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Reduction of Dehydroascorbic Acid by Bacteria

3. ENZYMIC ACTIVATION OF DEHYDROASCORBIC ACID

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In the previous communications in this series (Eddy, 1952; Eddy, Ingram & Mapson, 1952) it has been shown that the mechanism by which *Escherichia coli* reduces dehydroascorbic acid (DHA) to ascorbic acid (AA) includes any of various dehydrogenases possessed by the organism and cytochrome b_1 , and it has been suggested that there may be a further enzyme which acts on DHA. Because cell-free extracts of *Esch. coli*, however prepared, would not reduce DHA, this hypothetical activating enzyme could not be studied directly, but there remained several lines of attack which could strengthen the evidence for its existence. These were the use of leuco-Nile blue as the immediate hydrogen donor (Tarr, 1940; Jebb, 1949); the determination of the specific chemical configurations which are capable of combining with the enzyme; the investigation of the chemical specificity and kinetic behaviour of inhibitory substances, and experiments to find typical Michaelis-Menten relationships between the concentrations and rates of reduction of DHA and related reducible substances.

METHODS AND MATERIALS

Methods

The organisms used for this part of the work were two strains of *Esch. coli*: a reducing strain isolated in this laboratory and a strain (NCTC 7277) which we shall denote as the non-reducing strain, although in fact it does reduce DHA very slowly. The cells were grown and harvested as described previously (Eddy, 1952).

Thunberg tube experiments with Nile blue were carried out as follows. Into the body of the Thunberg tubes were introduced 3.2 ml. of 0.2M phosphate buffer (pH 6.2) made from Na_2HPO_4 and KH_2PO_4 , 0.1 ml. of M lithium lactate

(to give the optimum concentration—Eddy, 1952, Fig. 2), 0.2 ml. of 0.2% (w/v) aqueous Nile blue sulphate (British Drug Houses Ltd.) and 1 ml. of bacterial suspension. A solution of 2 mg. DHA in 0.5 ml. of water was placed in the hollow stopper. The tubes were evacuated and placed in a water bath at 35°. When the dye had become reduced the DHA was added from the stopper and the oxidation of the dye measured by visual comparison with a series of colour standards. In a parallel series of tubes, serial AA estimations were carried out by opening the tube, adding 5 ml. 20% (w/v) metaphosphoric acid, diluting to a suitable volume and filtering. Samples were then titrated with 2.6-dichlorophenol indophenol.

Measurements of electrode potential were made in a 50 ml. beaker which was fitted with a rubber bung carrying a platinum electrode, a mercury-calomel half-cell and gas inlet and outlet tubes. It was immersed in a water bath at 35°. Into the beaker were put 17.5 ml. 0.2M phosphate buffer (pH 6.2), 5 ml. of bacterial suspension and 0.5 ml. of M glucose. Air was removed by bubbling N_2 through the suspension. When the potential had reached a steady state after about 20–25 min., 2 ml. of DHA solution (4 mg./ml.) were added, and the ensuing potential changes measured. From a duplicate system samples were removed at intervals for AA determinations.

Experiments on the effect of inhibitors and H-acceptor concentration on the rate of reduction were carried out as described by Eddy (1952), the method involving serial titrations of AA on samples withdrawn from a reaction mixture containing H-donor, bacterial cells, DHA and buffer at pH 6.2, held at 35° under anaerobic conditions.

Materials

Of the compounds cited in Fig. 4, L-ascorbic acid, dihydroxymaleic acid, glyoxal, diacetyl, pyruvic acid and dihydroxytartaric acid (the hydrate of dioxosuccinic acid) were available commercially, as was also D-araboascorbic acid. The hydroxytetronic and reductic acids and the 3-hydroxy-1:2-dioxo-3-phenylbutyric acid lactone were given

by Professor T. Reichstein and Professor H. Dahl (Institute of Organic Chemistry, Basle University). The L-galactonic, L-gulonic and D-mannonic acid lactones and the D-galacturonic acid methyl ester were supplied by Dr F. A. Isherwood (for methods of preparation see Isherwood, Chen & Mapson, 1954). Butyrolactone was prepared by heating silver glutarate with I_2 (Sircar, 1928); dioxo-L-gulonic acid (as its Ca salt) by the first method of Penney & Zilva (1945), and methyl D-ribofuranoside by the HCl-catalysed methylation of D-ribose (Barker, 1948).

The unstable dehydro-compounds were prepared immediately before use by dissolving the enediols in 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.

