72. CYTOCHROME SYSTEM IN *BACTERIUM COLI COMMUNE*

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(*Received 25 May 1941*)

THE aerobic respiration of *Bact. coli*, like that of *Bacillus subtilis*, baker's yeast and other aerobic cells, is strongly and reversibly inhibited by KCN, H_2S, NaN_3 and CO, but, unlike that of *B. subtilis* or cells of baker's yeast, the inhibition with CO is not light-sensitive.

The absorption spectrum of cytochrome in *B. coli* differs markedly from that of *B. subtilis* or yeast cells. Thus, the band *a* is absent from *B. coli*, and is replaced by a hardly perceptible shading a_1 at 590 mμ and a feeble band a_2 at 628 mμ which is similar to the band a_2 of *Azotobacter*. This band in organisms exposed to CO moves to 634 mμ, on oxidation it is shifted to 645 mμ and in presence of KCN and O_2 it disappears completely[1] [Keilin, 1934; Fugita & Kodama, 1934]. The band a_2 belongs therefore to an autoxidizable haemochromogen compound which reacts with KCN and with CO. According to Lemberg & Wyndham [1937] this compound can be considered as a bile pigment haemochromogen with an open tetrapyrrolic chain kept together in a ring form by the central iron atom. Lemberg ascribes to a_2 the structure of a biliviolin-haemochromogen which is obtained by dehydrogenation of verdohaemochromogen. The component a_2 appears therefore to belong to a bile pigment derivative of haematin *a*, and, as in all the derivatives with an open tetrapyrrolic chain [Holden & Lemberg, 1939], its absorption spectrum is devoid of the Soret or γ-band.

The properties of a_2, namely its reactions with O_2, CO and KCN, suggest that in *B. coli*, as in *Azotobacter* [Negelein & Gärischer, 1934], this component may act as cytochrome oxidase. On the other hand, the failure to find any parallelism between the concentration of a_2 and the respiratory activity of cells containing this component leaves open the possibility of the existence in *B. coli* of yet another autoxidizable haematin compound acting as cytochrome oxidase and capable of oxidizing the component b_1.

The bands *b* and *c* of a typical cytochrome are replaced in *B. coli* by one band which lies at about 560 mμ and which represents the α-band of a non-autoxidizable haemochromogen compound b_1. This band is asymmetric and shows on its short-wave side a reinforcement which, according to Yamagutchi [1937], may represent the band *c* masked by an extension of the band b_1.

On freezing and cooling the suspension of *B. coli* to the temperature of liquid air, the short-wave side of the band b_1 becomes greatly intensified and appears at 551 mμ united by a shading to a much fainter band at 559 mμ. The α-band of cytochrome *c* which, under similar conditions, is also markedly intensified is, however, shifted from its usual position at 549·5 to 547 mμ. This clearly shows, even if we assume that the asymmetric band b_1 of *B. coli* is composed of two

[1] In some other strains of *B. coli* and in *Proteus vulgaris* Yamagutchi [1937] failed, however, to detect any reaction between a_2 and KCN.

fused bands belonging to two distinct compounds, that neither of them can be identified with the true components *b* and *c* of cytochrome.

The component b_1 in living intact cells of *B. coli* does not react with KCN, NaN_3 or CO, which however inhibit its oxidation.

Catalytic activity of cytochrome in Bact. coli

Unlike *B. subtilis* and many other aerobic cells, *B. coli*, even when free from metabolites, is incapable of oxidizing *p*-phenylenediamine or 'Nadi' reagent [Happold, 1930]. This may be partly explained by the absence from *B. coli* of cytochrome *c* which, as was previously shown [Keilin & Hartree, 1938], is essential for these catalytic oxidations. A washed suspension of *B. coli* can neither reduce nor oxidize added cytochrome *c*, which is not surprising considering that cytochrome *c*, being a comparatively large molecule, cannot diffuse through the cell wall of *B. coli* and thus come in contact with its intracellular oxidizing systems. The direct reaction between cytochrome *c* and the oxidizing systems of *B. coli* can only be determined when crushed micro-organisms, in which these systems are exposed, are brought into contact with a solution of cytochrome *c*.

The study of this inter-reaction was carried out in a series of spectroscopic and manometric experiments. For this purpose *B. coli* were grown in the usual way on a peptone-agar medium[1] in about thirty large Roux flasks. The organisms after 24 hr. growth were collected, washed several times in saline solution and mixed with 30 ml. of phosphate buffer solution at *p*H 7 to a thick suspension which was ground for about 1 hr. in a Booth & Green [1938] bacterial mill. The ground preparation thus obtained gave on centrifuging three distinct layers: the top layer (*A*) of opalescent fluid, the intermediate or granular layer (*B*) composed of crushed bacteria and the bottom layer (*C*) of intact bacteria. The first two layers (*A* and *B*), collected separately and dialysed for at least 14 hr. against a large amount of 0·02 *M* phosphate buffer solution (*p*H 7·3), were found to possess similar properties, except that the fraction *B* displayed a stronger absorption spectrum and a greater catalytic activity than fraction *A*. The fraction *B*, which was used for most of the observations recorded below, did not take up O_2 and its cytochrome remained in a completely oxidized state. If, however, substrates such as succinic, lactic or formic acids were added to this preparation, they became rapidly oxidized and the preparation took up O_2 vigorously. Cytochrome in such preparations shaken with air undergoes rapid oxidation and reduction.

