SUMMARY

1. A modified method for the preparation of phosphorylase by adsorption on $\text{Al}(\text{OH})_3$ is described. By this method, phosphorylase can be demonstrated in all animal tissues.

2. The phosphorylase activity and the intensity of the glycogen metabolism of a tissue are shown to be positively correlated. The same is true also of the phosphorolytic breakdown of glycogen.

3. Subcutaneous tissue possesses a highly active phosphorylase, and metabolizes glycogen at a high rate.

4. Phosphorylase is not found in the muscles of rats less than 10 days of age. In 14-day-old rats the enzyme is already comparatively active.

5. Brain tissue from new-born rats shows 60 % of the phosphorylase activity of the adult brain. Phosphoglucomutase in new-born rat brain, however, was either inactive or absent. This enzyme is the limiting factor in glycogen phosphorolysis up to 14–20 days after birth, after which time its activity approaches that found in the adult brain.

6. In intoxication by phosphorus, carbon tetra-

chloride and chloroform, liver phosphoglucomutase is inactivated, but phosphorylase activity is almost normal.

7. In adrenalectomized as well as in thyroidectomized rats no decrease in glycogen phosphorolysis by muscle could be demonstrated.

8. Some properties of the phosphorylase of muscle have been studied: on irradiation with ultra-violet light, or on heating to 55° for 20 min., complete destruction of the enzyme occurred. Adenylic acid diminished the inhibition caused by glucose in 2 % concentration. With 0.3 % glucose no lessening of inhibition was found when adenylic acid was added. With potato phosphorylase no inhibition of glycogen synthesis by 2 % glucose was found.

9. If synthesis by muscle phosphorylase is slowed down by low temperature or by ageing of the enzyme preparation, an iodine colour reaction such as is given by glycogen can be observed after 5–10 min. of synthesis.

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The Chemical Behaviour of Dehydro-*l*-ascorbic Acid *in vitro* and *in vivo*

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It is reasonable to assume that the function of *l*-ascorbic acid in the metabolism of the animal organism is that of a link in a co-ordinated chain of reactions. It might therefore be expected that, since ascorbic acid and dehydro-*l*-ascorbic acid constitute under certain conditions a thermodynamically reversible oxidation-reduction system, dehydroascorbic acid would become a component of

these co-ordinated reactions, although under prevailing conditions it may be present in too small quantities to be detected by the available technique. With this hypothesis in view one of us started some years ago an investigation on the behaviour of dehydroascorbic acid in the guinea-pig. It was then found that whilst ascorbic acid could be almost completely recovered from the organs and tissues

of the guinea-pig soon after injection, this was not the case when dehydroascorbic acid was used. It was therefore assumed that when dehydroascorbic acid (II) was present in the organism in large quantities a part of it was reduced in the body to ascorbic acid (I) but considerable quantities were at the same time converted irreversibly into 2:3-diketo-*l*-gulonic acid (III).

Before this could be demonstrated experimentally it was necessary to acquire further information on the subject. First, a direct method for estimating diketogulonic acid in small quantities in animal tissues had to be devised. In the second place, the chemistry of the conversion of dehydroascorbic acid had to be studied in greater detail than hitherto in order to be able to interpret correctly results obtained *in vivo*. The former has since been accomplished [Penney & Zilva, 1943]. The latter investigation is reported here. This communication is divided into three sections, one dealing with the purely chemical aspect of the problem performed *in vitro*, the second with the behaviour of dehydroascorbic acid and diketogulonic acid *in vivo*, and the third consisting of a general discussion of the results described in the first two sections.

The conversion of dehydroascorbic acid to diketogulonic acid was first postulated by Herbert, Hirst, Percival, Reynolds & Smith [1933] who observed that dehydroascorbic acid mutarotated in acid solution. For convenience we therefore often employ the expression mutarotation for the change of dehydroascorbic acid into diketogulonic acid, although the conversion was not measured by us polarimetrically.

I. EXPERIMENTS IN VITRO

Since this investigation pivots on the colorimetric determination of diketogulonic acid [Penney & Zilva, 1943] it was essential to elucidate as far as possible the chemical nature of the reactions involved. This estimation consists of two distinct phases, namely, the formation of the 2:4-dinitrophenylhydrazine derivative and the subsequent production of a red coloration on addition of alkali.

The colour forms the ultimate criterion for the assessment of diketogulonic acid.

Solutions of dehydroascorbic and diketogulonic acids were prepared as described [Penney & Zilva, 1943]. To 250 ml. solutions containing 100 mg. of each of these compounds and of ascorbic acid, three equivalents of 2:4-dinitrophenylhydrazine in 100 ml. 2 *N* HCl were added. After 5 days at 40° the bulky red precipitates were filtered off, washed with dilute HCl and water and dried *in vacuo* over H₂SO₄. Table 1 gives the crude yields of the preparations and their melting points after recrystallization from acetone-ethanol. The mixed m.p. was

Table 1. Crude yields and m.p. of 2:4-dinitrophenylhydrazine derivatives

Derivative from	Crude yield % of theory	m.p. corrected
Ascorbic acid	80	279° decomp.
Dehydroascorbic acid	85	280° "
Diketogulonic acid	70	281° "

unchanged. These melting points agree well with those obtained by Herbert *et al.* [1933] for ascorbic and dehydroascorbic acid derivatives (282° and 280° decomp. respectively), and by Drumm, Scarborough & Stewart [1937] for the dehydroascorbic acid derivative (280°). Roe [1937], on the other hand, found that the dehydroascorbic acid derivative melted at 257–259° decomp. This worker apparently did not recrystallize his product, which might explain the lower m.p. The m.p. of the diketogulonic acid derivative obtained by us was the same as those of the ascorbic and dehydroascorbic acid derivatives.

The N content of the diketogulonic acid derivative (Weiler) was 20.8% (calculated for osazone 21.0%), which is also in good agreement with those obtained by the above workers for the ascorbic and dehydroascorbic acid derivatives.

Small quantities of each of the derivatives in 50% acetone-ethanol were passed through a column of active alumina (Savory & Moore). In each case the adsorption was similar, a purple zone being obtained at the top of the column which could not be eluted with acetone, but was easily removed with acetic acid. No chromatographic separation could be obtained from a mixture of the three derivatives.

In view of the above results, little doubt remains that the same dinitrophenylhydrazine derivative is obtained from each of the three compounds. Indirect evidence further points to the fact that in this reaction, ascorbic acid is first oxidized to dehydroascorbic acid which is converted to diketogulonic acid by the opening of the lactone ring. That ascorbic acid is first oxidized to dehydroascorbic acid is shown by the fact that more than two equivalents of the dinitrophenylhydrazine are required

to obtain the maximum yield. Furthermore, as will be shown later, at the pH of the reaction mixture the mutarotation of dehydroascorbic acid is accelerated. All this evidence suggests that the dinitrophenylhydrazine ultimately condenses with diketogulonic acid in the case of all the three compounds, in support of which we found that when the H^+ concentration of the reacting solution was increased, the rate of the reaction as measured by the colorimetric method also increased (Table 2) as a consequence of the more rapid formation of diketogulonic acid.

