chlorinated metabolic product, while growth on media devoid of chloride in no way assisted the isolation of the red component. Variation of the KCl concentration showed that its elimination from the medium was quite regular after the initial period when the sugar metabolism was exceptionally rapid. Increased chloride concentration had no effect on the production of sclerotiorine and only affected the chlorine content of the other products of extraction

of the mycelium. It is suggested that the pigment is a metabolic by-product rather than the fundamental end-product of the chloride metabolism. The activating effects of yeast extract was no more than equivalent to the effect of increased KCl concentration.

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The Determination of 2:3-Diketo-*l*-gulonic Acid

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The first stage of the oxidation of *l*-ascorbic acid is of particular interest to the biochemist. The product, dehydro-*l*-ascorbic acid, retains its lactone ring and can be reduced to *l*-ascorbic acid by mild reagents such as H_2S , etc., in vitro, and can also be regenerated in vivo almost quantitatively. In aqueous solutions, dehydro-*l*-ascorbic acid undergoes mutarotation which is generally assumed to be due to the gradual opening of its lactone ring with the formation of 2:3-diketo-*l*-gulonic acid. This latter compound can still be converted into *l*-ascorbic acid by lactonization and reduction when treated with HI in vitro [Herbert, Hirst, Percival, Reynolds & Smith, 1933]. Regeneration apparently does not occur in vivo, since little if any antiscorbutic activity has been observed in solutions of dehydro-*l*-ascorbic acid which have undergone mutarotation for some time [Hirst & Zilva, 1933; Borsook, Davenport, Jeffreys & Warner, 1937].

Whilst dehydroascorbic acid can easily be determined by titration of the regenerated ascorbic acid after reduction with H_2S , no direct method is available for the determination of diketogulonic acid. In this communication conditions are described under which this compound, which so far has not been isolated, can be determined directly.

Herbert *et al.* [1933] have shown that both ascorbic acid and freshly oxidized ascorbic acid give derivatives with 2:4-dinitrophenylhydrazine of m.p. 282° (decomp.) and 280° (decomp.) respectively. We have found that diketogulonic acid obtained by mutarotation from dehydroascorbic acid also gives with 2:4-dinitrophenylhydrazine a red derivative or derivatives which on solution in NaOH produce an intensely red colour. This colour reaction forms the foundation on which the procedure for the direct determination of the diketogulonic acid is based.

2:4-Dinitrophenylhydrazine reacts also with phenylpyruvic acid [Penrose & Quastel, 1937] and with other α -keto acids [Lu, 1939; Sealock & Silberstein, 1940; Sealock & Scherp, 1941], and consequently the reaction cannot be considered specific. In actual practice, however, it has been found that extracts of the tissues of normal guinea-pigs, and untreated human urine give a very low and fairly constant colour with dinitrophenylhydrazine; thus it should be possible to detect the appearance of even small quantities of diketogulonic acid by this reaction. Furthermore, the results suggest that the procedure outlined by us is open to further improve-

ments as regards specificity and sensitivity should the necessity arise.

GENERAL TECHNIQUE

Reagents. (1) 2 g. 2:4-dinitrophenylhydrazine (laboratory reagent) were dissolved with warming in 1 l. 2*N* HCl (AnalaR). The stock solution was filtered before use.

(2) *N* NaOH (AnalaR).

(3) *L*-Ascorbic acid, 99–100% purity. Messrs Roche Products, Ltd.

(4) Dehydro-*L*-ascorbic acid was obtained by oxidizing *L*-ascorbic acid with the theoretical amount of 0.1*N* I_2 (AnalaR). Yield as determined by reduction with H_2S , 95–100%. The solutions were used immediately after preparation.

(5) 2:3-Diketo-*L*-gulonic acid was prepared by allowing a solution of dehydro-*L*-ascorbic acid to mutarotate for 18 days at room temperature without neutralizing the HI formed in the preparation of the dehydroascorbic acid [cf. Herbert *et al.* 1933]. After this time the ascorbic acid regenerated from the solution by reduction with H_2S reached a value varying from 10 to 15% of its original concentration, and this remained approximately constant for at least 10 days. The intensity of the colour given by the solutions with 2:4-dinitrophenylhydrazine increased during mutarotation and also became constant after about 18 days. Diketogulonic acid more than 10 days old was never used in this investigation.

