OXIDASE REACTION OF VARIOUS GROUPS OF BACTERIA.

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The oxidases of both animal and plant tissue have been extensively studied; they are found almost universally in all forms of life and exert undoubtedly an important function in the oxidation in living cells. The constitution of these enzymes and the mechanism of their action are not understood. They have been defined by Portier¹ as "agents found in life which have the power to oxidize certain substances in the presence of oxygen in gaseous or dissolved state." Duclaux² states that: "Oxidases are substances which at ordinary temperatures and under physiological conditions carry oxygen rapidly to materials upon which, without the intervention of oxidases, ordinary oxygen would act very slowly." The different oxidases are usually classified on the basis of the substance which is oxidized; that is, a tyrosinase is an oxidase which oxidizes tyrosine; an aldehydase one which oxidizes an aldehyde, etc. Whatever their constitution or mechanism of action, it is understood that oxidases are known to change the so called inactive molecular oxygen into oxygen in the active state, suitable for chemical union, and that without the aid of these agents as an expression of vital activity oxidation takes place very slowly, if at all. Hence, the importance of any study that may furnish a better insight into a chemical reaction so important to all forms of life.

From a survey of the literature it is evident that few investigators have studied or commented on this activity in the case of the ordinary microorganisms, though great attention has been focused upon the oxidases of animal and plant tissue.

¹ Portier, Les oxydases dans la séries animale, leur rôle physiologique, Thèse de Paris, No. 63, Paris, 1897.

² Duclaux, E., Traité de microbiologie, Paris, 1899, ii, 565-584.

Schönbein,³ as early as 1855, made a study of fungi and found positive oxidase activity. Roux,⁴ in 1899, demonstrated the existence of an oxidase in a culture of *B. coli*. Yeast was shown to be capable of producing oxidases by Tolomei,⁵ Effront,⁶ Buchner,⁷ Grüss,⁸ and Issajew.⁹ Dietrich and Liebermeister¹⁰ found this ferment in pustules. Lehmann and Sano,¹¹ in a study of thirty microbic species, detected the presence of a tyrosinase in three; namely, *B. putidum*, *B. phosphorescens*, and *Actinomyces chromogens*. Kastle's monograph on oxidases¹² provides an excellent bibliographic source and historical presentation of the development of knowledge concerning oxidases.

While estimating the bactericidal action of certain compounds of the triphenylmethane leuco bases, I observed that some of them were changed into their corresponding dyes in the presence of growing pneumococci, a chemical reaction not actuated by individual strains of *Streptococcus hæmolyticus*, *Staphylococcus aureus*, meningococcus, or *Bacillus typhosus*. The oxidative phenomenon was first noted with *p*-aminoleucomalachite green when pneumococci were grown on whole horse blood. Control tubes consisting of whole defibrinated horse blood and the leuco compound remained colorless for a period of 96 hours. With the pneumococcus a deep purple color developed, the shade being that of *p*-aminomalachite green. Obviously, the dye was

³ Schönbein, C. F., Ueber Ozon und Ozonwirkungen in Pilzen, *Phil. Mag.*, 1856, ii, series 4, 137; Ueber die Selbstbläuung einiger Pilze und das Vorkommen von Sauerstofferregern und Sauerstoffträgern in der Pflanzenwelt, *Verhandl. Naturf. Ges.*, 1855, i, 339; Ueber Ozon und Ozonwirkungen in Pilzen, *J. prakt. Chem.*, 1856, lxvii, series 3, 496.

⁴ Roux, Sur une oxydase productrice de pigment, sécrétée par le coli-bacille, *Compt. rend. Acad.*, 1899, cxxviii, 693.

⁵ Tolomei, G., Sopra un fermento solubile che si trova nel vino, *Atti Real. Accad. Lincei, Rend. sc. fis., mat. e. nat.*, 1896, v, series 5, 52.

⁶ Effront, J., Enzymes et leurs applications, Paris, 1898; English translation by S. C. Prescott, New York, 1902.

