THE RESPIRATION MECHANISM OF PNEUMOCOCCUS. III*

By M. G. SEVAG and LORE MAIWEG

(From the Robert Koch Institute, Berlin, Germany)

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In two previous communications on the respiratory mechanism of the pneumococcus[1] the following observations were made. A washed suspension of various types and strains of pneumococcus uses up oxygen in the presence of glucose, sodium lactate, ethyl and propyl alcohols, and glycogen. The oxygen thus consumed is quantitatively converted into hydrogen peroxide as shown by analysis after a 10–20 minute reaction period. Later a discrepancy occurs, since the per cent of hydrogen peroxide found is less in proportion to the volume of oxygen consumed. This discrepancy is caused by an intermediary reaction between hydrogen peroxide and pyruvic acid, these being formed simultaneously from glucose or lactic acid. In the case of lactic acid the reaction forms one molecule of acetic acid, one molecule of carbon dioxide, and one molecule of water. Besides these reaction products, we have isolated another acid of unknown constitution. The reactions involved in the formation of this acid we assume to be responsible for the excess of hydrogen peroxide found during the oxidation of lactic acid. Should lactic acid be quantitatively oxidized to hydrogen peroxide and pyruvic acid, they would react with one another at the time of their formation, and would be absent in the reaction mixture. However, since there is an excess of hydrogen peroxide, it must be assumed that the quantity of pyruvic acid formed is less than that of hydrogen peroxide, and the presence of this excess peroxide must be attributed to another source.

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[1] Sevag, M. G., *Ann. Chem.*, Berlin, 1933, **507**, 92; *Biochem. Z.*, Berlin, 1933, **267**, 211.

Because of the destruction of the enzyme system by the excess hydrogen peroxide, the consumption of oxygen begins to slow down after 20–30 minutes, and the system is paralyzed entirely after 60–80 minutes. This destruction is accounted for by the lack of catalase in the cellular substance, or the lack of ability of the organism to complete the reaction and to convert lactic acid and glucose quantitatively into hydrogen peroxide and pyruvic acid. These observations bring up the question: What will be the oxygen-consuming capacity of various organisms if we supply them with protective agents such as catalase and pyruvic acid?[2] The addition of 1 cc. of colorless, clear, blood catalase, or 1 cc. M/5 sodium pyruvate to the system increases the oxygen consumption up to 1772 per cent and with pyruvic acid up to 920 per cent within 300 minutes. The rôle of catalase and pyruvic acid as protective agents for enzyme systems is evident. Not only do they enable the organisms to carry on their respiratory functions, but also to maintain their reproductive functions and virulence for a longer period of time. The organism in a reaction system consisting of phosphate buffer and a fermentable substrate, after a 3 hour reaction period failed to grow in a new medium, and failed to kill mice. On the other hand, in the presence of catalase and pyruvic acid the organism maintained its ability to respire, and to grow in a new medium and to kill mice. Furthermore the activity of the organism respiring in a system containing glucose or lactic acid as substrate, and catalase or pyruvic acid as protective agent is many times greater than the activity of those organisms which respire in a similar system free from a fermentable substrate. In the absence of a substrate the organisms are deprived of a source of energy necessary for self-maintenance, whereupon they undergo oxidative autolysis, resulting in a diminution of the number of active reproductive forms. This is usually associated with a loss of virulence, although the organisms are still viable.

In the presence of protective agents the capacity of various types and strains of pneumococci to consume oxygen is practically of the same magnitude. The slight discrepancies do not offer sufficient basis for the formation of a definite opinion concerning their metabolic

[2] Sevag, M. G., *Naturwissenschaften*, 1933, **21**, 466–467.

differences. This similarity is only apparent, and is due to the early destruction of the organism by hydrogen peroxide. Noteworthy differences are first observed when we supply the respiring system of each organism with protective agents. They remove the hydrogen peroxide immediately after its formation, and hence the organisms are able to survive the experiment. By this means sharp differences can be observed (1) between various types, (2) between various strains belonging to the same type, (3) between virulent and avirulent organisms belonging to the same type, and finally (4) between young and old avirulent organisms of the same strain. It has also been shown that a young avirulent organism derived from Type I virulent (75) consumed many times more oxygen than the old avirulent organism derived from Type I virulent (Laux). We were not able to decide whether this difference was due to individual characteristics inherited from the various virulent parent strains, or was true in young and old avirulent mutants derived from a single virulent parent strain. In the present paper we have attempted to do this.

