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Oxidations Effected by the Plague Bacillus

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OXIDATIONS EFFECTED BY THE PLAGUE BACILLUS.

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INTRODUCTION.

IN a previous communication dealing with the metabolism of the plague bacillus the nutritional requirements of the organism were reported (Rao, 1939). In the present paper are described manometric experiments on the oxidations catalysed by suspensions of the bacillus. The main object has been to collect quantitative data on the ability of the organism to oxidize and thus utilize as energy sources the more important sugars, organic acids and amino-acids met with in the environment. This information serves also as a necessary preliminary to further investigations on aspects of bacterial oxidation and reduction. The experiments are concerned only with the question whether a given compound is oxidized and of the rate of oxidations under defined conditions. The nature of the oxidation products, the path of break-down, and other details being outside the scope are not considered. The use of washed suspensions for the study of bacterial metabolism (the so-called 'resting cell' technique) that is used here has the following important and recognized advantages over other methods: the bacterial cells are in a condition unfavourable for multiplication so that respiratory activity can be studied to a large extent in isolation from activity associated with growth: the suspension, which can be standardized, behaves like an enzyme solution: a single substrate can be studied at a time: manometric and microchemical methods can be applied to obtain quantitative results. The method is almost indispensable for the investigation of a micro-organism like the plague bacillus the metabolism of which is very little known.

TECHNIQUE.

Bacterial suspensions.—The plague strain '120/5H, virulent' (which has been selected and used for studying other aspects of metabolism as well) was used for all the experiments. Some of the experiments were repeated with another strain, 'I, virulent', of a different origin and were found to yield results similar in all

important respects. The strain was transferred from the stock blood-agar sub-culture into 200 c.c. acid-digest broth and grown for a week. The broth-culture, after testing purity, was stored in the refrigerator and was used for plating Roux bottles (5 c.c. culture per bottle) containing acid-digest broth at pH 6.9 with 2 per cent agar. After 48 hours' incubation at 27°C. the thick bacterial growth from the agar surface was dislodged and collected in 0.85 per cent saline by rolling a few spherical and smooth glass-beads. The suspension was centrifuged at 3,000 r.p.m. for 15 minutes and the sedimented bacteria were re-suspended in fresh saline. They were not washed further since certain oxidation catalysts are lost during the process (unpublished experiments). The suspension was shaken with glass-beads to break up clumps and its bacterial content was determined by drying 1.00 c.c. at 100°C. for one hour and weighing the residue accurately. The weight of the bacteria in the dry residue is obtained by deducting its content of NaCl (8.5 mg.) from the total weight. The suspension was diluted further with saline to contain 20 mg. bacteria, dry weight, per c.c. Each Roux bottle produces about 100 mg. bacteria or 5 c.c. standard suspension. The suspension was prepared fresh each day since its activity falls even in the refrigerator.

Manometric methods.—The oxidation reactions were studied with Warburg manometers which were shaken at the rate of 100 oscillations per minute in a water-bath maintained electrically at $27 \pm 0.05^\circ\text{C}$. Conical manometric vessels of 15 c.c. to 17 c.c. capacity with side bulbs were used. Each vessel contained the following quantities of solutions: the side bulb contained 0.50 c.c. bacterial suspension containing 10 mg. bacteria; the central well contained 0.10 c.c. 20 per cent KOH with a small roll of filter-paper; the main chamber contained 0.10 c.c. distilled water, 2.00 c.c. of the substrate solution (or in the controls 2.00 c.c. distilled water) and 0.30 c.c. of M/1.5 phosphate buffer at pH 7.0; the total volume being 3.00 c.c.; the gas-space consisted of air. After 15 to 20 minutes' shaking for temperature equilibration, the bacteria were tipped into the main chamber, which marked the zero minute of the reaction. A thermo-barometer and a control manometer (with bacteria but no substrate) were always run with every batch of manometers. Further details of manometric technique were exactly as described by Dixon (1934).

