5-nucleotidase activity in such human tissues as the thyroid gland, testicle or lungs. In the present experiments 5-nucleotidase was always very abundant in these tissues. The failure of Newman *et al.* may be explained by inactivation during histological fixation, and, which seems more important, by the fact that their work was carried out at pH 9.2, whereas 5-nucleotidase is optimally active near pH 7.8.

The physiological role of 5-nucleotidase is not known at present, but the existence of such an enzyme, specific for a substance so important and widely distributed as adenylic acid, seems to indicate a mechanism able to regulate the concentration of both phosphate and adenylic acid, which are so important for glycogenesis.

Since Robison's (1932) work it has been generally considered probable that the alkaline phosphatase plays a part in calcification of bones. As the 5-nucleotidase acts optimally near the physiological pH, where the alkaline phosphatase has a very low activity, it seems possible also that 5-nucleotidase may play a role in calcifications. It is of interest that certain tissues, e.g. aorta wall or thyroid gland, which are apt to undergo pathological calcifications, contain this enzyme, but not the alkaline phosphatase.

SUMMARY

1. At the physiological pH in all human tissues examined, with the exception of intestinal mucosa, the activity of the specific phosphatase, 5-nucleotidase, is much more pronounced than the activity of the non-specific alkaline phosphatase. This includes the tissues notably rich in alkaline phosphatase, e.g. ossifying cartilage, choroid plexus and kidney cortex.

2. The optimal pH for 5-nucleotidase from human tissues is pH 7.8.

3. The highest 5-nucleotidase activity in human tissues was found in the posterior lobe of the pituitary.

4. In adults the highest alkaline phosphatase activity (pH 9) was found in choroid plexus. The alkaline phosphatase activity of kidney cortex and intestinal mucosa was comparatively much lower.

5. The wide distribution of 5-nucleotidase seems to indicate a mechanism regulating phosphate and adenylic acid concentrations, which may perhaps play a part in the calcification of tissues.

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Observations on the Use of *Escherichia coli* for the Reduction and Estimation of Dehydroascorbic Acid

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Gunsalus & Hand (1941), working with suspensions of *Escherichia coli*, reported that the bacteria were capable of reducing dehydroascorbic acid to ascorbic acid in milk, wine and fruit juices, but seemed incapable of effecting the reduction with vegetable extracts. In these latter juices the *Esch. coli* acted as an oxidation catalyst and oxidized the ascorbic acid present.

Stewart & Sharp (1945), working with suspensions of *Esch. coli* and *Staphylococcus albus* and with a wide range of animal and plant extracts, were able to obtain a rapid and quantitative reduction of dehydroascorbic acid in such materials. Only after the complete removal of oxygen from these solutions, or under aerobic conditions if sodium cyanide was added, was there any reduction of dehydroascorbic

acid. They noted that, with the vegetable extracts in which the former workers had been unable to effect the reduction, complete reduction of dehydroascorbic acid to ascorbic acid occurred, provided that the ascorbic acid was extracted and titrated against indophenol dye at a pH not below 4.5. If the extract, prior to titration, was acidified to a pH of 3.5 or below, a rapid oxidation of ascorbic acid occurred. When, however, sodium cyanide was present in a concentration of 0.001M the phenomenon did not occur and the dehydroascorbic acid was rapidly reduced to ascorbic acid which, under these conditions, could be titrated at pH values below 3.5 without oxidation. They attributed this behaviour to the fact that *Esch. coli* forms a compound or complex when incubated with vegetable juices which can oxidize ascorbic acid in acid solution below pH 3.5.

Being interested in alternative methods of estimating dehydroascorbic acid in plant extracts, we have re-examined the results of using bacterial suspensions for this purpose and have reported them below.

METHODS AND REAGENTS

Preparation of bacterial suspensions. Suspensions of *Esch. coli* were prepared by growing the organisms in Roux bottles on 2% digest agar containing 0.5% glucose for 18 hr. at 37°. The culture was harvested and washed twice with Ringer solutions diluted 1 in 4 and prepared from glass-distilled water. After the final washing, the culture was resuspended in the diluted Ringer solution to give a concentration of about 10^{10} or 10^{11} organisms/ml. Brown's opacity tubes were used for this standardization. No culture more than 18 hr. old was used since cultures older than this had lower enzyme activity. The activities of these suspensions were found to be constant over long periods, but, in practice, no suspension more than 7 days old was used in these experiments.