Table 2. Colour produced by dehydroascorbic acid in the presence of 0.2 N and 0.04 N HCl at 38°, recorded as reciprocal colorimeter readings

HCl conc. N	Reciprocal colorimetric reading after				
	60	90	120	180	270 min.
0.2	0.0145	0.024	0.0325	0.0435	0.068
0.04	0.010	0.017	0.025	0.0325	0.0545

In another experiment the disappearance of dehydroascorbic acid was determined in the total absence of, and in the presence of, an excess of dinitrophenylhydrazine under the same conditions, i.e. in 0.4 N HCl at 38°. The solutions were withdrawn after various periods, cooled, and a slight excess of furfuraldehyde added to remove the remaining dinitrophenylhydrazine. Precipitation of this furfuraldehyde derivative was complete in 5 min. $KHCO_3$ was then added to neutralize partially the acid present and the final pH adjusted to 3.5 by the addition of McIlvaine's phosphate-citrate buffer. After filtration, the dehydroascorbic acid was determined in both cases by treatment with H_2S and subsequent titration with indophenol. The presence of furfuraldehyde in these experiments did not interfere with the determination of dehydroascorbic acid. The results are given in Table 3; it

Table 3. Recovery of dehydroascorbic acid after mutarotation in the presence and in the absence of 2:4-dinitrophenylhydrazine at 38°. HCl conc. = 0.4 N

	Dehydroascorbic acid found (%) after					
	30	60	90	120	150	180 min.
Dinitrophenylhydrazine present	80	64	53	44	41	32
Dinitrophenylhydrazine absent	80	65	55	46	40	34

will be seen that the quantity of dehydroascorbic acid remaining in each case was the same within the limits of the experimental error, thus demonstrating that dinitrophenylhydrazine does not react with dehydroascorbic acid directly. The necessity for converting ascorbic acid and dehydroascorbic acid

to diketogulonic acid before the reaction can take place accounts for the differential rate of the formation of the diketogulonic acid derivative, and therefore affords the possibility of determining quantitatively diketogulonic acid in the presence of the other two compounds under suitable conditions.

The evidence obtained concerning the mechanism of the formation of the deep red coloration on the addition of alkali to the dinitrophenylhydrazine derivative of diketogulonic acid was less instructive. Although the dinitrophenylhydrazine derivative of diketogulonic acid was soluble in NaOH, it was insoluble in $KHCO_3$ and Na_2CO_3 . A compound or a mixture of compounds melting about 85° could be obtained from the NaOH solution by acidification. This product, unlike the original dinitrophenylhydrazine derivative, was soluble in Na_2CO_3 and $KHCO_3$ solutions. The solution of the precipitated compound turned from yellow to deep red at pH 9.5–10.5. It is of interest to note that the dinitrophenylhydrazine of pyruvic acid similarly changes its colour over this range, which suggests the presence of a carboxylic group in the compound.

The fact that the dinitrophenylhydrazine derivative of diketogulonic acid did not react with $KHCO_3$ or Na_2CO_3 suggests the absence of a COOH group, and from this it may be concluded that the derivative possesses either a lactone or pyrazolone structure. Ohle & Böckmann's work [1934] on the reaction of araboascorbic acid with phenylhydrazine favours the former view. They found that in the presence of HCl both the open chain (IV) and the lactone (V) forms are present and that on simple recrystallization the former readily lactonizes.

It is quite likely that under the experimental conditions prevailing in the colorimetric determination of diketogulonic acid the corresponding open-chain compound (IV) is formed, which tends to lactonize in time or on crystallization. It is therefore the open-chain compound which in all probability reacts with NaOH to form the characteristic red colour.

Mutarotation of dehydroascorbic acid under different conditions

Before investigating whether any conversion of dehydroascorbic acid takes place *in vivo* it was necessary to study in detail the mutarotation of

this substance under different conditions so as to be able to interpret the results of metabolic experiments.

Mutarotation in acid medium at 25°. It was obviously of importance to ascertain the influence of concentrations of dehydroascorbic acid on its rate of mutarotation since it could not be assumed that the kinetics of the conversion to diketogulonic acid conformed to that of a reaction of the first order. By following colorimetrically the rate of mutarotation of three concentrations of dehydroascorbic

of HCl. In this experiment, 15 ml. HCl, of a concentration necessary to give the final normalities recorded in Fig. 2, were introduced into each of a series of tubes kept in a water-bath at 25°. Dehydroascorbic acid solutions (100 ml.) were prepared by the oxidation of ascorbic acid (100 mg.) with I_2 . The temperature of the dehydroascorbic acid solution was rapidly adjusted to 25° and 5 ml. then added to each tube. At given times, tubes were withdrawn, 2N $KHCO_3$ added to adjust the pH of the contents to 2 and the final volume made up to

Fig. 1.

Fig. 1. Influence of initial concentration of dehydroascorbic acid on the rate of diketogulonic acid formation at 25° in the presence of HI produced during the oxidation of ascorbic acid with I_2 . $\times-\times$ 50 mg./100 ml. $\triangle-\triangle$ 200 mg./100 ml. $\odot-\odot$ 1 g./100 ml.

Fig. 2.

Fig. 2. Influence of hydrogen-ion concentration on the rate of mutarotation of dehydroascorbic acid at 25°. $\odot-\odot$ In N HCl. $\triangle-\triangle$ In 0.5 N HCl. $\nabla-\nabla$ In 0.2 N HCl. $\times-\times$ In 0.1 N HCl.

acid, i.e. 50, 200 and 1000 mg./100 ml., it was observed that the velocity of conversion (%) was increased with increase of the concentration (Fig. 1). Further experiments, however, showed that this increase was not due to the increase in concentration of the dehydroascorbic acid but to the increase in the H^+ concentration of the medium, since the dehydroascorbic acid used in these experiments was prepared by oxidation of ascorbic acid with I_2 . This reaction produces HI, the concentration of which would rise as higher concentrations of ascorbic acid were employed. That the rate of mutarotation is actually accelerated by a rise in the H^+ concentration of the medium is seen from Fig. 2 which shows the effect of the addition of various concentrations

of HCl. In this experiment, 15 ml. HCl, of a concentration necessary to give the final normalities recorded in Fig. 2, were introduced into each of a series of tubes kept in a water-bath at 25°. Dehydroascorbic acid solutions (100 ml.) were prepared by the oxidation of ascorbic acid (100 mg.) with I_2 . The temperature of the dehydroascorbic acid solution was rapidly adjusted to 25° and 5 ml. then added to each tube. At given times, tubes were withdrawn, 2N $KHCO_3$ added to adjust the pH of the contents to 2 and the final volume made up to

50 ml. Diketogulonic acid was then determined directly by the colorimetric method, and indirectly by reduction of the residual dehydroascorbic acid with H_2S . When Cl_2 was used as the oxidizing agent instead of I_2 , identical results were obtained. Further confirmation that the rate of conversion (%) is influenced by H^+ concentration was obtained in an experiment in which ascorbic acid was oxidized with I_2 or with KIO_3 . In the former case the mutarotation was followed, with and without neutralizing the HI produced, whilst in the second case HCl equivalent to the HI produced by an equal quantity of ascorbic acid oxidized with I_2 was added to one of the neutral solutions of dehydroascorbic acid. It will be seen from Fig. 3 that the rate of conversion

(%) in each case was higher in acid solution than in neutral and was independent of the method of preparation.

