Obituary Notice

H. P. MARKS, 1894–1944

H. P. Marks, whose death occurred on 13 September 1944 after a short illness, had been a member of the Biochemical Society since 1921. After being trained as a chemist and serving in the Navy during the last war Marks became an attached worker at the National Institute for Medical Research (Medical Research Council) in 1922, and was appointed a full member of the staff in 1927. His move to Hampstead occurred at the time when interest in insulin was rapidly growing and Marks quickly became concerned with research work on the various aspects of the action of insulin, including its biological assay, and his work from that time on was mainly concerned with carbohydrate metabolism. During the last 10 years he had become interested in the influence of the pituitary gland on carbohydrate metabolism, and had published a number of papers on this aspect of the subject. For the two years immediately before his death he had been concerned with research work of a rather exacting nature on matters of national importance, and had not spared himself in the exhausting experiments which he had undertaken. His unexpected death was a great shock to his colleagues and friends, since he had been working with full enthusiasm until immediately before his final illness.

Among his many interests Marks was a microchemist of some standing and had visited Graz in 1925 to take a special course in this subject. His work on the standardization of insulin has been of the utmost importance in the provision of acceptable International Standard preparations of this important substance, and his interest in biological standardization in this connexion led Marks to make some useful contributions on the general question of biological assay.

Marks was rather shy and was not so well known among the members of the Biochemical Society as might have been expected from his scientific standing and experience, but his easy good nature led him to make friends of all those with whom he associated in his work and in other activities. During the present war his determination to contribute to the defence of the country led him to become both an Air Raid Warden and a member of the Home Guard, an undertaking that would have taxed the strength of a much younger man.

He leaves a widow and a young daughter, to whom the sympathy of the many friends and colleagues of 'H. P.' will go out in the present troubled times.

F. G. YOUNG

The Isolation of Barium and Calcium Diketo-*l*-gulonates and the Biological Significance of 2:3-Diketo-*l*-gulonic Acid

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To explain their observation that dehydro-*l*-ascorbic acid mutarotates in aqueous solution, Herbert, Hirst, Percival, Reynolds & Smith (1933) postulated the formation of 2:3-diketo-*l*-gulonic acid as a result of the gradual opening of the lactone ring. Their subsequent work and that of others have fully supported this theory, although Ghosh & Rakshit (1938) and Rosenfeld (1943) have suggested an alternative mechanism.

An outstanding characteristic of mutarotated solutions is that they apparently afford no anti-scorbutic protection to guinea-pigs, although *l*-ascorbic acid can be regenerated from the solutions by reduction with HI. In other words, the indication is that the ring closure effected *in vitro* does not take place *in vivo*. The use of mutarotated solutions in these biological experiments introduced some degree of uncertainty and it was obviously desirable to employ the pure substance which has hitherto not been isolated. Another point of interest is that when mutarotated solutions are reduced with H_2S an indophenol-reducing substance is produced which has been generally assumed to consist entirely of *l*-ascorbic acid derived from the residue of uncon-

verted dehydro-*l*-ascorbic acid. Although this assumption was made in determining diketo-*l*-gulonic acid indirectly, it was never demonstrated that the indophenol reduction was due to *l*-ascorbic acid alone. This point, too, could not be cleared up conveniently until diketo-*l*-gulonic acid was actually isolated.

This communication deals with the isolation of the barium and calcium salts of diketo-*l*-gulonic acid and with biological experiments which, in our opinion, settle in a satisfactory fashion the points raised above. Owing to its instability the free acid has not yet been obtained by us from the salts.

EXPERIMENTAL

Preparation of mutarotated solutions of dehydroascorbic acid

The following methods were adopted:

(1) 0·88 g. of ascorbic acid was oxidized with the theoretical quantity of N-KIO_3 (10 ml.). 5 ml. of N-NaOH were then added slowly in order to convert the dehydroascorbic acid into its mutarotated product. The resulting solution was slightly yellow but became colourless when adjusted to pH 3 with HI.

(2) 0·88 g. of ascorbic acid was oxidized with the theoretical amount of 0·5 N-I_2, $KHCO_3$ being added to adjust the pH to 1. It was found that the oxidation was not complete until some of the HI produced had been neutralized. After mutarotation for 4 days at room temperature, the pH of the solution was raised to about 3 by the further addition of bicarbonate.

