A Study of the Cytochrome System and some other Aspects of the Respiration of *Aerobacter aerogenes*

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The object of this work was the study of the inter-relationship between the components of cytochrome in *Aerobacter aerogenes*, the effect of metal deficiency on the development of cytochrome in relation to the metabolism of these micro-organisms, and the effect of azide both on their cytochrome system and their respiratory activities.

Whereas in aerobic cells with a typical cytochrome system comprising the components a_3 , a , b and c , much knowledge is now available on the properties of and the relationship between these components, very little is known as to the properties and functions of the components in an atypical cytochrome system.

A. aerogenes shows an atypical cytochrome system comprising the components a_2 , a_1 and b_1 and is thus suitable for the study of these problems. In this bacterium, the component a_2 is much more highly developed than in any known micro-organism, and is therefore easier to study.

Moreover, the fact that *Aerobacter* can be cultivated in a metal-deficient medium, as was shown by Waring & Werkman (1944), makes it possible to follow the effect of metal deficiency on the development and synthesis of the different components of cytochrome in relation to the respiratory activity.

Finally, since the introduction of azide as an inhibitor of respiration and of the activity of certain catalytic systems, an extensive literature on this subject has revealed that its inhibitory effect is more specific than that of cyanide. However, it was shown recently by Aubel & Szulmajster (1947) that in *Escherichia coli* azide does not affect the rate of oxygen uptake in presence of glucose. *A. aerogenes* has also been found suitable for further analysis of this phenomenon.

MATERIAL AND METHODS

Methods of cultivation. *A. aerogenes*, strain I, from the M.R.C. Unit for Chemical Microbiology, Cambridge, was used in the course of this work. It was cultivated either in a nutrient broth with glucose or in a special metal-deficient medium. The nutrient broth had the following composition: peptone, 10 g.; NaCl, 5 g.; 'Lemco' meat extract, 5 g.; glucose, 10 g.; and distilled water 1 l. It was sterilized by autoclaving. Incubation was carried out at 30° in plugged Erlenmeyer flasks, each fitted with a capillary tube reaching

to the bottom of the flask. Air was bubbled through the medium by means of a small mechanical pump, and cotton wool plugs in glass tubes acted as air filters. After 15–18 hr. incubation, the bacteria were harvested, washed twice with distilled water, and suspended in 0.1M-phosphate buffer, pH 6.8.

Metal-deficient medium was prepared according to Waring & Werkman (1944), with the modifications described below. For the preparation of 1 l. of medium, 4 g. Na_2HPO_4 , 1 g. KH_2PO_4 and 1 g. $(\text{NH}_4)_2\text{SO}_4$ were dissolved in about 200 ml. redistilled water in a separating funnel, shaken with about 15 mg. 8-hydroxyquinoline in 1 ml. CHCl_3 for 30 sec., left for 1 or 2 min. and extracted with several lots of 10 ml. CHCl_3 , until the CHCl_3 layer was no longer coloured by the metal complex. This treatment was repeated six times, the medium being finally extracted with six lots of CHCl_3 to remove all traces of the chelating agent. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g.) was added, the solution was boiled to remove CHCl_3 , made up to 1 l. in an Erlenmeyer flask, covered with a well fitting beaker and autoclaved. A 20% glucose solution was treated separately in the same way, autoclaved, and 50 ml. were added to 1 l. of the sterile medium. It was found necessary to sterilize the glucose separately, otherwise a brown insoluble pigment was produced which subsequently interfered with spectroscopic examination.

The inoculations were made in the following way: 250 ml. Erlenmeyer flasks, containing 100 ml. metal-deficient medium, were inoculated from a normal culture. After 24 hr. growth, 0.2 ml. of this was used to inoculate 1 l. of medium. The cultures were incubated at 30°, air being bubbled through them as before. The growth after 24 hr. was about one-third of that obtained from a purified medium to which iron had been added.

Spectroscopic methods. 0.5 ml. of a thick bacterial suspension in 0.1M-phosphate buffer pH 6.8 was placed in a small flat-bottomed glass tube giving a depth of 3–5 mm. This bacterial suspension was then examined by means of a Zeiss microspectroscope ocular fitted to a microscope, as described by Keilin & Hartree (1946). Aeration of the bacterial suspension was carried out by means of a fine glass capillary fitted to an air pump and frothing was prevented by a very small amount of octyl alcohol (octan-2-ol, 'caprylic alcohol') (about 0.001 ml./0.5 ml. of bacterial suspension).

To reinforce the absorption bands, the suspension of micro-organisms was mixed with an equal volume of glycerol and cooled in liquid air, as described by Keilin & Hartree (1949).

Manometric methods. After an initial dry-weight determination, an aqueous suspension of bacteria was diluted with 0.1M-phosphate buffer pH 6.8, to give a dry weight of 10 mg./ml. The experiments were carried out in Barcroft differential manometers at 30°. The bacterial suspension

(1–3 mg. dry weight) and the substrate were added in the right-hand flask to a sufficient amount of 0.1M-phosphate buffer to give a final volume of 3.0 ml. 20% (w/v) KOH (0.3 ml.) was placed in the centre well. The left-hand flask contained 3.3 ml. distilled water.