Fig. 3. — Oxidizing agent I_2 . ---- Oxidizing agent KIO_3 .

The results in Table 4 show conclusively that the rate of conversion (%) of dehydroascorbic acid to diketogulonic acid is independent of concentration at a given H^+ concentration. This being so it would

Table 4. Effect of initial concentration of dehydroascorbic acid on the mutarotation at 25° .

Initial conc. of dehydro- ascorbic acid mg./100 ml.	HCl conc. N	Diketogulonic acid (%) found after (hr.)		
		30	49	73
50	0.011	58	79	84
100		56	77	87
250		58	78	85
750		58	79	85
25	1.0	2	3	4
100		48	63	72
200		50	66	74
		50	65	76

be expected that the mutarotation should proceed as a reaction of the first order. By calculating all the results of this section according to the formula

$$k = \frac{2.3 \log a/(a-x)}{t}$$

(a =initial concentration of dehydroascorbic acid; x =decrease in concentration after time t ; k =velocity constant), it was found that for a given H^+ concentration the values of k were constant. The average values were: in N HCl, $k=0.0053 \text{ min.}^{-1}$; in 0.5 N HCl, $k=0.0031 \text{ min.}^{-1}$; in 0.2 N HCl, $k=0.0015 \text{ min.}^{-1}$; in 0.1 N HCl, $k=0.0010 \text{ min.}^{-1}$.

Since the conversion depends on the H^+ concentration the reaction is pseudo-unimolecular. It may also be added that so far the calibration used for

the above determinations is based on the supposition that the conversion of dehydroascorbic acid to diketogulonic acid was complete. If, however, equilibrium is reached as described by Herbert *et al.* [1933] the above figures will represent the sum of the velocity constants of the direct and reverse reactions.

The action of H_2S on diketogulonic acid solutions. When the mutarotation of dehydroascorbic acid reaches completion, treatment of the resulting solution with H_2S yields an amount of ascorbic acid corresponding to about 10% of the original content of dehydroascorbic acid. Herbert *et al.* [1933] suggested that this is due to an equilibrium between diketogulonic acid and dehydroascorbic acid. This hypothesis has been questioned by Borsook, Davenport, Jeffreys & Warner [1937] who found that by reducing a mutarotated solution of dehydroascorbic acid with glutathione at pH 7 reconversion of the diketogulonic acid did not take place as would have been expected in true equilibrium solution.

It was of interest to follow the mutarotation of a dehydroascorbic acid solution by determining the diketogulonic acid formed directly, by means of the colorimetric method and at the same time indirectly by difference after reducing the mutarotating solution with H_2S (on the assumption that the subsequent indophenol reduction was due to the formation of ascorbic acid from the dehydroascorbic acid of the mutarotating solution). Fig. 4 gives a graphic

Fig. 4. The course of the mutarotation of dehydroascorbic acid at 25° as determined by the colorimetric and indirect H_2S reduction methods. $\odot$ — $\odot$ Colorimetric method. $\triangle$ — $\triangle$ Indirect H_2S reduction method.

representation of an experiment in which the dehydroascorbic acid present (100 mg./ml.) was obtained by oxidation of a solution of ascorbic acid with I_2 . The difference between the results obtained by the two methods up to about 70% conversion falls within the limits of the experimental error.

After this a deviation eventually reaching a difference of about 10% was noted. This difference was to be expected, since, in the colorimetric determination, it was assumed that the conversion into diketogulonic acid was complete. An observation was, however, made in the course of this experiment which suggests that the change produced by H_2S in a mutarotated solution is rather more complex than might at first be expected. When diketogulonic acid was determined colorimetrically in the mutarotated solution after H_2S treatment a very low value, about 20% of that expected, was obtained. Apparently some chemical change in the diketogulonic acid had occurred on treatment with H_2S . The conversion of diketogulonic acid (III) to a tautomeric form (VI) of 2-keto-*l*-gulonic acid (VII), a compound capable of conversion into ascorbic acid [Reichstein & Grüssner, 1934], is one of the changes that can be visualized.

If this scheme be correct the presence of 10% dehydroascorbic acid in equilibrium with 90% diketogulonic acid need not therefore be postulated, and consequently the quantity of diketogulonic acid determined by the colour reaction may indeed indicate a 100% conversion. Whatever be the case the colorimetric method is applicable to the experiments to be described later, since mutarotated solutions which attained 'equilibrium' were employed in the calibration.

Influence of pH on the mutarotation of dehydroascorbic acid at 38°. It has already been shown that the concentration of acid in the medium influenced the rate of mutarotation. In the present experiments the mutarotation of dehydroascorbic acid was studied at higher pH values. The ascorbic acid was oxidized with I_2 and the solution was brought to 38° as rapidly as possible. 5 ml. dehydroascorbic acid solution were then added to each of a series of tubes containing 10 ml. McIlvaine's phosphate-citrate buffer and 20 ml. H_2O also at 38°. All buffer salts used in these experiments were recrystallized twice from glass-distilled water. For the determination of diketogulonic acid colorimetrically as well as indirectly by H_2S reduction, the tubes were removed at various intervals, cooled, and the contents transferred to a 50 ml. graduated flask, adjusted to pH 2 by addition of HCl, and made up to volume.

It will be seen from Fig. 5 that the rate of mutarotation as measured colorimetrically was markedly accelerated by an increase in pH. Whilst at pH 7.4 the maximum conversion of about 80% was attained in less than 15 min., only 30% conversion was recorded after 300 min. at pH 4. It may be

Fig. 5. The influence of pH on the rate of mutarotation of dehydroascorbic acid at 38°.

mentioned that at pH 4 or 5 the figures obtained by the two methods were almost identical, as would be expected from our previous observations (Fig. 4), since not more than 50% of the dehydroascorbic acid had been converted. It is also of interest to note that whilst at pH 4 and at pH 5 the mutarotation proceeds normally as a reaction of the first order, this is not true at pH 6.1. Above pH 6 it was found that diketogulonic acid began to decompose, the rate of disappearance increasing with increasing pH. The observation that maximum conversion of only 80% could be obtained at pH values greater than 6 could to some extent be explained by the decomposition of the diketogulonic acid. Borsook *et al.* [1937], employing electrometric methods at 35.5°, concluded that at pH 4.0–5.75 the mutarotation was a reaction of the first order, but at pH 6.43 the results obtained were too complicated for interpretation. This, as in our experiments, may have been due to the disappearance of diketogulonic acid at the latter pH. Ball [1937], using an electrometric technique which promoted the rapid establishment of the electrode potential, found the reaction at 30° to be of the first order between the pH values of 5.188 and 8.566. These results do not appear to agree completely with those obtained in our experiments and those of

Borsook *et al.*; since Ball used a technique involving rapid adjustment of electrode potential, a comparison is not strictly justified.