Factors influencing the reaction. The intensity of the colour produced could be increased by raising the quantity of dinitrophenylhydrazine used in the reaction. The amount of the reagent that could be employed was, however, limited by the fact that when certain quantities of the hydrazine were exceeded the addition of the alkali made the reacting solutions turbid. For the range of concentrations of diketogulonic acid used by us the addition of 1 ml. of the hydrazine solution produced a colour of suitable intensity without influencing the transparency of the solution. The use of 2:4-dinitrophenylhydrazine (laboratory reagent) recrystallized from ethyl acetate did not influence the colour reaction in any way. The time and temperature of heating of a mixture of diketogulonic, dehydroascorbic, and ascorbic acids with dinitrophenylhydrazine greatly affected the intensity of the colour; this will be discussed in detail later.

As would be expected the quantity of NaOH which it was necessary to add depended on the quantity of acid present in the reacting solution, but under the above conditions 4 ml. of *N* NaOH amply satisfied the requirement, and an increase to 6 ml. was without influence on the colour production.

After the addition of the *N* NaOH a brown colour developed immediately, which rapidly turned to orange in dilute and red in more concentrated

solutions. As the final colour is unstable it was necessary to measure its intensity at a given time soon after production.

Measurement of colour. The measurement was carried out in a Duboscq type of colorimeter. Owing to the instability of the colour it was, however, more convenient to have recourse to a photometric technique [King & Haslewood, 1937] instead of matching the colour directly. For this purpose a spectrum green colour filter (Ilford no. 604) was introduced into the eyepiece of the instrument and the standard was replaced by neutral density filters against which the absorption was measured. Although a range of these filters was employed all results were calculated for the 0.5 neutral density filter.

Effect of time and temperature on colour production. As already mentioned the time and temperature of heating diketogulonic, dehydroascorbic or ascorbic acids with dinitrophenylhydrazine greatly influenced the intensity of the colour production. This is probably due in part to the changes the last two compounds undergo to yield dinitrophenylhydrazine derivatives similar to, if not identical with, the derivative yielded by diketogulonic acid. The reaction was therefore studied after various periods of heating at 25°, 50° and 75°. To each of a series of tubes containing 9 ml. solution of the individual acids previously brought rapidly to about 2° above the desired temperature, 1 ml. of dinitrophenylhydrazine reagent was added and the tubes placed in the respective thermostats. Those mixtures which were submitted to high temperatures were lightly corked to avoid evaporation. The solutions were then shaken for a short time in order to adjust their temperature to that of the thermostat. At various intervals one of each of the series was withdrawn, cooled rapidly if necessary, treated with the alkali, diluted to 25 ml., and the colour measured as described above.

The results are given in Figs. 1, 2 and 3. On careful examination of these data it appeared to us that the colour produced after 1 hr. at 25° was the most suitable for the quantitative assessment of diketogulonic acid in the presence of the other two acids, for the following reasons. It will be seen from Fig. 1 that the intensity of the colour given by dehydroascorbic acid under these circumstances is only about 8% of that given by its equivalent of diketogulonic acid and that this percentage increased with time. At 50° and 75° (Figs. 2 and 3) on the other hand the reaction of dehydroascorbic acid is increased in 1 hr. to as much as 44% and 81% respectively of that of its equivalent of diketogulonic acid. Furthermore at 25° ascorbic acid even in quantities as high as 2 mg. does not react at all. In addition it might be mentioned that as the reaction proceeded there was a tendency for the formation of a reddish precipitate, especially at the higher temperatures, which was

Fig. 1. Colour production at 25°. $\bigcirc$ — $\bigcirc$ diketogulonic acid. $\times$ — $\times$ dehydroascorbic acid. In this and the following graphs, diketogulonic and dehydroascorbic acids are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

Fig. 2. Colour production at 50°. $\triangle$ — $\triangle$ ascorbic acid. Other symbols are as in Fig. 1.

soluble in *N* NaOH. At 25° the amount of this precipitate was very small. It is also of significance that in all these experiments the colour produced at any time when expressed as a percentage of the maxi-

mum colour obtained at 75° after 300 min., was the same in the two concentrations both for dehydroascorbic acid and diketogulonic acid. This was also found to be true for ascorbic acid when it was possible to obtain significant figures namely, at 75°.