⁷ Buchner, E., Ueber zellenfrei Gährung, *Ber. chem. Ges.*, 1898, xxxi, 568.

⁸ Grüss, J., Über Oxydaseerscheinungen der Hefe, *Woch. Brauerei*, 1901, xviii, 310, 318.

⁹ Issajew, W., Ueber die Hefeoxydase, *Z. physiol. Chem.*, 1904, xlii, 132.

¹⁰ Dietrich, A., and Liebermeister, G., Sauerstoffübertragende Körnchen in Milzbrandbacillen, *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxii, 858.

¹¹ Lehmann, K. B., and Sano, Über das Vorkommen von Oxydationsfermenten bei Bakterien und höheren Pflanzen, *Arch. Hyg.*, 1908, lxvii, 99.

¹² Kastle, J. H., The oxydases, *Bull. Hyg. Lab., U. S. P. H.*, No. 59, 1909.

oxidized from its leuco base by the activity of the growing pneumococci.

This observation presented an opportunity for studying the problem of the oxidase activity of bacteria by means of a simple technique. Leucomalachite green had been used for the detection of blood by Adler and Adler¹³ and later by Buckmaster¹⁴ in a study of pseudoperoxidase reaction between hemoglobin and its derivatives. The compound used by them was the leucotetramethyldiaminotriphenylmethane, whereas the *p*-aminoleucomalachite green made use of by a simple technique in the present study is the 4, 4'-bis dimethylamino-4-aminotriphenylmethane. We wish to present here, first, a study of the pneumococci in relation to the oxidase activity under various conditions of growth, and, second, an inquiry into the oxidases of other microbic species as determined by this indicator.

Technique.

Solution of the indicator is made by dissolving a weighed portion of the leuco base in dilute hydrochloric acid, which is added drop by drop on the dry chemical. After dissolving, water is added to make a 1:100 concentration and the excess of acid neutralized by dilute sodium hydroxide, enough alkali being used to produce just a slight opalescence. Sterilization can be effected by boiling, or autoclaving under nitrogen, or by means of a Berkefeld filter. For practical purposes, it was found sufficient to boil in an excess of hydrochloric acid, and then neutralize with sterile sodium hydroxide (N) after the solution became cool. The amount of leuco base used in the test represented a final dilution in the media of 1:10,000.

The usual method was to pipette 2 cc. of medium into a 10 mm. tube, add, in turn, 0.1 cc. of a 1:500 concentration of leuco base and then 0.1 cc. of an 8 hour culture of the bacteria in question. The inoculated tubes were incubated for varying lengths of time at 37.5°C. Controls of both medium and indicator were always run.

Conditions Which Influence the Reaction with Pneumococcus.

As stated above, the reaction was first noted when whole defibrinated horse blood was used as medium. Serum was found to give as suitable conditions as whole blood, the one requirement for both being

¹³ Adler, O., and Adler, R., *Z. physiol. Chem.*, 1904, xli, 59.

¹⁴ Buckmaster, G. A., The pseudo-peroxydase reaction between hæmoglobin, its derivatives and the leucobase of malachite green, *J. Physiol.*, 1908, xxxvii, p. xi.

that they be fresh. Contrarily, plain broth or meat infusion (plain broth with peptone) and 1 per cent peptone solution, each having a pH of 7.8, prevented the oxidation of the indicator by pneumococci. The difference is striking and perhaps significant. Pneumococci growing in serum cause oxidation of *p*-aminoleucomalachite green, whereas growth, seemingly as abundant on the broth, meat infusion, and peptone media as on serum, produced not the slightest oxidation of the indicator. The reaction is unique in that it appears to require something in fresh animal tissue (fresh serum) as a substrate. Although it is not our purpose to explain the reaction in this communication, the following experiments were performed in quest of a possible reason for the difference in action between media with and without serum.

Experiment 1.—Variation in the concentration of serum, plain broth, and meat infusion from 100 to 10 per cent, in gradients of 10.

Experiment 2.—Variation in the concentration of meat infusion, peptone solution, and plain broth in a quantity of horse serum that rendered possible the oxidation of this indicator.