The discrepancy between the figures obtained by the colorimetric method and by indirect H_2S reduction at pH 7.4 in McIlvaine buffer is shown in Fig. 6. It will be seen that when the diketogulonic

Fig. 6. The course of the mutarotation of dehydroascorbic acid at pH 7.4 (McIlvaine's phosphate-citrate buffer) and at 38° . $\odot$ — $\odot$ Diketogulonic acid as determined colorimetrically. $\triangle$ — $\triangle$ Diketogulonic acid as determined indirectly by H_2S reduction. ∇ — ∇ Indophenol-reducing capacity before H_2S reduction. $\times$ — $\times$ Diketogulonic acid as determined indirectly by H_2S reduction after correcting for initial indophenol-reducing capacity.

acid was determined directly a maximum value of about 80% was obtained in less than 15 min., after which time the diketogulonic acid content began to decrease, attaining a value of about 25% after 300 min. The indirect method of determining diketogulonic acid at this pH failed completely. Under these conditions the production of a substance which reduced indophenol without treatment with H_2S was observed. The reducing power gradually increased and eventually attained a maximum value. After treatment with H_2S the indophenol-reducing capacity was only slightly increased as will be seen from Fig. 6, in which values corrected for the initial reducing capacity are represented by an interrupted line.

That the rate of mutarotation at pH 4, 5 and 6 was affected by the pH of the solution *per se* and not by the constituents of the buffers, was indicated by the fact that identical results were obtained

when phthalate buffer was used instead of McIlvaine phosphate-citrate buffer. At pH 7.4, when phosphate-NaOH buffer was used, the conversion of

Fig. 7. The course of the mutarotation of dehydroascorbic acid at pH 7.4 (phosphate-NaOH buffer) and at 38° . Symbols as in Fig. 6.

Fig. 8. Influence of borate buffer on the mutarotation of dehydroascorbic acid at pH 7.4 and at 38° . Symbols as in Fig. 6.

dehydroascorbic acid into diketogulonic acid and the initial decomposition of the latter proceeded as in the presence of McIlvaine buffer. After about

150 min., however, when the value recorded for diketogulonic acid was 50%, the reaction became unreliable as the colour characteristic of diketogulonic acid was no longer obtained (Fig. 7). The production of an indophenol-reducing substance was also observed when this buffer was used, the indophenol-reducing capacity again being only slightly increased after H_2S treatment. In the presence of borate buffer ($\text{pH } 7.4$), almost complete conversion to diketogulonic acid occurred, and good agreement was found between results obtained by the direct colorimetric method and the indirect H_2S reduction method (Fig. 8). The resulting diketogulonic acid was relatively stable and no indophenol-reducing substance was formed from the mutarotating solution. It is of interest to note that diketogulonic acid solution, when added to borate buffer at $\text{pH } 7.4$, did not decompose. Since borate is known to combine with polyhydroxy compounds, it might be expected to have a stabilizing effect on the diketogulonic acid.

equivalent of 2.5 mg. ascorbic acid was placed in each stopper. The tubes were then evacuated, washed with O_2 -free nitrogen, re-evacuated and the contents mixed after they had attained the temperature of the bath. After various intervals the pH was adjusted to 2 and the diketogulonic acid determined by the colorimetric method as well as by the indirect H_2S reduction method.

A control experiment was carried out in air. The results are graphically represented in Fig. 9. It is seen at once that the course of the conversion of the dehydroascorbic acid into diketogulonic acid as measured colorimetrically was the same in both cases. Furthermore, the subsequent change in the diketogulonic acid formed in both experiments was similar to that already observed. On the other hand, the indophenol-reducing capacity of the mutarotated solution before and after H_2S treatment when carried out in the absence of air differed significantly from that observed under aerobic conditions. No indophenol-reducing substance was

Fig. 9. The influence of O_2 on the mutarotation of dehydroascorbic acid at $\text{pH } 7.4$ (McIlvaine's phosphate-citrate buffer) and at 38° . Symbols as in Fig. 6.

Mutarotation of dehydroascorbic acid at $\text{pH } 7.4$ in the presence and absence of air. The mutarotation of dehydroascorbic acid at $\text{pH } 7.4$ in the absence of air was determined in a series of evacuated modified Thunberg tubes maintained at 38° . The dehydroascorbic acid was prepared by oxidizing a solution of ascorbic acid in oxygen-free water with I_2 and neutralizing the HI formed with NaOH . McIlvaine's buffer was also prepared in oxygen-free H_2O and was adjusted to $\text{pH } 7.4$ electrometrically. 5 ml. buffer was introduced into each of the tubes and 1 ml. dehydroascorbic acid solution containing an

produced during the course of the anaerobic experiment, and after treatment with H_2S the indophenol titration was very low. This latter reduction was of the same order as the increase found after H_2S treatment when mutarotation was allowed to proceed in the presence of air. This observation offers further evidence concerning the unreliability of determining diketogulonic acid indirectly by H_2S reduction.

Effect of temperature on the mutarotation of dehydroascorbic acid. The mutarotation of dehydroascorbic acid in the presence of HI formed by the

oxidation of ascorbic acid by I_2 (100 mg./100 ml.) was studied colorimetrically at 25 and 38°. The conversion, as would be expected, proceeded at a higher rate at 38° than at 25° (Fig. 10).

mutarotate for 14 days at room temperature. After this time the conversion of dehydroascorbic acid to diketogulonic acid, as assessed colorimetrically and by H_2S reduction, was complete. This solution was

Fig. 10.

Fig. 10. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in the presence of 0.011 N HCl.

Fig. 11.

Fig. 11. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in N HCl.

In addition, the production of diketogulonic acid was determined in N HCl at 10, 20, 25, 30 and 35°. The results when substituted in the formula of a reaction of the first order gave a constant for each temperature. In Fig. 11 $\log k$ is plotted against $1/T$ according to the usual integrated form of the Arrhenius equation, and it will be seen that a linear relationship exists. The temperature coefficient k_{35}/k_{25} calculated from the above data is 2.2.

II. EXPERIMENTS IN VIVO

The biological experiments consisted of the determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid in the tissues, in the contents of the digestive tract and in the urine at various intervals after the administration of the last two compounds *per os* or parenterally to guinea-pigs weighing 300–350 g.

