Growth of *Leptospira* in Defined Media

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SUMMARY

Serum-free chemically defined media were developed for cultivating several *Leptospira* strains. *Leptospira canicola*, Ruebeusch strain, was the main test strain. Esterified fatty acids (monoolein, monostearin, methyl palmitate, methyl oleate) replaced serum. Fe^{++} over a narrow range replaced haemin. Thiamine was essential. Other vitamins added to thiamine-containing medium (nicotinic acid, pantothenate, putrescine, *p*-hydroxybenzoic acid, vitamin B_{12}) speeded or increased final growth. Acetate stimulated growth moderately. Ammonium sulphate was favourable over a wide concentration range.

INTRODUCTION

Many investigators (e.g. Woratz, 1955; Fulton & Spooner, 1956; Alston & Broom, 1958; Broom, 1959) have noted that leptospires generally require serum or serum fractions for growth; nevertheless, Savino & Rennella (1944) and Woratz (1957) serially transferred *Leptospira icterohaemorrhagiae* and *L. canicola* without serum or serum fractions. Woratz's medium contained gelatin and a tryptic digest of casein. Savino & Rennella could grow only 4 of their 12 strains in a defined medium containing 'activators'—a mixture of vitamins (nicotinic acid, nicotinamide, thiamine, pyridoxine, riboflavin, pimelic acid) and aspartic acid. The present paper describes a chemically defined medium which permits continued serial growth of various leptospires.

METHODS

Leptospira canicola, Ruebeusch strain, was the initial experimental organism. This strain, originally carried in Korthof medium (Alston & Broom, 1958) containing 10 % (v/v) pooled rabbit serum, was transferred to agar-free basal medium (BM-AF, Table 1) containing 1 % (v/v) serum. This culture served as inoculum for preliminary experiments. A standard loopful (3 mm. diam.) covered with a 10 mm. $\times$ 10 mm. coverglass had about 6 leptospires/high-dry dark field ($\times$ 440) after incubation for 7–14 days at 30°.

For counts by dark field, 0.01 ml. of culture was put on a slide and covered with a 22 mm. coverglass. As determined by ocular micrometer, the area of a high-power ($\times$ 530) field was 0.0962 mm.² The number of fields per coverglass was 484 mm.². (area of the coverglass)/0.096 = 5040. The count/ml. was thus obtained by multiplying the count per high-power field by 5040 $\times$ 100. Usually 20 fields were used for

a count, and duplicate counts agreed to within 10%. With sparse cultures counting was done at a $\times 100$ magnification.

The same concentration of serum in BM medium (containing 0.1% agar) permitted faint growth after 2 weeks as a faint line about 20 mm. below the surface (Dinger's phenomenon: Fletcher, 1928; Lawrence, 1951; Czekalowski, McLeod & Rodican, 1954). Serum at 0.1% (v/v) did not give visible growth in 2-3 weeks.

Cultures of *Leptospira canicola* intended as inocula were transferred serially in BM-AF medium containing 1% (v/v) serum. The initial nutritional experiments were undertaken in BM medium containing 1% (v/v) serum.

The ingredients required for 10 ml. of final medium were dissolved in 9 ml.; the inoculum brought it to 10 ml. Inoculation was by means of a Cornwall side-arm syringe (Becton-Dickenson, B-D, no. 1251) delivering 1 ml. through a short 15-gauge needle passing through a Pyrex filling attachment (Corning No. 3960). Connexions were made between the syringe and needle by means of B-D adapters H 468/L and L/606 inserted in 3 mm. diam. gum-rubber tubing. The inside delivery tip of the attachment was filed back to allow the needle to project freely. Inoculation was made after the autoclaved medium had cooled to about 37°, before solidification of the agar, and was forceful enough to mix the contents of the tube.

It was convenient to prepare BM medium 5-fold concentration without agar. The pH value was adjusted with Quadrol (*N,N,N',N'*-tetrakis (2-hydroxypropyl)-ethylenediamine; Wyandotte Chemicals Corp., Wyandotte, Mich., U.S.A.; Hutner, Cury & Baker, 1958), a few drops of volatile preservative added (Hutner *et al.* 1958), and the concentrate stored in the refrigerator. Test substances were prepared in ten-fold concentration and preserved similarly.

Since distillates from bleached cotton can inhibit fatty acid-sensitive organisms (Drea, 1946), the $\times 16$ 150 mm. Pyrex culture tubes were capped with inverted shell vials. Later experiments were made in 50 ml. screw-cap flasks containing 20 ml. medium. Media were autoclaved at 121° for 15 min.

