Reduction of Dehydroascorbic Acid by Bacteria

1. REDUCING MECHANISMS OF *ESCHERICHIA COLI*

By B. P. EDDY
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(*Received* 5 *July* 1951)

Tkachenko (1936) reported that dehydroascorbic acid (DHA) was reduced to ascorbic acid (AA) in cultures of *Lactobacillus acidophilus*, *L. bulgaricus* and *L. leichmannii*. The system has since been investigated by Esselen & Fuller (1939), Gunsalus & Hand (1941), Stewart & Sharp (1945) and Mapson & Ingram (1951). Esselen & Fuller (1939) investigated eleven strains of *Escherichia coli* and one each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salm. aertrycke* (*Salm. typhimurium*) and *Salm. enteritidis*. They did not obtain any convincing evidence of reduction except with two strains of *Esch. coli*. Gunsalus & Hand (1941) tested a number of strains of *Esch. coli* isolated from human faeces and found approximately half to be active in reducing DHA. They also found several strains of *Aerobacter cloacae* to be active, but obtained negative results with fifteen strains of streptococci and with baker's yeast. Stewart & Sharp (1945) tested a large number of organisms and found that coliforms and 'coccus forms' were most likely to reduce DHA. Of eighty-eight strains of coliform organisms investigated almost half were active. They finally selected strains of *Esch. coli* and *Staphylococcus albus*. Mapson & Ingram (1951) showed that DHA can be reduced quantitatively to AA by *Esch. coli*, and that this affords a means of estimating DHA.

None of these investigators, however, attempted to account for the distribution of ability to reduce DHA in terms of reducing systems possessed by the bacteria, nor have any investigations been made of the mechanism of DHA reduction. This paper describes an investigation of the mechanism of the reduction which involves, in essence, the addition to the DHA molecule of two atoms of hydrogen.

METHODS

The organism used was a strain of *Esch. coli* (*Bact. coli* Type I 44° negative) isolated in this laboratory and was the same as that used by Mapson & Ingram (1951). It was grown on digest agar (McCartney) in Roux bottles and, except when feeding with formate or lactate, 0·5% (w/v) glucose was incorporated in the medium. The cells were harvested and washed three times with Ringer solution (one-quarter strength) in which they were finally suspended at a concentration of about $7{\cdot}6 \times 10^{10}$ cells/ml. (standardized by opacity tube).

Dry weights were determined by drying 1 ml. of suspension to constant weight over a boiling-water bath. It was found that 1 ml. of suspension containing $7{\cdot}6 \times 10^{10}$ cells had a dry weight of 20 mg. ± 15% after deducting the weight of the solids of the Ringer solution.

The DHA was prepared by dissolving 200 mg. AA in 50 ml. of glass-distilled water, adding Br_2 in slight excess and shaking. The remaining Br_2 was removed with a stream of air bubbled through the solution.

Experiments on the rate of DHA reduction not involving measurements of hydrogen uptake were carried out as follows. Into a tube, with a two-hole bung carrying a gas inlet reaching to the bottom, were introduced 1 ml. of suspension + 0·5 ml. M-substrate (unless otherwise stated or unless hydrogen were being used as donator) + 23 ml. of 0·067 M-phosphate buffer of pH 6·2. The tube was fixed in a water bath at 35° and nitrogen (or hydrogen if used) blown through the mixture. At zero time 0·5 ml. DHA solution (2 mg.) was added. Samples (5 ml.) were removed at 5 min. intervals and, after mixing with an equal volume of 20% (w/v) metaphosphoric acid, were titrated for AA with 2:6-dichlorophenolindophenol.

Investigations involving gas uptakes were carried out using normal manometric (Warburg) technique, the flask contents being titrated for ascorbic acid at the end of the experiments, if necessary.