The dehydroascorbic acid employed in these experiments was prepared immediately before administration by the oxidation of ascorbic acid with the theoretical quantity of KIO_3 . Diketogulonic acid was obtained by allowing a solution of dehydroascorbic acid, adjusted to pH 2 with HCl, to

partially neutralized with NaOH immediately before use. Equivalents of 120 mg. ascorbic acid contained in approximately 4 ml. were used in each experiment. As the volume of the dose was high it was found convenient, when injecting, to introduce equal parts successively into each of the femoral and biceps muscles of the animals. All the guinea-pigs used in this investigation were previously kept for 5 days on a scorbutic diet to ensure depletion of ascorbic acid in their tissues, so that any formation of ascorbic acid after the administration of dehydroascorbic acid or diketogulonic acid could be detected. After administration of the dose, the animals were placed in metabolism cages for 15, 30 or 60 min., so that any urine voided could be collected for analysis. The animals were then killed after the appropriate time by stunning and bleeding, the blood being collected in oxalate. The tissues and the contents of the intestinal tract were then prepared for analysis as follows. The plasma obtained after centrifuging the blood was precipitated with an equal volume of 10% CCl_3COOH . The contents of the stomach and intestines were obtained by ligaturing the digestive tract at the appropriate places, removing the contents by washing

out with water, making up to a given volume, and finally extracting with an equal volume of 10% CCl_3COOH . The stomach and intestinal tissues, after drying between filter paper, and also the kidneys and liver were extracted with two parts by weight of 10% CCl_3COOH . Urine, if present, was removed from the bladder by means of a Pasteur pipette and, if necessary, added to that passed before death. The body was then skinned and the 'carcass' was prepared for analysis as described by Zilva [1935]. After removal of the hair, the skin was minced thoroughly and a sample was extracted with two parts by weight of 10% CCl_3COOH . Ascorbic acid and dehydroascorbic acid were then determined by titration with indophenol, the former before, and the latter after, H_2S treatment. Diketogulonic acid was assessed by a modified form of the colorimetric method already described by us [Penney & Zilva, 1943]. We found that the sensitivity of the original method could be increased by partial neutralization of the acid present in the test solution immediately after the addition of the di-

nitrophenylhydrazine reagent. A calibration curve was therefore constructed for this purpose by adding 0.7 ml. 2N KHCO_3 to the standard after the addition of 1 ml. dinitrophenylhydrazine. The test solutions were treated similarly, allowing for the presence of the CCl_3COOH acid in the extracts. The standard error of percentage deviation of this modified method was 10%. This modification was not necessary in the case of urine and plasma because in these instances higher values were under consideration. The solutions obtained in the determination of diketogulonic acid in the 'carcass' were turbid; a blank reading was obtained immediately after the addition of the dinitrophenylhydrazine reagent to the extract and subsequent treatment with alkali, and used as a correction to the final reading.

The average quantities of ascorbic acid, dehydroascorbic acid and diketogulonic acid present in the tissues, stomach and intestinal contents and urine 15, 30 and 60 min. after the administration of dehydroascorbic acid or diketogulonic acid *per os* or by injection are given in Tables 5-8, together

Table 5. *Effect of injection of 120 mg. dehydroascorbic acid into the guinea-pig*

		Quantity found													
		Liver		Kidney mg.	Stomach		Small intestine		Large intestine		'Car- cass' mg.	Skin mg.	Ex- creted in urine mg.	Plasma mg./ml.	Total mg.
After min.		Total mg.	mg./g.		Tissue mg.	Con- tents mg.	Tissue mg.	Con- tents mg.	Tissue mg.	Con- tents mg.					
Ascorbic acid															
15	Mean	11.4	0.91	0.99	0.35	0.0	2.2	0.1	1.3	0.0	12.6	4.2	0.0	0.05	33.8
	S.D.*	1.1	0.1	0.08	0.1	0.0	0.0	0.1	0.1	0.0	3.1	1.0	0.0	0.01	5.1
Dehydroascorbic acid															
	Mean	0.1	0.01	0.05	0.01	0.0	0.0	0.0	0.0	0.2	8.9	1.7	0.2	0.0	11.1
	S.D.	0.1	0.01	0.05	0.01	0.0	0.0	0.0	0.0	0.3	2.3	1.0	0.1	0.0	3.6
Diketogulonic acid															
	Mean	2.6	0.20	0.99	0.45	1.0	1.7	1.1	1.7	4.8	21.2	12.7	1.0	0.36	54.6
	S.D.	0.5	0.03	0.07	0.18	0.3	0.8	0.2	0.4	2.3	4.7	1.0	0.5	0.05	6.7
Ascorbic acid															
30	Mean	14.8	1.16	1.09	0.36	0.0	2.4	0.5	1.4	1.0	9.6	3.7	0.1	0.06	35.8
	S.D.	1.1	0.07	0.35	0.06	0.0	0.3	0.1	0.1	0.9	1.7	1.1	0.1	0.02	2.3
Dehydroascorbic acid															
	Mean	0.2	0.01	0.00	0.04	0.0	0.0	0.1	0.0	0.0	4.6	0.3	0.6	0.00	5.8
	S.D.	0.3	0.01	0.00	0.02	0.0	0.0	0.1	0.0	0.0	0.9	0.3	0.4	0.00	0.8
Diketogulonic acid															
	Mean	3.3	0.26	1.05	0.46	0.6	1.1	0.9	1.5	5.9	17.5	10.6	3.2	0.29	50.4
	S.D.	0.5	0.06	0.16	0.11	0.2	0.3	0.4	0.3	0.8	5.6	1.0	1.6	0.01	6.2
Ascorbic acid															
60	Mean	14.9	1.10	0.99	0.41	0.1	3.3	0.6	1.5	0.7	10.8	4.2	0.6	0.07	35.9
	S.D.	1.8	0.11	0.09	0.04	0.1	0.2	0.3	0.2	0.7	2.3	—	0.6	0.02	3.2
Dehydroascorbic acid															
	Mean	0.0	0.00	0.00	0.02	0.0	0.0	0.1	0.0	0.7	1.4	0.6	0.8	0.00	3.0
	S.D.	0.0	0.00	0.00	0.02	0.0	0.0	0.1	0.0	0.7	2.5	—	0.5	0.00	2.2
Diketogulonic acid															
	Mean	3.4	0.25	0.88	0.37	0.5	1.1	1.1	1.4	5.0	12.7	16.8	9.1	0.17	42.5
	S.D.	1.0	0.09	0.01	0.10	0.1	0.1	0.0	0.3	0.9	8.5	—	6.6	0.01	11.0

In this and in the following tables, it was assumed that the total volume of plasma from each guinea-pig was 15 ml.

* Standard deviation.