Experiment 3.—Variation of peptone solution and meat infusion in guinea pig serum.

The results of these experiments are given in Table I. The leuco compound is not oxidized by growing pneumococci in any concentration of the media without serum, while complete oxidation occurs in serum in as high a concentration as 70 per cent. In undiluted serum and in 80 and 90 per cent serum, definite inhibition of the oxidation reaction is manifest. The results in these dilutions indicate the existence of a reducing potential, perhaps an antioxidase, which counterbalances the oxidation by the microorganism. Experiment 2 emphasizes the reciprocal relationship; that is, at a definite concentration of broth and meat infusion media, in a quantity of serum in which a positive reaction would normally be obtained, no oxidation of the indicator is observed. It might be thought that the meat infusion, broth, and peptone media have a destructive action on the oxidizing enzymes of the serum; indeed, this mechanism might be explanatory of the difference between the action of these media and serum, were it not for the fact that undiluted serum inhibits or prevents the reaction in a similar way.

By chance, guinea pig serum was used in one of the experiments instead of horse serum, and the impression was gained that oxidation proceeded much more readily in guinea pig serum than in horse serum. To study the question further, Experiment 3 was carried out. The

TABLE I.
Influence of Different Media on Oxidase Production by Pneumococcus.

Medium.	Diluent.	Percentage of dilution.											
		Medium.											
		100	90	80	70	60	50	40	30	20	10	0	
		Diluent.											
		0	10	20	30	40	50	60	70	80	90	100	
Meat infusion (pH 7.8).	Buffer salt (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0	
Plain broth (pH 7.8).	“ “ (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0	
Fresh horse serum.	“ “ (pH 7.8).	1	1	2	4	4	4	4	4	4	4	0	
20 per cent fresh horse serum.	20 per cent meat infusion.	4	3	2	2	1	1	Tr.	0	0	0	0	
20 “ “ “ “ “	20 per cent of 1 per cent peptone solution.	4	3	2	1	0	0	0	0	0	0	0	
20 “ “ “ “ “	20 per cent plain broth.	4	3	2	1	0	0	0	0	0	0	0	
20 “ “ “ guinea pig serum.	50 per cent meat infusion.	4	4	4	4	4	4	4	4	3	2	0	
20 per cent fresh guinea pig serum.	50 per cent of 1 per cent peptone solution.	4	4	4	3	2	1	Tr.	0	0	0	0	

The figures 1, 2, 3, and 4 represent respectively 25, 50, 75, and 100 per cent oxidation as judged by the intensity of the color of the indicator. 0 means no visible oxidation.

All tubes contained 2 cc. of medium and 0.2 cc. of 1:500 *p*-aminoleucomalachite green.

Observation made after 48 hour period of growth.

results show definitely that guinea pig serum possesses the essential elements for the oxidation of the indicator by pneumococci to a greater degree than horse serum. If undiluted meat infusion is used as a basis for comparison it is seen that a 1 per cent mixture of meat infusion

with a constant amount of horse serum inhibits the reaction; whereas, 40 per cent of meat infusion is necessary to cause the same inhibition in guinea pig serum. Because of the result of the last experiment, sera from seven different animal species, under as nearly the same conditions as practicable, were studied. The experiment with each specimen was repeated three times with sera of rat, guinea pig, rabbit, horse, man, cat, and chicken, with the following results. In order of suitability for the oxidation reaction, the general grouping was: rat, guinea pig, and rabbit best; horse next best; man, cat, and chicken least suitable. There exists the possibility that the variation in the sera from different animal species may be due to the presence of hemoglobin, though reasonable care was exercised in securing the specimens of sera. The animals, with the exception of the horse, were bled from the heart by means of a syringe with a new needle, both of which had been washed out with normal physiological salt solution prior to use.

