BIOLOGY OF BACTERIUM LEPISEPTICUM.

II. THE STRUCTURE OF SOME IRON COMPOUNDS WHICH INFLUENCE THE GROWTH OF CERTAIN BACTERIA OF THE HEMOPHILIC, ANAEROBIC, AND HEMORRHAGIC SEPTICEMIA GROUPS.

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In a previous report, one of us has shown that certain virulent cultures of *Bacterium lepi-septicum* need, for optimum growth and maintenance, either a plain nutrient medium at a reduced oxygen tension or, under aerobic conditions, this same medium plus rabbit blood.¹ The blood was shown to function in high dilutions to the limit of the benzidine reaction, and also after autoclaving for 30 minutes at 115 pounds pressure. For these reasons it was concluded that the active substance contained in blood could not be a food, nor of vitaminic nature. But its behavior did suggest a function similar to that of the X factor, the presence of which Avery and his associates² regard as one of the essentials for optimum growth of hemophils, anaerobes, and pneumococci at atmospheric pressure.

Avery has described the X factor as follows: "The so called X factor, which is associated with the pigment fraction of blood, gives the peroxidase reaction, and is not destroyed by moist heat at 120°C. . . . Moreover, the X substance is active in such minute amounts as to suggest that it functions as a biocatalyst."³ It would seem then that the principle needed for good growth of virulent strains of *Bacterium lepi-septicum* resembles the X factor.

The X factor has been found in plant tissue² and in a solution of ferrous sulfate and gum arabic.⁴ A list of substances which function as X has been assembled

¹ Webster, L. T., *J. Exp. Med.*, 1925, xli, 571.

² Avery, O. T., *et al.*, *J. Exp. Med.*, 1921-24, xxxiv, xxxviii-xl.

³ Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 296.

⁴ Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 59.

from the literature by Novy.⁵ The chemical constitution of most of them is, however, so little understood that it seemed advisable to test the action of simple compounds in the hope that additional facts relating to the structure and behavior of the X mechanism might be recognized.

Technique.

A number of substances with some of the properties of hemoglobin have been used; sodium pentacyano-aquo-ferroate and its derivatives, ferrous and ferric oxides, and vegetable charcoal.

The peroxidase reaction was carried out with either benzidine chloride dissolved in distilled water, or benzidine dissolved in glacial acetic acid, plus superoxol (hydrogen peroxide, Merck). The presence of ferrous iron was detected by the Prussian blue reaction, or by titration with KMnO_4 . The amount of oxygen absorbed by a compound was determined by means of a Barcroft-Haldane apparatus as modified by Brodie.⁶

The biological test consisted in adding the given substance to tubes containing 5 cc. of plain meat extract broth, pH 7.4, and inoculating each with *Bacterium lepi-septicum*, Type D, in numbers varying exponentially from 10^9 to 10^0 . If growth occurred in tubes seeded with less than 100,000 bacteria, and if, after 3 or more weeks incubation at 37°C ., no Type G variants appeared in any culture, the test was considered positive and the compound was considered to function as the X factor.¹

Sodium Pentacyano-Aquo-Ferroate.

Baudisch's studies of the catalytic properties of iron⁷ led us to test the activity of sodium pentacyano-aquo-ferroate, an inorganic compound of known formula discovered by Hofmann.⁸ This substance, like hemoglobin, contains a ferrous radical, gives a strong peroxidase reaction, and absorbs oxygen at 37.5°C ., at an initial rate of 2.38 cc. per gm. per hour.⁹ It is derived from the inactive sodium ferrocyanide by the replacement of one NaCN group by H_2O . This H_2O group,

⁵ Novy, F. G., Jr., *J. Infect. Dis.*, 1925, xxxvi, 343.

⁶ Brodie, T. G., *J. Physiol.*, 1909-10, xxxix, 391.

⁷ Baudisch, O., and Welo, L. A., *J. Biol. Chem.*, 1924, lxi, 261.

⁸ Hofmann, K. A., *Ann. Chem.*, 1900, i, 312.

⁹ We are indebted to Dr. James A. Hawkins for measuring the oxygen absorption of these compounds. His readings for sodium pentacyano-aquo-ferroate were as follows:

Cell 14	=	-2.720	cc. O_2 consumed per gm. per hour.
" 23	=	-2.010	" O_2 " " " "
" 13	=	-2.41	" O_2 " " " "

although forming an integral part of the molecule, is associated with an available bond which endows the central iron atom with activity, catalase, oxygenase, and peroxidase properties.