As all the components of the oxidizing systems in this preparation are now exposed they can easily be brought into contact with cytochrome *c*.

Spectroscopic experiments

For spectroscopic study, a small amount of pure cytochrome *c* prepared from heart muscle as previously described [Keilin & Hartree, 1937] was added to fractions *A* or *B* of crushed bacteria in presence and in absence of substrate. Spectroscopic examination of these mixtures showed that the dialysed fractions (*A* or *B*) of crushed *B. coli*, unlike similar preparations of baker's yeast, do not oxidize reduced cytochrome *c* even when the mixtures are vigorously shaken with air. The same preparations in the absence of substrate do not reduce oxidized *c*. They reduce this compound, however, in the presence of substrates

[1] The nutrient medium is composed of 20 g. peptone, 20 g. 'Lab. Lemco,' 10 g. NaCl, 60 g. agar and 2 l. water.

such as succinic or lactic acids. Once reduced, cytochrome *c* does not undergo reoxidation on shaking the mixture with air, while components a_2 and b_1 of cytochrome proper to *B. coli* undergo normal and rapid reoxidation.

Manometric experiments

It has been shown [Keilin, 1930; Keilin & Hartree, 1938] that the addition of cytochrome *c* to enzyme preparations obtained from heart muscle, liver or kidney, greatly increases the rates of oxidation of different substances, such as cysteine, succinic acid, *p*-phenylenediamine or adrenaline, catalysed by these preparations. The addition of cytochrome *c* increases also the rates of oxidation of lactic acid or of *p*-phenylenediamine by enzyme preparations obtained from crushed cells of baker's yeast.

Similar experiments were carried out in Barcroft differential manometers at 39° with both fractions (*A* and *B*) of the preparation obtained from crushed *B. coli*. The enzyme preparations were suspended in 3 ml. *M*/15 phosphate buffer, *p*H 7·3. The substrates, such as succinic or lactic acids, or *p*-phenylenediamine, were added from dangling tubes which were dislodged only after the temperature of the manometers had been equilibrated and the taps closed. The final concentrations of substrate were *M*/50. The clear fraction (*A*) was used in these experiments in a higher concentration than the granular and more active fraction (*B*). In some experiments the enzyme preparation received 0·2 ml. of 1 % cytochrome *c*, or 10^{-3} *M* methylene blue and cyanide.

Table 1

B. coli enzyme prep. ml.	Cyto-chrome *c*	*p*-pheny-lenedia-mine	Succinic acid	Lactic acid	KCN	M.B.	O_2 uptake μl. in 25 min.
0·5 (*A*)	−	+	−	−	−	−	6
+	+	+	−	−	−	−	6
+	−	−	+	−	−	−	174
+	+	−	+	−	−	−	160
+	−	−	+	−	+	−	17
+	−	−	+	−	+	+	66
+	−	−	−	+	−	−	215
+	+	−	−	+	−	−	212
0·1 (*B*)	−	+	−	−	−	−	13
+	+	+	−	−	−	−	16
+	−	−	+	−	−	−	198
+	+	−	+	−	−	−	160
+	−	−	−	+	−	−	166
+	+	−	−	+	−	−	164
+	−	−	−	+	+	−	11
+	−	−	−	+	+	+	174

The results of these experiments, which are summarized in Table 1, show that the oxidation of succinic or lactic acid which is actively catalysed by preparations of crushed *B. coli* is not accelerated by the addition of cytochrome *c*. Under similar conditions cytochrome *c* greatly increases the rate of catalytic oxidation of these substances by crushed cells of baker's yeast. Moreover, crushed *B. coli*, even on addition of *c*, oxidized neither *p*-phenylenediamine nor other artificial chromogens such as adrenaline, Nadi reagent, quinol or catechol. The main conclusion which can be drawn from these experiments is that cells of *B. coli* contain only the systems which are capable of reducing cytochrome *c* but are completely devoid of a catalyst capable of oxidizing the reduced *c*. Such a catalyst is, however, present in cells containing the typical cytochrome system. It was shown recently [Keilin & Hartree, 1939] that these cells, in addition to

components *a*, *b* and *c* of cytochrome, contain also a fourth component described as a_3. This component is thermolabile and autoxidizable; it forms reversible compounds with KCN, H_2S, NaN_3 and CO and the absorption bands of its CO compound lie at about 591 and 430 mμ.

