued to gain weight for a period of 8 days after injection of emulsion but then lost weight rapidly over the next 7 days (Fig. 1). From day 12 to day 17 all animals showed pronounced flaccid hind-