In addition, it has been noted that hemoglobin influences the reaction; a small amount is stimulatory, a larger quantity inhibitory. Oxygen is essential for the reaction itself, no change taking place under anaerobic conditions. However, if air is admitted to an 8 hour culture which has been grown anaerobically in suitable medium, oxidation takes place almost immediately. H ion concentration between the limits of pH 6 and 7.8 increases the rate of oxidation in direct ratio to the H ion concentration,—the more acid the media, the more rapid the reaction. Virulence of the pneumococci does not seem to cause any change in the degree or rate of oxidation of the indicator. During the course of another investigation, the virulence of a strain of pneumococcus was so altered that the minimum lethal dose of various cultures for mice was as follows: 2, 1,800, 46,000, 120,000, and 3,000,000 organisms. The cultures were tested in varying amounts of meat infusion in a constant quantity of guinea pig serum. No difference in the reaction was discernible. It was also found that heating fresh serum for 30 minutes at 55°C. made it more suitable for the reaction, in part destroying the inhibiting power of undiluted serum. A concentration of 1 per cent dextrose in serum was found to increase the rate of oxidation by pneumococci.

Reaction with Different Microbic Species.

In applying the test to other species of microorganisms, we have made use of conditions for oxidase production which were optimum for pneumococci; namely, 10 per cent fresh horse serum, 10 per cent fresh horse serum with the addition of 1 per cent dextrose, and 10 per cent

TABLE II.
*Failure of the Oxidase Reaction.
Miscellaneous Group of Bacteria.*

Organism.	No. of strains.	Medium pH 7.8.			Plain broth.
		10 per cent horse serum.	10 per cent horse serum with 1 per cent dextrose.	10 per cent guinea pig serum.	
<i>Staphylococcus aureus.</i>	6	0	0	0	0
“ <i>albus.</i>	4	0	0	0	0
<i>Meningococcus.</i>	14	0	0	0	0
<i>B. typhosus.</i>	3	0	0	0	0
“ <i>paratyphosus</i> A.	2	0	0	0	0
“ “ B.	3	0	0	0	0
“ <i>coli communis.</i>	5	0	0	0	0
“ “ <i>communior.</i>	2	0	0	0	0
“ <i>dysenteriae</i> Flexner.	2	0	0	0	0
“ “ Shiga.	3	0	0	0	0
“ <i>friedländeri.</i>	1	0	0	0	0
“ <i>lactis aerogenes.</i>	2	0	0	0	0
“ <i>alkaligenes.</i>	1	0	0	0	0
“ <i>pyocyaneus.</i>	3	0	0	0	0
“ <i>proteus vulgaris.</i>	1	0	0	0	0
“ <i>cereus.</i>	1	0	0	0	0
“ <i>subtilis.</i>	1	0	0	0	0
“ <i>anthracoides.</i>	1	0	0	0	0
“ <i>violaceus.</i>	1	0	0	0	0
<i>Spirillum cholerae asiaticæ.</i>	1	0	0	0	0

fresh guinea pig serum. The work represented in the tables was done as a series of complete experiments. A sample of serum and indicator which was to be employed for the experiment was tested prior to its use to establish its suitability as a substrate for oxidase formation by growing pneumococci.

1. *Miscellaneous Group*.—Table II represents the results of the study of twenty different microbic species comprising 57 strains.¹⁵ It is at once apparent that, under the same conditions in which the pneumococcus causes complete oxidation of the aminoleucomalachite green, no oxidation of the indicator occurs with the other members of the microbic species tested.

TABLE III.
Positive Oxidase Reaction.
Pneumococcus of Human Origin.