Aerobic CO_2 output and respiratory quotients were measured by the method of Dixon & Keilin (1933). The special manometric flasks contained a total liquid volume of 3.6 ml.

Determination of dehydrogenase activity. The experiments were carried out at 30° in Thunberg tubes. Each tube contained 1 ml. 5.2×10^{-4} M-methylene blue, 0.3 ml. 0.1M-substrate, and enough 0.1M-phosphate buffer to give a total volume of 3 ml., with 0.3 ml. bacterial suspension in the hollow stopper. The tubes were evacuated and twice washed with O_2 -free N_2 .

Estimation of glucose, pyruvic acid and lactic acid. In the early experiments, glucose was estimated by two methods: the colorimetric method of Folin & Malmros (1929) as described by Umbreit, Burris & Stauffer (1945), which gives the total reducing value, and the method of Keilin & Hartree (1948) utilizing a specific glucose oxidase (notatin). As the two methods gave similar results, the simpler colorimetric method was used in later experiments. Pyruvic acid was estimated by the method of Friedmann & Hangen (1943), using toluene as the organic solvent, and lactic acid by the method of Barker & Summerson (1941), as described by Umbreit *et al.* (1945).

RESULTS

Cytochrome system in bacteria grown in metal-rich medium (nutrient broth)

Washed bacteria have a negligible respiration, and show either very feeble or no absorption bands, the cytochromes being in the oxidized state. The absorption band of component a_2 in the oxidized state is sometimes visible at 645 m μ . On the addition of a suitable substrate (5% glucose was used in the following experiments) the cytochrome is quickly reduced, and the characteristic absorption bands appear. *A. aerogenes* now shows three absorption bands in the visible region: band b_1 at 560 m μ , a_1 at 590 m μ , and a_2 at 630 m μ . Of these, the band b_1 is the strongest. It is a rather wide, asymmetric band, with a reinforcement on the side towards the shorter wavelengths. The a_1 band is very feeble, while the a_2 band is sharp and almost as strong as b_1 .

When the bacterial suspension was aerated, the bands b_1 and a_1 gradually disappeared, while the band a_2 at 630 m μ was replaced by one at 645 m μ . When the aeration was stopped, the cytochrome bands reappeared in their reduced form. At room temperature and in the presence of an excess of substrate, the reduction was almost instantaneous. To follow the progressive changes in the absorption bands during this process, it was necessary to slow down the rate of reduction by cooling the bacteria. The tube containing the suspension of micro-organisms was placed in a small glass cylinder of

6 cm. diameter, the base of which was made of plane glass. The tube was then surrounded by ice. With this arrangement it was possible to increase the time for complete reduction to about 1 min., and to measure with precision the rate of reappearance of the bands of reduced cytochrome. Band b_1 reappeared first. As it became stronger, so the band a_2 at 645 m μ faded and a shading appeared between 645 and 630 m μ . Finally, as b_1 reached its full intensity, the shading disappeared and a_2 returned to its original position (630 m μ) and intensity. In a typical experiment, the time for complete reduction was 105 sec.; the band b_1 reappeared after 30 sec. and the band a_2 began to move from 645 to 630 m μ at about 35–40 sec. A comparison between the time of reduction of components b_1 and a_2 is usually complicated by the facts that their intensities are not the same, and that their absorption bands do not behave in the same way on oxidation: the band b_1 disappearing at 560 m μ , while a_2 at 630 m μ is replaced by a band at 645 m μ . However, in the case of *A. aerogenes* used in this work, a_2 was almost as strong as b_1 . The fact that on reduction the band b_1 reappeared before the strong band a_2 at 645 m μ had shown any change in intensity or position, indicates that the component b_1 was certainly reduced before a_2 . During aeration, however, the band a_2 moved very rapidly from 630 to 645 m μ , before any change in the intensity of b_1 could be observed. Thus the component a_2 was oxidized before b_1 .

The absorption band a_1 was so faint that no definite conclusions about its reactions with the other components could be reached.

Since in all aeration experiments it became necessary to add octyl alcohol in order to prevent frothing, careful investigations were made to find out whether it had any action on the cytochrome or any other part of the respiratory system. Bacterial suspension (0.5 ml.) containing 5% glucose was cooled with ice under the spectroscope and about 0.001 ml. octyl alcohol was added. Under these conditions the time of complete reduction of the cytochrome bands, after aeration, was found to be 15 sec. The mixture was aerated intermittently over a period of 10 min., but the reduction time, after interrupting the air stream, remained constant at 15 sec. Octyl alcohol (0.04 ml.) was then added, and the time of reduction of the cytochrome bands again measured: it was found to be still 15 sec., and remained so for another 5 min. Finally a further 0.06 ml. of octyl alcohol was added. The reduction time immediately after this addition was still 15 sec., but the band a_2 seemed less sharp than before. However, 2 min. later, the time of reduction had increased to 20 sec., and after 2 hr. to about 4 min. In a second experiment, the bacterial suspension was treated with 0.02 ml. octyl alcohol at

room temperature, and then cooled with ice. Under these conditions, the reduction time was far greater than that of a control suspension which had received no octyl alcohol. Thus this reagent can safely be used in small amounts at 0° in studies of the oxidation and reduction of cytochrome, but is dangerous at higher temperatures. The effect of octyl alcohol was also studied manometrically (see below). Attempts to use tributyl citrate in place of octyl alcohol were unsuccessful. This substance strongly inhibited the reduction of cytochrome in *A. aerogenes*, even at 0°.