EXPERIMENTAL

The consumption of atmospheric oxygen was measured by means of a Barcroft-Warburg apparatus, using Brodie's solution as a manometer liquid. For the respiratory system the bacterial sediment was prepared as follows: To obtain the virulent organisms beef broth was inoculated with the heart's blood of an infected mouse and incubated for 10 hours, after which 0.1–0.5 cc. was introduced into 100 cc. of 0.1 per cent glucose bouillon pH 7.6, and incubated for 10 hours. Such 10 hour old cultures were used in all the experiments. They were centrifugalized as quickly as possible, and the sediment was suspended in M/15 phosphate buffer pH 8.0, and centrifugalized. The sediment from the second centrifugalization was suspended in the desired volume of the same buffer and used immediately.

The avirulent organisms were obtained by serial transplantation of the virulent organism in homologous rabbit antiserum. To obtain the bacterial sediment for the respiratory systems the above conditions of growth, etc., were strictly observed.

The cultures were examined for purity and morphology. On the basis of previous studies[3] the cultures containing more than 5 per cent of Gram-negative organisms were discarded.

The respiratory system consisted of 1 cc. M/5 substrate solution, 2.5 cc. M/15

[3] Wieland, H., and Sevag, M. G., *Ann. Chem.*, Berlin, 1933, **501,** 151. Sevag, M. G., *Z. Hyg. u. Infektionskrankh.*, 1933, **114,** 756; *Ann. Chem.*, Berlin, 1933, **507,** 92; *Biochem. Z.*, Berlin, 1933, **267,** 211.

TABLE I

*The Relation between the O_2 Consumed and H_2O_2 Found**

Pneumococcus	C.mm. O_2 consumed		Cc. N/100 $Na_2S_2O_3$		C.mm. O_2 recovered as H_2O_2		Per cent of O_2 recovered as H_2O_2	
	d-Glucose	Sodium lactate	*d*-Glucose	Sodium lactate	*d*-Glucose	Sodium lactate	*d*-Glucose	Sodium lactate
Virulent Type I (Laux)	865	1330	4.93	6.57	552	736	64	55
Young avirulent Type I (Laux) (avirulent for a week)	840	1820	4.87	9.75	545	1092	65	60
Old avirulent Type I (Laux) (avirulent since 1927)	650	1080	2.49	4.70	279	527	43	49
Virulent Type II (Erfurt)	1122	1192	5.33	5.61	597	628	53	52.7
Young avirulent Type II (Erfurt) (avirulent for a week)	1393	1238	8.18	8.17	912	915	65	74
Old avirulent Type II (Erfurt) (avirulent since 1928)	500	986	1.78	2.63	199	295	39.8	30

* The duration of the respiration period was 200 minutes.

TABLE II

*Increase of O_2 Consumption in the Presence of Catalase and Sodium Pyruvate**

Pneumococcus	*d*-Glucose			Sodium lactate			$CH_3COCOONa$	Respiration (without a substrate)	
	Alone	With $CH_3COCOONa$	With catalase	Alone	With $CH_3COCOONa$	With catalase		With catalase	Pneumococcus alone
	c.mm.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
Virulent Type I (Laux)	865	2768	4610	1330	3615	6810	1311	984	228
Young avirulent Type I (Laux) (avirulent for a week)	840	3585	9250	1820	3625	8300	437	450	84
Old avirulent Type I (Laux) (avirulent since 1927)	650	2280	3140	1080	3085	4080	143	280	55
Virulent Type II (Erfurt)	1122	2945	3750	1192	3055	5600	474	1482	199
Young avirulent Type II (Erfurt) (avirulent for a week)	1393	2928	5500	1238	3800	7648	1222	1324	69
Old avirulent Type II (Erfurt) (avirulent since 1928)	500	2534	2975	986	2246	2750	1589	1300	70

* The duration of the respiration period was 200 minutes.

phosphate buffer pH 8.0 (or 1.5 cc. buffer with 1 cc. of catalase, or 1 cc. M/5 sodium pyruvate solution), and 1.5 cc. bacterial suspension. To maintain the reaction at the original pH 8.0 we attempted to use M/8 phosphate buffer, but it was found that in this concentration of salt the organism undergoes marked and continuous autolysis, and hence it was found inadvisable to use a buffer solution stronger than M/15. For the same reason a concentration of sodium pyruvate necessary for the complete removal of H_2O_2, namely M/2, could not be used. With every experiment the controls consisted of 3.5 cc. phosphate buffer (or 2.5 cc. buffer with 1 cc. of catalase or 1 cc. of M/5 sodium pyruvate) and 1.5 cc. bacterial suspension.