Pneumococcus.	10 per cent horse serum.		10 per cent horse serum with 1 per cent dextrose.		10 per cent fresh guinea pig serum.	Plain broth.	1 per cent dextrose broth.
	Period of observation.						
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	72 hrs.
F 55	4	4	4	4	4	0	0
A 66	4	4	4	4	4	0	0
J 36	4	4	4	4	4	0	0
F 104	2	4	2	4	4	0	0
Lister G	2	4	2	4	4	0	0
Ca 81	4	4	4	4	4	0	0
Lister F	4	4	4	4	4	0	0
Melford	Tr.	4	2	4	4	0	0
V 1 16	4	4	4	4	4	0	0
Neufeld	4	4	4	4	4	0	0
169	Tr.	4	1	4	4	0	0
168	4	4	4	4	4	0	0
D 40	4	4	4	4	4	0	0
F 208	3	4	3	4	4	0	0
Jones	2	4	2	4	4	0	0
Lister A	2	4	3	4	4	0	0
“	Tr.	4	3	4	3	0	0

2. *Pneumococcus*.—As with the other bacteria, these strains were picked from single colonies and grown on serum broth and were stained to determine purity prior to use in the test.¹⁶

¹⁵ With the exception of the meningococcus, one strain each of the organisms given in the table were old stock cultures kindly furnished by Dr. Stanhope Bayne-Jones. The meningococci were stock strains from The Rockefeller Institute. The remaining cultures had been recently isolated from different sources.

¹⁶ We are indebted to Dr. O. T. Avery for the excellent collection of strains of both pneumococci and streptococci here studied. The latter organisms comprised a large portion of those described by Avery and Cullen (Avery, O. T., and Cullen, G. E., The use of the final hydrogen ion concentration in differentiation of *Streptococcus hæmolyticus* of human and bovine types, *J. Exp. Med.*, 1919, xxix, 215).

In contradistinction to the results obtained with the organisms of the above miscellaneous group, nine out of seventeen strains of pneumococci (Table III), irrespective of type, grown on 10 per cent horse serum, with or without dextrose, completely oxidize the leuco compound in a 24 hour period, all of them in 72 hours. The difference between the influence of horse serum and guinea pig serum is here emphasized. Whereas in 24 hours on the horse serum, nine out of

TABLE IV.
Positive Oxidase Reaction.
Streptococcus viridans of Human Origin.

<i>Streptococcus viridans.</i>	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.	
	Period of observation.					
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
B/26	Tr.	4	0	4	2	4
A/148	"	4	0	4	4	4
A/179	"	4	1	4	4	4
A/141	"	4	0	4	3	4
A/135	"	4	Tr.	4	4	4
380	"	4	"	4	4	4
A/4	"	4	"	4	4	4
R/0	"	4	"	4	4	4
B/38	"	4	"	4	2	4
B/39	1	4	1	4	2	4
B/26	1	4	1	4	4	4

seventeen strains produce a No. 4 reaction (100 per cent), in this same length of time with guinea pig serum, complete oxidation occurred in sixteen out of seventeen, the remaining one producing a reaction but slightly less (No. 3, or 75 per cent).

3. *Streptococcus viridans*.—*Streptococcus viridans*, like pneumococci, gave a positive reaction with this indicator, but seemingly at a lower rate (Table IV). Growth for 24 hours is marked by only a slight oxidation, which, however, becomes complete in 72 hours. Again, guinea pig serum enhances the reaction, and to such a degree that in the serum of this animal the demarcation between the strains of *Streptococcus viridans* and pneumococci becomes greatly obscured.

TABLE V.
Positive Oxidase Reaction.
Hemolytic Streptococci of Human Origin.

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.	10 per cent horse serum pH 7.8, with 1 per cent dextrose.	10 per cent guinea pig serum pH 7.8.		
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
79	Tr.	1	Tr.	4	1
47	"	3	"	4	1
267	0	1	0	2	1
E 7	0	2	0	4	1
43	0	2	0	4	2
A 49	0	4	0	4	3
E 208	0	1	0	4	1
E 223	0	1	0	4	1
239	0	1	0	4	3
E 200	0	2	0	4	2
151	0	1	0	4	1
95	0	0	0	0	1
66	0	2	0	4	1
38	2	2	2	4	2
10	0	2	0	4	2
Boston	0	1	0	4	2
149	0	1	0	4	1
46	0	2	0	4	1
56	0	2	0	4	1
E 238	0	2	0	4	1
8	0	2	0	4	1
27	0	1	0	4	1
92	0	2	0	4	1
89	Tr.	3	1	4	1
25	0	1	0	4	1
S 4	Tr.	2	Tr.	4	1
E 232	0	2	0	2	2
M 29	0	2	0	4	1
M 48	0	1	0	4	1
143	0	0	0	1	3
16	0	1	0	4	1
S 84	0	1	0	4	2
134	0	0	0	0	1
286	0	2	0	4	1
148	0	0	0	4	2
83	0	1	0	4	1
11	0	1	0	4	1
50	0	2	0	4	3
E 265	0	1	0	4	1

