Iron as a Growth Requirement for Pathogenic *Leptospira*

By S. FAINE*

*Microbiology Department, Otago University Medical School, Dunedin,
New Zealand*

SUMMARY: Virulent and avirulent strains of *Leptospira icterohaemorrhagiae* did not grow from inocula of fewer than 10^7 organisms/ml. in Korthof medium, but did grow when FeCl_3 or preparations containing iron porphyrins were added. With added FeCl_3 or haematin growth was somewhat slower than when the iron porphyrin-containing preparations were added.

Many authorities have recommended that the serum essential in culture media for pathogenic leptospires should contain small quantities of haemolysed erythrocytes, or that a solution of laked erythrocytes should be added as a 'source of haemoglobin' (Alston & Broom, 1958; Wolff, 1954). There has been no agreement about the need for haemoglobin nor suggestions about its possible functions. This report describes experiments made with Korthof medium which did not support growth of *Leptospira* but did so when FeCl_3 , haematin or preparations containing iron-porphyrin compounds were added.

METHODS

Organisms. The strains used were *Leptospira icterohaemorrhagiae* (Jackson) avirulent for guinea-pigs, and *L. icterohaemorrhagiae* (Field), virulent and in its 5th laboratory subculture since guinea-pig passage. They were obtained in 1953 from Dr J. C. Broom (*Leptospira* Reference Laboratory, Wellcome Laboratories of Tropical Medicine, London) and maintained in modified Korthof medium. Incubation was at 30° .

Counting method. A direct counting method (Faine, 1957) was used.

Culture medium. Broom's modification (Alston & Broom, 1958) of Korthof's medium (Korthof, 1932) was used but with the substitution of Neopeptone (Difco) for the Witte peptone recommended. All other ingredients were 'pure' or of analytical grade. The batch of medium used in these studies, designated K11, was prepared to contain 10% (v/v) pooled rabbit serum obtained by heart puncture, care being taken to avoid haemolysis. The medium was sterilized by filtration through a Seitz E.K. filter-pad; it contained $40\text{ }\mu\text{g. Fe/100 ml.}$ This concentration of Fe may be compared with that in a sample of a complete medium with added 'Hb solution' (see below) adequate for growth and which contained *c.* $190\text{ }\mu\text{g. iron/100 ml.}$

Sources of iron and porphyrins. Solutions containing haemoglobin ('haemoglobin solution' = Hb solution in the sequel) were prepared by repeatedly freezing and thawing washed packed rabbit erythrocytes, and were sterilized

* Present address: Department of Bacteriology, University of Sydney, Sydney, Australia.

by Seitz filtration. The haemoglobin content of this material was measured photometrically and dilutions were prepared from a stock standard solution. Solutions containing derivatives of haemoglobin were prepared from Hb solution as follows: containing carboxyhaemoglobin (COHb) by bubbling coal-gas through; containing methaemoglobin (MetHb) by adding 0.2% (w/v) potassium ferricyanide; containing 'boiled Hb' by boiling for 30 min.; containing 'heated Hb' by heating at 56° for 30 min. Haematin was prepared by adding NaOH to crystalline ox haemin. FeCl₃ was diluted from 'Analar' 60% (w/v) solution. Water distilled twice from glass was used to rinse glass-ware and for dilutions.

RESULTS

The need for iron or iron porphyrin compounds

It was found that the leptospire used would not grow well in medium K11, but that this supported luxuriant growth when a little Hb solution was added. In an experiment to find substitutes for the Hb solution, 16 ml. of medium K11 were inoculated with 0.5 ml. of a 3-day culture of *Leptospira icterohaemorrhagiae* (Jackson); the final concentration was 10⁶ organisms/ml.

Table 1. *Growth-promoting effect of FeCl₃ and material containing haemoglobin and certain derivatives*

The medium K11 (iron and porphyrin deficient) was inoculated with 10⁶ leptospire/ml.

Supplements were added to c. 10⁻⁴M final concentration, in duplicate series. The numbers of leptospire given are average of duplicates.

