support the view that the hydrocarbons are being actively metabolized. This is further borne out by the results obtained on the partially oxidized derivatives, such as long-chain monohydric alcohols and long-chain monocarboxylic acids. Methylene-blue reduction, moreover, points to the presence of a dehydrogenase enzyme present in the cells capable of oxidizing the long-chain hydrocarbons.

It seems that this organism offers very interesting possibilities for further studies on metabolism.

SUMMARY

1. The oxygen uptake of washed suspensions of *Proactinomyces opacus* is increased in the presence of *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane and paraffin wax.

2. Decyl, lauryl (dodecyl) and octadecyl alcohols give increased oxygen uptake, but amyl, *iso*amyl, *iso*hexyl, and heptyl alcohols are toxic.

3. The long-chain fatty acids (C_7 – C_{18}) are all actively metabolized at very low concentrations (0.0012M). At 1%, octanoate, decanoate, undecanoate and laurate (dodecanoate) are toxic.

4. The rate of methylene-blue reduction by the supernatant liquid obtained from the crushed cells of *Proactinomyces opacus* is increased in the presence of dodecane and hexadecane.

We are grateful to Mrs D. Oxford for suggesting this problem and to her and to Dr G. K. Fraser for their continued interest in this work. The work was carried out in the Botany Department, and we are grateful to Prof. J. R. Matthews for the facilities provided. We also wish to thank Messrs Hess Products Ltd., Littleborough, for gifts of fatty acids and to others for gifts of hydrocarbons of high purity.

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Reduction of Dehydroascorbic Acid by Bacteria

2. ROLE OF CYTOCHROME IN HYDROGEN TRANSPORT

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In a previous paper (Eddy, 1952) it was shown that the reduction of dehydroascorbic acid (DHA) to ascorbic acid (AA) by *Escherichia coli* can be effected with hydrogen supplied by various hydrogen donor/dehydrogenase systems in intact cells, but that corresponding cell-free systems are ineffective. It was therefore suggested that some additional factor, possibly a hydrogen carrier, is necessary.

Our attention was first directed to cytochrome because it was reported that some fifteen strains of streptococci were unable to reduce DHA (Gunsalus & Hand, 1941) and we found the same with species of *Lactobacillus* and *Clostridium*. These bacteria can produce reducing conditions of sufficiently high intensity for the reduction of DHA to be thermodynamically possible, but possess no cytochrome (Fujita & Kodama, 1934; Frei, Riedmüller &

Almasy, 1934). When a connexion between DHA reduction and cytochrome was in fact found in *Esch. coli*, comparison of the two properties was naturally extended over a range of bacterial types.

The present paper falls, therefore, into two parts, the first describing attempts to relate DHA reduction to the hydrogen carrier and the second being a survey of the types of bacteria able to effect the reduction.

METHODS

For spectroscopic work the organisms were grown in bulk on a solid medium containing 1% Protex, 1% peptone, 1% gelatin, 0.5% Yeastrel, 0.5% glucose, 1% $CaCO_3$ and 3% agar. After harvesting, the cells were washed twice with Ringer's solution (quarter strength) and suspended at the required concentration either in this solution or in phosphate buffer at pH 6.2.

Organisms to be tested for DHA reduction were grown in nutrient broth + 0.5% glucose in 1 oz. screw-capped bottles in which they were centrifuged, washed once with Ringer's solution and finally suspended in buffer. Their ability to reduce DHA was tested as described previously (Eddy, 1952) using 0.02M-glucose as hydrogen donor.