TABLE V—*Concluded.*

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.	10 per cent horse serum pH 7.8, with 1 per cent dextrose.	10 per cent guinea pig serum pH 7.8.		
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
72	0	2	0	4	1
E 256	0	2	0	4	2
271	0	2	0	2	1
134	0	2	0	4	2
59	0	2	0	2	1
44	0	2	0	4	1
C 67	0	2	0	4	1
2	Tr.	2	0	4	1
39	0	0	0	0	1
15	Tr.	1	0	4	2
140	0	1	0	4	2
48	0	1	0	4	1
C 251	Tr.	Tr.	0	1	1
29	0	1	0	4	1
144	0	1	0	4	2
150	0	3	0	4	1
S 6	0	3	0	4	2
S 71	0	3	0	4	3
S 93	0	3	0	4	Tr.
S 128	0	1	0	4	1
S 116	0	Tr.	0	4	1
S 97	0	1	0	4	1
S 85	0	2	0	4	1
S 35	Tr.	1	0	4	1
S 14	"	1	0	4	2
118	"	1	0	4	1
S 24	0	2	0	4	2
S 1	0	0	0	4	1
E 236	0	2	0	4	1
S 108	0	2	0	4	1
96	0	2	0	4	1
S 148	0	Tr.	0	4	1
209	Tr.	1	0	4	2
F 1	0	Tr.	0	4	1

4. *Hemolytic Streptococci of Human Origin.*—The study by this test of 73 strains of hemolytic streptococci of human origin demonstrated a difference from pneumococcus and perhaps in a slight degree from *Streptococcus viridans*; that is, with horse serum media, with or

without dextrose, the reaction was negative in a 24 hour period save in the case of one strain (No. 38); on horse serum without dextrose, the reaction after 72 hours ranged from nil with the majority of strains to a complete oxidation with one (No. A 49). But in horse serum to which dextrose had been added, all but ten strains (Nos. 267, 95, E 232, 134, 39, 59, 271, 143, and C 251, Table V) gave a No. 4 (100 per cent) reaction in 72 hours. *A priori* it seems likely that the influence of the dextrose with the serum is one of H ion concentration. That may be true with the hemolytic streptococci of human origin, but as shown above in the miscellaneous group (Table II), in spite of the low final H ion concentration of the media to which dextrose had been added, no oxidation of the indicator resulted. Although H ion concentration influences the rate of oxidation of *p*-aminoleucomalachite green by certain species of organisms that possess this oxidative faculty, in no case in which the reaction was negative for 72 hours was it possible to bring about the oxidation of the indicator by changing the H ion concentration of the serum. The specific oxidizing power seems to be an inherent characteristic of certain species of organisms. The degree of oxidation in guinea pig serum indicates that the hemolytic streptococcus of human origin possesses lower oxidizing activity than either *Streptococcus viridans* or pneumococcus.

5. *Hemolytic Streptococci of Milk Origin.*—With this group of streptococci the reaction is much less pronounced than with the bacteria studied above (Paragraphs 2 to 4) and the type of reaction approaches closely that of miscellaneous Group 1. All, excepting four strains, reacted either negatively or but slightly positively. One of these (No. M 53) was isolated by Jones¹⁷ from the milk of a cow showing no evidence of mastitis and was considered by him as perhaps of human origin. The uniformity of the reaction with guinea pig serum demonstrates clearly that while these strains are able to oxidize the indicator, the reaction is less pronounced than with the other species studied (Table VI).

