Iron and the Synthesis of Cytochrome c_3

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SUMMARY: *Desulphovibrio desulphuricans* (El Agheila Z), in lactate + sulphate media, required 10 m μ mole Fe/ml. for optimal growth; no absolute iron requirement was demonstrable in pyruvate + sulphate media but iron up to 20 m μ mole/ml. stimulated growth. In a sulphate-deficient pyruvate medium, in which this strain can grow, iron was not required nor did it stimulate growth. Organisms grown with limited iron formed limited amounts of cytochrome c_3 , and in the absence of iron were markedly deficient in this; their content of hydrogenase was reduced to a lesser extent and their content of desulphoviridin was only halved. Iron-deficient organisms were able to reduce sulphate only slowly. Less than 1 % of the iron added to cultures was accounted for as cytochrome c_3 , though only some 6 % of this iron remained in solution after growth.

The demonstration of cytochrome c_3 as a component of the anaerobic sulphate-reducing bacteria (see Postgate, 1956, for references) provided at least a partial explanation of their requirement for traces of iron (Butlin, Adams & Thomas, 1949). The present paper reports a study of the effect of iron on the synthesis of cytochrome c_3 , desulphoviridin (the porphyrin-like pigment which absorbs at 630 m μ), and of hydrogenase by a strain of these bacteria chosen because of its ability to grow without sulphate when pyruvate is present (Postgate, 1952).

METHODS

Organism. *Desulphovibrio desulphuricans* strain El Agheila Z (National Collection of Industrial Bacteria, NCIB, no. 8380) was a variant, containing hydrogenase, of the organism isolated from a sulphur-producing lake in Libya (Adams, Butlin, Hollands & Postgate, 1951). It was subcultured weekly under H₂ 95 % (v/v) CO₂ 5 % (v/v) at 30° in LSY medium (see below); its purity was checked as described by Postgate (1953).

Media. Medium LSY consisted mainly of lactate, sulphate and yeast extract; it was similar to medium C of Butlin *et al.* (1949) but was supplemented with 2.5 % NaCl. Medium PY was a sulphate-deficient medium containing pyruvate and yeast extract (Postgate, 1952). Medium PSY was a similar medium supplemented with Na₂SO₄ (18 μ mole/ml.).

Inocula. For routine subculture 0.05 ml. of parent culture was added to 5 ml. fresh medium. For growth tests in media of defined iron concentration a smaller inoculum was used to avoid carry-over; the parent culture was diluted *c.* 1/2500 to give 0.1 μ g. dry wt. organisms/ml. subculture (*c.* 4×10^5 viable bacteria/ml.), the concentrations of medium components being adjusted so that the final volume after all additions was 5 ml.; Na₂S (5 μ mole/ml.) was included in all media to adjust the redox potential to *c.* -200 mv. and thus to ensure growth of this relatively small inoculum (see Grossman & Postgate, 1953).

Iron-free reagents and glassware. Media and solutions to be added were prepared with analytical grade reagents and freed from iron with 8-hydroxyquinoline (Waring & Werkman, 1942), except that Na_2S solutions were assumed to be effectively iron-free owing to the low solubility product of FeS . Chromic acid-washed Pyrex glassware was freed from adsorbed iron as recommended by Waring & Werkman; all-glass Seitz filters, used for sterilizing Na_2S solutions and media PY and PSY, were freed from adsorbed iron by washing successively with chromic acid, distilled water, a solution of 8-hydroxyquinoline in water saturated with chloroform, chloroform, and iron-free distilled water.

Culture in media of defined iron concentration. Cotton-wool plugs are unsuitable for use with iron-deficient media because of their high iron content. On the other hand, aluminium or glass-capped test-tubes are not entirely suitable for use with cultures of exacting anaerobes in an anaerobic jar, since the gas phase in the jar cannot be changed without the risk of sweeping contaminant micro-organisms under the caps as the new gas phase enters the evacuated jar. This procedure was nevertheless adopted, cultures in test-tubes with aluminium caps being stood in McIntosh & Fildes anaerobic jars on filter-paper wetted with dilute CuSO_4 solution; cultures were frequently examined microscopically for contaminants, and doubtful ones tested as described by Postgate (1953). In fact, only one culture out of 300 or 400 used in this work contained a contaminant. Duplicate cultures were set up at all iron concentrations, and crucial tests were repeated three times.

Larger quantities of bacteria were grown in 'iron-free' Pyrex glass flasks with beakers as caps, or, when c. 2 l. of culture was required, in 'iron-free' flasks with cotton-wool plugs protected with washed gauze. The use of Na_2S as a supplement to the test media ensured growth of the relatively small inocula necessary to eliminate carry-over of iron, but it had the disadvantage that iron could not conveniently be tested at concentrations above 20 $\text{m}\mu\text{mole/ml}$. because at this concentration turbidity due to FeS appeared on addition of Na_2S . This observation supports Seidell (1940), who quoted a value of 70.1 $\text{m}\mu\text{mole/ml}$. for the saturation solubility of FeS at 18° but stated that this figure was probably too high.