Substrates.—In Table I are listed all the compounds which were tested for bacterial oxidation. They were chemically pure, natural or synthetic, samples supplied by Merck, Kahlbaum, or Schuchardt, or prepared in the laboratory. A few of them were further purified. The substrate concentration was 0.05 molar with three exceptions, which were: (1) ethyl alcohol which is inhibitory at this concentration, (2) cystine which is not soluble to the extent required at the necessary pH, and (3) cystein hydrochloride which was used at the same concentration as cystine for comparison. These three compounds were used at a uniform concentration of 4 mg. per vessel. Tyrosine which is insoluble was used as a fine suspension. All the substrates were neutralized to pH 7.0 (phenol red) with NaOH or HCl before use. In the absence of a complete series of samples of the optical isomers of the amino-acids little light could be thrown on optical specificity.

Expression of results.—Oxidation is indicated and measured by the increased oxygen uptake of the bacteria in the presence of the substrate. The O_2 -uptake of the bacteria in the absence of substrate was determined in a separate control

manometer for every parallel series of manometers, and subtracted from the O₂-uptakes observed with each substrate in that series to obtain that due to the oxidation of the substrates. This is expressed as cu. mm. oxygen (at N. T. P.) taken up by 10 mg. bacteria at a substrate concentration of 0.05 M. and 27°C. and corrected for control. Oxidation curves obtained by plotting O₂-uptake in cu. mm. against time of reaction in minutes are presented for all those substrates which are oxidized at appreciable rates. (In Graph 1 are given the oxidation curves for carbohydrates, in Graph 2 for amino-acids, and in Graph 3 for organic acids.) The rate of oxidation has been expressed as the metabolic quotient, Q_{O₂} (see Table I).

$$Q_{O_2} = \frac{\text{Cu. mm. oxygen absorbed}}{\text{mg. bacteria} \times \text{hours}}$$

= cu. mm. oxygen absorbed by 1 mg. bacteria in 1 hour. From the oxidation curves it can be seen that during the one hour that the reaction has been followed the rate varies considerably from point to point. The Q_{O₂} values of Table I have therefore been calculated for the entire period and represent the average rates of oxidation for the first hour.

TABLE I.

The rates of oxidation of substrates by the plague bacillus.

Substrate.	Q _{O₂}	Substrate.	Q _{O₂}
<i>d</i> (+)-mannose ..	36.1	<i>dl</i> -proline ..	9.79
<i>d</i> (+)-glucose ..	35.8	succinate ..	7.30
<i>d</i> (-)-fructose ..	34.6	<i>l</i> -cystine (4 mg.) ..	5.35
<i>d</i> (+)-galactose ..	31.5	<i>d</i> -glutamate ..	5.20
<i>dl</i> -lactate ..	29.1	<i>l</i> (+)-arabinose ..	4.90
hexosediphosphate ..	28.5	glycine ..	4.88
maltose ..	27.4	<i>dl</i> -cystine (4 mg.) ..	3.87
pyruvate ..	21.6	cystein HCl (4 mg.) ..	3.48
<i>d</i> -mannitol ..	18.1	citrate ..	3.21
<i>dl</i> -serine ..	14.6	<i>dl</i> -phenylalanine ..	3.20
<i>dl</i> -alanine ..	13.0	<i>l</i> -tyrosine ..	2.87
acetate ..	11.0	lactose ..	2.73
fumarate ..	10.3	ethyl alcohol (4 mg.) ..	2.61
<i>l</i> -malate ..	9.85	<i>d</i> (+)-tartrate ..	2.52

TABLE I—concl'd.

Substrate.	Q_{O_2}	Substrate.	Q_{O_2}
<i>l</i> (+)-rhamnose ..	2.43	glycerol ..	0
<i>dl</i> -methionine ..	1.92	formate ..	0
<i>d</i> (+)-xylose ..	1.59	propionate ..	0
sucrose ..	1.56	butyrate ..	0
<i>d</i> -dulcitol ..	1.19	capronate ..	0
<i>dl</i> -isoleucine ..	1.13	<i>dl</i> -aspartate ..	0
<i>dl</i> -valine ..	0.89	<i>l</i> -lysine ..	0
<i>dl</i> -leucine ..	0.16	<i>l</i> -histidine ..	0
raffinose ..	0	<i>d</i> -arginine ..	0
<i>d</i> -sorbitol ..	0	<i>l</i> -tryptophane ..	0