RESULTS

As growth-promoters were identified, a serum-free but complex medium was assembled (Medium 283, Table 1) which supported serial growth of *Leptospira canicola*, also *L. pomona* L-9 and *L. icterohaemorrhagiae* N.I.H. strain. Dissection of medium 283 to identify its active components led to several defined agar-free media (Table 1) which supported the growth of the three original strains and 9 of 11 additional strains of *Leptospira* (Table 2). The point of departure in developing the defined medium was the observation that a supplement of 1% (v/v) serum permitted growth of *L. canicola*, Ruebeusch strain, in BM and BM-AF media; 0.1% (v/v) serum was ineffective.

Proteose peptone (Difco) increased growth in the presence of 1% (v/v) serum but did not replace serum. Liver fraction 'L' (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.), soluble starch and haemin were inert or inhibitory at higher concentrations.

The Fe:Co ratio was critical; Table 3 shows a typical experiment.

The three original test strains did not grow as well in defined media as in Korthof medium containing 10% (v/v) serum; growth however did exceed that obtained

with 1% (v/v) serum; many strains (Table 4) grew well although less than in Korthof medium with 10% (v/v) serum.

In serum-free medium, 0.5% (v/v) of a blunderbuss mixture consisting of an acid-hydrolysate of gelatin, L-tryptophan, DL-methionine, alkali-hydrolysed yeast nucleic acid, acid-hydrolysed deoxyribonucleic acid and vitamins (complete

Table 1. *Serum-free and chemically defined media for leptospirae*

Media were adjusted to final pH 7.4–7.5. Sterilization was by autoclaving at 121° for 15 min.

	Quantities as mg. or ml./100 ml. final medium.					
	Basal medium (BM)	Transfer medium no. 283	Serum-free medium no. 198 A	Chem.-def. medium no. 198 B	Chem.-def. medium no. 198 E	Chem.-def. medium no. 198 F
KH ₂ PO ₄	10	10	10	10	10	10
MgSO ₄ ·7H ₂ O	40	40	40	40	40	40
Ca ⁺⁺ (as Cl ⁻)*	0.4	0.4	0.4	0.4	0.4	0.4
Agar, Special† (Noble, Difco)	100	100	.	.	.	.
HEDTA‡	.	2.0	2.0	2.0	2.0	2.0
TEM-4T§	.	3.5	3.5	.	.	.
Hycase SF	.	10	.	.	.	.
'Complete suppl. No. 9'	.	0.5 ml.	.	.	.	.
'Metals 45 A'	.	1.2 ml.	.	.	.	.
Fe ⁺⁺	.	.	0.1	0.1	0.1	0.1
Mn ⁺⁺	.	.	0.015	0.015	0.015	0.015
Zn ⁺⁺	.	.	0.15	0.15	0.15	0.15
Cu ⁺⁺	.	.	0.008	0.01	0.01	0.01
Na acetate. 3H ₂ O	.	.	5.0	5.0	5.0	5.0
Co ⁺⁺	.	.	0.0015	0.01	0.01	0.01
(NH ₄) ₂ SO ₄	.	.	.	20	20	20
Nicotinic acid	.	.	.	0.02	0.02	0.02
Thiamine HCl	.	.	0.04	0.04	0.04	0.04
Ca pantothenate	.	.	.	0.02	0.02	0.02
Pyridoxamine.HCl	.	.	.	0.02	0.02	0.02
Vitamin B ₁₂	.	.	.	0.00002	0.00002	0.00002
DL-Alanine	.	.	.	.	10	.
DL-Aspartic acid	.	.	.	.	50	.
DL-Isoleucine	.	.	.	1.0	1.0	.
DL-Leucine	.	.	.	1.0	1.0	.
DL-Methionine	.	.	.	.	0.5	.
DL-Phenylalanine	.	.	.	5.0	5.0	.
DL-Serine	.	.	.	.	1.0	.
DL-Tryptophan	.	.	.	.	2.0	.
DL-Valine	.	.	.	.	5.0	.
Glycine	.	.	.	.	1.0	.
L-Glutamic acid	.	.	.	.	10.0	.
L-Histidine HCl.H ₂ O	.	.	.	.	25	.
L-Proline	.	.	.	.	2.0	.
Monoolein	.	.	.	2.0	2.0	2.0

* Ca was weighed as CaCO₃ and dissolved in dilute HCl.

† Agar may be eliminated = BM – AF.

‡ HEDTA = hydroxyethylethylenediamine-triacetic acid.