Table 6. *Effect of oral administration of 120 mg. dehydroascorbic acid to the guinea-pig*

		Quantity found														
After min.		Liver		Kidney mg.	Stomach		Small intestine		Large intestine		'Car-cass' mg.	Skin mg.	Ex-creted in urine mg.	Plasma mg./ml.	Total mg.	
		Total mg.	mg./g.		Tissue mg.	Con-tents mg.	Tissue mg.	Con-tents mg.	Tissue mg.	Con-tents mg.						
15	Mean	3.5	0.27	0.30	0.47	Ascorbic acid										22.5
	S.D.	0.4	0.1	0.05	0.08	1.0	5.1	4.0	0.9	0.4	4.7	1.5	0.0	0.03	1.8	
						0.2	0.5	0.9	0.1	0.4	0.3	0.4	0.0	0.01		
						Dehydroascorbic acid										
	Mean	0.1	0.01	0.03	0.59	51.5	0.4	3.2	0.0	0.0	1.7	0.6	0.0	0.00	58.0	
	S.D.	0.1	0.01	0.03	0.03	3.9	0.3	2.6	0.0	0.0	1.7	0.4	0.0	0.00	6.5	
30						Diketogulonic acid										
	Mean	2.6	0.19	0.41	0.41	2.3	3.5	15.8	1.3	9.8	5.5	4.9	0.1	0.05	45.8	
	S.D.	0.9	0.03	0.02	0.06	2.1	0.4	9.7	0.1	2.0	1.6	1.7	0.1	0.01	11.5	
						Ascorbic acid										
	Mean	3.5	0.30	0.45	0.48	0.9	4.7	2.8	1.1	0.5	6.1	2.7	0.1	0.05	24.1	
	S.D.	0.6	0.05	0.18	0.12	0.5	0.9	1.6	0.2	0.8	2.9	1.2	0.1	0.02	5.9	
60						Dehydroascorbic acid										
	Mean	0.3	0.02	0.01	0.16	45.8	0.0	2.1	0.0	0.2	1.0	0.5	0.01	0.00	50.2	
	S.D.	0.1	0.01	0.01	0.18	18.1	0.0	0.8	0.0	0.4	0.9	0.1	0.01	0.00	16.5	
						Diketogulonic acid										
	Mean	1.9	0.17	0.37	0.40	3.3	1.9	12.2	1.2	13.1	6.6	5.9	0.7	0.05	48.4	
	S.D.	0.1	0.01	0.09	0.06	0.2	0.7	3.0	0.4	10.6	1.9	2.0	0.4	0.01	9.2	
1						Ascorbic acid										
	Mean	5.6	0.37	0.49	0.40	0.2	5.0	3.2	1.2	0.8	5.0	2.5	0.1	0.04	25.1	
	S.D.	1.4	0.02	0.17	0.11	0.3	1.4	0.6	0.2	0.8	0.7	1.2	0.1	0.01	5.5	
						Dehydroascorbic acid										
	Mean	0.2	0.01	0.01	0.06	5.0	0.2	1.2	0.0	0.0	1.0	0.6	0.0	0.00	8.2	
	S.D.	0.2	0.01	0.01	0.03	1.5	0.2	0.3	0.0	0.0	0.7	0.6	0.0	0.00	2.2	
2						Diketogulonic acid										
	Mean	2.4	0.14	0.37	0.26	1.5	2.2	14.2	1.5	18.8	2.7	5.9	0.8	0.04	51.3	
	S.D.	0.6	0.03	0.05	0.02	0.4	0.9	5.6	0.2	3.1	2.4	0.6	0.5	0.01	9.5	

Table 7. *Effect of injection of 120 mg. diketogulonic acid into the guinea-pig*

		Quantity found													
After min.		Liver		Kidney mg.	Stomach		Small intestine		Large intestine		'Car- cass' mg.	Skin mg.	Ex- creted in urine mg.	Plasma mg./ml.	Total mg.
		Total mg.	mg./g.		Tissue mg.	Con- tents mg.	Tissue mg.	Con- tents mg.	Tissue mg.	Con- tents mg.					
		Ascorbic acid													
15	Mean	0.9	0.08	0.08	0.11	0.0	0.8	0.0	0.5	0.0	1.8	0.6	0.0	0.00	4.8
	S.D.	0.1	0.01	0.01	0.02	0.0	0.2	0.0	0.1	0.0	0.2	0.2	0.0	0.00	0.3
Diketogulonic acid															
	Mean	2.6	0.20	1.19	0.48	0.7	0.9	0.9	1.3	3.8	43.4	16.0	7.5	0.55	86.6
	S.D.	0.5	0.01	0.06	0.06	0.1	0.1	0.1	0.3	0.7	0.3	1.0	1.1	0.03	2.3
Ascorbic acid															
30	Mean	1.0	0.07	0.09	0.08	0.0	0.9	0.0	0.5	0.0	1.4	0.6	0.0	0.00	4.5
	S.D.	0.3	0.01	0.00	0.02	0.0	0.2	0.0	0.1	0.0	0.8	0.2	0.0	0.00	1.3
Diketogulonic acid															
	Mean	3.2	0.21	1.28	0.43	0.9	1.0	0.7	1.4	4.7	26.6	15.4	11.5	0.45	73.8
	S.D.	0.7	0.01	0.12	0.08	0.6	0.2	0.2	0.3	1.6	4.3	1.0	5.5	0.01	9.6
Ascorbic acid															
60	Mean	0.9	0.07	0.10	0.09	0.0	0.9	0.0	0.6	0.0	1.1	0.6	0.1	0.00	4.3
	S.D.	0.2	0.01	0.03	0.01	0.0	0.1	0.0	0.1	0.0	0.8	0.3	0.2	0.00	1.0
Diketogulonic acid															
	Mean	2.3	0.17	0.90	0.35	2.1	1.1	0.6	1.2	4.6	11.7	8.9	9.3	0.23	46.5
	S.D.	0.4	0.02	0.17	0.06	0.5	0.1	0.1	0.1	0.9	8.4	2.7	7.2	0.05	18.1

No dehydroascorbic acid was detected in this experiment.

Table 8. *Effect of oral administration of 120 mg. diketogulonic acid to the guinea-pig*

		Quantity found													
After min.		Liver		Kidney mg.	Stomach		Small intestine		Large intestine		'Car-cass' mg.	Skin mg.	Ex-creted in urine mg.	Plasma mg./ml.	Total mg.
		Total mg.	mg./g.		Tissue mg.	Con-tents mg.	Tissue mg.	Con-tents mg.	Tissue mg.	Con-tents mg.					
		Ascorbic acid													
15	Mean	0.6	0.05	0.07	0.12	0.0	1.0	0.1	0.4	0.0	1.6	0.5	0.0	0.00	4.1
	S.D.	0.2	0.01	0.01	0.02	0.0	0.2	0.1	0.1	0.0	0.1	—	0.0	0.00	0.3
Diketogulonic acid															
	Mean	2.2	0.17	0.34	0.91	55.6	4.2	18.5	1.0	4.8	2.8	6.6	0.7	0.05	94.0
	S.D.	0.3	0.04	0.04	0.08	10.7	0.7	1.5	0.2	0.6	2.0	—	0.6	0.01	14.4
Ascorbic acid															
30	Mean	0.6	0.05	0.07	0.12	0.0	1.5	0.2	0.6	0.2	0.9	0.3	0.0	0.00	4.5
	S.D.	0.2	0.01	0.03	0.01	0.0	0.2	0.2	0.1	0.3	0.8	0.2	0.0	0.00	1.8
Diketogulonic acid															
	Mean	1.8	0.16	0.32	0.89	23.3	3.7	9.8	2.4	13.7	7.3	5.6	1.1	0.07	70.5
	S.D.	0.3	0.01	0.07	0.17	11.0	2.3	9.3	1.3	4.7	4.2	0.6	0.2	0.02	13.1
Ascorbic acid															
60	Mean	0.7	0.06	0.08	0.13	0.0	1.4	0.6	0.7	0.5	1.5	0.5	0.0	0.00	6.1
	S.D.	0.1	0.01	0.02	0.02	0.0	0.2	0.4	0.2	0.5	0.3	0.1	0.0	0.00	0.7
Diketogulonic acid															
	Mean	2.1	0.18	0.32	0.88	31.0	3.4	14.5	1.9	17.7	0.5	4.5	1.1	0.04	78.4
	S.D.	0.4	0.03	0.02	0.13	4.1	1.5	8.5	0.1	6.7	0.8	1.3	0.2	0.01	1.6

No dehydroascorbic acid was detected in this experiment.