TABLE III

*Percentage Increase of O_2 Consumption**

Pneumococcus	*d*-Glucose		Sodium lactate	
	With $CH_3COCOONa$	With catalase	With $CH_3COCOONa$	With catalase
	per cent	*per cent*	*per cent*	*per cent*
Virulent Type I (Laux)	129	469	109	429
Young avirulent Type I (Laux) (avirulent for a week)	316	1064	84	352
Old avirulent Type I (Laux) (avirulent since 1927)	260	380	186	270
Virulent Type II (Erfurt)	168	146	160	315
Young avirulent Type II (Erfurt) (avirulent for a week)	29	215	120	441
Old avirulent Type II (Erfurt) (avirulent since 1928)	120	290	†	58

* The duration of the respiration period was 200 minutes.

† As result of a possible combined effect of lactate, pyruvate concentration, and the acetic acid gradually accumulating, an accurate measurement could not be obtained.

The dry weight of bacteria (of 1.5 cc. bacterial suspension) used in all the experiments ranged from 8–12 mg. The results reported here, however, are recalculated for 10 mg. of dry weight for the purpose of immediate comparison.

DISCUSSION

The results in Table I show that during a 200 minute reaction period in the presence of glucose without a protective agent, the O_2 consumed by four different organisms ranges from 650–1393 c.mm., and in the presence of sodium lactate from 986–1820 c.mm. The volume of O_2