§ Supplied by Hachmeister Inc., P.O. Box 357, Pittsburgh, 80, Pa., U.S.A. TEM-4T is a mixture of diacetyl tartaric acid esters of glycerides from tallow.

|| Hycase SF = 'low-salt' acid-hydrolysed casein, Sheffield Chemical Co., Norwich, New York, U.S.A.

supplement No. 9; Hutner *et al.* 1957), supported growth provided that a semi-synthetic fat, TEM-4T (Shorb & Lund, 1959; a mixture of diacetyl tartaric acid esters of glycerides from tallow) was present. Addition of Hycase SF (Sheffield Chemical Co., Norwich, N.Y., U.S.A.), a 'salt-free' acid hydrolysate of casein, increased growth further (Table 5). Of the components of the 'complete supplement', the alkali-hydrolysed yeast nucleic acid had a slight effect, the vitamin mixture somewhat more.

Absolute requirements; lipids and vitamins. Thiamine was essential. Supplementation with nicotinic acid or pantothenate speeded growth. Putrescine also was

Table 2. *Strains of Leptospira investigated*

	Designation	Culture no.
<i>L. autumnalis</i>	Fort Bragg	5287*
<i>L. ballum</i>	Garcia	5303*
<i>L. bataviae</i>	EER (AM 2)	5304*
<i>L. canicola</i>	Ruebeusch	RU 43†
	Utrecht	5937*
	Undesignated	39660*
<i>L. grippotyphosa</i>	Andaman CH 31	5862*
<i>L. icterohaemorrhagiae</i>	Wijnberg	5309*
	Undesignated	39661*
	M 20	5938*
	NIH	NIH†
<i>L. pomona</i>	Johnson	5939
	Undesignated	L-9†

* Supplied through the courtesy of Dr A. H. Harris and Julia M. Coffey, Div. Labs., N.Y. State Department of Health. See *Annual Rep. Div. Laboratories and Research*, 1953, New York State Department of Health, Albany, N.Y., p. 149, 'Collection of Type Cultures'.

† Original strains from Communicable Disease Center, U.S. Public Health Service, Chamblee, Georgia, U.S.A.

Table 3. *Iron-cobalt relations for growth of Leptospira canicola (Ruebeusch strain)*

Medium: basal medium (100 ml.) + TEM-4T*, 3.5 mg. + HEDTA† 2 mg. + 'vitamin mix No. 12', 0.002 ml.; agar ('Noble', Difco, 0.1 %, w/v); pH 7.4–7.5. Fe⁺⁺ was added as Fe(NH₄)₂(SO₄)₂·6H₂O. Growth recorded after incubation for 14 days at 30°. Arbitrary scale: 0 = no growth; + + + + = growth as in Korthof + 10 % (v/v) serum medium. Quantities of Fe⁺⁺ and Co⁺⁺ are in mg./100 ml. medium.

Fe ⁺⁺ (mg.)	Co ⁺⁺ (mg.)				
	No addition	0.0125	0.025	0.05	0.10
	Relative growth				
No addition	0	0	0	0	0
0.031	0	0	0	0	0
0.062	0	0	0	0	0
0.12	++	++	++	++	++
0.25	++	++	+	+	0
0.5	0	+	+	0	0
1.0	0	0	0	0	0

* TEM-4T = a mixture of diacetyl tartaric acid and esters of glycerides of tallow (see Table 1).

† HEDTA = hydroxyethylethylenediamine-triacetic acid.

favourable but not so clearly as was nicotinic and pantothenic acids; *p*-hydroxybenzoic acid and vitamin B₁₂ also stimulated slightly.

In the presence of 1 % (v/v) serum, the optimum concentration of TEM-4T was 3.5 mg./100 ml.; Tween 80 supported growth from about 1 to 80 mg./100 ml. Tweens 60 and 40 were less satisfactory. Cholesterol at 0.5–4 mg./100 ml. and soybean lecithin from 0.6–5 mg./100 ml. were without effect in the presence of serum and did not replace serum.

In serum-free media, monacetin, methyl linoleate, and methyl linolenate at 0.5–

Table 4. *Growth responses of Leptospira strains in serum-free chemically defined media (198B, 198E, 283; see Table 1); and in Korthof medium + 10 % (v/v) pooled rabbit serum*

Flasks inoculated with 10⁵ leptospire/ml. Most inocula made from cultures in 11–15th transfer except *L. canicola* no. 5937 (6th transfer). *L. canicola* (Ruebeusch) inoculation made from culture in 38th transfer; in medium 283; *L. pomona* (L-9) and *L. icterohaemorrhagiae* (N.I.H.) from 28th transfer.