Table 9. *Analysis of tissues from control guinea-pigs*

		Quantity found													
		Liver		Stomach		Small intestine		Large intestine		'Car-cass' mg.	Skin mg.	Ex-creted in urine mg.	Plasma mg./ml.	Total mg.	
		Total mg.	mg./g.	Kidney mg.	Tissue mg.	Con-tents mg.	Tissue mg.	Con-tents mg.	Tissue mg.						Con-tents mg.
Ascorbic acid															
Mean		0.6	0.05	0.04	0.10	0.0	0.6	0.0	0.5	0.0	1.4	0.6	0.0	0.00	3.5
S.D.		0.3	0.02	0.02	0.05	0.0	0.2	0.0	0.1	0.0	0.6	0.0	0.0	0.00	0.5
Diketogulonic acid															
Mean		1.9	0.17	0.33	0.27	0.6	0.8	0.8	0.9	4.8	4.7	6.1	0.2	0.05	17.9
S.D.		0.2	0.01	0.08	0.02	0.1	0.1	0.3	0.2	1.9	1.8	0.0	0.1	0.01	6.9

No dehydroascorbic acid was detected in this experiment.

with the standard deviations. Each experimental group consists of three guinea-pigs. The concentrations of these substances in the liver are also given in the Tables. The approximate concentrations in the other tissues can be obtained from the mean weights of the organs, which were as follows: kidney 2.75 g., stomach 3.15 g., small intestine 11.0 g., large intestine 11.2 g., 'carcass' 164 g., skin 57 g. All the results are calculated as equivalents of ascorbic acid. A group of animals depleted of vitamin C for 5 days but not receiving either dehydroascorbic acid or diketogulonic acid was analysed and these

results were used as controls (Table 9). All the tissues of the control animals gave a slight colour reaction, but in all probability this was due to the presence of substances other than diketogulonic acid. As the experiments were repeated several times and data of a similar trend were obtained, only representative results are given in this communication.

Significance of the biological results

Sufficient evidence has been obtained to elucidate the behaviour of dehydroascorbic acid and diketo-

gulonic acid in the living guinea-pig. When dehydroascorbic acid was introduced into the organism by intramuscular injection it was very soon partly reduced by the organism to ascorbic acid and partly converted to diketogulonic acid. The former entered the tissues. The latter was mainly excreted by the kidneys but some of it was also found deposited in some of the tissues (Table 5). As will be seen from a separate experiment, the results of which are given in Table 10, as much as about 25 % of the quantity of dehydroascorbic acid injected

Table 10. *Excretion of diketogulonic acid in 24 hr. after the injection of dehydroascorbic acid or diketogulonic acid*

Day	Substance injected	Animal no. ...	Diketogulonic acid excreted in 24 hr. (mg.)		
			41	42	43
1	None		5.6	6.0	5.8
2	120 mg. dehydroascorbic acid		32.6	21.6	38.8
3	None		4.1	8.4	8.3
		Animal no. ...	44	45	46
1	None		5.8	8.7	8.2
2	120 mg. diketogulonic acid		23.5	36.8	35.1
3	None		7.6	11.7	7.2

can be excreted as diketogulonic acid in 24 hr., mostly during the first 3 or 4 hr. Similar results were obtained when diketogulonic acid was injected. Dehydroascorbic acid was estimated at the same time, and the results indicated that possibly small quantities of dehydroascorbic acid were excreted. The identity of diketogulonic acid in the urine after injection of dehydroascorbic acid was established by isolating and identifying the dinitrophenylhydrazine derivative as described previously. The crude yield was in large excess of that which would have been expected from the ascorbic acid present and was in fair agreement with the total quantity of ascorbic acid and diketogulonic acid found in the urine. M.P. after recrystallization from acetone-ethanol was 278°. Mixed m.p. with authentic derivative was 279°. N content (Weiler) = 20.7 % (calculated for osazone N = 21.0 %).

It will be seen from Table 5 that the concentration of ascorbic acid in the liver after injection of 120 mg. dehydroascorbic acid was very high (about 1 mg./g.). When 120 mg. ascorbic acid were injected we found that the ascorbic acid concentration was only about one-half this value. It appears that most if not all the unchanged dehydroascorbic acid which entered the blood stream and which had not mutarotated was reduced in the liver, since none was found in the blood after administration. Therefore when dehydroascorbic acid is injected the concentration of ascorbic acid in the blood is not the

only factor which controls the concentration in the liver.

When dehydroascorbic acid was administered *per os* (Table 6) it passed gradually into the system but part of it remained almost entirely unchanged in the stomach before being absorbed. About 50 % could be recovered as dehydroascorbic acid after 15 min. The dehydroascorbic acid in the stomach decreased with time, by absorption and passage into the small intestine, where, owing to the prevailing alkaline condition, it was converted to diketogulonic acid. No dehydroascorbic acid was found under the conditions of this experiment to reach the caecum, colon or rectum.

When diketogulonic acid was given *per os* (Table 8) it was found that most of the dose passed from the stomach along the intestine and no doubt would eventually have been excreted in the faeces either unchanged or as decomposition products. Since small quantities were found in the tissues of the digestive tract and in the urine, it may be presumed that minute quantities had passed into the blood stream although the presence of diketogulonic acid could not be demonstrated in the plasma. This may be explained by the fact that the little that entered the blood was excreted by the kidneys as fast as it passed through the intestinal tissues. Furthermore, as will be seen later, diketogulonic acid undergoes chemical change in the animal tissues. When the compound was injected the greater part was found in the 'carcass', skin and plasma (Table 7). Significant increases were also noted in the kidneys. 15 min. after injection the total amount found in the body of the animal was about 70 mg.; this quantity decreased with time and after 60 min. the amount recovered was about 30 mg., up to 15 mg. of which was found in the urine. No doubt diketogulonic acid is changed chemically in the body, as it is *in vitro* at pH 7.4 at 38° (cf. Fig. 9). Whether the compound was administered orally or by injection, no evidence was obtained indicating any significant lactonization of diketogulonic acid. Doubtful traces of ascorbic acid have been observed in some of the tissues and in the urine after the administration of diketogulonic acid. Their presence could be explained by the fact that mutarotated solutions were used, and these are known to yield small quantities of ascorbic acid when reduced by H₂S. Even if these minute amounts were derived from diketogulonic acid very large quantities of this open-chain compound would have to be administered in order to exert any antiscorbutic effect. It is therefore not surprising that Hirst & Zilva [1933] and Borsook *et al.* [1937] had observed no antiscorbutic effect when employing small doses of mutarotated dehydroascorbic acid.