Bacterial suspensions, in which the viable organisms were destroyed by heat treatment at 60° for 1 hr., had lost the whole of their activity.

Estimation of ascorbic acid. Ascorbic acid was estimated by titration against standardized indophenol dye after acidification and arrest of the reaction by the addition of HPO_3 to a concentration of 4%.

Dehydroascorbic acid was prepared by dissolving a known amount of pure crystalline L-ascorbic acid in glass-distilled water, adding 1 drop of bromine, and removing the excess bromine rapidly by vigorous aeration. To avoid loss of dehydroascorbic acid this process was carried out as rapidly as possible and was completed within a period of 5 min. from the time of adding the bromine.

Buffers. For the estimation of ascorbic acid in biological material it is usual to extract the fresh tissue with metaphosphoric acid. In such an extract the ascorbic acid may be estimated by direct titration, and a further sample of the solution taken for estimation of total ascorbic acid (ascorbic acid + dehydroascorbic acid). For this reason in most of the experiments reported in this paper we have studied the reduction of dehydroascorbic acid by *Esch. coli* in a 2% (w/v) HPO_3 solution, buffered to pH 6.2 by the

addition of NaOH. In some experiments a phosphate-citric acid buffer, 0.2M, was employed. All buffer solutions were made up with glass-distilled water unless otherwise stated.

Temperature. These experiments were performed at 35°, like those of Stewart & Sharp (1945).

Amidosulphonic acid ($\text{NH}_2\text{SO}_3\text{H}$) solutions were freshly prepared from the pure dry substance.

Nitrite was estimated colorimetrically by means of the Griess-Ilosvay reagent, in a photoelectric colorimeter. Nitrate was detected qualitatively by means of the diphenylamine reagent.

Determination of the rate of the reaction. (a) *With pure dehydroascorbic acid.* To 50 ml. of buffer solution, 2 ml. of a glucose solution, 25% (w/v) was added. The reaction flask was placed in a water bath at a temperature of 35° and a rapid stream of N_2 passed. After 10 min., a suitable amount of bacterial suspension was added (in most experiments 4 ml.) and, finally, a measured amount of dehydroascorbic acid, ascorbic acid or other substance under test was added. The rate of reaction was followed by removing samples at suitable intervals. The reaction was arrested by the addition of 5 ml. of 5% (w/v) HPO_3 to 5 ml. of reaction mixture and the reducing titre of the solution determined by titration against standard indophenol dye. The pH of the solution was approx. 2.0. (b) *With potato extract.* The plant material was extracted in a Waring blender with 2% HPO_3 , filtered and a 50 ml. portion neutralized to pH 6.2–6.3 with K_2HPO_4 , 40% (w/v). It was at once freed of O_2 by passing a rapid stream of N_2 through it for 5 min., the bacterial suspension was added, the reaction flask placed in the bath at 35° and the reaction followed by withdrawing samples at suitable intervals. The reaction was arrested in the usual way by the addition of HPO_3 .

EXPERIMENTAL

Reduction of dehydroascorbic acid

We have confirmed the results of Stewart & Sharp (1945) that *Esch. coli* rapidly reduces dehydroascorbic acid under anaerobic conditions at pH 6.2 and at a temperature of 35°. Unlike them, however, we have not been able to obtain a quantitative recovery of added dehydroascorbic acid as ascorbic acid. We have found that the percentage of dehydroascorbic acid recovered depends on at least two factors: (1) the concentration of dehydroascorbic acid added, and (2) the enzymic activity of the bacterial suspension. This is illustrated in Figs. 1a and 1b. For Fig. 1a the concentration of dehydroascorbic acid was varied and the quantity of bacterial suspension kept constant, while for Fig. 1b the concentration of dehydroascorbic acid was constant and the enzymic activities were varied by altering the quantity of bacterial suspension added. The key to these results lies in the different periods of time taken for the reaction to be completed. Whether the time is prolonged by increasing the concentration of dehydroascorbic acid or by reducing that of the bacterial suspension, the percentage of dehydroascorbic acid recovered as ascorbic acid falls progressively. This is clearly illustrated in Fig. 2.