Calibration curve. Calibration curves for diketogulonic acid and dehydroascorbic acid covering a range of 0.05–0.5 mg. in the case of the former and 0.35–0.5 mg. in that of the latter, were constructed from the figures obtained by keeping the reaction mixtures for 1 hr. at 25°. For convenience the diketogulonic acid is expressed as mg. of ascorbic acid from which it was prepared, since an absolute value cannot be obtained owing to the fact that it has not been isolated. These units are plotted against the reciprocal of the reading on the colorimeter scale ($1/R$). The results are the mean of the figures obtained from three different solutions determined in duplicate; the variation of the individual readings from the mean were within the limits of $\pm 3\%$.

As can be seen from the curve (Fig. 4) the relationship between the concentration and the reciprocal colorimeter readings is linear up to about 0.4 mg. of diketogulonic acid; beyond that concentration there is a slight deviation from Beer's Law. Since the intensity of the colour produced by dehydroascorbic acid is only about 1/12 of that produced by diketogulonic acid it was not possible to obtain values lower than 0.35 mg. It is seen that figures for the colour given by diketogulonic acid in pure solution approximating concentra-

tions as low as 1 part in 200,000 can be read off the calibration curve.

The curve does not pass through the origin. This can be explained in two ways. Either some absorp-

Fig. 3. Colour production at 75°. The symbols are as in Fig. 2.

Fig. 4. Calibration curve.

tion is produced by the reagents or Beer's Law is not obeyed in very dilute solution. We favour the former explanation, since some experimental evidence was obtained supporting this view. By measur-

ing the absorption of solutions of KMnO_4 with the spectrum green filter, a linear relationship between $1/R$ and the concentration of the KMnO_4 could be obtained, obeying Beer's Law even in dilute solution, i.e. the curve passing through the origin. When a blank solution containing 1 ml. of dinitrophenylhydrazine and 4 ml. N NaOH in 25 ml. was matched against a suitable dilution of KMnO_4 it was found that the value for the absorption of the concentration of KMnO_4 equivalent to the blank expressed in units of the 0.5 neutral density filter coincided with the zero value obtained from the calibration curve (0.007). All results including those given in Figs. 1, 2 and 3 were therefore corrected for zero displacement.

Procedure. A quantity of the solution containing 0.05–0.5 mg. diketogulonic acid is introduced into a 6×1 in. tube and the volume,

if necessary, is made up to 9 ml. with distilled water. 1 ml. of the dinitrophenylhydrazine reagent is then added and the tube placed in a thermostat at $25^\circ (\pm 0.2^\circ)$ for 1 hr. At the end of this time 4 ml. of N NaOH are added, the solution is transferred to a 25 ml. graduated flask and after 1 min. is made up to volume with the washings and with distilled water. After mixing the contents the colour of the solution is measured in the colorimeter as rapidly as possible (30 sec.) in order to avoid any error due to fading. Six readings are taken which should not deviate by more than $\pm 2\%$ from the mean. The quantity of diketogulonic acid present is then read off the calibration curve.

If dehydroascorbic acid is present it is determined by reduction with H_2S and titration with indophenol and the colorimetric equivalent is obtained from the calibration curve. The calculation is then made as follows:

$$\frac{1}{R_v} = \frac{1}{R} - \frac{1}{R_x} + \frac{1}{R_c}$$

R = Observed colorimetric reading.

R_x = Equivalent colorimetric reading for quantity of dehydroascorbic acid found.

R_c = Colorimetric reading due to blank (zero correction).

R_v = Colorimetric reading for diketogulonic acid.