Experiment 1.—Nine tubes containing 5 cc. of plain extract broth, pH 7.4, plus 1 cc. of a 10 per cent sterile solution of the aquo salt, nine tubes of broth plus 1 cc. physiological salt solution, and nine tubes of broth plus 1 cc. of a 10 per cent solution of autoclaved, laked rabbit corpuscles were inoculated with a 17 hour broth culture of *Bacterium leprosepticum*, Type D, in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation the cultures were examined for growth and later for the appearance of Type G variants.

The results are shown in Table I. Growth occurred in all the aquo salt and blood tubes, including those which had received the smallest

TABLE I.

Effect of Sodium Pentacyano-Aquo-Ferroate on the Growth of Bacterium leprosepticum, Type D.

Medium.	Benzidine test.	Reducing power.	Oxygen-absorbing power.	No. of bacteria per tube.									Type G variation.
				10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³	10 ⁻²	10 ⁻¹	10 ⁰	
Plain broth.....	0			++	++	++	0	0	0	0	0	0	++
Plain broth + laked blood corpuscles....	++	++	++	++	++	++	++	++	++	++	++	++	±
Plain broth + aquo salt.....	++	++	++	++	++	++	++	++	++	++	++	++	±

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

number of organisms, but only in such of the plain broth tubes as had been seeded with 100,000 or more. Later, Type G variants appeared in these cultures, but not in the blood or aquo salt tubes.

Further experiments demonstrated that this aquo salt was active as the X factor in dilutions of 1/100,000 gm. per cc. This dilution was also the limit of the benzidine reaction. The ferric salt was found to be active as well, but the presence of NO₂ and NH₃ groups in place of H₂O decreased its sensitivity.

Experiments with strains of *Bacillus avisepticus* yielded similar results.

TEXT-FIG. 1. Growth of *Bacterium leipsepticum*, Type D, in plain broth plus aquo salt.

In the work which followed a determination of the rate of bacterial growth was made by the serial counting method.¹

Experiment 2.—To one Erlenmeyer flask containing 150 cc. plain broth was added 18 cc. hemolyzed, washed rabbit blood corpuscles plus 12 cc. 0.85 per cent NaCl; to another, 20 cc. autoclaved red cells plus 10 cc. saline; to a third, 30 cc. of an aqueous 10 per cent solution of the aquo ferrous salt, and to a fourth, 30 cc. of the aquo ferric salt. Samples from the first three flasks gave strong benzidine reactions; in Flask 4 the test was weaker.

Each flask was now inoculated with 1 cc. of a 1:1,000,000 dilution of a 17 hour plain broth culture of *Bacterium leprosepticum*, Type D, and incubated at 37°C. Counts were made at half hour intervals for the first 6 hours; then at 24 and 48 hours. The numbers of bacteria per cc. in each flask at various time intervals are plotted in Text-fig. 1.

Following an inoculation of less than 10 bacteria per cc., the count in each flask increased immediately and logarithmically to reach a maximum of about 1 billion per cc. within 24 hours. The growth curves of the bacteria in the flasks containing broth and blood, and broth and aquo ferro salt paralleled one another closely; that from the ferric salt medium differed in that the rise was somewhat less abrupt. Evidently the aquo salt, by supplying the X factor, furnishes optimum conditions for the aerobic growth of *Bacterium leprosepticum*.

Experiment 3.—This experiment was planned to test the activity of the aquo ferrous salt as the X factor in the growth of organisms of the so called hemophilic group.

Three cultures were obtained from Dr. T. M. Rivers. The first, *B. influenza* No. 6,¹⁰ required for growth the presence of the X and V factors; the second, "J. R. L.,"¹¹ needed the V factor alone; and the third, "Dog,"¹² the X factor only. These strains were taken from blood agar slants and inoculated into 10 per cent rabbit blood broth tubes. After 24 hours incubation, 0.1 cc. from each tube was transferred to a tube containing 4 cc. of plain broth, pH 7.4, plus 1 cc. of a 10 per cent solution of the aquo salt. The media gave a strong benzidine test prior to inoculation. These first subcultures were then incubated overnight and on the following day 0.1 cc. was plated on chocolate agar to determine the presence of live organisms, and one loopful was transferred to the second series of aquo salt broth tubes.

This procedure was continued for six subcultures, each after an incubation period of 24 to 48 hours. Then 0.1 cc. of each culture was transferred to tubes of

¹⁰ Rivers, T. M., and Poole, A. K., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 202.