The explanation appears to be that dehydroascorbic acid is unstable and is converted to 2,3-diketogulonic acid by the opening of the lactone

reduce this correction to the minimum, highly active bacterial suspensions should be employed so as to give reaction times of short duration.

Fig. 1. (a) The effect of variation of dehydroascorbic acid (DHA) concentration on the percentage recovered as ascorbic acid (AA) using the same concentration of *Esch. coli*. ●—●, DHA 0.04 mg./ml.; ×—×, DHA 0.08 mg./ml.; ▲—▲, DHA 0.16 mg./ml.; ○—○, DHA 0.32 mg./ml. (b) The effect of altering the concentration of *Esch. coli* on the percentage DHA recovered as AA. ●—●, 4 ml. bacterial suspension in 50 ml. reaction mixture; ×—×, 2 ml. bacterial suspension in 50 ml. reaction mixture; ▲—▲, 1 ml. bacterial suspension in 50 ml. reaction mixture; ○—○, 0.5 ml. bacterial suspension in 50 ml. reaction mixture; DHA 0.08 mg./ml. reaction mixture.

ring at a rate governed by pH and temperature. The reaction leads to irreversible destruction of dehydroascorbic acid. This explains that our failure to obtain a quantitative recovery of dehydroascorbic acid, as ascorbic acid, is because the rate of destruction of dehydroascorbic acid, as determined chemically, corresponds with the decrease in the percentage of dehydroascorbic acid recovered with bacteria in a series of tests in which the time taken for completing the reaction was varied (Fig. 2). In these experiments the disappearance of dehydroascorbic acid was followed chemically by determining the ascorbic acid regenerated after treatment with hydrogen sulphide, after suitable intervals of time, under the same conditions of pH and temperature as those used for the study of the reduction with the bacteria.

Penney & Zilva (1943), experimenting under very similar conditions, observed a conversion of approximately 39 % of dehydroascorbic acid to diketogulonic acid in 40 min., as compared with approximately 30 % found by us within the same time (Fig. 2). Since, under the conditions of our experiments, the rate of loss of dehydroascorbic acid over a period of 40 min. was approximately constant, it is possible to correct for it. The procedure would then be to follow the course of the reduction by the bacteria, determining the time taken for completion of the reaction, and then to apply a simple correction to give the total dehydroascorbic acid originally present. To

Fig. 2. The DHA (%) recovered as AA as affected by the time taken for the reaction to be completed compared with the rate of destruction of DHA determined chemically (H_2S reduction). ○—○, values obtained by variation of DHA concn.; ×—×, values obtained by variation of *Esch. coli* concn. (abscissae represent time in min. for the bacterial reaction to be completed); ●—●, disappearance of DHA as determined by reduction with H_2S .

The reason for the slight discrepancy between our results and those of Stewart & Sharp (1945), who reported quantitative recoveries of added dehydro-

ascorbic acid, is not clear. Gunsalus & Hand (1941) found that quantitative recovery was obtained only if the reaction was complete within 15 min. These latter workers prepared dehydroascorbic acid either by iodine oxidation or by enzymic oxidation with cucumber juice, whilst Stewart & Sharp prepared it by oxidation with cucumber extract. Gunsalus & Hand reported a better recovery with iodine than with ascorbic oxidase, and ascribed this to the fact that the longer time needed for the oxidation of ascorbic acid with the enzyme allowed more dehydroascorbic acid to be destroyed. It seemed possible that the difference in the method of preparing dehydroascorbic acid (we oxidized with bromine), might have been the cause of our failure to obtain quantitative recovery of dehydroascorbic acid. Experiments in which dehydroascorbic acid was prepared by oxidation with iodine, however, yielded similar results to those in which bromine was used; a result in accord with those of Penney & Zilva who found the rate of conversion of dehydroascorbic acid to diketogulonic acid to be the same whether iodine or chlorine was used as the oxidizing agent.