Azide (0.003M) at room temperature has no observable effect on the cytochrome spectrum of the bacteria. At 0° different results were obtained after 2 months' interval on the same strain of *A. aerogenes*. Differences in the behaviour of the cytochrome bands were also noticed towards cyanide, at 2 months' interval (see below).

In a first series of experiments, when azide was added to a suspension of bacteria which showed the reduced cytochrome bands, no change of spectrum was observed. As soon as a few air bubbles had passed through the suspension, band a_2 moved from 630 to 645 m μ ., i.e. to its oxidized position; but the band b_1 , which in absence of azide disappears almost completely on oxidation, remained clearly visible at approximately half its original intensity, and could not be oxidized further by prolonged aeration. When the aeration was interrupted there was a marked delay in the return of the band a_2 to its normal reduced position at 630 m μ . In fact, the change was two to three times slower than was observed in absence of azide. However, it was no longer as intense as before. The band b_1 also did not return to its original strength. Hence, in the presence of azide, the band a_2 has a tendency to remain in the oxidized state, and the band b_1 to remain in the reduced state.

The action of azide was also studied in a suspension of bacteria in 50 % glycerol, frozen and cooled in liquid air. In the reduced state, azide did not bring about any change. To observe the oxidized state, the suspension was shaken vigorously in air, cooled to 0°, shaken again, and finally frozen and cooled in liquid air. Without addition of azide, the typical oxidized spectrum of *A. aerogenes* was seen. The very sharp b_1 band had almost completely disappeared, but the band a_2 was very sharp and strong at 645 m μ . If the same procedure was followed in the presence of azide, the band b_1 remained strong, but was slightly weaker than before, i.e. it was not fully reduced. The band of component a_2 was visible at 645 m μ ., but since it was not as strong as in the azide-free control it follows that a_2 was not fully oxidized.

In a second series of experiments, carried out 2 months later, the above results could not be

repeated: azide now had no effect on the absorption spectrum or on the rate of reduction of the cytochrome components. A new culture of the same strain of *A. aerogenes* I was obtained from the M.R.C. Unit for Chemical Microbiology, and cultivated under the standard conditions in broth. The resulting suspensions of bacteria showed the same reactions with azide as in the first series of experiments.

When pure carbon monoxide was bubbled through a bacterial suspension, in which the cytochromes were in the reduced state, the band a_2 moved from 630 to 635 m μ . and became slightly sharper. The bands b_1 and a_1 did not change.

Cyanide (0.002–0.001M) did not modify the reduced cytochrome spectrum, although on aeration the band a_2 disappeared completely. By freezing the aerated suspension in liquid air, it was possible to see a faint band a_2 at the position of the reduced compound. After 5–8 min. at room temperature, the band a_2 , which had disappeared on aeration, reappeared but did not return to its original intensity. These results could no longer be obtained when the behaviour of *A. aerogenes* had changed towards azide, in the course of 2 months: cyanide no longer affected the band a_2 , although it still strongly inhibited the respiration at 30°.

Urethane (0.05M) accelerated the oxidation, and slowed down the reduction of the cytochrome components, without modifying their intensities.

Hydrogen peroxide (0.02–0.1M) at room temperature in presence of 0.001M-hydroxylamine, which effectively inhibits the catalase in *A. aerogenes*, had no effect on the absorption bands of cytochrome.

Cytochrome in metal-deficient bacteria

Washed metal-deficient bacteria were white in appearance, whereas those grown in nutrient broth were distinctly brownish grey. By using optimal conditions of illumination, and very thick suspensions of metal-deficient bacteria, the band b_1 could be clearly seen; it had about one-third the normal intensity. An extremely feeble band a_1 was just perceptible, but there was no detectable trace of a_2 .

When the bacteria were mixed with glycerol and cooled in liquid air, the bands b_1 and a_1 were strongly reinforced, and it was possible to see a faint band a_2 at 630 m μ . Hence the intensity of band a_2 is much more dependent than that of a_1 or b_1 on the concentration of metals, of which iron is the most significant (Table 1). It should be noted that the band a_2 showed an extraordinary degree of variability, even in cultures grown under apparently identical conditions, while the intensities of the bands b_1 and a_1 remained remarkably constant.