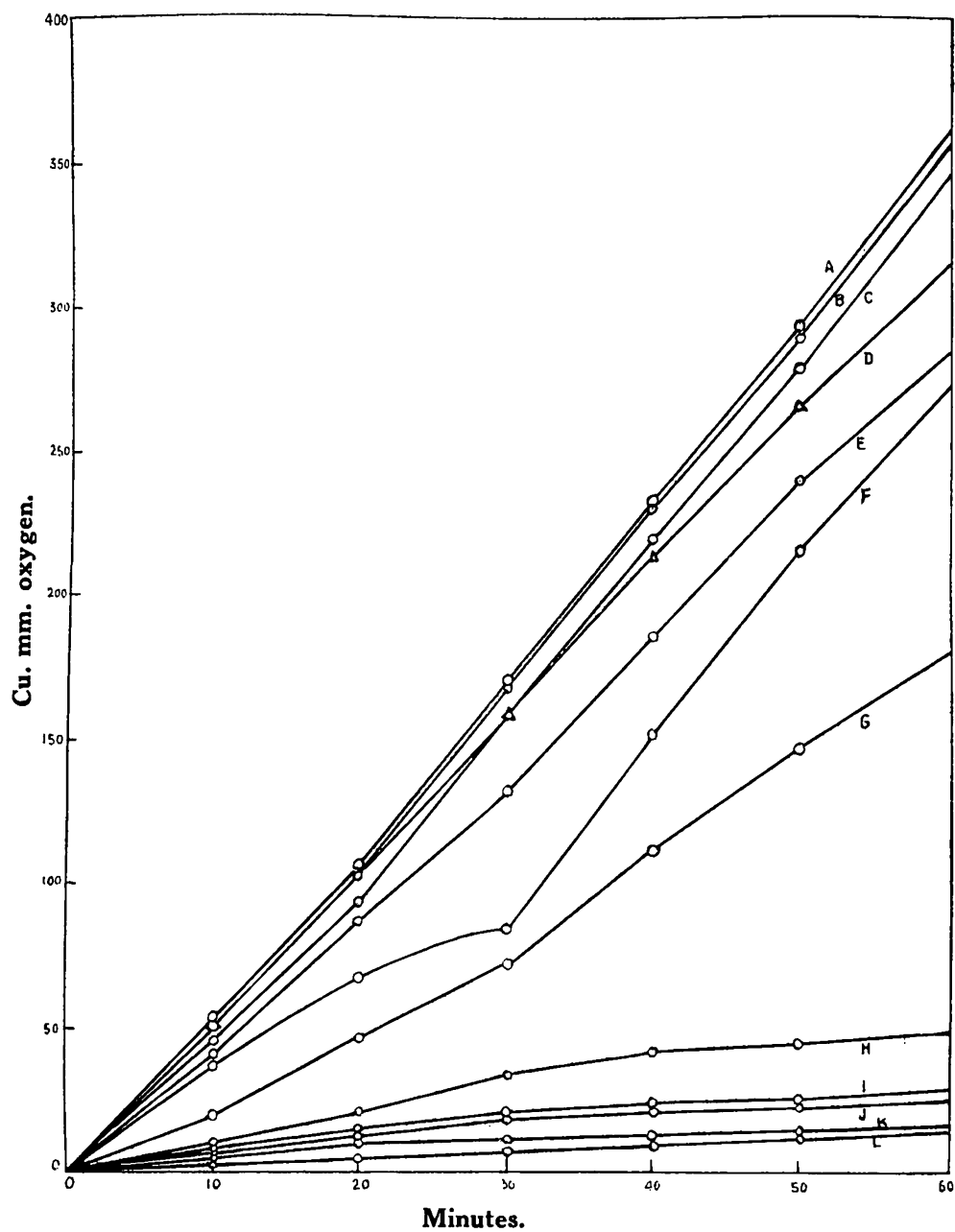
RESULTS AND DISCUSSION.

In Table I is given the list of compounds tested for oxidation by the bacillus arranged in the decreasing order of their Q_{O_2} values. In the discussion that follows the chemical structure of the substrate compounds is given so as to convey, as far as possible, the relation of structure to oxidizability. Towards 12 out of the 48 compounds tested the bacillus possesses no oxidative activity whatever. Several others are oxidized only to a slight extent. The highest activity recorded is displayed towards the hexose sugars (see Graph 1).