For viewing cytochrome spectra a Zeiss microspectroscope was used. Investigations at liquid air temperature were carried out as described by Keilin & Hartree (1949). For estimating changes in the intensities of cytochrome bands the microspectroscope was fixed vertically above one limb of a Duboscq colorimeter from which the prisms and eyepiece had been removed. A standard suspension of *Esch. coli* (1.5×10^{11} cells/ml.), with added dithionite, was placed in the colorimeter cup and the vernier scale set at 10 mm. The experimental suspension of equal density but without dithionite was placed in a cell of optical depth 10 mm., flooded with liquid paraffin and illuminated horizontally so that both spectra could be viewed simultaneously. The intensities of the spectra were then matched by altering the intensities of their respective light sources. After the addition of DHA the intensities of the spectra and of the cytochrome b_1 bands were rematched, altering the comparison spectrum by varying the intensity of the light source and the depth of suspension interposed.

RESULTS

A. Connexion between cytochrome and ability to reduce DHA in *Escherichia coli*

It seemed that, if cytochrome were needed to reduce DHA, the failure of enzyme preparations to effect the reduction might be due to the absence of cytochrome. Non-reducing cell-free dehydrogenase preparations of *Esch. coli* prepared by tryptic digestion and acetone treatment as described in a previous paper (Eddy, 1952), or mechanically in a Mickle tissue disintegrator, were examined spectroscopically after the addition of sodium dithionite. It was found that in all cases the cytochrome bands were either absent or were so weakened as to be barely visible; this supported the idea that reduction might be connected with cytochrome.

Unfortunately we have not been able to obtain cell-free preparations of the cytochrome from *Esch. coli*, with which to try to restore the reducing capacity of cell-free dehydrogenase preparations, and so we have had to use spectroscopic methods to detect any reaction between DHA and the cytochrome. The experiments have consisted essentially in generating the reduced cytochrome spectrum (b_1 band) by incubating a suspension of *Esch. coli* anaerobically, and then adding DHA (also anaerobically because the band disappears completely on aeration), and seeking to observe a weakening of the absorption band due to oxidation of the cytochrome by the DHA.

The first observations were made visually. They indicated that the intensity of the b_1 band was decreased on adding DHA, but only slightly. Because it was difficult visually to be certain of the

oxidation of the band, when it was so incomplete, attempts were made to confirm the observations photographically, using the following technique.

A suspension of *Esch. coli* containing approximately 1.5×10^{11} cells/ml. was prepared in phosphate buffer at pH 6.2. 2.5 ml. of this suspension were transferred to a glass cell of optical depth 5 mm. and the surface was flooded with liquid paraffin to maintain anaerobic conditions in the system. 0.1 ml. 0.0625M-sodium formate was added (at the final concentration the dehydrogenase activity

Fig. 1. Effect of dehydroascorbic acid on the fully reduced cytochrome b_1 band in *Esch. coli*. 1 mg. DHA was added to 2.5 ml. suspension containing 1.5×10^{11} cells/ml.

would be about half maximal), the cytochrome allowed to become fully reduced and the spectrum photographed. Urethane (0.1 ml. 50%, w/v), DHA (0.1 ml. 1% (w/v); to give maximal rate of reduction with respect to DHA concentration) and finally sodium dithionite, were added in turn and the spectrum photographed after each addition. Both the urethane and DHA solutions were previously de-aerated by evacuation and held under liquid paraffin until required. A definite weakening of the cytochrome b_1 band was recorded photographically after the addition of DHA in such experiments.

Attempts were then made to measure the degree of oxidation of the cytochrome b_1 band, by comparing it in the microspectroscope with that of a standard suspension of *Esch. coli* in which the cytochrome had been completely reduced with dithionite. Using a suspension in which the bands had become fully reduced without added hydrogen donor, and to which urethane had then been added, it was estimated that the addition of DHA resulted in approximately 30% diminution in the intensity of the cytochrome b_1 band in 3 min. (Fig. 1), indicating 30% oxidation of the reduced cytochrome. When the urethane was omitted, and sufficient glucose included to reduce all the added DHA, the cytochrome (b_1 band) changed from 70 to

90% reduced in 2.5 hr. and could then be re-oxidized to the same extent as previously by the addition of more DHA.