No correction is necessary for ascorbic acid since no colour is obtained after an hour at 25° with quan-

ties less than 2 mg. and such high concentrations usually fall outside the range.

Table 1 gives the degree of accuracy that can be attained by determining diketogulonic acid in concentrations of about 0.5–5 mg. in 100 ml. solution.

Table 1. *Determination of diketogulonic acid in the presence and in the absence of dehydroascorbic and ascorbic acids*

Diketogulonic acid in the presence of dehydroascorbic and ascorbic acids					
Diketogulonic acid		Dehydro-ascorbic acid		Diketogulonic acid	
		Added	Ascorbic acid		
Added mg./100 ml.	Found mg./100 ml.	mg./100 ml.	mg./100 ml.	Added mg./100 ml.	Found mg./100 ml.
5.5	5.5	3.3	0	4.4	4.4
5.0	5.0	5.5	0	3.3	3.3
4.4	4.4	3.3	0	2.2	2.3
3.8	3.7	5.5	0	1.1	1.1
2.5	2.6	5.5	0	0.55	0.6
2.2	2.3	3.3	22.0	3.3	3.4
1.2	1.1	4.4	22.0	2.2	2.3
1.1	1.1	5.5	22.0	1.1	1.1
0.55	0.5	5.5	22.0	0.55	0.6

In this and in the following tables diketogulonic acid and dehydroascorbic acid are expressed as equivalent quantities in mg. of ascorbic acid from which they were prepared.

Effect of pH on the colour production. In studying the colour production in the presence of buffer solutions it was observed that the intensity of the colour produced by a given concentration of diketogulonic acid varied with the quantity of buffer added. This point is illustrated by the results given in Table 2. It was evident that the reason for the

Table 2. *Effect of buffering capacity on colour production*

Amount buffer, ml. in 9 ml. test solution containing 0.3 mg. diketogulonic acid	1	2	3	4	5
% Colour produced	102	108	113	114	122
Phosphate buffer pH 7.4; 2.72 g. KH_2PO_4 + 15.9 ml. N NaOH in 200 ml. solution					

The colour intensity for 0.3 mg. diketogulonic acid obtained from the calibration curve = 100%.

deviation in the colour production was the pH of the reacting solution after the addition of the dinitrophenylhydrazine. This difficulty can be overcome by adjusting the pH of the test solution to about 2. Table 3 gives the results of an experiment in which diketogulonic acid (0.3 mg./9 ml.) in a number of buffered solutions of various pH's as well as in solutions containing different quantities of HCl, was determined by the above procedure before and after the adjustment of the test solution to pH 2. It is seen that the intensity of the colour produced increased with the rise in the pH of the test solution.

Table 3. *Influence of pH of test solution on colour production*

Buffer or acid added	Quantity ml. in 9 ml. test solution	pH	Colour production %	
			Before adjustment of pH	After adjustment to pH 2
Phosphate-citrate buffer*	2	4.0	105	100
" "	2	5.0	107	101
" "	2	6.0	109	101
" "	2	7.0	117	100
Borate buffer†	2	7.4	115	99
Phthalate buffer‡	2	6.0	107	101
N HCl	0.5	—	94	101
"	1.0	—	88	100
"	2.0	—	74	101

* McIlvaine [1921].

† Clark & Lubs [1916].

‡ Palitzsch [1915].

The colour intensity for 0.3 mg. diketogulonic acid obtained from the calibration curve = 100%

As the results of the preceding experiment (Table 2) show, this difficulty does not hold if the buffering capacity of the test solution is low, since the addition of the phenylhydrazine reagent then brings about a pH similar to that which obtains in the pure diketogulonic acid solution, which is used as the standard for comparison, after the addition of this reagent.