Glucose, fructose, and mannose, are oxidized at approximately equal rates, and galactose at a slightly lower rate. The disaccharide maltose (or glucose-4- α -glucoside) is oxidized vigorously, lactose (or glucose-4- β -galactoside) moderately, and sucrose (or 1- α -glucoside-2- β -fructofuranose) slightly. The trisaccharide raffinose (or 2- β -fructofuranose-1- α -glucose-6- α -galactoside) is not oxidized.* Oxidative activity towards the pentoses is considerably less than that towards the hexoses. Arabinose is oxidized moderately; the methyl-pentose, rhamnose, at half this rate, and xylose at a still lower rate. Of the sugar-alcohols mannitol is oxidized rapidly, but dulcitol is oxidized slightly, while sorbitol is not attacked. Hexosediphosphate (1: 6-diphosphofructofuranose) is oxidized at a rate slightly below that of the hexoses. This is a fact of considerable interest in view of the recent demonstration that in the biological break-down of sugar through phosphorylated intermediates hexosediphosphate holds a central position.

*A rapid oxidation observed with a sample of raffinose was found to be due to the presence of a trace of glucose as an impurity. The sugar when freed from this impurity was not oxidized.

GRAPH 1.



OXIDATION OF CARBOHYDRATES.—A-mannose; B-glucose; C-fructose; D-galactose; E-hexosediphosphate; F-maltose; G-mannitol; H-arabinose; I-lactose; J-rhamnose; K-xylose; and L-sucrose.

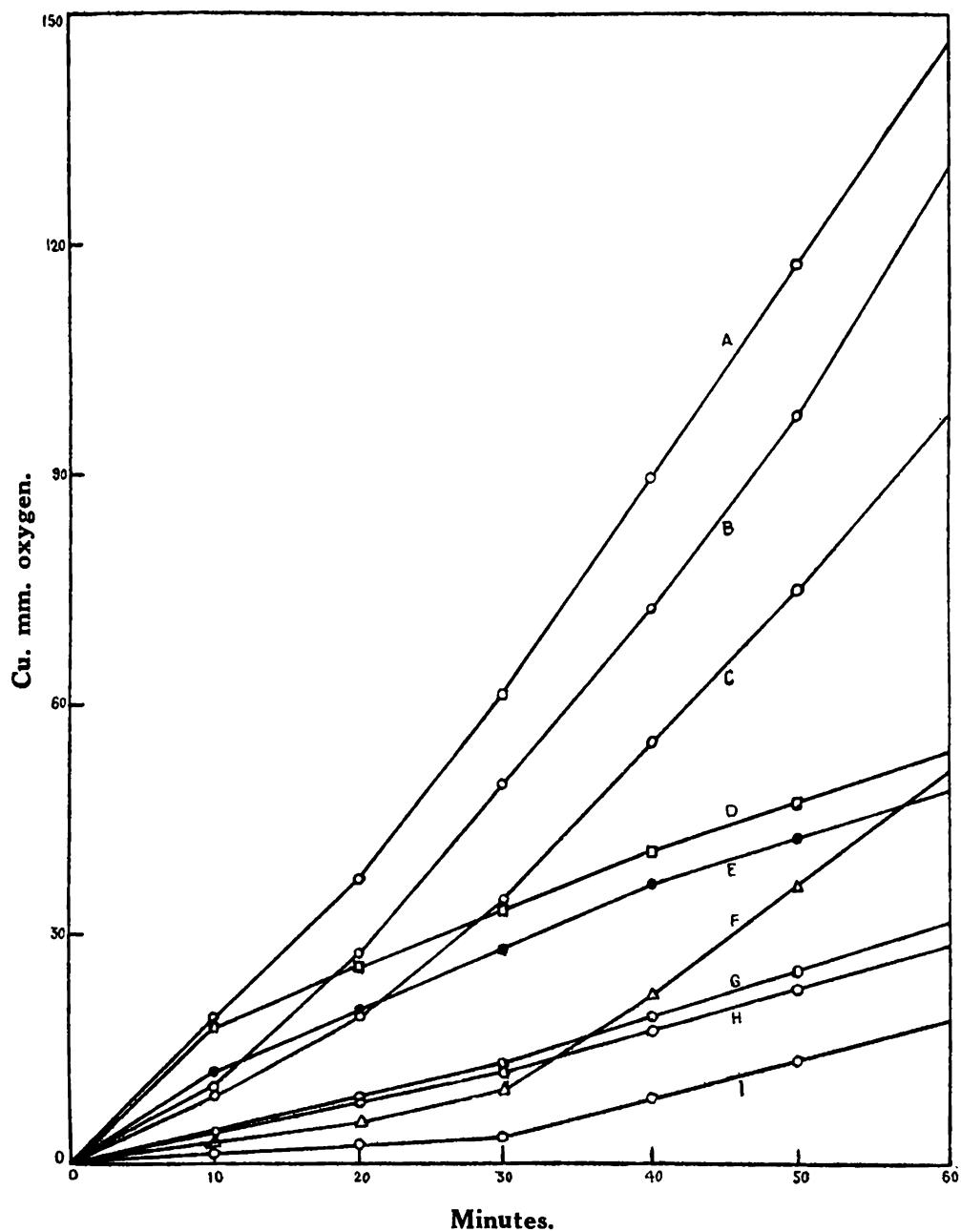
The amino-acids are oxidized by the plague bacillus at rates which are in general lower than those of carbohydrates (*see* Graph 2). Glycine [$\text{CH}_2(\text{NH}_2).\text{COOH}$] is oxidized at a moderate rate. Alanine [$\text{CH}_3.\text{CH}(\text{NH}_2).\text{COOH}$] is attacked rapidly. Aspartic acid [$\text{HOOC}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$] is not oxidized but glutamic acid [$\text{HOOC}.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$] is oxidized. Serine [$\text{CH}_2\text{OH}.\text{CH}(\text{NH}_2).\text{COOH}$] has the highest oxidation rate among the amino-acids. Cystine [$\text{HOOC}.\text{CH}(\text{NH}_2).\text{CH}_2\text{S}-\text{SH}.\text{CH}(\text{NH}_2).\text{COOH}$] and cysteine [$\text{CH}_2\text{SH}.\text{CH}(\text{NH}_2).\text{COOH}$] are both oxidized. *l*-cystine is oxidized at a higher rate than *dl*-cystine indicating that the natural isomer (*l*-) is preferentially attacked. Methionine [$\text{CH}_3.\text{S}.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$] is attacked at a slow rate. The amino-acids with aromatic nuclei, phenylalanine [$\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$], and tyrosine [$\text{HO}.\text{C}_6\text{H}_4.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$], are both oxidized at nearly equal rates though the latter is almost insoluble at pH 7. Proline (α -pyrrolidine carboxylic acid) is oxidized rapidly. Tryptophane (β -indole- α -aminopropionic acid) is, however, not attacked. It is of interest to compare this with the known observation that the indole test is always negative with the plague bacillus. Isoleucine and valine are slightly and leucine is hardly oxidized. Lysine, histidine, and arginine, are left untouched. It should be noted that the amino-acids essential for growth, viz., proline, phenylalanine, and cystine (Rao, *loc. cit.*), are all oxidized by the bacillus.