	Period of incubation (days)		
	4	7	18
	Leptospire (millions)/ml.		
Medium + H ₂ O	2	7	1
Medium + Hb solution	110	309	167
Medium + heated Hb solution	99	295	—
Medium + COHb solution	145	324	—
Medium + MetHb solution	130	—	—
Medium + haematin	34	232	280
Medium + boiled Hb solution	82	164	—
Medium + FeCl ₃	44	286	125

The inoculum was contained in 1/30 the volume of medium to be inoculated. The inoculated medium was distributed in duplicate 1 ml. volumes. To each of two control tubes was added 0.01 ml. H₂O. To each of other pairs was added 0.01 ml. of a c. 0.01 M solution containing one of the following: Hb, boiled Hb, heated Hb, COHb, MetHb, haematin or FeCl₃. The organisms were counted in each tube after incubation for 4, 7 and 18 days at 30° (Table 1). Growth was slower with added haematin or FeCl₃ than with the other supplements. Similar results were obtained with two virulent strains of *L. icterohaemorrhagiae*. Growth in medium K11 was not enhanced by heating it at 56° for 30 min., nor by adding 1, 10 or 100 µg. cyanocobalamin/ml., or 10⁷ boiled washed leptospire/ml.

Optimum concentration of added iron or other supplement

The optimum concentration of Hb solution or Fe to promote growth was found by adding serially diluted solutions to inoculated K11 medium. Duplicate tenfold serial dilutions of Hb solution and of FeCl_3 containing the equivalent of 10^{-2} M-Fe were made in sterile double glass-distilled water. To tubes containing 0.98 ml. of medium K11 were added 0.01 ml. of the appropriate dilution of Hb solution or FeCl_3 and 0.01 ml. of a 1/10 dilution of a 4-day culture of *Leptospira icterohaemorrhagiae*. This resulted in a range of Fe concentrations from 10^{-4} to 10^{-8} M (as Hb solution or FeCl_3). Each tube contained in 1.0 ml. of medium 10^4 leptospores and 10^{-3} ml. of medium carried over from the inoculating culture. Control tubes contained supplements of double glass-distilled water alone. The organisms were counted at intervals (Table 2), but not all tubes were counted at the same time. The optimum supplement concentration was equivalent to *c.* 10^{-5} M-Fe as haemoglobin in the Hb solution or as FeCl_3 . Growth was faster with added Hb solution than with added FeCl_3 at equivalent Fe concentrations. There was a threshold concentration of added iron which was just above the concentration in un-supplemented medium.

Effect of size of inoculum

It has frequently been stated that a large inoculum of 1/6–1/10 volume should be used in routine subculture of leptospores (Wolff, 1954; Alston & Broom, 1958; Rimpau, 1950; Wiesmann, 1952); this would be equivalent to *c.* 10^7 organisms/ml. Inocula of *c.* 100 washed or unwashed organisms/ml. grew typically and satisfactorily in Korthof medium. The function of a large inoculum would seem to be to supply iron or some iron-porphyrin contained in the transferred leptospores or medium. Half a washed suspension of organisms from a 5-day culture in adequate medium was serially 10-fold diluted in duplicate 1 ml. volumes of medium K11. The other half was similarly diluted in medium K11 containing the equivalent of 10^{-5} M haemoglobin as Hb solution. Final concentrations of inoculum were 10^8 to 10^2 organisms/ml. Growth up to *c.* 10^8 leptospores/ml. occurred in 2–13 days in all media with added Hb solution. From one to four generations occurred in *c.* 5 days in medium K11 without added Hb solution and inoculated with 10^8 or 10^7 organisms/ml., but there was no growth in tubes inoculated with 10^6 organisms or less/ml., within 20 days. In a similar experiment boiled organisms to 5×10^7 /ml. added to each tube of K11 medium prolonged survival of inocula of 10^6 organisms/ml. as compared with the controls, but did not promote growth nor alter the results in other tubes.

DISCUSSION

There has hitherto been little or no evidence that haemoglobin, a constituent of many recipes for culture media for *Leptospira*, is an essential nutrient requirement for these organisms; nor has there been any indication of its possible functions. Unless special precautions are taken, at least traces of

Table 2. *Effect of different concentrations of FeCl₃ or Hb solution (measured as haemoglobin) supplements on multiplication of Leptospira icterohaemorrhagiae.*

Cultures in deficient medium supplemented with 10⁻⁴ to 10⁻⁸M-Fe measured as haemoglobin in Hb solution or FeCl₃. Inoculum 10⁴ leptospire/ml.; carry-over 10⁻³ ml. medium.

