IRON AS A GROWTH-PROMOTING FACTOR FOR LEPTOSPIRA — IN VITRO

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Ever since the time of the detection of the first Leptospira (1916),[1] rabbit serum has been the essential constituent of the culture medium supporting growth of this organism. Schüffner[2] noted that growth was often improved when rabbit serum was slightly haemolysed. Since then many authorities[3,4] have recommended small quantities of haemolysed erythrocytes to be added to a medium such as Korthof's medium to improve cultivation conditions. Fulton and Spooner[5] studied the respiratory pattern of *L. icterohaemorrhagiae* extensively and demonstrated that a cytochrome system is involved in their respiration. Faine[6] (1959) observed that the addition of small amounts of $FeCl_3$ or haemoglobin supported growth of small inocula, whereas a deficient medium could not maintain growth.

Although the nutritional and biochemical requirements of leptospira have received much attention recently,[5,7-9] the aspect of iron as a growth requirement and its participation in the metabolism of leptospira needs further investigation. The present report describes experiments made with Korthof's medium, supplemented with small amounts of ferrous and ferric iron, in which the growth patterns of *L. icterohaemorrhagiae, L. pomona, L. canicola,* and *L. sejrϕ* were studied. Serial micro-iron estimations on subcultures at various stages after inoculation were carried out in an attempt to determine the possible role of iron in the metabolism of these organisms.

MATERIAL AND METHODS

Organisms

The different strains used were *L. icterohaemorrhagiae, L. pomona, L. canicola, L. sejrϕ,* all obtained from Prof. H. D. Brede, formerly at the University of Cologne, Germany, and maintained in a modified Korthof's medium at 27°C. incubation.

Counting Method

Direct examination by dark-field microscopy. The number of organisms is indicated by + markings in tabular form. (+ = 100 organisms per low-power field; ± = less than 10 organisms per low-power field.)

Culture Method

Broom's modification[3] of Korthof's medium[10] was used. Apart from the serum, all other ingredients were of analytical grade. To each 8·0 ml. of Korthof's medium, 1·0 ml. of sterile rabbit serum (obtained by heart puncture) was added. The medium was then placed in a waterbath at 56°C. for 30 minutes and, after cooling, 0·1 - 0·2 ml. of the stock iron solution was added so as to obtain a concentration of 250 - 350 μg. Fe^{+++}/100 ml. at pH 7·2.

Stock Iron Solution

This was prepared according to Wong's method. Crystallized ferrous ammonium sulphate [$FeSO_4.(NH_4)_2SO_4.6H_2O$, Mohr's salt], 0·7 G., was dissolved in about 50 ml. of

distilled water. To it was added 20 ml. of 70% sulphuric acid, the solution was warmed slightly and then decinormal solution of potassium permanganate (about 8 ml.) was added to oxidize the ferrous salt completely. The solution was diluted to 1,000 ml. with distilled water so that 1 ml. contained 100 μg. Fe^{+++} Small quantities (about 10 ml.) of the stock iron solution were sterilized in an autoclave at 15 lb. pressure for 20 minutes before use and were found to be resistant to heat, unlike some other iron preparations tried.

In a separate batch, 0·4980 G. of ferrous sulphate ($FeSO_4.7H_2O$) was dissolved in water to which 1·0 ml. of concentrated sulphuric acid was added, and made up to a litre so that 1 ml. = 100 μg. Fe^{++} Sterilization of this solution was carried out in a Koch's steaming apparatus.

Iron Estimations

Before iron estimations leptospira were removed from the medium by filtration through a collodion membrane of pore-size 0·1 μ, described by Hindle and Elford.[11] The micro-estimation for iron with O-tolidine of Budtz-Olsen[12] was found to be suitable for this purpose, because less than 1 ml. of medium was needed for a double estimation.

Iron estimation done on uninoculated culture media was found to be in the region of 45 μg. Fe^{+++}/100 ml. (similar to that reported by Faine[6]), while, after the addition of 0·1 - 0·2 ml. stock iron solution, the range was between 250 and 350 μg. Fe^{+++}/100 ml.

Haemoglobin Solution

Citrated sheep cells were washed 4 times with physiological saline. 1·0 ml. of the washed cells were made up to 50 ml. with distilled water, and the 2% solution thus obtained was sterilized by Seitz filtration before use. To each tube containing Korthof's medium, 0·1 - 0·2 ml. of the 2% haemoglobin solution was added.

RESULTS

(a) Change in the Growth Rate

Ferric iron (in the form of oxidized Mohr's salt) was found by Faine[6] to influence the growth rate in a manner similar to that reported for haemoglobin and iron-containing porphyrins; he also compared the effect of ferric chloride on the growth rate of these organisms. Parallel experiments initially started with ferric chloride, yielded no reproducible results, and, in view of the many practical difficulties encountered, experimentation with this salt was abandoned.