RESULTS

Specificity of the hydrogen donator. Since the reduction of DHA to AA simply involves the addition of hydrogen, one might assume that a suitable hydrogen-donating system only would be necessary. A number of substrates of some of the dehydrogenases known to be possessed by *Esch. coli* have therefore been tested for their ability to act as hydrogen donators for the DHA-reducing mechanism. Table 1 gives a list of these substances, together with the respective rates of the reaction, with two different suspensions of *Esch. coli*, expressed as hydrogen equivalents (µl.) of DHA reduced/mg. dry wt. of bacteria/hr. ($Q_{H_2}^{DHA}$).

The results show that the DHA-reducing mechanism is not specific towards any single hydrogen donator/dehydrogenase system. There are, however, large differences between the rates of reduction by different systems. Formate, lactate and hydrogen were selected as substrates for use in further work.

Table 1. *Ability of* Esch. coli *to use various substrates as* H-*donators for the reduction of dehydroascorbic acid* (*DHA*)

(In each case the endogenous reduction has been deducted. $Q_{H_2}^{DHA}$ represents the rate of reduction of DHA expressed as equivalent volumes of H_2 (μl.) transferred from donator to acceptor/mg. dry wt. of bacteria/hr.)

	Suspension 1			Suspension 2		
Substrate	Substrate concn.	pH	$Q_{H_2}^{DHA}$	Substrate concn.	pH	$Q_{H_2}^{DHA}$
—	—	6·2	4	—	—	9·5
Succinate	0·02 M	6·2	15	0·04 M	6·2	9·5
Malate	0·02 M	6·2	9	0·04 M	6·2	13
Lactate	0·02 M	6·2	23	0·02 M	6·2	38·5
Ethanol	2 % (v/v)	6·2	12	2 % (v/v)	6·2	17·5
Hydrogen	Continuous stream	6·2	25	Continuous stream	7·0	19
Formate	0·02 M	6·2	27	0·02 M	6·2	17
Glucose	0·02 M	6·2	55	0·02 M	7·0	76
Glycine	—	—	—	0·1 M	6·2	32·5

Cell-free enzyme preparations. A cell-free formic dehydrogenase preparation was made by digesting *Esch. coli* cells with trypsin (Stickland, 1929) and a hydrogenase preparation was made by acetone treatment (Bovarnick, 1941). These preparations actively reduced methylene blue, but were completely inactive towards DHA. Mechanical disintegration of the cells, or freeze-drying, also resulted in inactive preparations. The following substances were added in unsuccessful attempts to restore the activity; methylene blue, pyocyanine, glutathione, coenzyme II, yeast extract and boiled and unboiled whole yeast.

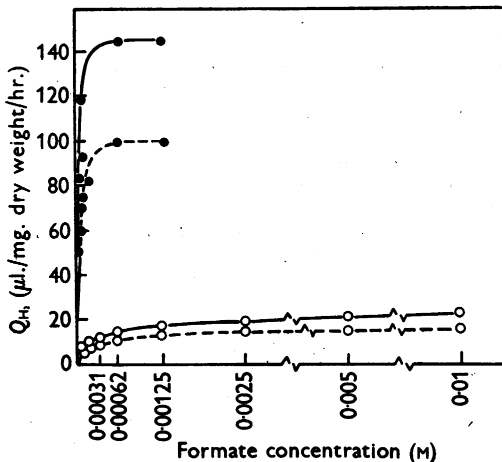

Fig. 1. Effect of formate concentration on rates of reduction of methylene blue and dehydroascorbic acid (DHA) by washed suspensions of *Esch. coli* (—, suspension 1; - - -, suspension 2). ●—●, $Q_{H_2}^{MB}$ (rates of methylene-blue reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.); ○—○, $Q_{H_2}^{DHA}$ (rates of DHA reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.).

Effect of hydrogen-donator concentration on the rate of DHA reduction. In an attempt to throw further light on possible additional links in the chain of reduction reactions, the effects of substrate concentration on the rates of DHA and methylene-blue reduction were next compared. Fig. 1 shows the rates of reduction of DHA and methylene blue for samples of a single suspension, plotted as $Q_{H_2}^{DHA}$ and $Q_{H_2}^{MB}$ against formate concentration; the experiment was later repeated with a different suspension of the same strain of *Esch. coli*.