These observations appeared to reveal a connexion between the cytochrome of *Esch. coli* and its ability to reduce DHA. It was soon evident, however, that some organisms possessing cytochrome were unable to effect this reduction: e.g. baker's yeast had been reported to be ineffective by Gunsalus & Hand (1941) and we confirmed this observation several times. It therefore seemed necessary to compare the ability to reduce DHA with the cytochrome complement in a representative range of bacterial types.

B. A survey of DHA-reducing ability and cytochrome complement in different genera

Some information was already available. The cytochrome absorption spectra for a variety of bacteria had been listed by Fujita & Kodama (1934) and by Frei *et al.* (1934). These lists were a guide in choosing types for test, though we always checked the quoted complement against that actually observed in the strains we used. Some information had also been published about the types of organisms able to reduce DHA, and as Stewart & Sharp (1945) had reported that various staphylococci and coliform bacteria are best able to bring about this reduction, we began by testing related organisms.

Representative species of *Eberthella*, *Erwinia*, *Escherichia*, *Proteus*, *Salmonella* and *Serratia* were found to reduce DHA. Gunsalus & Hand (1941) reported that several strains of *Aerobacter cloacae* could also effect the reduction, though a British strain and two strains of *A. aerogenes* tested by us were found to be ineffective. Strains of three species of *Staphylococcus* were also found to be able to reduce DHA. The nomenclature in this paper follows *Bergey's Manual of Determinative Bacteriology*, 5th ed. (1939).

Species from most of the above genera had been examined by Fujita & Kodama (1934) and found to exhibit the cytochrome b_1 band in place of the b and c bands of other types. Hence a series of types characterized by other cytochrome complements was next examined—species of *Clostridium* (1), *Lactobacillus* (1), *Acetobacter* (2), *Pseudomonas* (1), *Bacillus* (8), *Micrococcus* (7) and *Sarcina* (1)—and all were found to be unable to reduce DHA.

These observations suggested a correlation between ability to reduce DHA and the possession of cytochrome b_1 , and our belief in this connexion was strengthened on careful examination of several apparently irregular cases:

(1) It had been reported (Tkachenko, 1936) that DHA was reduced by *Lactobacillus acidophilus*. As species of this genus possess no cytochrome, we

carefully examined three strains of this species, and found no evidence of reducing power. Reference to the technique employed by Tkachenko (1936) has led us to regard his evidence as inconclusive.

(2) On examination of a strain designated *Staphylococcus citreus* which failed to reduce DHA, it was found to possess a spectrum with well defined cytochrome b and c bands, and further systematic examination revealed that it was, in fact, *M. conglomeratus*. It thus fell into line with the other *Micrococcus* species examined, namely *M. aurantiacus*, *M. candidans*, *M. flavus*, *M. freudenreichii*, *M. luteus*, *M. lyso-deikticus* and *M. ureae*.

(3) Among the Gram-negative bacilli related to the coliforms and included in the family Enterobacteriaceae according to *Bergey's Manual*, 5th ed. (1939), Fujita & Kodama (1934) described one species (*Bacillus prodigiosus* syn. *Serratia marcescens*) as possessing cytochromes b and c . As we found species of *Serratia* able to reduce DHA, we examined their spectra and found that they possessed only a single band, and that the band remained single even on cooling in liquid air, a treatment which sharpens the bands and makes it possible to distinguish readily cytochromes b and c (Keilin & Hartree, 1949).