Consideration of the total recovery of ascorbic acid and dehydroascorbic acid only after the ad-

ministration of dehydroascorbic acid shows that a great part of the dose is not accounted for. This, as can now be seen, is due to the fact that considerable quantities of dehydroascorbic acid are converted to diketogulonic acid. The *in vitro* experiments (cf. Fig. 9) explain the reason for this rapid conversion. At pH 7.4 and 38° the conversion of a great part of the dehydroascorbic acid into diketogulonic acid can take place within the first few minutes. As already mentioned it was observed in the preliminary experiments which led to this investigation that whilst most of the ascorbic acid could be accounted for in the body soon after injection this was not the case when dehydroascorbic acid was injected, and it was then suspected that part of the latter was converted into diketogulonic acid. This hypothesis is now fully supported by experimental evidence.

DISCUSSION

The main object of this investigation was to obtain experimental evidence which might offer some support to the hypothesis concerning the mode of action of ascorbic acid as outlined in the introduction. It was assumed that ascorbic acid was a link in a co-ordinated chain of reactions, in which it was alternately oxidized to dehydroascorbic acid and reduced to its original form. The presence of the reversibly oxidized product in minute quantities as a component available for further interaction was postulated accordingly. It is of interest now to consider how our results can be co-ordinated with this hypothesis.

Our *in vitro* and *in vivo* experiments have shown that dehydroascorbic acid tends to be converted to diketogulonic acid with comparative ease both in the presence and in the absence of O₂, the rate being influenced by the pH of the medium. On the above hypothesis therefore the reactions would proceed according to the following scheme:

Our present evidence and that of Hirst & Zilva [1933] and of Borsook *et al.* [1937] does not favour the view that diketogulonic acid can be reconverted to ascorbic acid in the body. This is accomplished *in vitro* only by such drastic methods as treatment with HI [Herbert *et al.* 1933]. It is, however, considered that the concentration of dehydroascorbic acid must at any given time in the above reaction be extremely low, and in consequence the diminution in the quantity of the ascorbic acid concerned through the conversion of dehydroascorbic acid into diketogulonic acid would be very slow. This may account for the long time that elapses before the appearance of scurvy. In animals not capable of synthesizing the vitamin and at the same time not receiving any in the diet, the total content of the ascorbic acid of the body, after the tissues have

been depleted, is very small, yet a considerable time has to elapse after the depletion before even latent scurvy can be diagnosed. The daily ascorbic acid lost during this period must therefore be so minute as to fit in with the requirements of the above hypothesis. Admittedly our results indicate no more than a bare possibility of the existence of the scheme outlined above, but they do in our opinion definitely indicate that possibility.

Another point of theoretical interest deserves mention. It was observed that when large doses of dehydroascorbic acid were given orally an increase in ascorbic acid in the tissues of the stomach was found, thus showing that dehydroascorbic acid was reduced there while passing into the blood stream. This observation offers an explanation for the economic utilization of small doses of dehydroascorbic acid. Demole [1933] and Hirst & Zilva [1933] found that dehydroascorbic acid was somewhat less active than ascorbic acid. The latter workers compared the biological response of guinea-pigs on a scorbutic diet receiving a daily oral dose of 0.25, 0.5 and 1 mg. dehydroascorbic acid with that of guinea-pigs receiving similar doses of ascorbic acid. The response of the former group was only very slightly less than that of the latter, and this was noticeable only in the two lower doses. Our present experiments suggest that doses of dehydroascorbic acid of the above order would pass quickly through the stomach wall where they would be converted into ascorbic acid. Dehydroascorbic acid in small doses would therefore begin functioning as ascorbic acid within a short time and any loss through conversion to diketogulonic acid would in consequence be very small. Large doses of dehydroascorbic acid, on the other hand, might be delayed in the stomach or in the small intestine before passing into the blood stream and would thus incur a proportionately higher loss through mutarotation.

The observations made in connexion with the parenteral introduction of dehydroascorbic acid and diketogulonic acid have, as far as we can see at present, no bearing on practical nutrition. The oral consumption of dehydroascorbic acid and even of diketogulonic acid is in all probability encountered in practice. It may occur when the tissues of vegetables, particularly those containing oxidizing enzymes, are disintegrated before being eaten. If large quantities are consumed the dehydroascorbic acid as we have seen may remain for some time in the stomach and in the small intestine before passing into the blood stream. The destruction of the antiscorbutic potency in the case of dehydroascorbic acid would be rather higher than that of ascorbic acid under similar conditions, but since this applies to quantities of vitamin C which are far greater than the physiological requirement it remains a point of theoretical interest only.

SUMMARY

(a) *In vitro experiments*

1. Diketogulonic acid in the presence of HCl forms a derivative with dinitrophenylhydrazine. When ascorbic acid and dehydroascorbic acid are present they are first converted to diketogulonic acid which then reacts with the reagent.

2. The percentage of dehydroascorbic acid which is converted to diketogulonic acid in a given time in the presence of mineral acids is independent of the initial concentration of the former and proceeds as a reaction of the first order at any pH value below about pH 4 investigated by us. The conversion is accelerated by increase in temperature, the temperature coefficient k_{35}/k_{25} being 2.2. At pH values higher or lower than about pH 4 the rate of conversion is accelerated.

3. Diketogulonic acid undergoes a chemical change in the presence of H_2S . At pH 7.4 the rate of formation of diketogulonic acid from dehydroascorbic acid, as measured by the colour reaction, is high and its decomposition proceeds at a considerable rate. This is independent of the presence of air.

The indirect determination of diketogulonic acid by H_2S treatment is not reliable under all conditions.

(b) *In vivo experiments*

1. When diketogulonic acid was injected intramuscularly into guinea-pigs which had previously received a scorbutic diet for 5 days, about 60% of the dose was recovered after 15 min., mainly from the 'carcass', skin and plasma. After 1 hr. only about 30%, of which as much as one-third was

present in the urine, was recovered. It was evidently destroyed in the animal body.

2. When diketogulonic acid was administered orally only small quantities, if any, entered the blood. The greater part of the dose passed from the stomach along the intestine and some no doubt was excreted in the faeces.

3. When dehydroascorbic acid was injected it was partly converted into diketogulonic acid and partly reduced to ascorbic acid, some of which filled the tissues of the unsaturated guinea-pigs and some was excreted by the kidney. The liver contained abnormally high quantities of ascorbic acid. It is suggested that when large quantities of dehydroascorbic acid are introduced into the blood stream reduction takes place mainly in the liver.

4. When dehydroascorbic acid was administered *per os* it was gradually absorbed, but a considerable part of the dose remained at first (15–30 min.) unchanged in the stomach. In the small intestine, owing to alkalinity, it was converted to diketogulonic acid.

5. When 120 mg. diketogulonic acid were injected, about 25 mg. were excreted in 24 hr. The greater part of this passed through the kidney during the first 4 hr. Injected dehydroascorbic acid (120 mg.) was similarly excreted by the kidney as diketogulonic acid, in almost the same quantities.

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