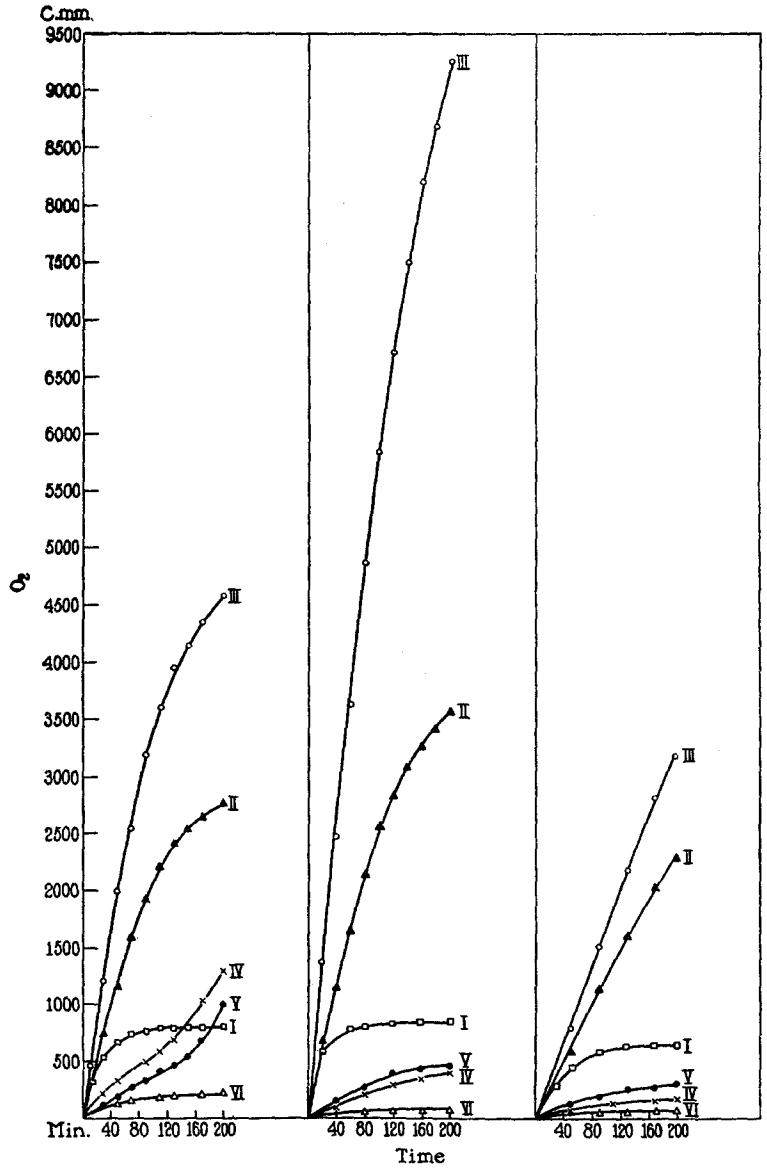

FIG. 1. Oxygen consumption during the oxidation of *d*-glucose by:
(*a*) Pneumococcus virulent Type I (Laux).
(*b*) Pneumococcus young avirulent Type I (Laux), avirulent for a week.
(*c*) Pneumococcus old avirulent Type I (Laux), avirulent for a week.
—□— = I, *d*-glucose alone + pneumococcus.
—▲— = II, *d*-glucose + $CH_3COCOONa$ + pneumococcus.
—○— = III, *d*-glucose + catalase + pneumococcus.
—×— = IV, $CH_3COCOONa$ alone + pneumococcus.
—●— = V, catalase alone + pneumococcus.
—△— = VI, pneumococcus alone.

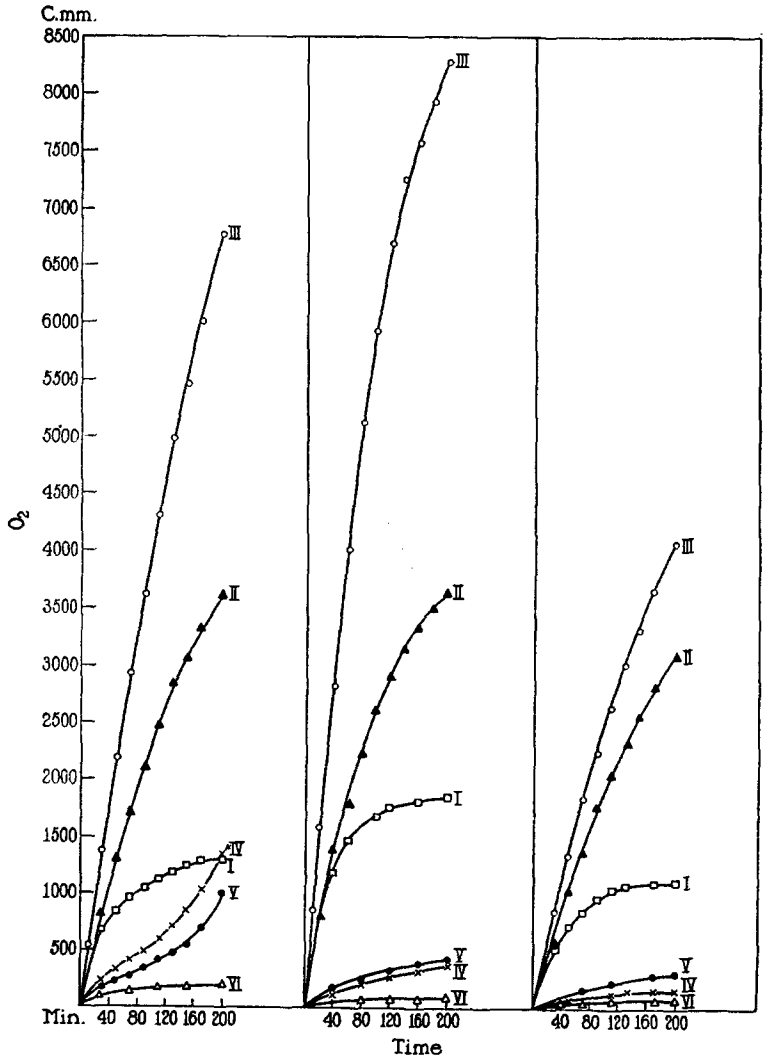

FIG. 2. Oxygen consumption during the oxidation of sodium lactate by:
(*a*) Pneumococcus virulent Type I (Laux).
(*b*) Pneumococcus young avirulent Type I (Laux), avirulent for a week.
(*c*) Pneumococcus old avirulent Type I (Laux), avirulent since 1927.

—□— = I, sodium lactate alone + pneumococcus.
—▲— = II, sodium lactate + $CH_3COCOONa$ + pneumococcus.
—○— = III, sodium lactate + catalase + pneumococcus.
—×— = IV, $CH_3COCOONa$ alone + pneumococcus.
—●— = V, catalase alone + pneumococcus.
—△— = VI, pneumococcus alone.