Preparation of barium diketogulonate

0·8 g. of BaI_2 (approximately 80 % of the theoretical) was added to each of the above mutarotated solutions. Ethanol was then added until its final concentration was 80 % by volume. The flocculent white precipitate was collected after cooling to 5°, washed thoroughly with absolute ethanol at 5°, and finally with cooled ether. The adhering ether was allowed to evaporate at room temperature. Yield = 60 %. (From mutarotated solutions prepared by method (1)—found: C, 21·8; H, 4·4; Ba, 20·7 %. From mutarotated solutions prepared by method (2)—found: C, 21·7; H, 4·5; Ba, 20·9 %. Ba diketogulonate ($C_{12}H_{14}O_{14}Ba.8H_2O$) requires: C, 21·7; H, 4·5; Ba, 20·7 %.) The salt was kept at 0°; when allowed to stand at *c.* 20° in air, in N_2 or *in vacuo*, the white substance turned yellow, the change in colour being accelerated by a slight rise in temperature. At −20°, however, no discoloration developed in 14 days.

Oxidation with I_2 *in alkaline solution.* When I_2 in alkaline solution is added to diketogulonic acid the carbon chain is ruptured with the formation of oxalic and *l*-threonic acids (Herbert *et al.* 1933). The I_2 used in the oxidation of the isolated Ba salt, the amount of total acid and the quantity of oxalic acid formed were estimated as follows.

A known quantity of the salt (about 0·1 g.) was dissolved in water and 20 ml. of 0·1 N-I_2 added, followed by 25 ml. of 0·1 N-NaOH. After standing for 15 min., the solution was acidified with 20 ml. of 0·1 N-HCl and the excess I_2 back-titrated with 0·1 N-$Na_2S_2O_3$. The I_2 used in the oxidation was thus obtained by difference. The amount of acid produced was determined by titrating the resulting solution with 0·1 N-NaOH. Oxalic acid formed as the result of the oxidation was estimated by precipitation as calcium oxalate and by subsequent titration with $KMnO_4$. 0·166 g. of the Ba salt required 9·9 ml. 0·1 N-I_2 for oxidation into oxalic and threonic acids and 19·75 ml. 0·1 N-NaOH to neutralize the acids formed. Calc. for $C_{12}H_{14}O_{14}Ba.8H_2O$: 10·05 ml. 0·1 N-$I_2$ and 20·1 ml. 0·1 N-NaOH. Oxalic acid produced: 94 % of theoretical.

Colorimetric estimation with 2:4-*dinitrophenylhydrazine.* The salt was assayed for diketogulonic acid by the method of Penney & Zilva (1943*a*). Amount present (calc. on above formula for Ba salt), 0·50 mg.; found, 0·49 mg.

Reaction with HI. 0·8 g. of the Ba salt was treated with the calculated quantity of H_2SO_4 and the $BaSO_4$ removed by filtration. The filtrate was evaporated with 2·5 ml. of 2 N-HI. To promote rapid evaporation, the solution was spread over a large glass plate in a very thin film. A current of warm air was directed over the surface of the plate and when evaporation was complete, the brown residue, washed in turn with chloroform, acetone and ethanol, gave ascorbic acid as a buff-coloured powder (purity = 97 % by indophenol titration). Yield = 35 %, m.p. 188° (decomp.). Mixed m.p. with authentic sample of ascorbic acid 190° (decomp.). $[\alpha]_D^{18°}$ in water = +22° (c = 0·305).

Preparation of the 2:4-*dinitrophenylosazone.* The 2:4-dinitrophenylosazone was prepared as previously described (Penney & Zilva, 1943*b*). Crude yield = 80 %, m.p. 280° (decomp.). Mixed m.p. with derivative prepared from mutarotated solution unchanged. (Found: N, 20·9 %; $C_{18}H_{14}O_{12}N_8$ requires 21·0 %.)

Specific rotation of the Ba *salt in* 0·1 N-HCl. $[\alpha]_D^{22°} = -6·8°$ (c = 1·00), whence $[\alpha]_D^{22°}$ for diketogulonic acid in 0·1 N-HCl = −11·8°.

Preparation of calcium diketogulonate

The procedure was similar to that adopted in the preparation of the Ba compound. The Ca salt was precipitated with CaI_2 and ethanol from a mutarotated solution prepared by method 1 (above). Yield = 50 %. (Found: C, 25·7; H, 5·1; Ca, 7·2 %. $C_{12}H_{14}O_{14}Ca.8H_2O$ requires: C, 25·5; H, 5·3; Ca, 7·1 %.)

Oxidation with I_2 *in alkaline solution.* 0·105 g. of the Ca salt required 7·45 ml. 0·1 N-I_2 for oxidation to oxalic and threonic acids and 14·6 ml. 0·1 N-NaOH to neutralize the acids formed. Calc. for $C_{12}H_{14}O_{14}Ca.8H_2O$: 7·5 ml. 0·1 N-$I_2$ and 15·0 ml. 0·1 N-NaOH.

Colorimetric estimation with 2:4-*dinitrophenylhydrazine.* Amount present (calc. on above formula for Ca salt), 0·45 mg.; found, 0·44 mg.

Preparation of the 2:4-*dinitrophenylosazone.* Crude yield, 75 %, m.p. 280° (decomp.). Mixed m.p. with derivative prepared directly from mutarotated solution, unchanged.