¹¹ Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 429.

¹² Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 149.

TABLE II.

Growth of Influenza Bacilli in Media Containing Aquo Salt.

Culture.	Subculture No.	Aquo salt.	Control plates.	Aquo salt + yeast.	Subculture No.	Control plates.	Yeast.	Subculture No.	Control plates.	Blood.	Control plates.	Plain broth.
Infl.										++	++	0
J. R. L.										++	++	0
Dog.										++	++	0
Infl.	1	±	+									
J. R. L.	1	±	+									
Dog.	1	++	++									
Infl.	2	±	+									
J. R. L.	2	±	+									
Dog.	2	++	++									
Infl.	3	±	+									
J. R. L.	3	±	+									
Dog.	3	++	++									
Infl.	4	±	+									
J. R. L.	4	±	+									
Dog.	4	++	++									
Infl.	5	±	+									
J. R. L.	5	±	+									
Dog.	5	++	++									
Infl.	6	±	+									
J. R. L.	6	±	+									
Dog.	6	++	++									
Infl.	7	±	+	++	1	++	++	1	++	++	++	0
J. R. L.	7	±	+	++	1	++	++	1	++	++	++	0
Dog.	7	++	++	++	1	++	++	1	++	++	++	0
Infl.	8	±	+	++	2	++	0	2	0	++	++	0
J. R. L.	8	++	++	++	2	++	++	2	++	++	++	0
Dog.	8	++	++	++	2	++	±	2	+	++	++	0
Infl.	9	±	+	++	3	++	0	3	0	++	++	0
J. R. L.	9	±	+	++	3	++	++	3	++	++	++	0
Dog.	9	++	++	++	3	++	0	3	0	++	++	0

±, survival, very slight growth; +, moderate growth; ++, good growth.

aquo salt broth, aquo salt plus 1.0 per cent yeast extract broth, and 1.0 per cent yeast extract broth. Blood and plain broth tubes served as controls. Plates were smeared the next day and subcultures were made from each tube to a second series of similar media. Three consecutive transfers were carried out in this manner.

Table II shows the results. The Dog culture grew well in aquo salt broth, but *Bacillus influenzae* and J.R.L., although surviving for nine subcultures, grew very poorly. All three cultures grew profusely in broth containing aquo salt plus yeast extract, and only J.R.L. grew in the media containing yeast extract alone. From these experiments it was concluded that the aquo salt functions as the X factor in the growth of *Bacillus influenzae* and other hemophils as well as in the case of bacteria of the hemorrhagic septicemia group.

Strains of *Bacillus welchii* and *Bacillus sporogenes* which failed to grow in tubes of aerobic broth sealed with vaseline, but which did multiply luxuriantly when the oxygen tension in the broth was reduced mechanically by boiling and sealing, showed similar abundant growth when aquo salt was added to aerobic broth sealed with vaseline.

Further experiments were made with oxides of iron which were being studied by Welo and Baudisch.¹³ Fe_3O_4 , natural magnetite, was first tried and, although quite insoluble, was found to give a positive benzidine reaction and to function as the X principle for the growth of *Bacterium leprosepticum*.

Iron Oxides.

Fe_3O_4 , Lefort's oxide, was then made by precipitating one part FeSO_4 and one part $\text{Fe}_2(\text{SO}_4)_3$ with NaOH . This substance possesses a definite cubic crystal structure, a magnetic susceptibility of 0.152, absorbs oxygen at an initial rate of 0.0043 cc. per gm. per hour,¹⁴

¹³ Welo, L. A., and Baudisch, O., *J. Biol. Chem.*, 1925, lxxv, 215.

¹⁴ Dr. Hawkins' readings for the oxides were the following.

Fe_3O_4 Cell 14 = 0.0045 cc. O_2 consumed per gm. per hour.	
" 23 = 0.0043 "	O_2 " " " "
" 13 = 0.0044 "	O_2 " " " "
Fe_2O_3 active Cell 14 = 0.0072 cc. O_2 consumed per gm. per hour.	
" 23 = 0.0092 "	O_2 " " " "
" 13 = 0.0076 "	O_2 " " " "
Fe_2O_3 inactive Cell 14 = 0	
" 23 = 0	
" 13 = 0	

and gives a positive benzidine reaction in broth. Oxidation at 330° removed all trace of ferrous iron, but did not affect the crystal structure. The magnetic susceptibility and initial rate of oxygen absorp-

TABLE III.