Neither of the groups of American workers give data showing the extent of the decrease in recovery of dehydroascorbic acid with longer reaction times, although both state that destruction of dehydroascorbic acid occurs under such circumstances. It is possible, however, that in their experiments the rate of destruction of dehydroascorbic acid was, for some unknown reason, slightly slower than in ours, and that the small percentage loss of dehydroascorbic acid that occurred in 15 min. was within the experimental error of their method of estimation.

Reduction of dienols other than ascorbic acid

Stewart & Sharp examined the reduction by *Esch. coli* of the oxidized forms of other non-specific reductants of indophenol dye. Glucose solutions previously treated with acid or alkali, sterilized evaporated milk, and canned fruit and vegetable juices were examined. No reduction of substances other than dehydroascorbic acid was observed in these products. They did, however, find that *D*-isodehydroascorbic acid was reduced at a slower rate than *L*-dehydroascorbic acid.

We have determined the rate of reduction of a number of known dehydroenediol compounds. These included the oxidized forms of *D*-isoascorbic acid, hydroxytetrone and reductic acids as well as reductone and reductone-like substances produced in an alkali-treated glucose solution.

The results with *D*-isoascorbic acid, reductic and hydroxytetrone acids are shown in Fig. 3*a*. We found *D*-isodehydroascorbic acid to be reduced at a slower rate than dehydroascorbic acid, though somewhat faster than the rate given by Stewart & Sharp. The oxidized forms of both hydroxytetrone

and reductic acid were reduced much more slowly than dehydroascorbic acid. However, if these substances were present with ascorbic acid some interference would result. Thus, in the minimum time for complete reduction of dehydroascorbic acid the percentage reduction of an equivalent concentration of the oxidized forms of reductic and hydroxytetrone acids would be 28 and 9 respectively. The estimation of dehydroascorbic acid by means of *Esch. coli* must, therefore, be used with discretion if substances of this type are known to be present.

Fig. 3. The rate of reduction by *Esch. coli* of the oxidized forms of certain enediols compared with DHA. *a*, x-x-x, *L*-ascorbic acid; o-o-o, *D*-isoascorbic acid; ▲-▲-▲, reductic acid; ●-●-●, hydroxytetrone acid. *b*, x-x-x, *L*-ascorbic acid; +-+, reductone (enoltartronaldehyde); ■-■-■, glucoreductone solution.

When pure reductone (enol of tartronaldehyde) was incubated over a period of 60 min. with *Esch. coli* no reduction of the oxidized form occurred.

Using a solution of glucoreductone prepared by treatment of glucose with alkali (Harris & Mapson, 1947) (this contains reductone and unidentified reducing substances closely akin to the indophenol reductants (cf. Mapson, 1943) produced in dried foods during processing or storage), some regeneration of the reducing power was effected by *Esch. coli* (Fig. 3*b*). Regeneration of 36% of the original indophenol titre occurred within 40 min., suggesting that one of the reductants in such a solution was reduced by *Esch. coli*, whereas the others were not. This confirms the result obtained by Mapson (1943) who showed that a glucoreductone solution, after oxidation by iodine and reduction by hydrogen sulphide, behaved as a mixture of reductants, one of which was reduced by hydrogen sulphide more rapidly than the others, and was apparently present to the extent of 35% of the total indophenol reducing titre. The present experiments with *Esch. coli* lead to the same conclusion. Whereas, however,

prolonged treatment with hydrogen sulphide completely restored the whole reducing power of the solution, with *Esch. coli* one only of these reductants was reduced.