Spectrophotometry. The application of Barer's (1955) procedure to the spectrophotometry of intact *Desulphovibrio desulphuricans* is described elsewhere (Postgate, 1956). The cytochrome c_3 content of the bacteria was measured at 554 $\text{m}\mu$., since, though the Soret peak at 419 $\text{m}\mu$. would have been a far more sensitive index of c_3 content, the green protein desulphoviridin absorbs at 411 $\text{m}\mu$. and would be expected to interfere at low concentrations of c_3 . Organisms suspended in strong serum albumin solution were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ in 0.5 cm. optical cells and the absorption relative to reduced controls was determined at 554 $\text{m}\mu$.; the specific extinction coefficient of cytochrome c_3 was taken as 4.2 and the absorption due to c_3 calculated from a hypothetical linear scatter curve using the arithmetical extrapolation below* (d = cell density in mg. dry wt./ml.):

$$\text{mg. } c_3/\text{g. air-dry wt.} = \frac{2}{4.2 \times 10^{-3}d} \left[\epsilon_{554} - \left(\epsilon_{570} + \frac{\epsilon_{538} - \epsilon_{570}}{2} \right) \right].$$

Several spectra of intact bacteria in the 500–600 m μ . range showed that this expression could legitimately be used provided the absolute optical density of the test suspension was below 2.0.

Cytochrome c_3 contents of bacteria are also quoted as μ -units (μ U.)/g.; 1 μ U. would equal 1 μ mole if the millimolar extinction coefficient were 54, the value to be expected for a bifunctional haematohaematin.

Desulphoviridin contents of bacteria were estimated by plotting the spectrum of intact organisms between 600 and 660 m μ . and estimating the height of the inflexion at 630 m μ .; they are expressed in arbitrary units: optical density at 630 m μ . /g. dry wt. bacteria.

Hydrogenase activity. The hydrogenase content of the organisms was judged by the rate of reduction of methylene blue: 2.6–3 mg. dry wt. organisms in buffer (KH_2PO_4 0.5 %, w/v, pH 6.80 $\pm$ 0.02, + NaCl 2.5 %, w/v) were incubated in conventional Warburg manometers under H_2 at 37° for at least 1 hr. before tipping in 7.5 μ mole substrate; this pre-incubation was necessary to obtain maximum reduction rates.

Sulphide estimation. Cultures for sulphide determination were grown *in vacuo* in sealed 'iron-free' Pyrex tubes. Immediately after opening them, sulphide was determined iodometrically as described by Grossman & Postgate (1955), applying their correction: iodine equivalent to 2.2 μ mole S'' /ml. absorbed by 1 mg. dry wt. bacteria/ml.

Bacterial densities. These were measured turbidimetrically in the E.E.L. colorimeter (Evans Electroselenium Ltd.) calibrated with the Hildenborough strain of these bacteria.

RESULTS

Iron requirement of Desulphovibrio desulphuricans, Strain El Agheila Z. This strain required 10 μ mole Fe/ml. for optimal growth in medium LSY (Fig. 1). Little iron remained after growth with a suboptimal iron concentration. The supernatant fluid from a 5-day culture in medium LSY + 7.5 μ mole Fe/ml. was acidified, concentrated tenfold and analysed for iron with *o*-phenanthroline (Sandell, 1944); it contained only 0.48 μ mole Fe/ml., equivalent to 6.4 % of the amount added initially.

Strain El Agheila Z had no demonstrable *requirement* for iron in medium PSY, but iron up to 20 μ mole/ml. stimulated growth; growth was not linearly related to iron concentration. In medium PY the organism had again no requirement for added iron, and iron up to 20 μ mole/ml. had little or no effect on growth (Fig. 1).

Sulphate reduction by iron-deficient cells. The bacterial densities reached in iron-deficient media PSY and PY were similar, and suggested that, even in the presence of sulphate, only 'sulphate-deficient' growth occurred. The rate of sulphide formation in medium PSY with and without iron was therefore determined. The results are recorded in Table 1: with 20 μ mole Fe/ml. all the available sulphate was reduced in less than 100 hr.; without Fe only about half was reduced after 550 hr. Though a lower bacterial density was reached in the absence of Fe, this was nevertheless half that reached in its presence and could

not account for the tenfold lowering in rate of sulphide formation. Mucopolysaccharide formation, mentioned by Grossman & Postgate (1955), occurred in both media and rendered determinations of optical density invalid after prolonged incubation. It was formed at an earlier stage in medium PSY than in medium PY; detailed quantitative data were not obtained.