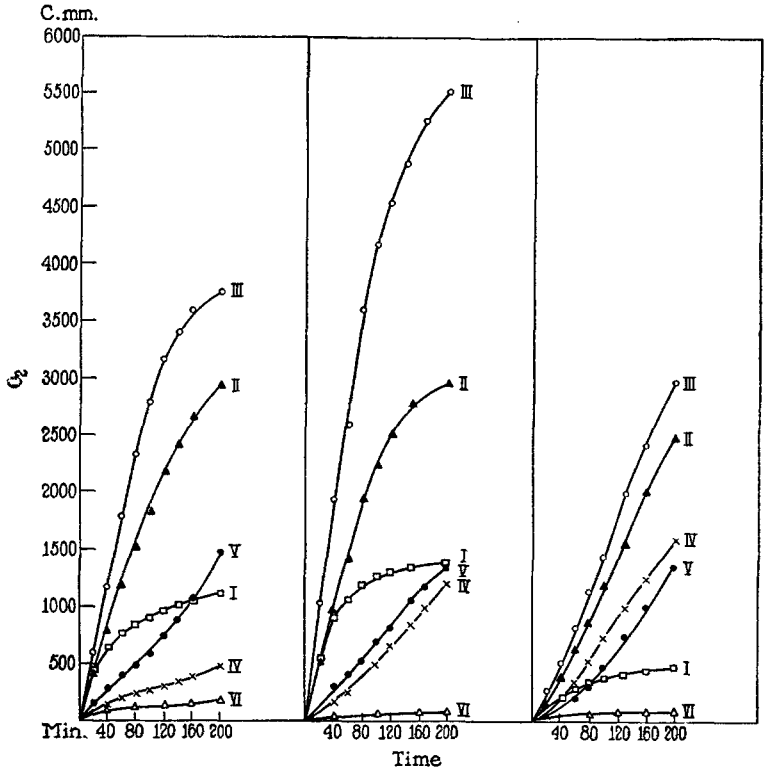

FIG. 3. Oxygen consumption during the oxidation of *d*-glucose by:
(*a*) Pneumococcus virulent Type II (Erfurt).
(*b*) Pneumococcus young avirulent Type II (Erfurt), avirulent for a week.
(*c*) Pneumococcus old avirulent Type II (Erfurt), avirulent since 1928.
—□— = I, *d*-glucose alone + pneumococcus.
—▲— = II, *d*-glucose + $CH_3COCOONa$ + pneumococcus.
—○— = III, *d*-glucose + catalase + pneumococcus.
—×— = IV, $CH_3COCOONa$ alone + pneumococcus.
—●— = V, catalase alone + pneumococcus.
—△— = VI, pneumococcus alone.