Period of incuba- tion (days)	Added iron (molarity)											No added material H ₂ O blank
	As haemoglobin in Hb solution					As FeCl ₃						
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸		
	Total μ g. Fe/100 ml. medium											
	No. leptospire (millions)/ml.											
3	0.007	0.005	0.002	—	—	—	0.007	0.005	0.007	0.002	0.001	
5.5	0.12	0.25	—	—	0.002	—	—	—	—	—	—	
7	10	20	0.005	—	—	0.015	0.33	0.005	0.005	—	—	
9	23	192	0.015	0.005	0.01	0.012	0.54	0.01	0.03	0.02	—	
12	143	116	0.04	0.03	0.05	0.03	9.9	0.2	0.2	0.02	0.1	
16	75	162	27.5	—	0.6	0.6	2.6	0.65	0.6	0.03	0.25	
18	—	—	—	0.76	50	0.12	43.5	1.0	1.5	0.02	2.1	
23	80	69	120	—	18	5.0	151	5.0	3.3	0.02	0.5	
26	—	—	120	<0.001	7.0	6.0	80	7.0	0.75	<0.001	3.0	

haemoglobin are probably present in the serum which is generally used in all media for *Leptospira*. The original Korthof medium (Korthof, 1932) did not recommend the addition of laked erythrocytes, but Broom's modification does (Alston & Broom, 1958). Babudieri (1943) found that adding laked erythrocytes made no difference to the ability of *Leptospira* to survive for long periods in culture media. Czekalowski, McLeod & Rodican (1953) were unable to show catalase or unequivocal oxidase activity in their organisms and later suggested that media containing laked erythrocytes were better than others because this material supplied catalase (Czekalowski, McLeod & Rodican 1954). Fulton & Spooner (1956) showed the spectrum of cytochrome *c*, and Faine (1958) demonstrated oxidase but not catalase activity, in *Leptospira icterohaemorrhagiae* (Jackson); the 'Field' strain and many other serotypes of *Leptospira* contained oxidases as well as catalase. Haemoglobin may be a source of Fe and porphyrin for the building of these enzymes. The *Leptospira* used in the present experiments grew very slowly and to a limited extent unless Fe, or material containing iron porphyrins, were added. The faster growth rate with Hb solution and some derivatives of it than with haematin or Fe suggested that *Leptospira* may synthesize more slowly from medium constituents + Fe those cellular components, presumably Fe porphyrin enzymes, for which the iron porphyrin compounds from laked erythrocytes are more effective precursors. Haematin was a constituent of the defined and partially defined media proposed by Savino & Rennella (1943). Tap water, recommended for use in many commonly used media, such as the original medium of Vervoort-Schüffner (see van Thiel, 1948) would provide significant amounts of Fe. On the other hand, Fe might be removed by chelation with phosphates during the preparation of these media. Traces of Fe might be introduced by filtration through filter pads (Hunter, 1943). The optimum Fe supplement of 10^{-5} M corresponds approximately to the concentration of haemoglobin in modified Korthof medium (Alston & Broom, 1958) supplemented with Hb solution as used here.

A heavy inoculum of culture equivalent to 1/6–1/10 of the volume of medium to be inoculated has been recommended and used by many workers (Alston & Broom, 1958; Wolff, 1954). Enough *Leptospira* organisms might thus be transferred for growth to become visible after only 4–5 divisions, which is the degree of growth obtained in medium K11 inoculated with 10^7 or more washed organisms/ml. Personal experience shows that *c.* $1-5 \times 10^7$ leptospires/ml. can be seen with the naked eye in tubes or bottles of 10–20 mm. internal diameter. Thus iron or iron porphyrin deficiency may not be important when media are very heavily inoculated. Conversely, large inocula are not necessary for growth in media made adequate by addition of iron or iron porphyrin-containing material because 10^2 washed leptospires/ml. grew to usual numbers, *c.* 10^8 /ml., in medium K11 supplemented with Hb solution.

This work was assisted by a grant from the New Zealand Medical Research Council. I am greatly indebted to Dr Frank H. Sims, Auckland Public Hospital, for arranging iron estimations, to Dr J. B. Howie, Pathology Department, Otago University Medical School for Hb estimations and to Miss Janet Oldham for technical assistance.

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(Received 23 September 1958)