A series of experiments was carried out in which each of the constituents of the tap water was added to the glass-distilled water buffer mixture separately. These included calcium and magnesium salts, iron

Fig. 4. *a*, the influence of a constituent of tap water on the oxidation of AA by *Esch. coli*. ○—○, 2% HPO₃ buffered to pH 6.2 with NaOH made with glass-distilled water; △—△, 2% HPO₃ buffered to pH 6.2 with NaOH made with tap water. AA estimated by titration in acid solution. *b*, the oxidation of AA in acid solution caused by the action of *Esch. coli* on some constituent of tap water. x—x, AA estimated by titrations in neutral solutions; ●—●, AA estimated by titrations in acid solution, pH 2.0.

The bacterial reduction method is thus, under these conditions, not as specific for dehydroascorbic acid as was claimed by Stewart & Sharp. It is, however, true that if any of these substances were present in the form of the dehydro derivatives, they would interfere less than in the corresponding chemical method of reduction by hydrogen sulphide.

Interference due to nitrite

When pure ascorbic acid is added to a phosphate buffer solution of pH 6.2, made with glass-distilled water and incubated with or without the bacterial suspension at a temperature of 35° under anaerobic conditions, there is no apparent oxidation of the ascorbic acid when samples are mixed with metaphosphoric acid prior to titration. When the buffer solutions were made with ordinary tap water and the above procedure followed, there was at first an apparent rapid oxidation of ascorbic acid, followed by a rapid regeneration of all of the ascorbic acid, and this despite the fact that dehydroascorbic acid seemed to have been produced in relatively large amounts as indicated by decrease in dye-reducing power (Fig. 4*a*).

This oxidation of ascorbic acid was however only apparent and was caused by the interaction of some constituent in the reaction mixture with ascorbic acid in acid solution, because when the titrations were carried out in neutral solution there was no evidence of any such oxidation (Fig. 4*b*).

and copper salts, and carbonates and sulphates. None of these had any effect, but when nitrate in a concentration of 0.001 M was added, the resultant effect was similar to that observed with tap water (Fig. 5).

Fig. 5. The oxidation of AA in acid solution produced by a substance formed by the interaction of *Esch. coli* and nitrate. ●—●, 0.01 M-NaNO₃; ○—○, 0.001 M-NaNO₃; x—x, 0.0005 M-NaNO₃.

That nitrate itself was not the oxidizing agent was shown by the fact that, in acid solution, nitrate does not oxidize ascorbic acid; but the well known fact that *Esch. coli* possesses nitratase (Stickland, 1936),

an enzyme causing the reduction of NO_3^- to NO_2^- , led us to examine whether the production of this substance by *Esch. coli* could be the cause. It can easily be shown that while nitrite will not oxidize ascorbic acid, nitrous acid oxidizes ascorbic acid very rapidly.

We therefore followed the apparent oxidation of ascorbic acid by *Esch. coli* in the presence of added nitrate and examined, in a parallel experiment, the production of nitrite. The results are illustrated graphically in Fig. 6. They show clearly that the apparent loss of ascorbic acid can be attributed quantitatively to the rapid production of nitrite from the nitrate under the anaerobic conditions, and that the loss of ascorbic acid occurs by oxidation

Fig. 6. Correlation between the production of nitrite by *Esch. coli* and the oxidation of ascorbic acid in acid solution. ●—●, ascorbic acid estimated by titration in acid solution, pH 2.0; ▲—▲, ascorbic acid estimated by titration in neutral solution; x—x, production of nitrite. Reaction mixture contained ascorbic acid 0.080 mg./ml. with 0.001 M- NaNO_3 in phosphate buffer pH 6.2.

when the solution containing nitrite is acidified. After the disappearance of the nitrite by the continued activity of the bacteria, there is no further loss of ascorbic acid on acidification of the solution. Moreover, as Fig. 6 shows, there is no oxidation of ascorbic acid if the titrations are carried out in neutral solution.

Similar results to these were obtained with extracts made from fresh potato tissue. Fresh tissue was extracted with 2% metaphosphoric acid in glass-distilled water in a modified Waring blender, a description of which is given by Barker & Mapson (1950). The extract was filtered to remove starch and protein and adjusted to pH 6.2 with sodium hydroxide. When this extract was treated with *Esch. coli* suspension under anaerobic conditions, and samples removed into metaphosphoric acid

prior to titration, there was first a rapid fall in the ascorbic acid followed by a regeneration of ascorbic acid within a period of 1 hr. The fall in ascorbic acid was, as in the previous experiments, correlated with the production of nitrite from the nitrate present in the extract, and the rise again in the indophenol titre was due to the disappearance of nitrite. No oxidation of ascorbic acid occurred if the extract was titrated against indophenol dye in neutral solution.