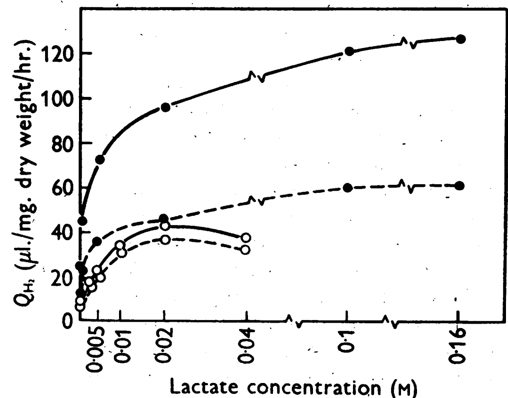

Fig. 2. Effect of lactate concentration on rates of reduction of methylene blue and dehydroascorbic acid (DHA) by washed suspensions of *Esch. coli* (—, suspension 1; - - -, suspension 2). ●—●, $Q_{H_2}^{MB}$ (rates of methylene-blue reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.); ○—○, $Q_{H_2}^{DHA}$ (rates of DHA reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.).

Comparison of the curves shows that in the DHA-reducing system the rate of reaction at any given substrate concentration is far below that for methylene-blue reduction. The K_m for methylene-blue reduction is $3{\cdot}3 \times 10^{-5}$, whereas that for DHA

reduction is $1 \cdot 5 \times 10^{-4}$ approximately (the substrate/rate relation does not quite follow the Michaelis and Menten curve in the latter case). In addition, at formate concentrations above 0·01 M, the rate of DHA reduction decreases with increasing concentration.

Fig. 2 shows the results of similar experiments with lactate as substrate. The lactic dehydrogenase values are calculated from Yudkin (1937) and give a K_m of 5×10^{-3}. The K_m for DHA reduction is (very roughly) 8×10^{-3}.

With lactate, as with formate, the rate of DHA reduction was low compared with that of methylene-blue reduction, but the difference was much greater in one suspension than in the other, although these suspensions had given comparable results in the formate experiments. The inhibitory effect of higher lactate concentrations was more pronounced than with formate.

Effect of increasing the hydrogenase activity. By replacing the glucose in the culture medium with formate or with lactate at the same concentration, the hydrogenase activity of washed suspensions (measured manometrically by the rate of hydrogen uptake with methylene blue as acceptor) was increased by 100 and 250% respectively. When the rates of DHA reduction by these suspensions using hydrogen as donator were determined, however, it was found that they were much diminished (Table 2).

The results in Table 2 confirm that, with glucose-fed organisms, there is a factor reducing the rate of hydrogen transfer when DHA is the acceptor. The relatively low rates of reduction with formate-fed and lactate-fed organisms further suggest that if some additional factor is required, there is more of it available in cells which are grown on a medium containing glucose.

Table 2. *Effect of lactate and formate feeding on the hydrogenase and dehydroascorbic acid (DHA) reducing activities of* Esch. coli

(The hydrogenase activities ($Q_{H_2}^{MB}$) are given as µl. H_2 taken up/mg. dry wt./hr. $Q_{H_2}^{DHA}$ = µl. taken up/mg. dry wt./hr. with DHA as acceptor.)

Substrate for cell growth	$Q_{H_2}^{MB}$ (30°)	$Q_{H_2}^{DHA}$ (35°)
Glucose	110	25·3
Formate	220	3·6
Lactate	385	8·6

Quantitative relations between hydrogen used and DHA reduced. Because of the differences between the $Q_{H_2}^{MB}$ and $Q_{H_2}^{DHA}$ values shown in Table 2, experiments were carried out to relate the hydrogen used to the amount of DHA reduced. The hydrogen uptakes were measured manometrically for 25 min., and the flask contents and those of control flasks containing nitrogen were titrated for ascorbic acid. The results are shown in Table 3.