The growth response evaluated in 4 stock strains of leptospira is shown in Table I. Inocula of 1:10 were carried out in control and test cultures, the latter receiving a supplement of 0·05 ml. of iron solution. Cultures were examined by dark-field microscopy at 6, 15, 25, 33, 52, and 60 days. The growth curve in control cultures agreed well with that reported by previous workers[7,13] for *L. icterohaemorrhagiae* and *L. pomona*. Test cultures, after an initial lag period of about 4 days, showed a logarithmic phase reaching an optimal growth density at the 20th day in the case of *L. icterohaemorrhagiae, L. pomona* and *L. canicola,* while that of *L. sejrφ* was reached at about the 25th day. After a period of 60 days, satisfactory growth densities were still observed in all 4 cultures to which iron was added, while the control tubes showed scanty growth or total expiration.

TABLE I. GROWTH RESPONSE IN 4 STRAINS OF LEPTOSPIRA

	6	15	25	33	52	60
L. pomona						
K.M. (Control)	+	++	+	+	0	0
K.M. + 0·05 ml. Fe +++	++	+++	+++	++	++	++
Days	6	15	25	33	52	60
L. canicola						
K.M. (Control)	+	++	+	+	±	±
K.M. + 0·05 ml. Fe +++	++	+++	++++	+++	+++	++
Days	6	15	25	33	52	60
L. icterohaemorrhagiae						
K.M. (Control)	+	++	+	±	0	0
K.M. + 0·05 ml. Fe +++	++	+++	+++	+++	++	++
Days	6	15	25	33	52	60
L. sejrø						
K.M. (Control)	±	±	+	±	0	0
K.M. + 0·05 ml. Fe +++	±	+	++	++	++	++
Days	6	15	25	33	52	60

K.M. = Korthof's medium

± = less than 10 organisms/low-power field.
0 = expired.
+ = 100 organisms/low-power field.

The beneficial effect of haemoglobin on the growth of leptospira is well established. A comparison between the effect of ferric iron and haemoglobin on the growth rate of three strains is represented in Table II. Inocula of 1:10 in control cultures and test cultures supplemented with 0·1 ml. of iron solution and 0·1 ml. of 2% haemoglobin solution respectively, were examined by dark-field microscopy at 3, 9, 14, 20, 33 and 42 days. An enchanced growth rate was observed in the test cultures, containing iron and haemoglobin, exhibiting also a marked degree of parallelism. Both reached their optimal growth density between the 15th and 25th day, showing definite ability of survival for longer than 42 days.

TABLE II. THE EFFECT OF FERRIC IRON AND HAEMOGLOBIN ON THE GROWTH RATE OF 3 STRAINS OF LEPTOSPIRA

	L. icterohaemorrhagiae					
K.M. (Control)	+	+	+	±	0	0
K.M. + 0·1 ml. Fe +++	++	+++	+++	+++	++	++
K.M. + 0·1 ml. Hb.	+	+++	+++	+++	++	++
Days	3	9	14	20	33	42

	L. canicola					
K.M. (Control)	+	+	+	+	+	±
K.M. + 0·1 ml. Fe +++	+++	+++	+++	+++	+++	+++
K.M. + 0·1 ml. Hb.	++	+++	+++	+++	+++	+++
Days	3	9	14	20	33	42

	L. pomona					
K.M. (Control)	+	+	+	+	+	0
K.M. + 0·1 ml. Fe +++	++	+++	+++	+++	+++	+++
K.M. + 0·1 ml. Hb.	+	++	++	++	++	++
Days	3	9	14	20	33	42

	L. icterohaemorrhagiae					
K.M. (Control)	±	±	+	+	±	0
K.M. + 0·1 ml. Fe++ Mohr's salt	±	±	±	−	−	0
K.M. + 0·1 ml. Fe +++	+	++	+++	+++	+++	++
Days	3	6	9	14	21	42

K.M. = Korthof's medium

± = less than 10 organisms/low-power field.
0 = expired.
+ = 100 organisms/low-power field.

Included in Table II is a graph (*L. icterohaemorrhagiae*) which indicates the effect on growth of ferrous ammonium sulphate ($FeSO_4.(NH_4)_2SO_4.6H_2O$) added in the same concentration, i.e. 0·1 ml., to a test culture. Poor growth was obtained together with expiration of the culture within 2 weeks, indicating the inability of the iron in this form to stimulate growth. When the iron salt was oxidized, however, in acid medium with concentrated H_2O_2, the effect on the growth rate was strikingly similar to that obtained with the stock iron solution. Such a solution has the advantage of being free of Mn^{++} ions, an important trace element in the consideration of metallo-porphyrin-enzyme complexes, but primarily indicates that the valency state in which iron is added to the medium, may be of importance.