DETERMINATION OF DIKETOGULONIC ACID IN BIOLOGICAL MATERIAL

Blood and plasma. In view of the observations made in the preceding section it became evident that in preparing trichloroacetic acid extracts of tissues for the determination of diketogulonic acid, the quantity of the acid present in the extract would have to be considered. Fortunately it was found that up to 4 ml. of 5% trichloroacetic acid the intensity of the colour was not affected. An equal volume of 10% trichloroacetic acid was therefore added to guinea-pig whole blood or plasma into which a known quantity of diketogulonic acid had been previously introduced. After removing the precipitate by centrifugation, aliquot portions of the

Table 4. *Determination of diketogulonic acid in whole blood and plasma*

Whole blood mg./100 ml.		Plasma mg./100 ml.	
Added	Found	Added	Found
2.5	2.37	2.5	2.4
5.0	5.0	5.0	5.4
10.0	9.5	10.0	10.2
15.0	14.3	15.0	16.0
20.0	20.4	25.0	24.0
33.0	32.5	34.0	35.0
40.0	39.2	50.0	49.5
66.0	64.0	67.0	68.0
100	101	80.0	82.0
200	196	100	100

extract, not exceeding 4 ml. were submitted to the procedure described above. As the extracts of the whole blood or of the plasma gave a small but significant colour (equivalent to about 2 mg./100 ml.) with dinitrophenylhydrazine, it was necessary to determine the intensity of this colour in a control experiment, so that a correction could be made. Table 4 gives 10 representative determinations. The standard error of percentage deviation in concentrations from 2.5 to 200 mg./100 ml. of blood or plasma was found by us to be 3½ %.

Liver, kidney and muscle. The tissues obtained from normal guinea-pigs, the added diketogulonic acid and 2 parts by weight of 10 % trichloroacetic acid were ground with sand in a mortar, squeezed through muslin, reground, and centrifuged in the usual way. The sensitivity of the determination in this case was conditioned by the fact that the use of too much extract produced turbidity after the addition of the alkali. We nevertheless found that it

was possible to determine 0.15 mg./g. of tissue with a standard error of percentage deviation of 4½ % after correcting for the blank. Results chosen at random are given in Table 5. The approximate equivalents of diketogulonic acid yielded by the blanks were, muscle 0.1 mg./g.; kidney 0.15 mg./g.; liver 0.2 mg./g.

Urine. The diketogulonic acid was added to freshly voided human urine and the determination was carried out directly on the urine without previous extraction. When necessary a correction was made for the blank. This was never higher than the equivalent of 5 mg. of diketogulonic acid per 100 ml. of urine. Table 6 gives some results obtained with

Table 5. *Determination of diketogulonic acid in liver, muscle and kidney*

Liver mg./g.		Muscle mg./g.		Kidney mg./g.	
Added	Found	Added	Found	Added	Found
0.15	0.14	0.15	0.15	0.15	0.16
0.20	0.21	0.20	0.21	0.20	0.21
0.25	0.26	0.25	0.26	0.25	0.23
0.50	0.51	0.50	0.52	1.00	1.04
0.75	0.75	0.65	0.64	1.00	0.98
1.00	1.02	1.00	0.95	1.50	1.56
1.00	0.98	1.00	1.04	2.00	1.98
1.25	1.30	1.25	1.30	2.00	2.09
1.50	1.60	1.50	1.52	3.00	3.00
1.50	1.60	2.00	1.94	3.00	3.15

Table 6. *Determination of diketogulonic acid in urine*

Added, mg./100 ml.	2.5	5.0	10.0	10.0	15.0
Found, mg./100 ml.	2.5	5.0	10.2	9.6	15.2
Added, mg./100 ml.	20.0	20.0	25.0	30.0	40.0
Found, mg./100 ml.	20.0	19.4	24.7	29.0	40.0

concentrations 2.5–40 mg./100 ml. of urine. The standard error of percentage deviation of all the urine determinations carried out by us was 3 %.

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Urea as a Partial Protein Substitute in the Feeding of Dairy Cattle

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The belief that certain simple nitrogenous compounds such as urea are capable of supplying to the ruminant a significant proportion of its nitrogen requirement is many years old. The theory most commonly advanced to account for the utilization of

these simple compounds is that they are built up into protein by certain micro-organisms of the rumen which use them as a source of nitrogen for their own multiplication. The protein so formed is subsequently broken down in the small intestine to