Experiments with dehydroascorbic acid

If similar experiments to those described in the previous section are carried out with dehydroascorbic acid instead of ascorbic acid, the production of

Fig. 7. The reduction of dehydroascorbic acid by *Esch. coli* in the presence and absence of nitrate. ▲—▲, 0.001 M- NaNO_3 , AA estimated by titration in acid solution pH 2.0; ○—○, 0.001 M- NaNO_3 , AA estimated by titration in neutral solution; x—x, no nitrate, AA estimated by titration in acid solution, pH 2.0; ●—●, no nitrate, AA estimated by titration in neutral solution.

nitrite delays the reduction of the dehydroascorbic acid until all the nitrite has disappeared. The results, illustrated in Fig. 7, show that the delay is not real but is an artifact, caused by the oxidation of the ascorbic acid by the nitrous acid formed when the solution is acidified. Only when nitrate is present and when the ascorbic acid formed is estimated by titration in acid solution is there any apparent delay in the reduction of the dehydroascorbic acid. In the absence of nitrate, the same result is obtained whether the titrations are carried out in acid or in neutral solution.

Elimination of interference due to nitrite

(a) *Titration in neutral solution.* The simplest way to eliminate interference due to nitrite when estimating ascorbic acid in pure solution is to carry out

the titration in neutral solution. However, when dealing with extracts of biological material, this procedure is not to be recommended for the indophenol method of assay is less specific for ascorbic acid at neutrality and the end point of the titration is much more difficult to determine. Moreover, as pointed out by Stewart & Sharp, if the titration is to be carried out in neutral solution, the reducing action of the bacteria on indophenol dye must be prevented by some means. This can only be done by heat treatment or by the addition of non-acid poisons. Such procedures are more likely to lead to oxidation of ascorbic acid than procedures in which the bacteria are destroyed by the addition of acids such as metaphosphoric which eliminate bacterial activity and at the same time stabilize the ascorbic acid. For these reasons we have investigated other means whereby the interference due to nitrous acid may be removed.

(b) *The use of substances reacting with nitrous acid.* Ascorbic acid may be protected from the oxidative effect of nitrous acid by the presence in the solution of substances which react with the latter. The data in Table 1 show the protective effect on ascorbic acid

Table 1. Protection of ascorbic acid from oxidation by nitrous acid by urea and amidosulphonic acid

Exp. no.	Solution tested	Ascorbic acid by indophenol titration (mg./ml.)
1	Ascorbic acid in 4% HPO_3	0.063
	Ascorbic acid + 0.001 M-NaNO ₂ in 4% HPO_3	0
	Ascorbic acid + 0.001 M-NaNO ₂ + urea 20% (w/v)	0.051
2	Ascorbic acid in 4% HPO_3	0.078
	Ascorbic acid + 0.001 M-NaNO ₂ in 4% HPO_3	0
	Ascorbic acid + amidosulphonic acid 1% in 4% HPO_3	0.078
	Ascorbic acid + 0.001 M-NaNO ₂ + amidosulphonic acid 1% in 4% HPO_3	0.078
3	Ascorbic acid in 4% HPO_3	0.087
	Ascorbic acid + 0.001 M-NaNO ₂ in 4% HPO_3	0.005
	Ascorbic acid + amidosulphonic acid 2% in 4% HPO_3	0.087
	Ascorbic acid + 0.001 M-NaNO ₂ + amidosulphonic acid 2% in 4% HPO_3	0.087
4	Potato extract in 2% HPO_3	0.0250
	Potato extract + amidosulphonic acid 1% in 2% HPO_3	0.0255
	Potato extract + 0.001 M-NaNO ₂ + 1% amidosulphonic acid in 2% HPO_3	0.0252

of urea and the more highly reactive amidosulphonic acid, $\text{NH}_2\text{SO}_3\text{H}$ (cf. Brooks & Pace, 1940). Urea in a concentration of 20% (w/v) in metaphosphoric

acid only partially protects ascorbic acid from a concentration of nitrous acid of 0.001 M, but amidosulphonic acid in a concentration of 1% (w/v) gives complete protection, and does not itself reduce indophenol dye. Amidosulphonic acid may therefore be added to metaphosphoric acid to eliminate the induced oxidation of ascorbic acid by nitrous acid.