Table 1. *The influence of the composition of the medium on the intensity of the absorption bands of cytochrome*

Media	Absorption bands		
	a_2	a_1	b_1
Metal-deficient medium	Traces only	+	+++
Metal-deficient medium + iron	+++	++	+++++
Synthetic medium + iron	++++	++	+++++
Nutrient broth	+++++	++	+++++

*Effect of azide on respiration of
Aerobacter cultivated in metal-rich medium*

The Q_{O_2} of the organism in presence of glucose at 30° was 110 and was independent of substrate concentration within the range 0.1–0.001M. With pyruvate the optimal substrate concentration was 0.03M, while with succinate it was 0.08M, the Q_{O_2} 's being 74 and 26 respectively.

Fig. 1. The action of various concentrations of azide on *Aerobacter* respiration in presence of 0.01M-glucose and 0.1M-phosphate buffer pH 6.8 and with 1 mg. dry-weight bacteria.

With glucose as substrate, azide produced an increase in oxygen uptake, which was maximal (more than 100%) at a concentration of 0.003M (Fig. 1). This increased rate of oxygen uptake persisted for at least 3 hr. The oxidation of lactate or pyruvate was not markedly influenced by 0.003M-azide at pH 6.8, whereas the oxidation of succinate and acetate was inhibited by about 65%; it is known that the inhibition produced by azide varies with pH (Keilin, 1936), being stronger in more acid solutions. The effect of this substance on the oxidation of glucose, pyruvate and succinate was therefore investigated at various pH's (Fig. 2). The increased oxygen uptake brought about by azide in the presence of glucose was maximal at pH 7.3; at pH's 5.5 or 7.8 azide had no effect. With pyruvate,

azide increased the oxygen uptake by 20% at pH 7.3, but produced an inhibition of about 40% at pH 5.5. With succinate, azide inhibited the oxygen uptake at all pH values between 5.5 and 7.8.

Fig. 2. The effect of azide on the oxidation of glucose, pyruvate and succinate, at various pH values, in presence of 0.1M-phosphate buffer. ●—●, Values obtained without azide; ○—○, values obtained in the presence of 0.003M-azide (1 mg. dry-weight bacteria).

Table 2. *The effect of azide on the oxygen uptake in presence of various sugars. The experiments were carried out at 30° in presence of 0.1M-phosphate buffer, pH 6.8*

Substrates (0.01M)	Q_{O_2}	
	Without azide	+0.003M-azide
Glucose	117	245
Fructose	119	116
Mannose	127	180
Galactose	51	54
Xylose	19	8
Dulcitol	14	7
Sorbose	14	14

The effect of azide was investigated in the presence of several sugars as shown in Table 2. The most marked increase (> 100 %) was obtained with glucose while mannose showed an increase of nearly 40 %. It is unlikely that this effect was due to glucose impurities. The oxygen uptake with fructose, which was equivalent to that of glucose, was not influenced by azide. With certain substrates which were slowly oxidized (xylose and dulcitol) azide exerted an appreciable inhibition.

Effect of other inhibitors on respiration

In order to obtain some information as to the mode of oxidation of glucose in the presence of azide, oxygen uptake, carbon dioxide output, glucose utilization and pyruvic and lactic acid formation, were estimated in the same experiment. The manometric technique was that of Dixon & Keilin (1933) which allows the direct determination of oxygen uptake and carbon dioxide output. Glucose was added from a dangling tube at the beginning of the experiment, and the reaction was stopped at the end of the experiment by tipping in 0.2 ml. 4N-sulphuric acid from a side arm. Residual glucose, and pyruvic and lactic acids were estimated in trichloroacetic acid filtrates. The results of a typical experiment are shown in Table 3. In the

Table 3. *The action of azide on glucose breakdown*

(The experiment was carried out in presence of 0.1M-phosphate buffer, pH 6.8, with 6.5 mg. dry-weight bacteria, during 30 min. at 30°, and with 12 µg.mol. of glucose as substrate. Results are given in µg.mol.)

	Without azide	With 0.003 M-azide
Oxygen taken up	8.25	17
Glucose utilized	4.06	8.06
Pyruvic acid formed	4.7	6.7
Lactic acid formed	1	0.8
Carbon dioxide output	6.25	14.5
R.Q.	0.76	0.85

absence of azide, the glucose carbon utilized during the experiment (24.36 µg.atoms) was recovered almost quantitatively as pyruvic and lactic acids and carbon dioxide (23.35 µg.atoms). In the presence of azide, 48.36 µg.atoms of carbon were utilized, but the carbon recovery was only 37.00 µg.atoms. It is possible that this discrepancy in the carbon balance was due to the formation of acetic acid, which was not estimated. In this experiment oxygen and glucose utilization and carbon dioxide production were all doubled by azide. Pyruvic acid production was increased by 40 %, whereas the yield of lactic acid dropped slightly. The quantity of pyruvic acid found during the oxidative breakdown of glucose was extremely high, both in the presence and in the absence of azide.

It was thought that the increased oxygen uptake brought about by azide might be due to a direct oxidation of glucose by an enzyme similar to glucose oxidase, with the production of hydrogen peroxide. The azide-catalase test, as described by Keilin & Hartree (1945), was used for the detection of hydrogen peroxide. A suspension of bacteria with 0.1M-glucose and 0.1M-phosphate buffer, pH 6.5, was placed under the microspectroscope and aerated vigorously for 30 min. Azide-catalase was then added, but the characteristic 'reduced azide-catalase', which is produced specifically by hydrogen peroxide, could not be detected. Neither was it formed if azide was present during the aeration. Attempts to show the presence of hydrogen peroxide in the clear supernatant after centrifuging down the bacteria were also unsuccessful, in presence of either 0.0001M-hydroxylamine, which inhibits catalase, or azide.