Specific rotation in 0·1 N-HCl. $[\alpha]_D^{21°} = -8·3$ (c = 1·00), whence $[\alpha]_D^{21°}$ for diketogulonic acid in 0·1 N-HCl = −12·1°.

BIOLOGICAL EXPERIMENTS

Antiscorbutic activity of calcium diketogulonate and regenerated l-*ascorbic acid*

In testing the possible biological activity of diketogulonic acid, the Ca salt was employed; it was prepared twice weekly and stored at −20°. Colorimetric estimations showed that

under these conditions of storage the substance was stable for at least 7 days. Regenerated *l*-ascorbic acid was prepared as described above, the bulk sample being at least 90% pure according to the indophenol titration.

The biological tests were carried out by the technique usually employed in this laboratory (Kellie & Zilva, 1941) except that the milk reconstituted from the commercial dried powder was not autoclaved but was dissolved in boiling water and aerated for 5 min. 60 ml. of this were reducing compound corresponding to a yield of about 4% calculated as ascorbic acid, this dose would be approximately equivalent to 0·5 mg. ascorbic acid. Such a daily dose of the vitamin affords a high degree of protection against scurvy.

(3) Regenerated *l*-ascorbic acid equivalent to 0·5 mg. of authentic *l*-ascorbic acid was administered daily as 2·5 ml. of a solution of 11 mg. regenerated ascorbic acid in 50 ml. of water.

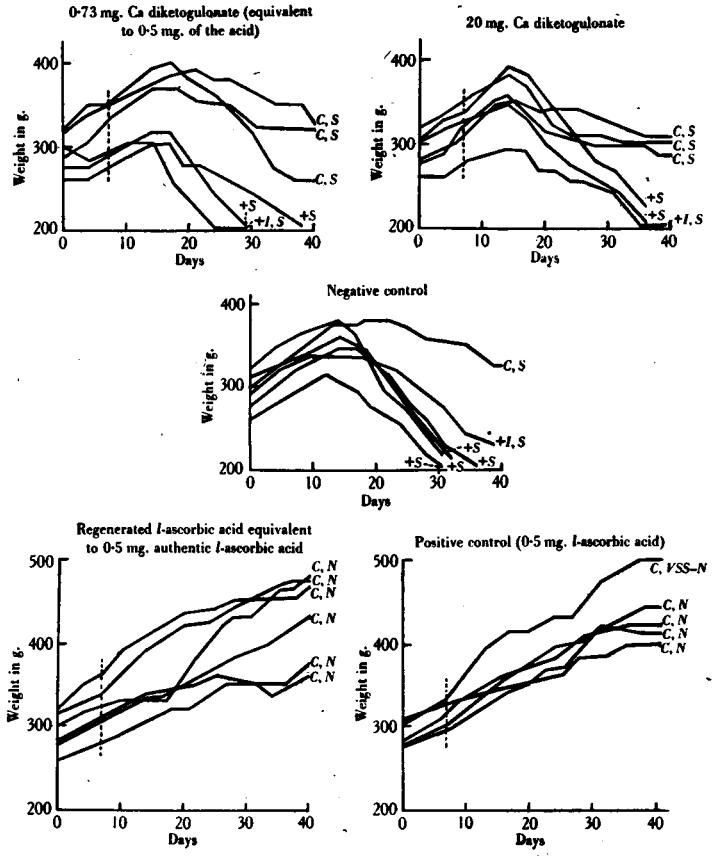

Fig. 1. Antiscorbutic and growth-promoting effects of diketogulonic acid and related substances. *C* = killed by chloroform; + = died; *N* = normal; *VSS* = very slight scurvy; *S* = scurvy; *I* = intercurrent disease; commencement of administration of dose is indicated by a vertical dotted line.

offered to each guinea-pig daily. The various doses prepared immediately before use were administered in the following way:

(1) An equivalent of 0·5 mg. diketogulonic acid was administered daily to each animal as 2·5 ml. of a solution containing 14·5 mg. Ca diketogulonate in 50 ml., the final volume including 5 ml. M/100 citric acid to adjust the pH to approximately 4. This small dose, to be effective, would require almost complete reduction *in vivo*, which is improbable.

(2) 160 mg. Ca diketogulonate were dissolved in 20 ml. of water containing 2 ml. M/100 citric acid (pH = *c.* 4) and 2·5 ml. of this solution administered daily. Since Ca diketogulonate on treatment with H_2S yields an indophenol-

(4) 10 mg. *l*-ascorbic acid were dissolved in 50 ml. of water and 2·5 ml. (0·5 mg. *l*-ascorbic acid) were administered daily to each of the animals in the positive control group.