(c) *The use of substances inhibiting the nitrataze enzyme of Esch. coli.* Confirmation that our interpretation of the observations made in the preceding section of this paper was correct was obtained in the following experiments. Stickland showed that the enzyme, nitrataze, is sensitive to cyanide, since the reduction of nitrate to nitrite by *Esch. coli* is suppressed in the presence of 0.001 M-potassium cyanide. In an experiment, the results of which are shown in Fig. 8 in which both nitrate and cyanide were present,

Fig. 8. The oxidation of ascorbic acid and reduction of dehydroascorbic acid by *Esch. coli* in the presence of 0.001 M-NaNO₃ and 0.001 M-KCN. ●—●, AA added originally; x—x, DHA added originally. All estimations carried out in acid solution, pH 2.0.

there was no induced oxidation of ascorbic acid on acidification of the solution and the reduction of dehydroascorbic acid proceeded rapidly. In parallel experiments, in which cyanide and nitrate but no ascorbic acid were added, direct tests showed that the production of nitrite was completely suppressed.

One feature of these experiments is worthy of note; that, despite a rapid reduction of dehydroascorbic acid, the final percentage recovered was lower than in those experiments in which cyanide was absent, Fig. 9. Herein lies the chief objection to the use of cyanide. We have shown by the chemical hydrogensulphide method that, under the conditions of these experiments (pH 6.2, 35°), the addition of

0.001 M-cyanide, a concentration necessary to inhibit the production of nitrite by the bacteria, accelerates the disappearance of dehydroascorbic acid by converting it into 2:3-diketogulonic acid, and it is presumably to this effect that the lowered percentage recoveries of dehydroascorbic acid are due. Because of this, the use of cyanide is to be avoided.

Fig. 9. Percentage recovery of dehydroascorbic acid as ascorbic acid by reduction by *Esch. coli* in presence and absence of KCN (0.001M) with glucose 1% (w/v) as substrate. ○—○, no KCN; ×—×, 0.001M-KCN.

Fig. 10. Reduction of dehydroascorbic acid by *Esch. coli* in the presence of 0.001M- NaNO_3 and 0.001M- NaN_3 compared with the reduction in absence of nitrate or azide. ×—×, nitrate + azide; ●—●, control. All estimations of AA in acid solution, pH 2.0.

In our search for an alternative inhibitor, we found that azide, in a concentration of 0.001 M, will, like cyanide, effectively inhibit the conversion of nitrate to nitrite by *Esch. coli*, without affecting the

enzymic systems associated with the reduction of dehydroascorbic acid. Moreover, it possesses the advantage over cyanide that it does not affect the rate of conversion of dehydroascorbic acid to 2:3-diketogulonic acid. The result of an experiment illustrated in Fig. 10 shows the same rapid reduction of dehydroascorbic acid and the same percentage recovery of dehydroascorbic acid, as ascorbic acid, with azide in the presence of added nitrate, as in the control solution in which neither azide nor nitrate were present. Further, in other experiments in which ascorbic acid was added with azide and nitrate, there was no oxidation of the ascorbic acid on acidification of the solutions, showing that no nitrite had been produced.