It was also shown that the catalytic activities of a tissue preparation measured by the velocity of oxidation of *p*-phenylenediamine in presence of an excess of *c* appear to depend on the concentration of the component a_3. This component seems, therefore, to possess all the properties of cytochrome oxidase and directly or indirectly must be responsible for the catalytic oxidation of cytochrome *c*. It is now conceivable that the failure of *B. coli* to oxidize cytochrome *c* is due to the absence from these micro-organisms of the component a_3 which invariably accompanies the cytochrome *a*.

The results obtained for *B. coli* will probably apply to cells of some other micro-organisms (bacteria and yeast) as well as some cells of higher organisms, in which the cytochrome system is devoid of components *a*, a_3 and *c*.

The fact that cytochrome *c* cannot act as a carrier between the dehydrogenases of *B. coli* and their cytochrome oxidase does not exclude the possibility that it may act as a link uniting into an artificial system the dehydrogenases of *B. coli* with cytochrome oxidase of heart muscle. To test such a possibility, the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed bacteria were determined in presence and absence of cytochrome *c* and of 0·1 ml. of a fresh heart-muscle preparation, which can rapidly oxidize the reduced *c*. The results of these experiments, summarized in Table 2, clearly show

Table 2

B. coli enzyme 0·05 ml. (*B*)	Cytochrome *c*	Cytochrome oxidase 0·1 ml.	Lactate	Formate	O_2 uptake μl.
+	−	−	+	−	136
+	+	−	+	−	156
+	+	+	+	−	174
+	+	+	−	−	50
+	−	−	−	+	140
+	+	+	−	+	123
+	−	−	+	−	168
+	+	+	+	−	138
+	+	+	+	+	285

that the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed *B. coli* are not affected by the addition of cytochrome *c* and cytochrome oxidase prepared from heart muscle. The substrate molecules, activated by their specific dehydrogenases, appear to react with molecular O_2 only through the cytochrome system proper to *B. coli* and not through the cytochrome system of heart muscle added to the *B. coli* preparation. These results suggest that the components of each of the insoluble dehydrogenase systems, together with those of cytochrome, are intimately bound to the protein of a single colloidal particle, forming thus a unit of a complete oxidizing system. The efficiency of such a system is naturally assured by the mutual accessibility of its components, which depends on their spatial distribution and orientation. This view explains the different degree of inhibition by CO of the oxidation by resting *B. coli* of succinic, lactic and formic acids [Cook *et al.* 1931] without postulating the existence of three different cytochrome oxidases. In fact the three different values of *K* (partition constant of oxidase between O_2 and CO) obtained by these workers in their experiments with the oxidation of these

three substances may simply be due to the different degree of accessibility between each of the dehydrogenases and the components of cytochrome bound in the same colloidal particle. The efficiency of the oxidizing systems of the cell and the co-ordination of the multiple reactions simultaneously proceeding within the cell must therefore depend not only on the integrity of all the components of these systems but also on that of the colloidal structure of the cell which supports them and regulates their mutual accessibility.

SUMMARY AND CONCLUSIONS

1. Contrary to the view of Yamagutchi, *Bact. coli* are completely devoid of cytochrome *c*.

2. The preparations obtained from crushed *B. coli* in which all the oxidizing systems are exposed are not capable of oxidizing reduced cytochrome *c* added to them. *B. coli* and probably some other cells are completely devoid of systems or catalysts capable of oxidizing cytochrome *c*.

3. It is therefore not surprising that the preparations of crushed *B. coli*, even on addition of cytochrome *c*, oxidize neither *p*-phenylenediamine nor other chromogens and that the oxidation of succinic and lactic acids by these preparations is not accelerated by the addition of *c*.

4. The failure by *B. coli* to oxidize cytochrome *c* is due to the absence from the cells of this micro-organism of the component a_3 which invariably accompanies cytochrome *a* and which directly or indirectly is responsible for the oxidation of cytochrome *c*.

REFERENCES

Booth & Green (1938). *Biochem. J.* **32**, 885.
Cook, Haldane & Mapson (1931). *Biochem. J.* **25**, 534.
Fugita & Kodama (1934). *Biochem. Z.* **273**, 186.
Happold (1930). *Biochem. J.* **24**, 1737.
Holden & Lemberg (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 133.
Keilin (1930). *Proc. roy. Soc.* B, **106**, 418.
—— (1934). *Nature, Lond.*, **133**, 290.
—— & Hartree (1937). *Proc. roy. Soc.* B, **122**, 298.
—— —— (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1939). *Proc. roy. Soc.* B, **127**, 167.
Lemberg & Wyndham (1937). *J. roy. Soc. N.S.W.* **70**, 343.
Negelein & Gärischer (1934). *Biochem. Z.* **268**, 1.
Yamagutchi (1937). *Bot. Mag., Tokyo*, **51**, 457.