The biologically important organic acids tested are oxidized at rates which are in general higher than those of amino-acids but lower than those of sugars (*see* Graph 3). Lactic acid [$\text{CH}_3.\text{CHOH}.\text{COOH}$] has next to the hexoses the highest oxidation rate. Pyruvic acid [$\text{CH}_3.\text{CO}.\text{COOH}$] is also oxidized at a high rate. Though acetic acid [$\text{CH}_3.\text{COOH}$] is oxidized at a good rate none of the other monobasic saturated acids tested, formic, propionic, butyric, and capronic, are attacked. Fumaric acid [$\text{HOOC}.\text{CH}=\text{HC}.\text{COOH}$], malic acid [$\text{HOOC}.\text{CH}_2.\text{CHOH}.\text{COOH}$], and succinic acid [$\text{HOOC}.\text{CH}_2.\text{CH}_2.\text{COOH}$], are oxidized at nearly equal rates. But citric acid [$\text{HOOC}.\text{CH}_2.\text{C}(\text{OH})(\text{COOH}).\text{CH}_2.\text{COOH}$] and tartaric acid [$\text{HOOC}.\text{CHOH}.\text{CHOH}.\text{COOH}$] are oxidized to a slight extent only.

Of the remaining compounds glycerol [$\text{CH}_2\text{OH}.\text{CHOH}.\text{CH}_2\text{OH}$] is not oxidized. Ethyl alcohol [$\text{CH}_3.\text{CH}_2\text{OH}$] is inhibitory at 0.05 M. concentration but is oxidized at lower concentrations.