Interpretation of results. The results are given in Fig. 1. At the outset attention should be drawn to the behaviour of the animals in the negative control group. It will be seen that instead of succumbing to scurvy within about 30 days, as animals on our scorbutic diet usually do, these animals, although suffering from acute scurvy, lived a little longer. This was found to be due to traces of vitamin C in the commercial milk powder which were not entirely inactivated by the aeration of the hot solution. The presence of these minute quantities of the vitamin in the basal diet did not, however, vitiate the experiment. The groups of

animals receiving doses of calcium diketogulonate equivalent to 0·5 mg. of diketogulonic acid and to 0·5 mg. of *l*-ascorbic acid respectively behaved in the same way as the animals in the negative control group. It is therefore evident that the isolated salt of diketogulonic acid, like its equivalent of the mutarotated solution, cannot be reduced in the body; or if it is reduced, as it is to some extent by H_2S *in vitro*, the indophenol-reducing substance produced is not identical with *l*-ascorbic acid, as has hitherto been supposed. That Ba diketogulonate, like the mutarotated solution of dehydroascorbic acid, can be regenerated into fully active *l*-ascorbic acid in good yield on reduction with HI, will be seen from the similarity of the biological response of the experimental guinea-pigs to the regenerated compound with that of the animals to ordinary synthetic *l*-ascorbic acid.

CONCLUSIONS

The properties of the isolated Ba and Ca salts of diketo-*l*-gulonic acid establish the correctness of the view of Herbert *et al.* (1933) that the mutarotation observed in acid solution of dehydro-*l*-ascorbic acid is due to the gradual formation of diketo-*l*-gulonic acid. The analytical data obtained show that eight molecules of water are associated with each molecule of the salts. The white colour of the compounds and their tendency to turn yellow on storage suggest that four of the molecules of water may be accounted for by hydration of the keto-groups.

The biological experiments show beyond doubt that ring closure with the production of *l*-ascorbic acid does not take place *in vivo*; the mechanism involved in its formation *in vitro* by treatment with HI is obscure. It would, however, appear that ring closure may precede reduction, since we found that 2-keto-*l*-gulonic acid, a probable reduction product of diketo-*l*-gulonic acid, which can be converted to *l*-ascorbic acid by the action of HCl (Reichstein & Grüssner, 1934), could not be lactonized by HI by the procedure employed by us in the case of diketo-*l*-gulonic acid. Several other points of interest arise from the biological tests. 20 mg. Ca diketogulonate, which on reduction with H_2S yields an indophenol-reducing substance equivalent to 0·5 mg. of *l*-ascorbic acid, failed to offer any antiscorbutic protection. In this connexion, it is of interest to note that when the reduced Ca diketogulonate solutions were analyzed by Lugg's formalin method (1942) as used in this laboratory (Snow & Zilva, 1943, 1944), data were obtained which indicated that about half of the indophenol-reducing capacity was due to *l*-ascorbic acid. A dose of 20 mg. of the Ca salt would according to this be equivalent to 0·25 mg. *l*-ascorbic acid, which is known to offer considerable antiscorbutic protection. That this was not so is seen from the fact that no response was observed in the group of guinea-pigs receiving this dose. From all these observations it is therefore difficult to decide whether the animal organism is incapable of reducing diketogulonic acid or whether an indophenol-reducing substance not identical with *l*-ascorbic acid is formed on reduction *in vivo*.

In conclusion, mention must be made of the alternative view on the chemical changes taking place during the mutarotation of dehydro-*l*-ascorbic acid advanced by Ghosh & Rakshit (1938) and extended by Rosenfeld (1943). The latter worker maintains that dehydro-*l*-ascorbic acid does not change initially into diketo-*l*-gulonic acid but into an intramolecular stabilization product having an enol lactone structure. Our experimental evidence does not accord with such a hypothesis.

SUMMARY

1. The Ba and Ca salts of 2:3-diketo-*l*-gulonic acid have been isolated and characterized.

2. The Ca salt offered no antiscorbutic protection to guinea-pigs in daily doses equivalent to 0·5 mg. of 2:3-diketo-*l*-gulonic acid.

3. A daily dose of 20 mg. of the Ca salt which, according to direct indophenol titration, yields an equivalent of 0·5 mg. of *l*-ascorbic acid after reduction with H_2S, also failed to offer any antiscorbutic protection to guinea-pigs.

4. *l*-Ascorbic acid, regenerated from Ba diketo-*l*-gulonate by reduction with HI, was chemically and biologically identical with authentic *l*-ascorbic acid.

One of us (J. R. P.) is indebted to the Medical Research Council for a whole-time grant. We wish to express our thanks to the late Mr W. F. Boston, who, with the kind permission of Prof. I. M. Heilbron, carried out the microanalyses. Our thanks are also due to Dr F. Bergel of Messrs Roche Products, Ltd. for gifts of 2-keto-*l*-gulonic and *l*-ascorbic acids.

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