Whereas with glucose-fed organisms the hydrogen uptake is equivalent to the DHA reduction in hydrogen less that in nitrogen, with formate-fed and lactate-fed organisms this is not the case. With

Table 3. *Relation between hydrogen uptake and dehydroascorbic acid (DHA) reduction in suspensions of glucose-fed, formate-fed and lactate-fed* Esch. coli

Substrate for cell growth	Gas	(1) Gas uptake (µl.)	(2) H_2 uptake (1) (H_2) − (1) (N_2) (µl.)	(3) DHA reduction (mg.)	(4) H_2 equiv. of (3) (H_2) (µl.)*	(5) DHA reduction in H_2 (3) (H_2) − (3) (N_2) (mg.)	(6) H_2 equiv. of (5) (µl.)*
Glucose	N_2	−3·0	—	0·084	—	—	—
Glucose	H_2	32·5	35·5	0·354	45·0	0·270	34·5
Formate	N_2	+2·5	—	0·173	—	—	—
Formate	H_2	38·0	35·5	0·341	43·5	0·168	21·5
Lactate	N_2	0·0	—	0·139	—	—	—
Lactate	H_2	47·0	47·0	0·373	47·5	0·234	30·0

* 0·1 mg. DHA is equivalent to 12·8 µl. H_2 at N.T.P.

Table 4. *Suppression of endogenous reduction of dehydroascorbic acid (DHA) by hydrogenase system in washed suspensions of* Esch. coli

($Q_{H_2}^{MB}$ = hydrogenase activity expressed as µl. H_2 taken up/mg. dry wt./hr. with methylene blue as acceptor.)

Substrate for cell growth	(1) Total DHA reduction in N_2 (mg.)	(2) DHA equiv. of H_2 uptake (mg.)	(3) DHA reduction not due to hydrogenase (mg.)	(4) DHA reduction in N_2 (mg.)	(5) (3) as percentage of (4)	(6) Suppression of DHA reduction in N_2 (%)	(7) $Q_{H_2}^{MB}$
Glucose	0·354	0·28	0·074	0·084	88	12	110
Formate	0·341	0·28	0·061	0·173	35	65	220
Lactate	0·373	0·37	0·003	0·139	2	98	385

the latter, the hydrogen uptake is equivalent to the total DHA reduced and with the former, to a figure somewhere between the nitrogen and hydrogen values.

Table 4 suggests that this happens because the hydrogenase system suppresses the endogenous reduction of DHA. Comparison with the respective $Q_{H_2}^{MB}$ values shows that the precentage suppression increases with increasing hydrogenase activity, indicating competition between the two reducing systems. Thus in lactate-fed organisms, with a high $Q_{H_2}^{MB}$, the endogenous system is wholly suppressed, and all the reduction is at the expense of the hydrogen/hydrogenase system, while in glucose-fed organisms the reduction by the endogenous system must first be deducted in order to obtain equivalence.

Table 5. *Rates of dehydroascorbic acid (DHA) reduction in the presence of one or more hydrogen donators expressed as equivalent volumes of* H_2 *(µl.) transferred from donator to acceptor/mg. dry wt./hr.* ($Q_{H_2}^{DHA}$)

Hydrogen donator	$Q_{H_2}^{DHA}$
(1) None	15
(2) 0·01 M-Formate	46·5
(3) 0·02 M-Lactate	52
(4) (2) + (3)	63
(5) Hydrogen (continuous stream)	30·5
(6) (2) + (5)	45
(7) (3) + (5)	59
(8) (2) + (3) + (5)	62
(9) 0·02 M-Glucose	84

Effect of mixed substrates. Table 5 shows the effect on the rate of DHA reduction of the presence of more than one dehydrogenase substrate, each at its optimal concentration (cf. Figs. 1 and 2) or, as is the case with hydrogen, at saturation. The rates of DHA reduction are not additive, but appear to have a maximum $Q_{H_2}^{DHA}$ of 63, except in the case of glucose.