RESULTS

Chemical reaction between leuco-Nile blue and DHA. Before carrying out experiments similar to those of Tarr (1940) and Jebb (1949) it was desirable to find out whether there was a chemical reaction between reduced Nile blue and DHA. This was done as follows. The dye was reduced with bacteria under a layer of soft wax in centrifuge tubes, the cells were centrifuged down and DHA solution (previously deoxygenated by evacuation) was injected through the wax with a hypodermic syringe. A gradual restoration of colour took place showing that there is a chemical reaction; however, the rate was much less than that between oxygen and reduced Nile blue.

The rates of reaction in the presence of living and dead cells of the DHA-reducing strain of *Esch. coli* were next compared. The dye was reduced by a bacterial suspension in an atmosphere of nitrogen

in a Thunberg tube and the cells were killed by heat before adding DHA solution from the stopper. The rate of oxidation of the dye was considerably lower than in unheated controls, but the full intensity of the colour corresponding to complete re-oxidation was not attained (Fig. 1). This was probably because some of the dye became adsorbed on the cells. The large difference between the two rates of oxidation of the leuco-dye is taken as evidence that there is an enzyme-catalysed reaction between DHA and reduced Nile blue in addition to a slower direct reaction.

Quantitative relationship between dye oxidation and DHA reduction. Previous workers (Jebb, 1949) found that oxidation of leuco-Nile blue by a hydrogen-acceptor was followed, after a lapse of time, by reduction. DHA behaved similarly in these tests. With glucose-grown cells the dye remained at its maximum level of oxidation for a period and was then quickly reduced, whereas with lactate-grown cells the dye attained a lower maximum level of oxidation and almost immediately began to be slowly reduced. The experiments were therefore repeated and the oxidation of leuco-Nile blue in Thunberg tubes observed simultaneously with measurements of the quantity of AA produced in duplicate tubes.

Fig. 1. Oxidation of leuco-Nile blue by dehydroascorbic acid. Experiments carried out in Thunberg tubes. Each tube contained 3.2 ml. 0.2M phosphate buffer pH 6.2; 0.1 ml. κ lithium lactate; 1.0 ml. bacterial suspension and 0.2 ml. 0.2% (w/v) Nile blue. Hollow stopper contained 2 mg. dehydroascorbic acid in 0.5 ml. water; temp. 35°. When dye reduced one tube heated at 100° for 2 min. to inactivate bacteria and dehydroascorbic acid then added. ●—●, control (unheated); ○—○, cells heated at 100° for 2 min.

Fig. 2. (a) The effect of *Esch. coli* on oxidation of leuco-Nile blue by dehydroascorbic acid. (b) Reduction of dehydroascorbic acid in presence and absence of leuco-Nile blue. Experimental conditions as for Fig. 1 with lactate as H-donor and ascorbic acid concn. 2.3×10^{-3} M. ○—○, reducing strain, glucose-grown, Nile blue present; ○—○—○, reducing strain, glucose-grown, Nile blue absent; ●—●, reducing strain, lactate-grown, Nile blue present; ●—●—●, reducing strain, lactate-grown, Nile blue absent; x—x, non-reducing strain, glucose-grown, Nile blue present; x—x—x, non-reducing strain, glucose-grown, Nile blue absent.

Cells of the reducing strain, grown on either lactate or glucose, and of the non-reducing strain grown on glucose, were tested and the results, which were reproducible, are shown in Fig. 2. Two features are of interest. The first is that although in the presence of leuco-Nile blue both glucose-grown and lactate-grown cells reduce DHA at the same rate, with the latter, Nile blue so formed becomes reduced almost immediately, whereas with the former it does not become reduced until all the DHA has been reduced. The second is that whereas Nile blue diminishes the rate of reduction of DHA by glucose-grown and lactate-grown cells of the reducing strain it increases the rate by the non-reducing strain. This is presumably due to the chemical reaction between DHA and reduced Nile blue.