Fig. 3. The action of octyl alcohol (OA) on the respiration of *Aerobacter* at 30°, in the presence or in the absence of azide, and with glucose as substrate and 0.1 M-phosphate buffer pH 6.8. Octyl alcohol (0.03 ml.) was added in the manometric flask at the time indicated by the arrow.

At 30°, it was found that 0.02–0.05 ml. octyl alcohol in 3.0 ml. bacterial suspension completely inhibited the oxygen uptake of *A. aerogenes* with glucose, either in the absence or in the presence of azide (Fig. 3). As these results were not in agreement with spectroscopic experiments carried out at 0°, the manometric experiments were repeated at the latter temperature. Under such conditions 0.02–0.05 ml. octyl alcohol did not produce any inhibition of the oxygen uptake, either in the absence, or in the presence of azide (Fig. 4), and furthermore azide alone gave only a very slight increase in the oxygen uptake.

A mixture of carbon monoxide (95 %) with oxygen (5 %) produced an inhibition of about 14 % in the

respiration of *A. aerogenes* in presence of glucose, but 90 % carbon monoxide with 10 % oxygen had no effect. In these experiments the control vessels contained nitrogen in place of carbon monoxide.

Fig. 4. The action of octyl alcohol on the respiration of *Aerobacter* at 0°, in the presence and in the absence of azide, and with glucose as substrate and 0.1 M-phosphate buffer pH 6.8. Octyl alcohol (0.03 ml.) was added to the manometric flask at the time indicated by the arrow. Curve A, *Aerobacter* respiration; curve B, *Aerobacter* respiration + octyl alcohol; curve C, *Aerobacter* respiration + octyl alcohol + 0.003 M-azide; curve D, *Aerobacter* respiration + 0.03 M-azide.

Potassium cyanide (0.001 M) inhibited the oxygen uptake with glucose, pyruvate or succinate by about 95 %, while 0.0001 M-cyanide produced 50 % inhibition. This effect could be reversed by adding a small amount of methaemoglobin from a dangling tube into the manometric flask. Methaemoglobin has a very great affinity for cyanide with which it forms the stable cyan-methaemoglobin (Haurowitz, 1931).

Effect of inhibitors on respiration of metal-deficient bacteria

With glucose as substrate, bacteria cultivated in metal-deficient media were found to have an oxygen uptake similar to that of bacteria grown (a) in a metal-deficient medium to which had been added 40 μg. iron/l. in the form of ferrous ammonium sulphate or (b) in nutrient broth. The oxidation of pyruvate was found to be decreased by 20 % with metal-deficient *Aerobacter* and that of acetate and succinate by 60–65 %. Bacteria grown on the deficient medium with added iron (40 μg./l.) oxidized these substrates at the normal rate.

Sodium azide (0.001 M) increased the oxygen uptake with glucose as substrate at pH 6.8 as it did with normal cells, but at pH 5.5, although it increased the oxygen uptake by about 65 % with

normal bacteria, it produced at first a small increase in oxygen uptake and then almost completely inhibited the respiration. Carbon monoxide (90 %) with oxygen (10 %), which did not affect the respiration of cells grown under normal conditions, inhibited the oxygen uptake of metal-deficient bacteria by 50–60 %. With a carbon monoxide: oxygen ratio of 19:1 an inhibition of about 65 % was obtained. This inhibition could not be reversed by light, although the intensity of the mercury vapour lamp used was sufficient to abolish the carbon monoxide inhibition of the respiration of baker's yeast. The respiration of metal-deficient bacteria was found to be much more sensitive to small concentrations of cyanide than that of bacteria grown under normal conditions: 0.0001 M-cyanide, which only produced 50 % inhibition with normal cells, inhibited the oxygen uptake of metal-deficient *Aerobacter* by 90 %; 2:2'-dipyridyl and 8-hydroxy-quinoline (0.005 M) increased the oxygen uptake by about 25 % with both normal and metal-deficient cells while 0.001 M-phenylthiourea produced a similar but smaller effect.

Dehydrogenase activity in Aerobacter grown in metal-rich medium

The results of experiments on the anaerobic reduction of methylene blue are summarized in Tables 4–6. They show: (1) that with glucose as substrate the rate of reduction was nearly four times faster in the presence of 0.003 M azide; (2) that azide inhibited the reduction by more than 50 % in the presence of succinate or acetate; and (3) that in the presence of fructose or galactose azide had practically no effect, while with mannose the reduction was stimulated by azide although not as much as with glucose.