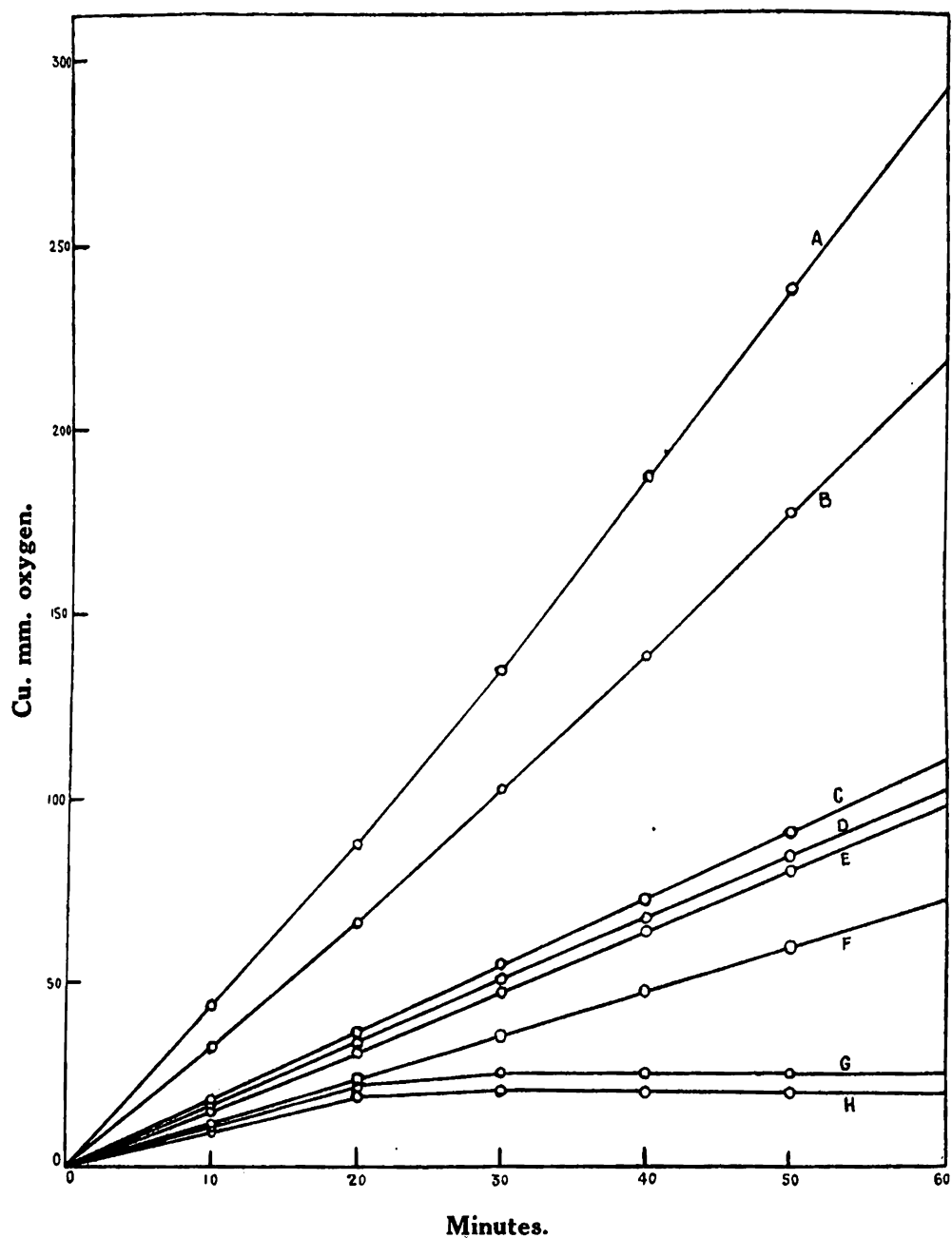
It is pertinent to inquire how far the oxidations studied are paralleled by the fermentation reactions described in the bacteriological literature. Oxidation in which molecular oxygen is the final hydrogen-acceptor is different from fermentation or break-down in the absence of oxygen, particularly with reference to the end-products. However, a basis of comparison is possible since the fundamental condition for the occurrence of either reaction is the *activation* of the substrate. When an organism ferments a substrate it should be expected to oxidize it as well and vice versa, though the production of acidity which accompanies the fermentation of carbohydrates and some other compounds, and which is taken as the main evidence for the occurrence of fermentation in bacteriological tests, may not take place with several other types of substrates. Petrie (1929) has summarized in a tabular

GRAPH 2.