DISCUSSION

As already stated, the results suggest that the mechanism of reduction of DHA by *Esch. coli* is not very specific with regard to the hydrogen-donating mechanism, since any of several dehydrogenases possessed by the organism which were tested, and hydrogenase itself, can serve to effect the reduction; also the rates of reduction vary considerably with the different hydrogen-donators and also from one suspension to another. This may be due, at least in part, to the differing amounts of enzymes present and to their various pH and substrate-concentration relationships. Among the dehydrogenases tested are representatives of the aerobic (glycine), cytochrome-linked (succinic, lactic and formic) and coenzyme-linked (malic, ethanol and glucose) types.

The bulk of the evidence presented, however, suggests that the hydrogen-donating mechanism does not constitute the whole system, as is the case when methylene blue is reduced, but that one or, more probably, several factors are involved, and that different treatments may result in the loss of one or other of these factors.

The work comparing the effect of hydrogen-donator concentration on the rates of DHA and methylene-blue reduction, and that using mixed substrates, has shown that the rate of DHA reduction is limited, but not by the potential rate of hydrogen transfer. When glucose is added as hydrogen donator, the rate of reduction is higher than that which is achieved by the combined effects of formate, lactate and hydrogen. It may be that there are two mechanisms for DHA reduction and that for one of these only glucose can act as hydrogen donator.

The fact that cell-free dehydrogenase and hydrogenase preparations are active towards methylene blue but not towards DHA shows that in this milieu DHA is not spontaneously reducible and suggests that a DHA-activating enzyme may be necessary before reduction can take place. Further evidence for a DHA-activating enzyme is the fact that although D-*iso*dehydroascorbic acid and the oxidized forms of hydroxytetronic and reductic acids are reduced, the rates of reduction are much slower than that of DHA (Mapson & Ingram, 1951). The experiments with formate-fed and lactate-fed cells indicate that a factor is required which is best produced if the organisms are fed on glucose. Since the yield of cells is greater on formate or lactate than on glucose, it may be suggested that the missing factor in this case is not of great importance in general metabolism and is, therefore, probably not one of the recognized hydrogen carriers.

Previous work on coupled oxidations and reductions has, however, shown that a hydrogen carrier is necessary before the reactions will take place *in vitro* (Green, Stickland & Tarr, 1934) and certain aspects of the present work, such as the failure of acetone powders and mechanically disintegrated preparations of *Esch. coli* to effect the reduction, suggest that a hydrogen carrier may be essential in this reaction. Further experiments have indicated that this is the case, and work is in progress to substantiate these indications.

SUMMARY

1. Hydrogenase and formic, lactic, succinic, malic, ethanol, glucose and glycine dehydrogenases, with their substrates, can act as hydrogen-donating mechanisms for dehydroascorbic acid (DHA) reduction by *Escherichia coli*.

2. Cell-free dehydrogenase preparations will not reduce DHA, indicating that some essential factor or factors is absent from them. When absent, the

factor is not replaceable by methylene blue, pyocyanine, glutathione, coenzyme II, yeast extract, or boiled or unboiled whole yeast.

3. The effect of hydrogen-donator concentration on the rate of DHA reduction, as compared with the rate of methylene-blue reduction, indicates that the rate of hydrogen transfer may be greatly reduced in the DHA-reducing system.

4. Growth on lactate or formate in place of glucose increases the hydrogenase activity, but decreases the DHA-reducing activity.

5. When more than one hydrogen donator is present, the rates of DHA reduction are not additive. The rate of reduction when formate, lactate and hydrogen are present together is not as great as when glucose is present alone.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The writer wishes to thank Dr M. Ingram and Dr L. W. Mapson for the interest they have taken in the work.