<i>Leptospira</i> spp. species and strain	Defined medium							
	198B		198E		283		Korthof 10% (v/v) rabbit serum	
	Max. growth (leptospire × 10 ⁻⁶ /ml.)		Max. growth (leptospire × 10 ⁻⁶ ml.)		Max. growth (leptospire × 10 ⁻⁶ ml.)		Max. growth (leptospire × 10 ⁻⁶ ml.)	
	Days		Days		Days		Days	
<i>L. bataviae</i> no. 5304	14	40	10	120	.	.	10	360
<i>L. canicola</i> no. 39660	10	85	14	44	.	.	17	140
<i>L. canicola</i> no. 5937	14	65	7	35	.	.	10	220
<i>L. canicola</i> Ruebeusch	14	25	.	.	7	10	10	610
<i>L. grippityphosa</i> no. 5862	.	.	10	100	.	.	10	600
<i>L. icterohaemorrhagiae</i> no. 5938	14	45	10	66	.	.	14	360
<i>L. icterohaemorrhagiae</i> no. 39661	10	100	10	80	.	.	10	170
<i>L. icterohaemorrhagiae</i> N.I.H.	7	60	.	.	7	10	10	600
<i>L. pomona</i> No. 5939	17	33	14	92	.	.	17	800
<i>L. pomona</i> L-9	.	.	.	.	14	.	17	1200

Table 5. *Hycase SF*/TEM-4T† relationships for growth of Leptospira canicola*

Basal medium: HEDTA 2 mg./100 ml.; metals 45A 1.2 mg./100 ml.; 'complete supplement no. 9', 0.5 ml./100 ml., 'Noble' agar 0.1 % (w/v); pH 7.4–7.5.

TEM-4T (mg./100 ml.)	Hycase conc. (mg./100 ml.)				
	No addition	40	20	10	5
10	+	+	±	±	±
5	±	++	++	++	+
2.5	0	0	±	+	±
1.25	0	0	0	0	±

* Hycase SF = low-salt acid hydrolysed casein (see Table 1).

† TEM-4T = mixture of diacetyl tartaric acid esters of glycerides of tallow (see Table 1).

8 mg./100 ml. were unsatisfactory fatty-acid sources. Monoolein, methyl oleate, methyl palmitate or monostearin could replace TEM-4T; monoolein was best. A mixture of 2 mg. each of monoolein, monostearin and methyl palmitate/100 ml. allowed earlier but not consistently heavier growth.

Table 6. *Serial transfer of Leptospira strains in serum-free chemically defined media*

Inoculum size†	Media									
	198A		198B		198E		198F		283	283SS*
	10 %	50 %	10 %	50 %	10 %	50 %	10 %	50 %	10 %	10 %
Species	No. of successful transfers									
<i>L. autumnalis</i> no. 5287	7‡	19	.	23	.	3‡	12‡	23	.	.
<i>L. ballum</i> no. 5303	.	2‡	.	2‡	.	3‡	.	1‡	.	.
<i>L. bataviae</i> no. 5304	21‡	21	25	24	16	.	6‡	18	.	.
<i>L. canicola</i> no. 39660	17	18	25	23	21	21	14‡	23	.	.
<i>L. canicola</i> no. 5937	22	19	1‡	7‡	10	.	1‡	10	.	.
<i>L. canicola</i> (Ruebeusch)	22	23	25	24	1‡	14	20	23	38	39
<i>L. grippotyphosa</i> no. 5862	16‡	17‡	4‡	11	19	15	22	19	.	.
<i>L. icterohaemorrhagiae</i> no. 5938	16‡	14‡	23	24	23	19	9‡	23	.	.
<i>L. icterohaemorrhagiae</i> no. 3228	17	22	.	4‡	.	4‡	.	4‡	.	.
<i>L. icterohaemorrhagiae</i> no. 39661	.	18	22	24	21	19	22	25	.	.
<i>L. icterohaemorrhagiae</i> no. 5309	.	4‡	.	3‡	.	3‡	.	5‡	.	.
<i>L. icterohaemorrhagiae</i> (N.I.H.)	14	25	18	.	1‡	3‡	17	18	29	.
<i>L. pomona</i> no. 5939	20	22	25	22	22	18	13‡	23	.	.
<i>L. pomona</i> (L-9)	6	23	.	3‡	.	3‡	17	13	29	.

* Semi-solid medium.