(b) Utilization of Iron

It has been stated that leptospiral cultures very infrequently survive longer than 3 months in artificial media.[14] In cultures supplemented with ferric iron, survival from 3 to 6 months was constantly found, enabling us to study iron-uptake over a period of time by serial iron estimations.

After the logarithmic growth phase was completed in all 4 strains at about the 20th day, an iron estimation on the organism-free medium was carried out and again repeated about 2 weeks later, because slight decreases in growth, especially in *L. icterohaemorrhagiae*, could be observed at about the 40th day. As a control, an uninoculated culture medium was treated in the same way. Table III indicates that according to our methods no iron utilization could be determined in 3 different experiments. The tendency to a higher iron content on older cultures can be explained by slight evaporation of the medium. So far no similar reports have been found in the literature with which these results could be compared.

TABLE III. IRON CONTENT OF KORTHOF'S MEDIUM AFTER FILTRATION IN μg. Fe^{+++}/100 ml.

Strain	*First estimation*	*Second estimation*	
L. pomona	291	325	Time interval 13 days
L. canicola	305	335	
L. icterohaemorrhagiae	273	283	
L. sejrø	311	317	
Mean	295	315	
Control	302	300	
L. pomona	310	300	Time interval 12 days
L. canicola	335	325	
L. icterohaemorrhagiae	308	310	
L. sejrø	303	321	
Mean	314	314	
Control	335	326	
L. pomona	310	308	Time interval 12 days
L. canicola	320	318	
L. icterohaemorrhagiae	315	308	
L. sejrø	300	292	
Mean	312	307	
Control	315	323	

DISCUSSION

Growth patterns of stock cultures grown in Korthof's medium with a total iron concentration of 250 - 350 μg. Fe^{+++}/100 ml. showed a generally delayed lag phase, an increased logarithmic phase, and maintenance of an adequate growth density for more than 60 days, if the iron added was in the ferric state. If ferrous iron to the same concentration was added, a growth response similar to that reported by other workers was obtained,[5,7,13] in respect of the duration of the lag phases and logarithmic phase, while the total density and survival rate appeared only slightly increased.

These results seem to indicate that ferric iron is not only an adequate substitute for haemoglobin in its growth-promoting activity *in vitro,* but also retains its effectiveness longer than that of haemoglobin, which is soon lost.[15] Less luxuriant and different growth patterns observed with ferrous as opposed to ferric iron, together with the slower but more prolonged effect of ferric iron, seem to suggest its utilization and incorporation into respiratory enzyme systems.

To determine the possible iron-uptake of these organisms, chemical estimations were resorted to after filtration of a culture through a collodion membrane. This procedure did not affect the total iron content of the medium as indicated by control tests. In spite of the fact that dense growths of cultures were removed from the medium, tests have failed to show any iron-utilization in 3 successive experiments (Table III). Because of the slow growth rate and low metabolic activity of the organisms *in vitro,* it can be assumed that the amount of iron incorporated by the organism would be in the region of a few micrograms per ml. of medium and would therefore fall outside the range of the test employed.

According to Fulton and Spooner[5] replacement of rabbit albumin by versene (sodium salt of EDTA) inactivating metallic ions by chelation, did not inhibit respiration of *L. icterohaemorrhagiae* in its presence. They further found that respiration of *L. icterohaemorrhagiae* was not stimulated by haemoglobin, confirming Marshall's[9] observation. The mode of action of iron would therefore seem to be rather on the slower processes affecting growth than on respiratory stimulation. Mino's[16] observation that leptospira can live in guinea-pig blood cultures from 16 to 24 months could be explained on this basis, namely, that sufficient iron is present for growth utilization.

A narrow limit of iron concentration has not been observed as found in the case of toxin production by *C. diphtheriae.* Addition of more than 300 μg. of iron from the stock solution will cause rapid changes in the pH of the medium because the solution itself is acidic. No evidence of iron-adaptation has been observed. Initial luxuriance of cultures did not seem to affect the survival rate of cultures in Korthof's medium at 27°C. *L. icterohaemorrhagiae* showed the shortest beneficial effect, while *L. pomona* and *L. canicola* showed the longest, the latter 2 strains having been successfully subcultured after a period of 11 months. As a routine, subcultures were successfully carried out every 2 months in Korthof's medium supplemented with iron.

SUMMARY

The growth patterns of *Leptospira icterohaemorrhagiae, L. pomona, L. canicola* and *L. sejrφ* have been studied with regard to ferrous iron, ferric iron, and haemoglobin. Ferric iron was found to have a prolonged influence on growth and reproduction of these organisms. The effect seems to be on growth rather than respiration. The addition of iron to Korthof's medium not only represents an improved culture medium, but also appreciably reduces the amount of subculturing necessary to preserve the strain. Chemical analysis failed to demonstrate any uptake of iron.

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