The use of the method with plant extracts

Our experience of the method of estimating dehydroascorbic acid in plant tissues has so far been confined to potato. Good recoveries of dehydroascorbic acid added in known amounts to extracts of this tissue have been obtained. These results will be reported in a forthcoming paper. One possible source of loss of dehydroascorbic acid has, however, been noted. After extraction of the tissue with metaphosphoric acid, dehydroascorbic acid may be lost during the neutralization of the extract to pH 6.2 preparatory to adding the bacterial suspension. Such a loss invariably occurs when the extract is neutralized with sodium hydroxide. This is due to the practical difficulty of preventing parts of the solution from becoming temporarily alkaline, with consequent rapid destruction of dehydroascorbic acid. Losses as high as 40% have been observed from this cause. Such losses may, however, be prevented if the metaphosphoric acid extract is neutralized with a solution of potassium hydrogen phosphate (40%, w/v) added slowly, with constant agitation.

SUMMARY

1. The reduction of dehydroascorbic acid by *Escherichia coli* has been reinvestigated. Complete recovery of dehydroascorbic acid as ascorbic acid was not obtained owing to small concurrent loss of dehydroascorbic acid by conversion to diketogulonic acid. Loss due to this cause can, however, be calculated and allowed for and dehydroascorbic acid may accordingly be estimated quantitatively by this method.

2. The difficulty associated with this method of estimating dehydroascorbic acid in vegetable extracts, reported by earlier workers, has been shown to be due to the reduction of nitrate to nitrite by *Escherichia coli* under anaerobic conditions. On acidification, the nitrous acid formed rapidly oxidizes ascorbic acid.

3. Interference in the estimation of ascorbic acid by nitrous acid may be overcome by the use of amido-sulphonic acid, which reacts rapidly with nitrous acid and so protects ascorbic acid from oxidation.

4. The nitratase enzyme of *Escherichia coli* may be completely inhibited by potassium cyanide or sodium azide, without affecting the enzyme systems associated with the reduction of dehydroascorbic acid.

5. The presence of potassium cyanide in a concentration of 0.001 M accelerates the conversion of dehydroascorbic acid to diketogulonic acid, but sodium azide in a similar concentration has no effect. Sodium azide may, therefore, be used to prevent the formation of nitrite by *Escherichia coli* without any disadvantageous effect on the reduction of dehydroascorbic acid to ascorbic acid.

6. The rate of reduction of the oxidized forms of other enediols has been studied. These include D-isoascorbic acid, hydroxytetronic acid, reductic acid, reductone (enol of tartronalddehyde) and the reductants present in a glucoreductone solution. All these substances with the exception of reductone were reduced, but at a slower rate than dehydroascorbic acid.

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The Stimulant Involved in the Germination of *Orobancha minor* Sm.

1. ASSAY TECHNIQUE AND BULK PREPARATION OF THE STIMULANT

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Among the parasitic plants, the subfamily Rhinanthoideae and the closely related Orobanchaceae are unusual in that all the species of these groups are root parasites. Those of the former group have green leaves and are only partially dependent upon other plants, but the Orobanchaceae are complete parasites which lack chlorophyll and have a much reduced and modified structure. They consist of little more than an erect flowering shoot above the ground and they produce many thousands of minute seeds, each no more than about 0.3×0.2 mm. in external dimensions. The base of the shoot is attached below ground to the root system of the host plant.

A number of species of *Orobancha* have considerable economic importance in that they damage a variety of crops, particularly in Mediterranean climates. Even in this country, *O. minor*, the lesser broomrape, may cause partial or complete failure of clover. The seeds of many species of these parasites

normally germinate only when they are in the immediate vicinity of a host root. According to Lindley (1853) this condition was first demonstrated by Vaucher with *Orobancha*. The phenomenon was subsequently investigated by Koch (1887) again with *Orobancha*, by Heinricher (1898) with *Lathraea* and by Pearson (1912) with *Striga*.

It has been shown that the influence of the growing root is due to a chemical stimulant which is released from it and which is required by the seed in germination. Saunders (1933) induced germination in *Striga lutea* seeds by irrigating them with water that had percolated through sand containing growing maize roots, and Barcinsky (1934) and Chabrolin (1934) reported similar results with *Orobancha cumana* and *O. speciosa* respectively. It was further shown by these two workers that a dry residue could be obtained from aqueous extracts of roots and other parts of host plants which, when redissolved in water, promoted the germination of the parasite seed.