Measurements of electrode potential. Green, Stickland & Tarr (1934) emphasized that leuco-Nile blue does not necessarily play a major part in reducing the hydrogen acceptor under examination; but rather its function is to show whether addition of the hydrogen acceptor to the bacterial suspension raises the overall oxidation-reduction potential. However, in the present experiments (see Fig. 2), the 95% 'plateau' of the curve for the glucose-grown reducing strain suggested that the potential attained in this system extends well beyond the range for which Nile blue is useful. For this reason,

and also because Nile blue appeared to be inhibiting the reduction of DHA, the Nile blue was omitted and the oxidation-reduction potential followed electrometrically. The AA produced was measured simultaneously on samples withdrawn from a duplicate system. The results (Fig. 3) show that the maximum E_H reached by the glucose-grown cells after the addition of DHA (+20 mv, an increase of 260 mv) was attained in 5 min., after which it began to fall. With lactate-grown cells, the maximum potential reached was 20 mv lower, i.e. 0 mv (an increase of 150 mv) and was attained in 25 min. As might be expected from the results with Nile blue, the potential with the non-reducing strain rose in a similar fashion to that with lactate-grown reducing strain but, within the duration of the experiment, showed little sign of attaining its maximum value, still less of declining.

The specificity of the reduction. Earlier observations (Mapson & Ingram, 1951) showed that besides DHA, the oxidized forms of D-araboascorbic (D-isoascorbic), hydroxytetrone and reductive acids, are also reducible. These observations have been confirmed and extended by showing that 3-hydroxy-1:2-dioxo-3-phenylbutyrolactone is also reduced.

Because the three last compounds lack carbon atoms 5 and 6 of DHA (see Fig. 4a), the remainder of the molecule has been investigated. In trials with diacetyl and dihydroxytartaric acid (the hydrated form of dioxosuccinic acid) the latter was not reduced; and results with the former were inconclusive because the compound produced by a reduction analogous to that of DHA would be the enol form of acetoin, which would be immediately converted into the keto form. The detection of acetoin, however, involves initial oxidation to diacetyl (Voges-Proskauer reaction).

Specific inhibition of the reduction. As a potential competitive inhibitor, a compound of related chemical structure is needed which is not itself reduced by the system. It is desirable, too, to test it against the substrate at a substrate concentration which does not saturate the system: $3.5 \times 10^{-4} M$ DHA is appropriate, as will appear later (cf. Fig. 8). Ascorbic acid was an obvious possibility, since the products of enzyme action often combine with the enzyme; but the constant rate of reduction of DHA at adequate concentrations (cf. Mapson & Ingram, 1951), and the fact that the equilibrium of the reaction is so greatly in favour of AA that the reaction appears to be irreversible, suggest that AA does not interfere. Observations made to confirm this with higher ratios of AA:DHA were not accurate because the presence of much AA gives a large blank in the titrations which measure the reduction of DHA, but it was quite definite that there was no inhibition with 4 times as much AA as DHA. Dihydroxymaleic acid behaved similarly.

Fig. 3. (a) Effect of dehydroascorbic acid (initial concn. $1.84 \times 10^{-3} M$) on the steady-state oxidation-reduction potential attained by washed suspensions of *Esch. coli* + 0.02 M glucose + 0.14 M phosphate buffer (pH 6.2) under N_2 at 35°. (b) Reduction of DHA under identical conditions. ○—○, reducing strain glucose-grown; ●—●, reducing strain lactate-grown; ×—×, non-reducing strain glucose-grown.

(a) Compounds reduced by *Esch. coli*C atom
no.

Dehydro-L-ascorbic acid

Dehydro-D-arabo-ascorbic acid

Dehydrohydroxy-tetronic acid

Dehydroreductic acid

3-Hydroxy-1:2-dioxo-3-phenyl-butyro- γ -lactone(b) Compounds inhibiting the reduction of DHA by *Esch. coli* L-Galactono- γ -lactone L-Gulono- γ -lactone D-Mannono- γ -lactone(c) Compounds neither reduced, nor inhibiting the reduction of DHA, by *Esch. coli*

L-Ascorbic acid

Dioxo-L-gulonic acid

D-Galacturonic acid methyl ester

Methyl D-ribo-furanoside

γ -Butyrolactone

Dihydroxy-maleic acid

Dioxosuccinic acid

Dihydroxytartaric acid

Glyoxal

Diacetyl

Pyruvic acid

Fig. 4.

These observations were consistent with the view that the critical centre of the molecule is the 1:2-diketo grouping, and that compounds with this grouping might inhibit. The most promising substance seemed to be oxidized reductone (*enol* tartronaldehyde) because this is not reduced by *Esch. coli* (Mapson & Ingram, 1951). Further investigation showed, however, that after oxidation with bromine, reductone could not be regenerated by hydrogen sulphide, and it seemed certain that the oxidized form is completely unstable. Glyoxal, diacetyl, pyruvic acid, 2:3-dioxogulonic acid and dihydroxytartaric acid were also tested and, at 10 times the molarity of the DHA (i.e. at $3.5 \times 10^{-3}M$) none of them diminished the rate of reduction of DHA by *Esch. coli*.