Fig. 1. Effect of iron on growth of *Desulphovibrio desulphuricans* in lactate or pyruvate media. Cultures of *D. desulphuricans* strain El Agheila Z were incubated in lactate or pyruvate media of known iron content ($\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4$ for media PSY and LSY; FeCl_2 for medium PY), and the amounts of growth determined after 5 days of incubation. ○ = medium LSY; ◐ = medium PSY; ● = medium PY.

Table 1. *Sulphide formation by Desulphovibrio desulphuricans (El Agheila Z) in pyruvate medium with and without added iron*

Iron-deficient medium PSY (see text) was distributed in iron-free tubes, inoculated (c. 3 µg. dry wt. organisms/ml.), sealed *in vacuo* and incubated at 30°. Pairs of tubes were opened and the amounts of growth and sulphide concentrations were determined at intervals.

Incubation time (hr.)	Without added iron		With 20 mµmol. $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2/\text{ml.}$	
	Dry wt. organisms µg./ml.	Sulphide µmole/ml.	Dry wt. organisms µg./ml.	Sulphide µmole/ml.
72	48	—	260	5.43
120	90	2.33	295	8.1
144	150	2.18	240	8.85
216	140	2.70	300*	9.2
230	130*	4.62	290*	8.85
360	105*	3.90	185*	9.75
550	c. 50*	4.50	c. 30*	9.25

* The optical densities of these suspensions were diminished by mucopolysaccharide formation; hence these readings are low.

Iron deficiency decreased the activity of suspensions of bacteria to an even greater extent. Organisms grown in an iron-deficient medium reduced sulphate in the presence of hydrogen at only about one-fiftieth of the rate observed with organisms grown with iron (Table 3). The decline may in fact have been greater than this, since the quoted Q values imply a net difference between blank and control vessels of 3 to 6 mm.³ H₂ absorbed over 1½ hr., a difference which is close to the limit of sensitivity of the manometric procedure used. The decline in rate of sulphate reduction could not be due to deficiency in hydrogenase, since there was only a fivefold to eightfold decrease in the hydrogenase activity of iron-deficient organisms (below).

Effect of iron on content of cytochrome c₃. Waring & Werkman (1944) showed that iron-deficient *Aerobacter indologenes* was deficient in cytochrome. The fact that the iron requirement of strain El Agheila Z depended on the test medium permitted a quantitative study of this effect in *Desulphovibrio desulphuricans*. Bacteria grown in medium LSY containing 5, 7.5 or 10 mμ-mole Fe/ml. contained cytochrome c₃ in concentrations proportional to the iron concentration (Table 2). Organisms harvested from pyruvate media (PY and PSY) contained plentiful cytochrome c₃ when iron was present in the medium; in the absence of iron cytochrome c₃ was present only in very small amounts and was undetectable unless very heavy suspensions were used in the spectrophotometer. Provided iron were present, however, cytochrome c₃ was found both in the presence and absence of sulphate.

Table 2. *Cytochrome c₃ and desulphoviridin contents of Desulphovibrio desulphuricans grown in media of defined iron concentration*

Batches (250 ml. to 1 l.) of *D. desulphuricans* strain El Agheila Z were grown in iron-deficient media with known amounts of added iron (as FeCl₃ or Fe(NH₄)₂(SO₄)₂), centrifuged, and the content of cytochrome c₃ and desulphoviridin/g. dry wt. organisms was determined spectrophotometrically (see text).

Medium	Added iron (mμmole/ml.)	cytochrome c ₃ content		Desulphoviridin content (optical density value/g.)
		(mg./g.)	(μU./g.)	
LSY	5	1.19	0.091	6.4
LSY	7.5	1.35	0.104	5.1
LSY	10	1.87	0.144	6.1
PY	—	0.121	0.0093	4.0
PY	20	2.3	0.177	8.7
PSY	20	1.83	0.141	7.8
PSY	—	0.0195	0.00153	3.8

Effect of iron on porphyrin synthesis. Iron deficiency sometimes leads to accumulation of porphyrin in culture media (see Lascelles, 1955); no porphyrin was detected in cultures of El Agheila Z in iron-free media PSY or PY.

Effect of iron on content of desulphoviridin. The amount of this pigment/unit wt. of organisms was little affected by the iron content of the culture medium (Table 2), in contrast to the amount of cytochrome c₃. This observation is consistent with Postgate's (1956) view that desulphoviridin is a porphyrprotein and not a haematin as supposed by Ishimoto, Koyama & Nagai (1954*a, b*).