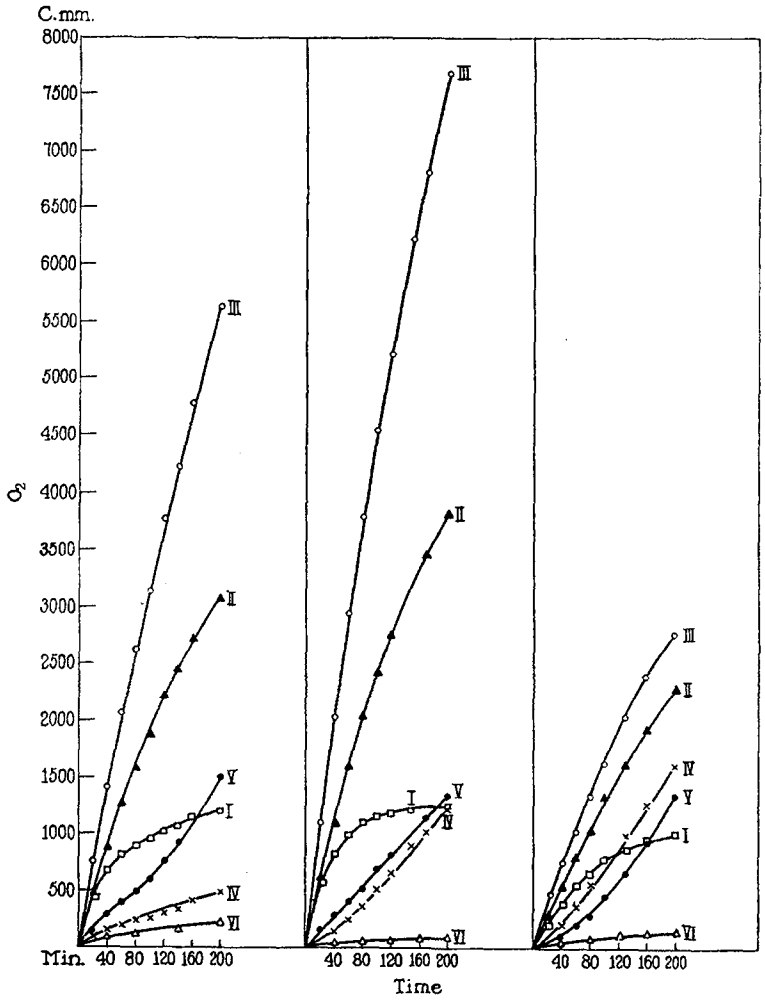

FIG. 4. Oxygen consumption during the oxidation of sodium lactate by:
(*a*) Pneumococcus virulent Type II (Erfurt).
(*b*) Pneumococcus young avirulent Type II (Erfurt), avirulent for a week.
(*c*) Pneumococcus old avirulent Type II (Erfurt), avirulent since 1928.
—□— = I, sodium lactate alone + pneumococcus.
—▲— = II, sodium lactate + $CH_3COCOONa$ + pneumococcus.
—○— = III, sodium lactate + catalase + pneumococcus.
—×— = IV, $CH_3COCOONa$ alone + pneumococcus.
—●— = V, catalase alone + pneumococcus.
—△— = VI, pneumococcus alone.

consumed by each organism, however, is neither a fixed quantity nor are the relationships presented constant. They vary within a certain limit from specimen to specimen, the differences in each series of experiments not exceeding 20–30 per cent. In the absence of a protective agent the ability of a given organism to use more oxygen than another depends on its ability to produce more pyruvic acid from glucose and lactic acid, its sensitiveness to hydrogen peroxide, and its resistance or tendency to autolysis. The same is true of different cultures of the same organism. These properties are often variable. For such reasons we are not able to make sharp differentiations. The results in the table show that with glucose 39–65 per cent of the oxygen consumed can be recovered as hydrogen peroxide, and with lactate 30–74 per cent. These values are by no means absolute. They vary from one experiment to another, but the variations are of negligible magnitude, and do not disturb the above relationships. It is to be noted that the ability of a virulent organism and its recently derived avirulent form to produce excess hydrogen peroxide is considerably greater than that of the old avirulent form of the same strain.

The addition of protective agents to the respiratory system brings about marked differences in the O_2 consumption capacities of the various organisms. The most efficient protective agent is catalase, partly because of the fact that a small amount of a highly active enzyme preparation can be introduced without increasing the salt content of the system. It destroys two molecules of H_2O_2, yielding one molecule of oxygen and two molecules of water. The O_2 so produced involves a liberation of energy which may be utilized by the cell. On the other hand, in only a few cases does pyruvic acid approximate catalase as a protective agent. Its use is confined to a certain concentration, for the reason that a higher concentration brings about the autolysis of the organism. Consequently in the presence of insufficient pyruvate some H_2O_2 is usually found in the reaction mixture. Furthermore the reaction products with pyruvate are acetic acid and CO_2 in the form of $NaHCO_3$. These gradually inhibit or paralyze the system. Thus differences in organisms which, studied in a catalase-containing system, are seen to possess markedly different activities,

may not be apparent when pyruvate is substituted for catalase. Nevertheless as a protective agent it has proved its usefulness by assuring the satisfactory growth of organisms such as old avirulent Type II (Erfurt) during a shorter period.

CONCLUSIONS

From an examination of Tables I to III, and Figs. 1 to 4 and the consideration of various observations, the following facts are evident.

A virulent pneumococcus on being transformed into its avirulent form consumes many times more oxygen than the parent organism; but this gain of activity is a temporary property. After a time it degenerates into a form which consumes very much less oxygen than either the virulent or the recently derived avirulent form. In a comparative study of the metabolic functions, and oxidation products of various virulent and avirulent pneumococci, these phenomena should receive consideration.

The change that takes place in the structure of the enzyme responsible for carbohydrate biosynthesis during the shift from the virulent to the avirulent form may be associated with the changes in the enzyme structure already demonstrated in connection with these metabolic studies.