Octyl alcohol greatly increased the rate of reduction of methylene blue with glucose as substrate, both at 30 and at 0° (Table 7). As the respiration was completely inhibited in a Barcroft manometer where regular shaking ensured the dispersion of octyl alcohol, it was important to repeat the reduction experiments, shaking the Thunberg tubes in the thermostat. However, even under these conditions, the methylene-blue reaction was still as markedly increased as in the experiments where the tubes were stationary.

Dehydrogenase activity in metal-deficient bacteria

The rate of methylene-blue reduction in presence of glucose was not markedly altered by metal deficiency. But with succinate and acetate the reaction rate was slowed down to about one-third, and with formate the reaction was almost completely abolished (Table 8).

DISCUSSION

The properties of the component a_2 in *A. aerogenes* are similar to those of cytochrome a_3 , which is generally believed to be identical with cytochrome oxidase (Keilin & Hartree, 1939); thus a_2 , like a_3 , reacts with carbon monoxide and cyanide. More-

Table 4. *The action of different concentrations of azide on the anaerobic reduction of methylene blue in the presence of 0.01 M-glucose, 0.1 M-phosphate buffer pH 6.8, at 30°*

Time of reduction of methylene blue (sec.)			
Without azide	With azide		
	0.003 M	0.01 M	0.02 M
300	80	90	120

Table 5. *The action of 0.003 M-azide on the reduction of methylene blue with glucose, succinate and acetate, at 30°*

(0.1 M-phosphate buffer, pH 6.8.)

Time of reduction of methylene blue (sec.)		
Substrates	Without azide	With azide
Glucose (0.01 M)	1320	360
Succinate (0.04 M)	900	2100
Acetate (0.04 M)	900	1920

Table 7. *The effect of octyl alcohol (OA) on the reduction of methylene blue in presence of 0.1 M-phosphate buffer, pH 6.8, without and with the addition of azide, and with glucose (0.01 M) as substrate*

Temp. (°)	Time of reduction of methylene blue (sec.)			
	Without azide		+ 0.003 M-azide	
30	420	+ 0.002 ml. OA	120	+ 0.02 ml. OA
		55		45
		+ 0.1 ml. OA		+ 0.1 ml. OA
30	420	30	120	25
0	1620	+ 0.002 ml. OA	1380	+ 0.02 ml. OA
		540		660

over, by following the reduction and the oxidation of the cytochrome bands under the microspectroscope, it has been observed that the band a_2 is reduced after b_1 and oxidized before it. These two arguments strongly support the view that cytochrome a_2 functions as cytochrome oxidase.

The variations in intensity of the a_2 band, and also in its general aspect, are marked in different species. Thus, the bands a_2 in *Azotobacter* ($Q_{O_2} = 5000$) and in *Acetobacter* ($Q_{O_2} = 1000$) are much weaker and narrower than in *Aerobacter* ($Q_{O_2} = 100$). It follows that there is no relation between the Q_{O_2} and the strength of the band, but it is possible that the catalytic activity of the component a_2 varies in different species.

In metal-deficient bacteria, the concentration of cytochrome a_2 is reduced to a minute trace (only perceptible after cooling the bacteria to the temperature of liquid air), whereas cytochromes b_1 and a_1

Table 6. *The action of 0.003 M-azide on the methylene blue reduction in presence of various sugars, at 30°, and with 0.1 M-phosphate buffer pH 6.8*

Substrates (0.01 M)	Time of reduction of methylene blue (sec.)	
	Without azide	With azide
Glucose	170	50
Fructose	165	145
Mannose	180	105
Galactose	190	170
Xylose	900	> 1800

are reduced by only 50–70 %. If the component a_2 is the only cytochrome component which reacts directly with molecular oxygen, and is thus one of the indispensable steps in the respiration of this organism, it is difficult to understand why, in iron-deficient cells, its concentration is decreased more markedly than that of the other cytochrome components, while at the same time the rate of oxygen uptake is unchanged. These facts point rather to the conclusion that there is yet another autoxidizable catalyst present which links the

Table 8. *The effect of iron deficiency on the reduction of methylene blue with glucose, succinate, acetate and formate*

(6 mg. dry-weight bacteria were used per Thunberg tube (0.1 M-phosphate buffer, pH 6.8).)

Substrate (0.01 M)	Time of reduction of methylene blue (sec.)	
	Bacteria grown in normal medium, or in metal-deficient medium + iron	Metal-deficient bacteria
Glucose	180	200
Succinate	170	515
Acetate	165	550
Formate	300	> 1800

cytochrome system with oxygen. Since in metal-deficient cells the sensitivity of the oxygen uptake towards cyanide and especially towards carbon monoxide is greatly increased, it is safe to assume that the supposed oxidase is a metal protein, the metal of which would most likely be iron or possibly copper. It is not an unreasonable supposition that component a_1 is this autoxidizable catalyst, for, under certain circumstances, it has been observed to react with carbon monoxide and cyanide (Warburg & Negelein, 1933). But it is also possible that the oxidase is of a type which is not detectable by direct spectroscopic examination of the cells. There is, however, an alternative possibility which must be considered: normal cells contain a great excess of a_2 and even if the amount of this catalyst is very much reduced, it is still sufficient to insure normal respiration. This would explain the increased sensitivity of metal-deficient cells towards inhibitors.