6. *Hemolytic Streptococci of Cheese Origin.*—It is interesting and perhaps significant that the members of this group react in a fashion

¹⁷ Jones, F. S., Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections, *J. Exp. Med.*, 1918, xxviii, 253.

TABLE VI.
Variations in the Oxidase Reaction.
Hemolytic Streptococci from Milk.

Streptococci from milk.	10 per cent horse serum pH 7.8.	10 per cent horse serum pH 7.8, with 1 per cent dextrose.	10 per cent guinea pig serum pH 7.8.		
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Indian Head.	0	0	0	0	0
Eagle Lunch.	Tr.	0	1	1	3
Munster Brand.	"	0	0	1	4
Philadelphia No. 1.	0	0	0	Tr.	1
" Cream.	Tr.	0	0	0	Tr.
Walker-Gordon.	0	0	0	1	1
Ancre Brand.	0	0	0	0	2
Philadelphia O.	0	0	0	0	1
Eagle Brand.	0	0	0	0	2
Sheffield (C).	0	0	0	0	3
" (F).	Tr.	Tr.	0	1	1
Crawford Farms.	0	0	0	0	3
Sheffield (E).	0	1	0	4	3
M 3	0	0	0	0	2
M 26	0	2	0	4	2
M 65	0	2	0	2	1
M 43	1	Tr.	1	0	2
M 95	Tr.	0	0	1	1
M 53	0	4	0	4	2

TABLE VII.
Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cheese.

Streptococci from cheese.	10 per cent horse serum pH 7.8.	10 per cent horse serum pH 7.8, with 1 per cent dextrose.	10 per cent guinea pig serum pH 7.8.		
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Ch 3	Tr.	0	0	1	3
Ch 12	"	0	1	0	3
Ch 2	"	0	Tr.	Tr.	3
Ch 14	"	0	0	0	3
Ch 10	"	0	0	1	2
Ch 15	0	0	0	0	3
Ch 22	0	0	0	0	1
Ch 11	0	0	0	0	3

similar to those of milk origin. The limited number of strains studied does not warrant the conclusion that the strains from milk and cheese origin can be characterized as identical. However, the streptococci from these sources are differentiated in the present study both from streptococci of human origin and streptococci of udder of cow and mastitis (Table VII).

TABLE VIII.
Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cow's Udder and Mastitis.

Organism.	10 per cent horse serum pH 7.8.	10 per cent horse serum pH 7.8, with 1 per cent dextrose.	10 per cent guinea pig serum.		
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Streptococci from udder.					
V 3	Tr.	1	0	4	1
V 7 5/6	0	2	0	4	1
V 9	1	4	0	4	3
V 5	0	2	0	4	2
V 2	0	2	0	4	2
V 1	0	2	0	3	1
V 8	0	2	0	4	1
V 20	0	0	0	4	1
Streptococci from mastitis.					
C 69	0	0	0	4	2
C 53	0	2	0	4	1
C 57	0	2	0	4	1
C 67	0	2	0	4	1
C 42	0	2	0	4	1

7. *Hemolytic Streptococci from Udder and Mastitis.*—Whatever the truth concerning the origin of the hemolytic streptococci isolated from milk and cheese, the test is not differential for organisms from human or bovine sources. Strains obtained from the udder or from mastitis of cows give manifestly the same reaction as those of human origin (Table VIII).

DISCUSSION.

It has been shown that certain bacterial species in common with both plant and animal tissue are capable of producing an oxidase as

determined by the oxidation of *p*-aminoleucomalachite green. We are not prepared to state that the effect on this indicator of pneumococci is characteristically that of an oxidase. However, if we hold to Portier's definition of an oxidase,—an enzyme capable of changing gaseous oxygen into active oxygen,—we may assume that the oxidation of *p*-aminoleucomalachite green under the conditions reported in this paper is an indication of the presence of such an enzyme.