Effect of Ferric Oxides on the Growth of Bacterium leipsepticum, Type D.

Medium.	Benzidine.	Ferrous iron.	Initial rate of oxygen absorption.	No. of bacteria per tube.										Type G variants.
				10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³	10 ⁻²	10 ⁻¹	10 ⁰		

			<i>cc. per gm. per hr.</i>										
Plain broth....	0	0	0	++	++	++	++*	0	0	0	0	0	++
Plain broth + blood.....	++	++	—	++	++	++	++	++	++	++	++	++	±
Plain broth + Fe ₃ O ₄	++	++	0.0043	++	++	++	++	++	++	++	++*	0	±
Plain broth + active Fe ₂ O ₃ .	++	0	0.008	++	++	++	++	++	++	++	+	0	±
Plain broth + inactive Fe ₂ O ₃	0	0	0	++	++	++	++*	0	0	0	0	0	++

—, no determination.

* Growth after 48 hours incubation.

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

tion increased slightly.¹⁴ The benzidine test continued positive. Further heating to 550° changed the crystal structure unit to a rhombohedral form, destroyed its magnetic and oxygen-absorbing properties,¹⁴ and its power to oxidize benzidine. Catalase activity was present before and after heating.

The biological test was carried out on these three oxides as follows:

Experiment 4.—About 0.5 gm. of each of these substances, Lefort's oxide (Fe_3O_4), active Fe_2O_3 , and inactive Fe_2O_3 , was added to each of nine tubes containing 5 cc. of plain broth, pH 7.4. These three series together with a control set of plain broth and blood broth tubes were inoculated with a 17 hour plain broth culture of *Bacterium leprosepticum* in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation they were examined for growth. Later the variant G forms were sought for by plating methods.

Table III shows the results of this experiment. Growth occurred in every tube to which blood, Fe_3O_4 , or active Fe_2O_3 had been added, and no G variants appeared for 2 weeks. In the plain broth and inactive Fe_2O_3 series bacterial multiplication occurred only in those tubes which had received an inoculation of 100,000 organisms or more. G forms were abundant in these after 72 hours. Further quantitative tests showed that at least 0.1 gm. of the active oxides per cc. of broth was necessary for a positive reaction.

Bacillus avisepticus behaved in a similar manner. The hemophilic and anaerobic strains used in the previous experiments grew well in the presence of the active Fe_2O_3 , while no growth occurred when the inactive oxide was used.

DISCUSSION.

Two types of inorganic iron compounds of known structure have been found to show the so called X type of biocatalytic activity, (1) pentacyano iron salts and (2) certain iron oxides. In each instance definite physical and chemical properties may be related to the growth-promoting power. Sodium pentacyano-aquo-ferroate contains an unsaturated bond which is associated with oxygen-absorbing and peroxidase properties, as well as with growth-promoting activity. And Fe_3O_4 , although quite insoluble, has similar characteristics. When this latter substance is heated just enough to oxidize all ferrous iron, a form of Fe_2O_3 is obtained which retains the same crystal structure, oxygen-absorbing, peroxidase, and growth-activating properties. Further heating of this Fe_2O_3 alters its crystal structure and destroys its oxygenase, peroxidase, and biocatalytic action.

The contrast in growth-promoting power of these two similar iron oxides indicates anew the close association of this property with that

of oxygen absorption and of benzidine oxidation. Avery regards the iron-containing X substances as peroxidases which destroy metabolic, toxic, organic peroxides, and which, in some more subtle way, activate cell respiration. Novy, on the other hand, considers that the mechanism of all chemical, animal, and vegetable X substances is to reduce the oxygen tension of the medium to a level suitable for growth.⁵

The data so far accumulated indicate that, though many X substances have characteristics in common with respiratory ferment, the only property common to all is that of being able to absorb oxygen.

The effect of these biocatalysts on the appearance of bacterial variants should be noted. Their presence in the media insures optimum growth and inhibits variation, while without them growth is retarded and variants are abundant after 48 hours. Apparently the occurrence of this type of bacterial variation is intimately associated with the presence of excess oxygen in the medium.

CONCLUSIONS.

Inorganic iron substances of known chemical structure have been tested for their ability to function as the X factor in the growth of bacteria of the hemophilic, anaerobic, and hemorrhagic septicemia groups.

Each iron compound possessing this biocatalytic activity showed oxygen-absorbing and peroxidase properties.

The interrelation of these mechanisms is discussed.