The inhibition of the respiration of *A. aerogenes* by cyanide is due to its combination with cytochrome a_2 , and perhaps with some other metal catalysts. But it is now obvious that the inhibition of respiration is not due to any effect on catalase, as was supposed by Aubel, Rosenberg & Szulmajster (1950) in the case of *Escherichia coli*, since azide, a powerful inhibitor of catalase, is without effect.

Azide did not inhibit the respiration of *A. aerogenes* in the presence of glucose or other sugars; but when succinate or acetate were used as substrates, azide (0.003M) inhibited the rate of oxygen uptake by more than 60%. Aubel & Szulmajster (1947) reported similar results with *Esch. coli*. One might suppose that the oxidation of glucose did not proceed through the same final chain of reactions as did succinate or acetate. However, with these two latter substrates both the rate of reduction of methylene blue and the oxidation measured manometrically were inhibited by azide (0.003M) to the same extent. It is thus very likely that the same catalytic system was inhibited in each experiment which indicates that the inhibition does not take place at the final steps of the aerobic oxidation but rather at the dehydrogenase level.

Metal deficiency, like azide, produced a decrease of about 60% in both the methylene-blue reduction and the oxygen uptake, with succinate or acetate as substrates. As this effect could be abolished by addition of iron only to the culture medium, an iron catalyst, concerned in the oxidation of these two substrates, is apparently involved at the dehydrogenase level.

At room temperature, where the rate of reduction of the cytochromes is very rapid, no action of azide on the absorption bands, or on the rate of their reduction, could be detected spectroscopically. At 0°, on the other hand, where the rate of reduction was considerably slower, azide was seen to decrease

this rate. However, this action is far less marked than that which may be observed for example in the cytochrome oxidase system of heart-muscle preparations in which azide completely inhibits the oxidation of all cytochrome components. Moreover, the feeble action of azide on cytochrome reduction in *A. aerogenes* disappeared as a result of repeated subculturing over a period of 2 months.

Azide (0.003M) doubled the quantity of glucose metabolized, the oxygen taken up and the carbon dioxide produced. The quantity of pyruvic acid formed was increased by 40% while that of lactic acid dropped slightly. This action of azide was found to be specific for glucose: with fructose and galactose, two sugars which were oxidized at the same rate as glucose, azide did not produce any increase in oxygen uptake or in the rate of methylene blue reduction. At neutral pH, azide had no marked action on the oxidation of pyruvate, a substance which was found in very large amounts during glucose oxidation, and was presumably an essential intermediate in the aerobic glucose breakdown. At acid pH, pyruvic oxidation was inhibited by azide, while the oxygen uptake with glucose was either increased or not affected. Thus it can be concluded that azide exerts two opposing actions on the oxidation of glucose at acid pH: a stimulation during the first steps of the oxidation leading to pyruvate, followed by inhibition during pyruvate breakdown.

Although the oxidation of glucose is stimulated by azide when either oxygen or methylene blue are used as final oxygen acceptors, the anaerobic fermentation of glucose is not affected by azide. Oxygen or some other hydrogen acceptor appears to be indispensable.

Aubel *et al.* (1950), studying the action of azide and other inhibitors on the respiration of *Esch. coli*, concluded that the oxidation of glucose and pyruvate did not go through the tricarboxylic acid cycle. Their view was based mainly on the facts that azide, malonate and fluoride, which produced little or no inhibition of glucose and pyruvate oxidation, strongly decreased the rate of oxidation of succinate and fumarate. In the case of *A. aerogenes* used in the course of the present work, the oxidation of pyruvate at neutral pH was stimulated by azide, whereas that of succinate was inhibited. However, this does not in itself prove that succinate is not an intermediate in pyruvate breakdown. Thus at acid pH the oxidation of pyruvate was inhibited while that of glucose was unchanged or even stimulated. Nevertheless, there is some evidence that pyruvate is an intermediate in glucose breakdown. It should be borne in mind that the inhibition by azide of the oxidation of succinate or acetate did not, in the present experiments, exceed 65%, and that the residual activity might well be adequate to bring about the

oxidation of these substances, should they be formed as intermediates.

The specific action of azide on glucose suggested that a special oxidizing enzyme, of the glucose oxidase type (Keilin & Hartree, 1948), might be concerned. However, hydrogen peroxide could not be detected during the oxidation of glucose by the bacteria.

Clifton (1937), Clifton & Logan (1939), and others found that azide prevented the assimilation of the substrate to build up cellular material, thus permitting the oxidation of the substrate to proceed to completion. The oxygen uptake was not markedly increased at the beginning of their experiments, but it remained constant and eventually accounted for complete oxidation of the substrate. However, in the experiments done in the course of the present work, azide increased the rate of respiration as soon as it came into contact with the bacteria.