(4) The situation in the genus *Bacillus* was particularly interesting. Our examination of strains of eight species (*B. cereus*, *B. licheniformis*, *B. megatherium*, *B. mesentericus*, *B. mycoides*, *B. polymyxa*, *B. pumilus* and *B. subtilis*) showed that none could reduce DHA though, on the other hand, Keilin (1934) had earlier reported that *B. megatherium* possessed a cytochrome b_1 band similar to that in *Esch. coli* and similar reports had been made in respect of *B. subtilis* (Frei *et al.* 1934). Further, Chaix & Roncoli (1951) have recently observed that young cultures of several *Bacillus* species exhibit a single broad absorption band at 550–560 $m\mu$. which they provisionally identify as that of cytochrome b_1 . Examination of the spectra of our *Bacillus* strains (in the state in which we tested them for DHA reduction) showed that they mostly possessed single cytochrome bands similar to those described by Chaix & Roncoli. At first, these observations weakened our belief in the connexion between DHA reduction and cytochrome b_1 . However, careful examination showed that the cytochrome bands in these *Bacillus* strains were broader than the typical b_1 bands of staphylococci and the Enterobacteriaceae. On heating a suspension of *B. pumilus* to 80° for 20 min. in the presence of dithionite, the long-wavelength side of the band disappeared, leaving a narrow band in the position of the typical c band. Again, examination of the ' b_1 ' band in *B. cereus* at the temperature of liquid air showed a band centred at 552 $m\mu$. with faint shading on either side (possibly indicative of

two additional bands analogous to the spectrum found in old cultures of *B. subtilis* which contains cytochromes *b* and *c*), whereas under these conditions the coliforms show a single band from 553 to 560 $m\mu$. with a barely perceptible maximum at 557 $m\mu$. These observations lead us to conclude that the single band in *Bacillus* species is probably not identical with the classical b_1 band of the coliform organisms, which conclusion agrees with the inability of the *Bacillus* strains to reduce DHA.

It seems, therefore, that ability to reduce DHA is confined to some strains within the genus *Staphylococcus* and the family Enterobacteriaceae, which contain typical cytochrome b_1 .

Variation in DHA-reducing capacity among strains. It has become certain, however, that a considerable proportion of organisms apparently containing a perfectly normal cytochrome b_1 are unable to reduce DHA. On further investigation of the two groups of reducing organisms mentioned above, it was found that some members of each were unable to reduce DHA. Six strains each of *Staph. albus* and *Staph. aureus* were provided by Dr R. M. Fry (Public Health Laboratory, Cambridge) and two strains of *Staph. citreus* (7415, 7990) were obtained from the National Collection of Type Cultures (N.C.T.C.). Of these, one strain of *Staph. albus* failed to reduce DHA. Table 1 gives the

(1945) stated that, of the strains of coliform organisms they tested, about half did not reduce DHA.

With a strain of *Esch. coli* (N.C.T.C. 7277) which is unable to reduce DHA, experiments similar to those in section 4 did not reveal any alteration in the intensity of the cytochrome band on addition of DHA. Attempts to make similar observations with strains of *Staphylococcus* were indecisive because the cytochrome bands were too weak.

DISCUSSION

The results in the first half of the preceding section, suggesting on spectroscopic evidence that cytochrome b_1 is oxidized by DHA, support, and are supported by, those in the second half, which indicate that a grouping of bacteria according to their complement of cytochromes will delimit a group of genera within which the ability to reduce DHA is confined. Taken together, the observations show that DHA can be reduced by hydrogen transported by cytochrome b_1 but not by other cytochromes.

These relations, be it noted, are those which obtain when dehydrogenases of the anaerobic type effect the reduction of DHA. With other hydrogen-donating systems different relations may hold: for example, glycine can act as a hydrogen donor for

Table 1. *Ability to reduce dehydroascorbic acid among members of the family Enterobacteriaceae*

(Numbers quoted are those of N.C.T.C. strains; other strains were supplied by Dr W. J. Dowson, Botany School, Cambridge.)