In the present report the attempt at the quantitative estimation of the degree of oxidase activity, as judged by the intensity of the color of the oxidized leuco base, has been essentially an approximation. However, sufficient work has since been done with a quantitative colorimeter technique to establish this approximation as a fair index of oxidase activity. In addition, despite considerable difficulty with electrodes, the potential in a typical oxidation and reduction set-up has revealed the possibility of using the method in a more exact study. The feasibility of the measurement of oxidation-reduction potential in biological fluids has been shown by Potter¹⁸ in the case of viable yeast, and Gillespie¹⁹ in the case of bacteria in water-logged soils. More recently, Clark²⁰ states that he has confirmed Gillespie's work in the main and has been able to develop a system of oxidation and reduction indicators which may be used colorimetrically to determine oxidation-reduction potentials.

The indicator described here separates a relatively small group of microbic species into a group distinct from other known pathogens. The line of demarcation is definite; that is, under the most favorable conditions found by us under which oxidation of the indicator by pneumococci occurs (dextrose in serum) there is not the slightest trace of oxidation discernible with any of the strains studied, except the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. It is not believed that the other bacteria are incapable of oxidation of this character, but rather that the oxidation potential produced by the

¹⁸ Potter, M. C., Rate of fermentation as measured by difference of potential, *Proc. Univ. Durham Phil. Soc.*, 1912, iv, 230.

¹⁹ Gillespie, L. J., Reduction potentials of bacterial cultures and of water-logged soils, *Soil Science*, 1920, ix, 199.

²⁰ Clark, W. M., Studies on oxidation-reduction. I. Introduction, *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.

growing organism is not effective for the indicator in question. This view is strengthened by the fact that there is considerable variation in the degree of oxidation among the species giving a positive reaction with this leuco base. *Pneumococcus* gives the most intense reaction, *Streptococcus viridans* next, and *Streptococcus hæmolyticus* follows, the result varying in part with the source of the organism. The findings with the hemolytic streptococci of human origin and cow's udder and mastitis are not only most pronounced relatively speaking but are indistinguishable from one another, while the organisms from milk and cheese though apparently similar to those just mentioned are of lower oxidative power. We consider these results as a preliminary study. Further work has been confirmatory, but an insufficient number of strains of organisms has been studied to make generalization advisable.

SUMMARY AND CONCLUSIONS.

1. A simple technique is described for studying the oxidase action of bacteria by means of the oxidation of *p*-aminoleucomalachite green.
2. It is shown that pneumococci under aerobic conditions produced an oxidase when grown on suitable medium. The sera of any of seven different animal species constitute such a medium, the degree of oxidation by the pneumococcus depending upon the animal from which the serum was taken—rat, guinea pig, rabbit, horse, man, cat, and chicken in order of diminishing suitability.
3. Conditions favoring the oxidation of *p*-aminoleucomalachite green by a single strain of pneumococci are: the presence of a slight amount of hemoglobin, dextrose, H ion concentration on the acid side, and heating of fresh serum for 30 minutes at 56°C. Conditions preventing the oxidation are: sterilized meat infusion, 1 per cent peptone, plain broth, a high concentration of hemoglobin, and absence of oxygen. In a quantitative fashion, meat infusion, 1 per cent peptone, and plain broth interfere with the suitability of serum as a substratum of oxidase production by the pneumococcus.
4. Twenty-three microbic species were studied with reference to oxidative power. They were grown upon 10 per cent horse serum, with and without dextrose, upon 10 per cent guinea pig serum, and upon plain broth. Only three of the twenty-three gave evidence of oxida-

tive power as tested by *p*-aminoleucomalachite green; namely, the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. Among the strains, of these three pneumococci gave the most intense reaction, after which *Streptococcus viridans* and *Streptococcus hæmolyticus* follow in the order named, but with a noticeable variation among the different strains of *Streptococcus hæmolyticus*.

5. Hemolytic streptococci of human and bovine origin were studied. The only variation in the type of reaction was manifested by the streptococci of milk and cheese origin. Strains from these sources showed definitely the least oxidase activity. Streptococci from mastitis and cow's udder were indistinguishable by the test from the hemolytic streptococci of human origin.