A new mode of action of azide has been found in the last few years: Hotchkiss (1944) and Spiegelman, Kamen & Dunn (1946) mentioned that azide prevented aerobic phosphate uptake in yeast, while Spiegelman, Kamen & Sussman (1948) presented some evidence that triosephosphate dehydrogenase was concerned. Recently, Loomis & Lipmann (1949), working with a kidney enzyme preparation and glutamate as substrate, also found that azide prevented the esterification of inorganic phosphate. If azide reduces the formation of energy rich phosphate, one might think that it leads to an increased rate of substrate utilization, to provide the cells with the necessary energy. However, in the experiments of Spiegelman *et al.* (1948), as in those of Loomis & Lipmann (1949), no marked increase in the rate of substrate breakdown was observed.

SUMMARY

1. *Aerobacter aerogenes*, grown in a normal medium (nutrient broth), shows the absorption bands of reduced cytochrome occupying the following positions: a_2 , 630 m μ .; a_1 , 590 m μ .; and b_1 , 560 m μ . On oxidation, the absorption bands a_1 and b_1 disappear, while a_2 moves from 630 to 645 m μ .

2. The concentration of cytochrome a_2 in *Aerobacter* is higher than in any other micro-organism so far examined, the band a_2 being almost as strong as b_1 . The band a_1 is very faint.

3. On aeration, cytochrome a_2 is oxidized before b_1 , and by stopping the aeration, it is reduced after b_1 . Moreover, cytochrome a_2 combines with carbon monoxide and cyanide. These two facts support the view that in *A. aerogenes* a_2 acts as cytochrome oxidase.

4. In *Aerobacter* grown in metal-deficient medium the band a_2 becomes hardly detectable, whereas the intensities of a_1 and b_1 are reduced to one-half or one-third of their usual intensities.

5. The respiration in presence of glucose is not affected by metal deficiency.

6. If normally cytochrome a_2 is present in great excess, it might still, in metal-deficient cells, be present in sufficient amount to allow normal respiration.

7. Azide does not combine with any of the cytochrome components of *A. aerogenes*.

8. Azide (0.003M) increases the oxygen uptake with glucose by more than 100 %, and the anaerobic reduction of methylene blue by 300–400 % at 30°. This effect is very feeble at 0°.

9. Azide (0.003M) increases the rate of glucose utilization and that of carbon dioxide production by about 100 %, and the amount of pyruvate found at the end of the experiment by 40 %.

10. Azide has no effect on the respiration or on the methylene-blue reduction in presence of fructose or galactose.

11. Azide (0.003M) inhibits by about 60 % the oxygen uptake and the rate of methylene-blue reduction in presence of succinate or acetate.

12. The oxygen uptake and the methylene-blue reduction by metal-deficient cells is reduced by about 60 % with succinate and acetate as substrate. Since the addition of iron to the metal-deficient medium is sufficient to abolish this effect, it follows that an iron catalyst, necessary for the anaerobic reduction of methylene blue, is apparently involved in the oxidation of succinate and acetate.

13. Octyl alcohol (0.02 ml. in 3 ml. bacterial suspension) completely inhibits the oxygen uptake in the presence of glucose and increases the rate of methylene-blue reduction by eight to ten times at 30°, with the same substrate.

14. At 0° the same amount of octyl alcohol has no effect on the oxygen uptake, but it still increases the rate of methylene-blue reduction by more than 100 %, with glucose as substrate.

15. Octyl alcohol (0.001–0.01 ml. in 0.5 ml. bacterial suspension) slows down the rate of reduction of the cytochrome components at room temperature, but has no effect at 0°.

16. Hydrogen peroxide could not be detected during the respiration of *A. aerogenes* in presence of glucose.

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REFERENCES

- Aubel, E., Rosenberg, A. J. & Szulmajster, J. (1950). *Biochim. Biophys. Acta*, **5**, 228.
- Aubel, E. & Szulmajster, J. (1947). *C.R. Acad. Sci., Paris*, **224**, 680.
- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
- Clifton, C. E. (1937). *Enzymologia*, **4**, 246.
- Clifton, C. E. & Logan, W. A. (1939). *J. Bact.* **37**, 523.
- Dixon, M. & Keilin, D. (1933). *Biochem. J.* **27**, 86.
- Folin, O. & Malmros, H. (1929). *J. biol. Chem.* **83**, 115.
- Friedmann, T. E. & Hangen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Haurowitz, F. (1931). *Hoppe-Seyl. Z.* **138**, 68.
- Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 153.
- Keilin, D. (1936). *Proc. roy. Soc. B*, **121**, 165.
- Keilin, D. & Hartree, E. F. (1939). *Proc. roy. Soc. B*, **127**, 167.
- Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 148.
- Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 210.
- Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 230.
- Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.
- Loomis, W. F. & Lipmann, F. (1949). *J. biol. Chem.*, **179**, 503.
- Spiegelman, S., Kamen, M. D. & Dunn, R. (1946). *Fed. Proc.* **5**, 99.
- Spiegelman, S., Kamen, M. D. & Sussman, M. (1948). *Arch. Biochem.* **18**, 409.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*. Minneapolis: Burgess.
- Warburg, O. & Negelein, E. (1933). *Biochem. Z.* **262**, 237.
- Waring, W. S. & Werkman, C. H. (1944). *Arch. Biochem.* **4**, 75.