OXIDATION OF AMINO-ACIDS.—A-serine ; B-alanine ; C-proline ; D-L-cystine ; E-glycine ; F-glutamic acid ; G-phenylalanine ; H-tyrosine ; and I-methionine.

GRAPH 3.



OXIDATION OF ORGANIC ACIDS.—A-lactic acid ; B-pyruvic acid ; C-acetic acid ; D-fumaric acid ;
E-malic acid ; F-succinic acid ; G-citric acid ; and H-tartaric acid.

form 12 sets of observations by different workers on the fermentation reactions (acid production) of the plague bacillus. The observations relating to such of the compounds studied here have been re-cast in Table II and presented side by side with the oxidation rates :—

TABLE II.

A comparison of oxidation with fermentation reactions of the plague bacillus.

Observations on fermentation adapted from Petrie (1929).

Substrate.	Oxidation.	Fermentation.	
	Q_{O_2}	Number of times acid production observed.	Total number of observations.
Glucose ..	35.8	12	12
Fructose ..	34.6	11	11
Galactose ..	31.5	10	11
Maltose ..	27.4	10	12
Mannitol ..	18.1	11	12
Arabinose ..	4.90	3	5
Lactose ..	2.73	0	12
Rhamnose ..	2.43	1	1
Xylose ..	1.59	1	2
Sucrose ..	1.56	0	12
Dulcitol ..	1.19	1	9
Raffinose ..	0	0	6
Sorbitol ..	0	0	4
Glycerol ..	0	3	6

A strict comparison of the two types of data is difficult on account of the fact that many of the observations on fermentation are not consistent, due either to real differences in the activities of the different strains or apparent differences caused by wide variations in the technique, and of the fact that the observations are purely qualitative. A general parallel between oxidative and fermentative abilities of the plague bacillus is evident. Manometric experiments clearly show a definite measurable action on substrates like lactose and sucrose though fermentation tests reveal no action whatever.

The study of oxidation conclusively indicates which of the several compounds occurring in the culture media are available to the plague bacillus as energy sources and which of them are metabolized. Glucose or lactic acid are the best (and the least expensive) carbon sources that can be used to supplement media. Of the amino-acids the following only are of service as nitrogen and energy sources, and consequently only these can be added to enrich media: serine, alanine, proline, cystine, glutamic acid, glycine, phenylalanine, tyrosine, and methionine. The two synthetic protein-free media described by the author previously (Rao, *loc. cit.*) can be modified in the light of these facts.

SUMMARY.

1. The oxidation of forty-eight compounds (carbohydrates, amino-acids, and organic acids) by suspensions of strain '120/5H, virulent' of the plague bacillus has been studied manometrically in air at 27°C. and 0.05 M. substrate concentration. The metabolic quotient, Q_{O_2} , has been calculated in each case and oxidation curves have been constructed.

2. The oxidative activity of the bacillus is greater towards carbohydrates (Q_{O_2} 36.1 to 1.59) than towards organic acids (Q_{O_2} 29.1 to 2.52) or towards the amino-acids (Q_{O_2} 14.6 to 0.16). Only thirty-six of the compounds tested are oxidized.

3. Substrates which undergo pronounced oxidation fall in the following series of relative oxidation rates:—

Carbohydrates: mannose > glucose > fructose > galactose > hexosediphosphate > maltose > mannitol > arabinose > lactose > rhamnose > xylose > sucrose > dulcitol.

Amino-acids: serine > alanine > proline > cystine > glutamate > glycine > phenylalanine > tyrosine > methionine.

Organic acids: lactate > pyruvate > acetate > fumarate > malate > succinate > citrate > tartrate.

4. In the light of these experiments it is concluded that the best energy and carbon sources to supplement media are glucose and lactate. The amino-acids which are available to the bacillus as nitrogen sources are: serine, alanine, proline, cystine, glutamic acid, glycine, phenylalanine, tyrosine, and methionine.

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