It seemed probable therefore that the ring structure was necessary and, since no further 5-membered ring compounds incorporating diketo groupings were available, we tried the γ -lactones of L-gulonic, L-galactonic and D-mannonic acids (see Fig. 4b). All of these inhibited the reduction of DHA (Fig. 5), in greater or lesser degree, without affecting the rate of methylene blue reduction under comparable conditions. The results of more detailed tests with $1.75 \times 10^{-3}M$; $3.5 \times 10^{-4}M$ and $1.2 \times 10^{-4}M$ DHA, and $3.5 \times 10^{-3}M$ and $7 \times 10^{-3}M$ L-gulono- γ -lactone are shown in Table 1. The activities are expressed as $Q_{H_2}^{DHA}$ ($\mu l. H_2$ at s.t.p. transferred/mg. dry wt. bacteria/hr.). In Fig. 6 the reciprocals of these values are plotted against the reciprocals of the respective DHA concentrations. It is clear that the inhibition is of the competitive type, since the maximum rate of reduction is unaffected but the K_m increases as the inhibitor concentration is raised.

As these compounds differ at carbon atom 5, this confirmed that the grouping there is of secondary importance. The necessity for the ring structure was verified when it was found that the methyl ester of D-galacturonic acid had no inhibitory effect, since

this compound (in the straight chain form which exists in the equilibrium mixture) is otherwise identical with L-galactono- γ -lactone at the four critical carbon atoms.

The inhibition by these γ -lactones shows that the diketo grouping cannot be essential, but since butyrolactone, at a molar concentration found effective for the other inhibitors, had no effect on

Fig. 5. Effect of aldonic acid γ -lactones ($3.5 \times 10^{-3}M$) on the rate of reduction of dehydroascorbic acid (initial concn. $3.5 \times 10^{-4}M$) by washed suspensions of *Esch. coli* using glucose as H-donor at 35° ; with $0.15M$ phosphate buffer pH 6.2. $\times-x$, control, no lactone; $\bullet-\bullet$, D-mannono- γ -lactone; $\blacksquare-\blacksquare$, L-galactono- γ -lactone; $\blacktriangle-\blacktriangle$, L-gulono- γ -lactone.

Fig. 6. Reciprocal plot of the effect of various concentrations of L-gulono- γ -lactone and dehydroascorbic acid on the rate of reduction of the dehydroascorbic acid by washed suspensions of *Esch. coli* + $0.02M$ glucose at 35° ; in $0.15M$ phosphate buffer pH 6.2. $Q_{H_2}^{DHA} = \mu l. H_2$ transferred/mg. dry wt./hr. $\times-x$, no lactone; $\bullet-\bullet$, $3.5 \times 10^{-3}M$ lactone; $\circ-\circ$, $7.0 \times 10^{-3}M$ lactone.

Table 1. Inhibition of the reduction of dehydroascorbic acid (DHA) by L-gulono- γ -lactone

Experiments carried out at 35° under N_2 with $0.02M$ glucose as H-donor in phosphate buffer pH 6.2. Results expressed as $Q_{H_2}^{DHA} = \mu l. H_2$ at s.t.p. transferred/mg. dry wt. bacteria/hr.

Concn. of DHA ($M \times 10^4$)	Concn. of lactone ($M \times 10^3$)	$Q_{H_2}^{DHA}$
17.5	0	45.5
17.5	3.5	34.0
17.5	7.0	25.0
3.5	0	42.0
3.5	3.5	14.5
3.5	7.0	9.0
1.2	0	29.0
1.2	3.5	5.5
1.2	7.0	3.5

the rate of reduction of DHA, it is clear that the nature of the groups on carbon atoms 2 and 3 is an important factor. Further, the carboxylic carbonyl group cannot be replaced by a hydrogen atom and a methoxy group, since methyl D-ribofuranoside did not inhibit.

Effect of hydrogen-acceptor concentration on the rate of reduction. The effect of DHA concentration on the rate of reduction was examined using both glucose-grown and lactate-grown cells of the reducing strain of *Esch. coli*. Similar experiments were made with dehydrohydroxytetronic acid using glucose-grown cells. In the experiments with DHA both lactate and glucose were used as hydrogen donors but only glucose for dehydrohydroxytetronic acid.