Species	Reducing strains	Non-reducing strains
<i>Aerobacter aerogenes</i>	—	(2 unknown)
<i>A. cloacae</i>	(U.S. strains)	(1 unknown)
<i>Eberthella belfastiensis</i> (<i>Bact. coli anaerogenes</i>)	4450	4174
<i>Erwinia aroideae</i>	(1 unknown)	—
<i>Erwinia carotovora</i>	(4 unknown)	—
<i>Escherichia coli</i> (<i>Bact. coli</i> Type I)	8196, (1 unknown)	8179, 8333
<i>Esch. coli</i> (<i>Bact. coli</i> Type II)	8164	—
<i>Esch. coli</i> (irregular)	—	7275, 7277
<i>Esch. freundii</i> (<i>Bact. coli</i> Inter. I)	—	8165, 8166
<i>Esch. freundii</i> (<i>Bact. coli</i> Inter. II)	6072	6071
<i>Klebsiella ozaenae</i>	—	5053
<i>K. pneumoniae</i>	—	2794, (1 unknown)
<i>K. pneumoniae A</i>	—	5054
<i>K. pneumoniae B</i>	—	5055
<i>K. pneumoniae C</i>	—	5056
<i>K. rhinoscleromatis</i>	—	5047
<i>Proteus vulgaris</i>	(1 unknown)	—
<i>Salmonella pullorum</i>	(1 unknown)	—
<i>Serratia marcescens</i>	(1 unknown)	(1 unknown)
<i>Serratia kielenensis</i>	4619	—

occurrence of DHA-reducing ability in the strains of species from the Enterobacteriaceae which we have examined; about half are inactive. Similar results had in fact been recorded previously. Gunsalus & Hand (1941) and Stewart & Sharp

DHA reduction (Eddy, 1952, Table 1), but the dehydrogenase probably involved is an autooxidizable flavoprotein. Such a system might not be expected to show any relation to cytochrome, but the fact that cytochrome is not an essential inter-

mediate does not exclude the possibility that the dehydrogenase is capable of reducing it.

It is not known why the cytochrome b_1 band is not fully removed by DHA. It may be either that the residual level of dehydrogenase activity in our experiments was sufficient to keep the cytochrome 70% reduced or, alternatively, that the oxidation-reduction potential of the AA/DHA system, as constituted in this case, was the same as that of 70% reduced cytochrome b_1 .

The inability of many organisms containing cytochrome b_1 to effect the reduction raises a further problem. As there is no evidence that this is due either to their cytochromes or cytochrome-reducing mechanisms, we suppose it to be due to lack of a mechanism linking oxidation of the cytochrome with reduction of DHA. In the previous paper (Eddy, 1952) theoretical considerations were given in favour of an enzyme activating DHA so that it can be reduced. The failure of some coliform bacteria to reduce DHA lends weight to these suggestions, and preliminary experiments have shown that the oxidation of leuco-Nile blue by DHA varies both in rate and magnitude, according to whether DHA-reducing or DHA-non-reducing strains of bacteria are present.

SUMMARY

1. In cell-free dehydrogenase preparations from *Escherichia coli*, which are unable to reduce dehydroascorbic acid (DHA), the cytochromes are either absent or greatly decreased in amount.

2. When DHA is added anaerobically to a suspension of *Escherichia coli* the intensity of the fully reduced cytochrome b_1 band becomes diminished by approximately 30%.

3. Amongst organisms examined so far, ability to reduce DHA is confined to members of the genus *Staphylococcus* and the family Enterobacteriaceae, these being the groups in which the b and c cytochrome bands are replaced by the b_1 band.

4. Approximately half the members of the Enterobacteriaceae tested failed to reduce DHA, and it is suggested that a DHA-activating enzyme may be necessary in addition to cytochrome b_1 .

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Studies in Immunochemistry

11. THE ACTION OF DILUTE ALKALI ON THE N-ACETYLHEXOSAMINES AND THE SPECIFIC BLOOD-GROUP MUCOIDS

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The increasing use within recent years of the colorimetric method introduced by Morgan & Elson (1934) for the detection and quantitative determination of the *N*-acetylhexosamines calls for a more detailed study of the conditions necessary to obtain reliable and reproducible results and demands a more thorough understanding of the structural changes involved before the results can

be applied to complex structures containing *N*-acetylhexosamine components, such as the blood-group mucoids.

Ehrlich (1901) observed that mucins and mucoid substances give rise to an intense purple colour after heating with dilute alkali for a short time and the addition of an acid solution of *p*-dimethyl-aminobenzaldehyde. If the alkali treatment is