REFERENCES

Bovarnick, M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **47**, 191.
Esselen, W. B. & Fuller, J. E. (1939). *J. Bact.* **37**, 501.
Green, D. E., Stickland, L. H. & Tarr, H. L. A. (1934). *Biochem. J.* **28**, 1812.
Gunsalus, I. C. & Hand, D. B. (1941). *J. biol. Chem.* **141**, 853.
Mapson, L. W. & Ingram, M. (1951). *Biochem. J.* **48**, 551.
Stewart, A. P. & Sharp, P. F. (1945). *Industr. Engng Chem.* **17**, 373.
Stickland, L. H. (1929). *Biochem. J.* **23**, 1187.
Tkachenko, E. S. (1936). *Biochimia*, **1**, 579. Cited in *Chem. Abstr.* (1937). **31**, 7461.
Yudkin, J. (1937). *Biochem. J.* **31**, 865.

Oxidation of Phenylacetic Acid by *Penicillium chrysogenum*

By D. J. D. HOCKENHULL, A. D. WALKER, G. D. WILKIN AND F. G. WINDER
Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks

(*Received* 4 *June* 1951)

Phenylacetic acid (PA) has been shown by Singh & Johnson (1948) to be almost wholly consumed by *Penicillium chrysogenum* in fermentations producing penicillin. Only a small fraction entered the penicillin produced. The present study has indicated that the rest is wholly or partly oxidized, beginning with the acetic acid residue.

This type of oxidation in moulds has not hitherto been described. Supniewski (1942) has, however, claimed that his 'Bacille pyocyanique' was capable of oxidizing PA by way of benzoic acid.

EXPERIMENTAL

Methods

(1) *Determination of phenylacetic acid.* As acetic acid was a normal contaminant of the fluids examined, it was necessary to steam-distil them before extraction with ether, following the procedure described by Friedemann (1938), but using Hockenhull's (1949) modification of the apparatus. Results had a precision of $\pm 5\%$. Benzoic acid interfered because it behaved in almost the same way as PA.

(2) *Growth conditions.* The material for use in these studies was obtained by growing the mould upon the synthetic medium of Jarvis & Johnson (1947). (Lactose, 22·5; glucose, 7·5; ammonium acetate, 3; PA, 2; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0·25; $FeSO_4.7H_2O$, 0·10; $CuSO_4.7H_2O$, 0·004; $MnSO_4.4H_2O$, 0·004; Na_2SO_4, 0·5; $CaCO_3$, 10 g./l. distilled water.) The medium was adjusted to pH 6·3 with 10N-NaOH before sterilization for 15 min. at 15 lb./sq.in. It was distributed in 60 ml. quantities into 250 ml. conical flasks. The flasks were each inoculated with 3% of a vegetative inoculum and incubated at 25° on a rotary shaker at 180 rev./min.

The inoculum was prepared as follows: A suspension of 10^7 to 10^8 spores/ml. in 0·1% aqueous Calsolene oil HS (Imperial Chemical Industries Ltd.) was made up from a 6- to 8-day culture on glycerol-molasses-peptone agar of the organism which had been derived from *P. chrysogenum* Q176 by ultraviolet light mutation. This suspension (3 ml.) was inoculated into 60 ml. of a development medium (containing corn steep liquor (C.S.L.), brown sugar and 0·1% PA) in a 250 ml. conical flask. This was incubated for 40 hr. at 25° on a rotary shaker at 180 rev./min.

(3) *Respiration of mould suspensions in presence of PA.* The preparations used for respirometric work with Warburg apparatus were made as follows. Mycelium from the 60 ml. culture described above was centrifuged, washed in 60 ml. 0·05M-sodium phosphate buffer, pH 7·2, centrifuged and again suspended in 60 ml. buffer in a 250 ml. conical flask. Four 6 mm. glass beads were added and the flask was shaken at 25° for 16 hr. to reduce O_2 consumption in the absence of substrate. The felt was again centrifuged off and finally resuspended in a further 60 ml. of buffer. In the presence of PA this suspension gave an increase in respiration of 50–200% over the blank.

The Warburg vessels usually contained 2 ml. suspension (20–30 mg. dry wt.). The standard substrate concentration