Fig. 7 shows the curves obtained in a typical experiment, from which the rates were calculated as $Q_{H_2}^{DHA}$. At higher concentrations of DHA the curves are linear; but at lower concentrations the rate of reaction falls with time, and the correspondingly

Fig. 7. Effect of dehydroascorbic acid concentration on the rate of its reduction by washed suspensions of glucose-grown *Esch. coli* + lactate (0.02M) at 35° and pH 6.2 (0.15M phosphate buffer). ●—●, 1×10^{-3} M DHA; ×—×, 2.5×10^{-4} M DHA; ■—■, 6×10^{-5} M DHA.

small titrations make it difficult to determine the initial rates accurately.

Fig. 8 shows the relations derived from these experiments, fitted Michaelis curves being drawn through the observed points, and Table 2 gives the observed and calculated maximum velocities and the calculated Michaelis constants.

The K_m values for DHA fall clearly into two groups according to the nature of the carbon source on which the cells were grown; the K_m for glucose-grown cells being only about one-tenth of that with cells grown upon lactate. The higher maximum velocities obtained with glucose-grown cells confirmed earlier observations (Eddy, 1952) that cells grown on glucose have much greater DHA-reducing activity than those grown on other sources of carbon.

Fig. 8. Relationship between H-acceptor concentration and rate of reduction by washed suspensions of *Esch. coli*: (a) dehydroascorbic acid, H-donor glucose; (b) dehydroascorbic acid, H-donor lactate; (c) dehydrohydroxytetronic acid, H-donor glucose. Temp. 35°, pH 6.2. $Q_{H_2} = \mu\text{l. H}_2$ transferred/mg. dry wt./hr. Fitted Michaelis curves are drawn through the experimental points. ×—×, cells grown on glucose; ●—●, cells grown on lactate.

Table 2. Observed and calculated maximum velocities and the Michaelis constants (K_m values) for the reduction of dehydroascorbic acid and dehydrohydroxytetronic acid by washed suspensions of *Esch. coli*

Experiments carried out in 0.15M phosphate buffer, pH 6.2 under N_2 ; temp., 35°. $Q_{H_2} = \mu\text{l. H}_2$ at s.t.p. transferred/mg. dry wt./hr.

H-acceptor	C-source in growth medium (0.5%, w/v)	H-donor (0.02M)	Observed maximum velocity Q_{H_2}	Calculated maximum velocity Q_{H_2}	Calculated K_m (M)
DHA	Glucose	Glucose	51	52	3×10^{-5}
DHA	Glucose	Lactate	32	33	5×10^{-5}
DHA	Lactate	Glucose	20	20	3×10^{-4}
DHA	Lactate	Lactate	19	16	3×10^{-4}
Dehydrohydroxytetronic acid	Glucose	Glucose	8.5	10	1.5×10^{-3}

DISCUSSION

Previous workers who have used the dye technique to demonstrate enzymic activation were fortunate in finding that there was no spontaneous reaction between reduced dye and hydrogen acceptor. This has not been the case with DHA which reacts, directly, with leuco-Nile blue. In spite of this difficulty, however, we have been able to show that reducing strains of *Esch. coli* possess a heat-labile enzyme which activates DHA before it is reduced. The potentiometric experiments did not clarify the situation but they confirmed what had been noted with Nile blue; namely that the effect of DHA on the oxidation-reduction potentials of bacterial suspensions was more rapid and greater with the glucose-grown reducing strain than with the lactate-grown reducing strain or with the glucose-grown non-reducing strain.

Although the activation is evidently not absolutely specific to DHA, the specificity is nevertheless close. The groups at carbon atoms 5 and 6 affect only the rate of the reaction. Compounds with a double bond between carbon atoms 2 and 3, such as ascorbic acid, apparently do not interfere. Hence, the specific structure would appear to be

That the γ -lactones of L-gulonic, L-galactonic and D-mannonic acids inhibit the reduction suggests that the diketo grouping is not essential, but that a dihydroxy grouping is effective, and this makes it seem likely that the structure which combines with the enzyme is in fact not that of DHA but of its hydrated form:

This might indeed be expected on general chemical grounds. Though all the possibilities have not been covered, owing to lack of the requisite compounds, it is clear that at least two of the four hydroxyl

groups can be replaced by hydrogens, but not all of them; and that the ---O--- bridge of the ring can be replaced by $\text{---CH}_2\text{---}$.