† Inoculum size; % values refer to volume of culture transferred to volume fresh medium.

‡ Died.

Amino acids. The values given in this section are mg. compound/100 ml. medium. Slight growth stimulation was obtained with glycine (0.8–3 mg.), DL-alanine or L-glutamic acid (12.5–50 mg.), L-lysine HCl or DL-methionine (1.6–6.25 mg.), L-proline or DL-valine (2.5–20 mg.), and DL-aspartic acid (50–100 mg.). Moderate stimulation was obtained by L-histidine (free base) (12.5–50 mg.), DL-isoleucine (1.2–5.0 mg.), DL-leucine (0.65–2.5 mg.), DL-serine (0.008–0.125 mg.), and DL-tryptophan (1.25–10 mg.). Marked stimulation was obtained with DL-phenylalanine (0.6–20 mg.). L-Arginine HCl (0.4–3.1 mg.), DL-threonine (0.078–2.5 mg.), and L-tyrosine (0.078–0.156 mg.) were inert.

A mixture of metals '45A' (Hutner *et al.* 1957) supported growth. Fe⁺⁺ alone supported growth only in a narrow range (0.125–0.5 mg.). Hycase became unnecessary for growth with adequate Fe⁺⁺. Individually added to Fe, Co (0.001–0.04 mg.), Cu (0.0015–0.24 mg.), Mn (0.015–0.02 mg.), or Zn (0.005–0.34 mg.) augmented growth. Since the metal requirements were critical, metal-buffering with the chelating agent of hydroxyethylethylenediamine-triacetic acid (HEDTA;

Provasoli & Pintner, 1960) was inhibitory at low concentrations of serum or TEM-4T. HEDTA 2 mg. was optimal in the presence of low concentrations of serum or in chemically defined media containing the standard metal mixture (Table 1). Acetate was moderately stimulating. Ammonium sulphate increased growth remarkably over a very wide range (1.95–125 mg.). Table 4 shows the growth responses of various leptospiral strains in the experimental media developed. Table 6 indicates the number of successful transfers made when either an equal volume of inoculum was added to fresh media or a 1:10 ratio of inoculum to fresh medium was used.

DISCUSSION

Previous workers were unable to replace serum with amino acids and vitamins (Chang, 1947*a, b*; Green, Camien & Dunn, 1950), or synthetic ion-exchange resins, charcoal or polyvinylpyrrolidone (Marshall, 1949; Fulton & Spooner, 1956; Johnson & Wilson, 1960). That lipid requirements were the major obstacle had been foreshadowed; defatted fraction V of blood serum no longer stimulated respiration of *Leptospira icterohaemorrhagiae* (Helprin & Hiatt, 1957) or supported growth of treponemes (Oyama, Steinman & Eagle, 1953). Our work confirms that of Woratz (1957) and Ivler (1960) that oleic acid esters promote growth of *Leptospira*. As their media had complex natural materials, one could not tell whether the fatty-acid requirement was absolute. A close parallel to our results are those of Power & Pelczar (1959), who found TEM-4T an advantageous source of essential fatty acids for the Reiter treponeme; a mixture of palmitic, stearic and oleic acids permitted the same degree of growth.

That the Fe concentration is critical probably accounts for the seeming dependence of these organisms on compounds such as haemoglobin. Strains not having catalase may appear to need exogenous haeme. Fe seems adequate for strains with catalase. Faine (1960) found appreciable amounts of catalase in certain pathogenic *Leptospira* strains. The optimum concentration of Fe for *Leptospira canicola* agrees well with that reported by Faine (1959) for *L. icterohaemorrhagiae*.

Thiamine, found stimulatory by others, e.g. Savino & Rennella (1944), may well be an absolute requirement for all leptospires.

The results with amino acids obtained by other investigators are difficult to compare with ours; different basal media were used, the presence of serum complicates matters, and the purity of amino acids has improved in recent years. Growth of a wide range of strains in chemically defined media was achieved here without introducing new factors, merely by applying information scattered in the literature. Whether strains which will not grow in the present defined media require additional growth factors or are exceptionally sensitive to nutritional imbalances remains to be decided. Another problem is to improve defined media so that they permit growth equal to or greater than that in media which contain complex natural materials such as serum.

More specific, more conveniently prepared antigens for diagnosis of leptospiral diseases are needed as recognition grows of the gravity and economic importance of these infections in animals and man. The availability of defined media may lead to better media for diagnosis by direct cultivation and to practical antigen-free media for the preparation of diagnostic antigens.

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