Effect of iron on content of hydrogenase. Waring & Werkman (1944) showed that *Aerobacter indologenes* was deficient in hydrogenase when grown in iron-deficient media; it was of interest to see if this were also true of *Desulphovibrio desulphuricans*. Table 3 lists some Q values for the reduction of methylene blue in hydrogen and shows that growth without Fe led to a fivefold to eightfold decline in the hydrogenase content.

Table 3. *Effect of iron on the hydrogenase content of Desulphovibrio desulphuricans*

D. desulphuricans strain El Agheila Z organisms were harvested from iron-deficient media PY or PSY in 'iron-free' vessels (see text) after incubation for 3–5 days at 30° under $H_2 + 5\%$ CO_2 . The rates of hydrogen absorption with sulphate or methylene blue as electron acceptor were compared with the rates for organisms harvested from similar media but with 15 to 20 $m\mu$ mole Fe/ml. added. Vessels were pre-incubated 1½ hr. before addition of substrates; $-Q$ values ($mm^3 H_2$ absorbed/mg dry wt. organisms/hr.) quoted from three experiments.

Growth medium	With iron			Without iron		
	$-Q_{H_2}^{MB}$	$-Q_{H_2}^{SO_4''}$	$-Q_{H_2}^{endogenous}$	$-Q_{H_2}^{MB}$	$-Q_{H_2}^{SO_4''}$	$-Q_{H_2}^{endogenous}$
PY	192	—	—	48	—	—
PSY	230	38	4.8	29	3.2	2.6
PSY	95	45	0.5	31	3.1	2.6

DISCUSSION

Recovery of iron as cytochrome c_3 . The molar extinction coefficient of cytochrome c_3 , together with its relatively high iron content, provide reasons for assuming the presence of two haemin groups per molecule (Postgate, 1955). The recovery of added iron as cytochrome c_3 can therefore be calculated. For example: a culture of El Agheila Z in medium LSY + 5 $m\mu$ mole Fe/ml. had a final bacterial density of 250 μ g. dry wt./ml. and the organisms contained 0.091 μ U. c_3 /g.; hence the cytochrome accounted for 0.9% of the added iron. Similar calculations for El Agheila Z grown with other concentrations of iron gave these values: recovery as c_3 from 7.5 $m\mu$ mole Fe/ml.: 0.78%; 10 $m\mu$ mole Fe/ml.: 0.88%.

Clearly a large proportion of the added iron was not used for cytochrome c_3 synthesis although over 90% of it was removed from solution. It was not detectable as haematin, since treatment of the whole organisms with NaOH, pyridine and dithionite did not increase the intensity of the α -band of c_3 , though it shifted 2 or 3 $m\mu$. towards the violet; the fate of the remainder of the added iron is thus unknown. Higher concentrations of cytochrome c_3 were observed with the Hildenborough strain grown on media of unknown iron content by continuous culture procedures; the organisms used by Postgate (1956) for the preparation of cytochrome c_3 contained about 0.20 μ U. c_3 /g.

Though the concentration of cytochrome c_3 in the organisms was low compared with the amount of iron added, it was roughly proportional to the amount of iron added and was not influenced by the presence of sulphate even though the cytochrome probably acts as a carrier in sulphate reduction. Thus strain El Agheila Z of *Desulphovibrio desulphuricans* differed markedly from the yeast

studied by Slonimski (1955), which formed cytochrome *c* and many other enzymes of the respiratory cycle, adaptively, in response to the presence of its terminal electron acceptor oxygen.

Biological function of cytochrome c_3 . Evidence for the view that cytochrome c_3 acts as a carrier between sulphate reduction and cellular oxidation processes in *Desulphovibrio desulphuricans* has been discussed elsewhere (Ishimoto *et al.* 1954*b*; Postgate, 1956); the present work supports this view in that organisms grown in media freed from iron by 8-hydroxyquinoline are deficient in cytochrome c_3 and can reduce sulphate only slowly. But neither this observation nor earlier evidence demonstrates conclusively that cytochrome c_3 is the natural carrier for the reduction of sulphate. The oxidation of cytochrome c_3 , observed when whole organisms are incubated with sulphate, may only reflect a general change in the redox potential of organisms which are metabolizing sulphate. The ability of methyl and benzyl viologen to substitute for cytochrome c_3 in the reduction of sulphite, thiosulphate, etc., by enzymically active cell preparations suggests that the carrier action of c_3 may be merely a reflexion of its low redox potential and have no real metabolic significance. The fact that cytochrome c_3 acts as a carrier in the hydrogen-oxygen reaction, which can scarcely be of great metabolic importance for so strict an anaerobe as *Desulphovibrio desulphuricans*, does not strengthen confidence in the view that it is the biological carrier with other terminal electron acceptors.

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