Typical Michaelis-Menten relations held for cells grown on different sources of carbon, and supplied with various hydrogen-donating substrates. With lactate-grown cells the maximum rate of reduction of DHA is lower, and the Michaelis constant is higher, than with glucose-grown cells. Hence it seems probable that both the total activity of the enzyme and its affinity for its substrate are affected by the source of carbon used for growth of the bacteria. It is noteworthy that the nature of the substrate subsequently offered to the cells as the primary hydrogen donor is much less important than the carbon source during growth, since the change from glucose to lactate as substrate only produced changes in the Michaelis constant which appeared to be within the experimental error. This might be expected if, as we suppose, the various substrate-dehydrogenase systems reduce cytochrome b_1 which is their common intermediate hydrogen carrier for the DHA-reducing system; while the different maximum rates of DHA reduction, with different substrates, may be a reflexion of the relative ease with which the different dehydrogenase systems are able to reduce cytochrome b_1 .

Although the enzyme has not been prepared in a cell-free state, the typical specificity and inhibition relationships, the normal Michaelis-Menten kinetics, and the less critical dye-reduction and potentiometric experiments all indicate that, before it can be reduced by bacteria, DHA must be enzymically activated. For the activating enzyme the name 'dehydroascorbase' seems appropriate, with the reservations about function mentioned below: it emphasizes the analogy with nitratase and tetrathionase, and the alternative dehydroascorbic acid reductase is already in use for a plant enzyme which catalyses the reaction between DHA and reduced glutathione and which is obviously different from the bacterial enzyme. Cytochrome b_1 , which is reduced by various dehydrogenases, is an intermediary in the reduction of DHA, but the possibility that there may be additional intermediates is not excluded. In the presence of oxygen as an alternative hydrogen acceptor, the system does not operate unless cyanide is added (Stewart & Sharp, 1945). There is an obvious parallel here to the reduction of nitrate, tetrathionate and trimethylamine oxide, especially as cytochrome b_1 may be implicated in nitrate reduction (Sato & Egami, 1949) but the DHA enzyme is not sensitive to cyanide or azide, nor is it adaptive. The above conclusions apply, strictly speaking, only to *Esch. coli*, but we may assume that other DHA-reducing organisms behave similarly. Further, although the

ability to reduce DHA is restricted to the genus *Staphylococcus* and the family Enterobacteriaceae, apparently because of the limited distribution of cytochrome b_1 , there is no other evidence that the systematic distribution of 'dehydroascorbase' is similarly restricted.

On this basis, it seems odd that we should have been able to distinguish so plainly between reducing and non-reducing strains, because there is no apparent reason why strains with intermediate activities of enzyme should not exist, but we have in fact not found any, although a few of our 'non-reducing' strains did reduce DHA very slowly. Nor is it clear why bacteria should possess such an enzyme, since it seems unlikely that DHA would be an important metabolite. Of course, the reduction of DHA might conceivably be the fortuitous activity of an enzyme with a totally different function *in vivo*, but experiments with a variety of substances have not produced any evidence that this is so; also, we have failed to correlate the distribution of ability to reduce DHA, between reducing and non-reducing strains, with any other biochemical activity. The natural significance of the property therefore remains obscure.

SUMMARY

1. There is a slow chemical reaction between dehydroascorbic acid and reduced Nile blue. This reaction is catalysed by washed cells of some strains of *Esch. coli*.

2. The effect of dehydroascorbic acid on the oxidation-reduction potential of bacterial suspensions was more rapid and greater with a glucose-grown reducing strain than with a lactate-grown reducing strain or a glucose-grown non-reducing strain. With heated cells the changes were greatly retarded.

3. Dehydro-D-araboascorbic, dehydrohydroxy-tetronic and dehydroreductic acids and 3-hydroxy-1:2-dioxo-3-phenylbutyro- γ -lactone are reduced by a strain of *Esch. coli* which can reduce dehydroascorbic acid.

4. The reduction of dehydroascorbic acid by *Esch. coli* is inhibited competitively by the γ -lactones of L-gulonic, L-galactonic and D-mannonic acids; but not by ascorbic acid, dihydroxymaleic

acid, dioxogulonic acid, butyrolactone, D-galacturonic acid methyl ester or methyl D-ribofuranoside. The specific grouping is apparently

where X or X' and Y or Y' must be hydroxyl groups and Z can be either ---O--- or $\text{---CH}_2\text{---}$.

5. Michaelis-Menten relationships hold for both dehydroascorbic acid and dehydrohydroxytetronic acid. When the cells are grown on glucose, the maximum velocity is greater and the K_m is lower, than with lactate.

6. These observations suggest that there is a specific thermolabile enzyme which activates DHA (and related substances) before reduction and for which the name 'dehydroascorbase' is proposed.

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