THE OXIDATION OF CITRAMALIC ACID AND β-HYDROXYGLUTARIC ACID TO ACETOACETIC ACID AND THE POSSIBLE SIGNIFICANCE OF CITRAMALIC ACID IN PLANT METABOLISM

by

A. C. HULME

Department of Scientific and Industrial Research, Food Investigation, Ditton Laboratory, East Malling, Maidstone (England)

An acid isolated from the peel of apple fruits was found to have the formula $C_3H_6O_3(COOH)_2$ and to contain one hydroxy group. In the process of identifying this acid its oxidation was studied as well as that of the oxidation of synthetic citramalic acid and β-hydroxyglutaric acid. The oxidation of an (OH) group was expected in each case to produce the corresponding keto acid, acetoacetic acid from citramalic acid, acetonedicarboxylic acid from β-hydroxyglutaric acid. Since both these keto acids are very unstable it was decided to oxidise the parent acid and immediately condense the resulting keto acid with 2.4-dinitrophenylhydrazine in the hope that at least some 2.4-dinitrophenylhydrazone might be obtained undecomposed for characterisation by melting point, paper chromatography, light absorption spectra etc. The ultimate object was to use such data to establish the identity of the "unknown" isomer from the apple peel.

The results obtained proved conclusively that both DL-citramalic acid and β-hydroxyglutaric acid as well as the acid from apple peel yielded, on oxidation (isolated as the 2.4-dinitrophenylhydrazone) acetoacetic acid with the probable intermediate formation in the case of β-hydroxyglutaric acid of β-ketoglutaric acid. During the process there was always considerable further breakdown to acetone.

Similarly, although on oxidation citric acid should yield acetone dicarboxylic acid, the main keto acid recovered (as the 2.4-dinitrophenylhydrazone) was acetoacetic acid.

EXPERIMENTAL

The method of isolation, in a pure form, of L-citramalic acid from the peel of apples and the method used to synthesise DL-citramalic and β-hydroxyglutaric acids have already been described (HULME[1]).

Chromatographic methods. All paper chromatograms were run by downward displacement in glass tanks with greased, well fitting lids, the tanks being situated in a room kept at a constant temperature of 20° C (± 0.1°). The solvent system used was *n*-butanol 70 parts, ethanol 10 parts, 0.5 *N* NH_4OH 20 parts (EL HAWARY AND THOMPSON[2]). Whatman No. 3 MM filter paper was used throughout. After running, the chromatograms were dried in a current of air at room temperature. The 2.4-dinitrophenylhydrazones travel as clearly visible yellow spots but to obtain a good photographic reproduction of the completed chromatogram it was found advantageous to spray with *N* NaOH. This causes the yellow spots to turn deep brown due to the property the nitrophenylhydrazones possess of forming deeply coloured water-soluble salts in NaOH (see later). The colour change on the paper is transitory so that photographs must be taken immediately after spraying. When chromatograms were to be used for the elution of the spots for subsequent examination this spraying

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technique was, of course, omitted. For subsequent examination of the various spots appearing on the completed chromatograms of the nitrophenylhydrazones, these yellow spots were eluted with 10% Na_2CO_3 by the method described by EL HAWARY AND THOMPSON[2].

Acetone dicarboxylic acid (A.D.A.) and acetoacetic acid (A.A.). The first named acid was obtained by the oxidation of citric acid (v. PECHMANN[3]); acetoacetic acid was prepared by hydrolysis of commercial ethyl acetoacetate (PLIMMER[4]).

Preparation of 2.4-dinitrophenylhydrazones from acetone dicarboxylic acid and acetoacetic acid. Several methods for the preparation of nitrophenylhydrazones of keto acids appear in the literature but preliminary experiments showed that the method giving minimal decomposition in the present instances was that used by CLIFT AND COOK[5] with the following modifications: (1) All initial preparative work was carried out at 1° C. (2) After drying the recrystallised phenylhydrazones *in vacuo*, they were extracted with a minimum of cold ether and then centrifuged off at 1° C. They were then redried *in vacuo*. This treatment removes traces of acetone formed during the preparation.

Analysis of the 2.4-dinitrophenylhydrazones prepared from A.D.A. and A.A. Nitrogen content (Weiler and Strauss, Oxford). A.D.A. compound: found 18.5; calc. for the pure compound, 17.2%. A.A. compound: found 20.2; calc. for the pure compound, 19.9%. M.p. (corrected); A.D.A. compound, 126–126.5° C; A.A. compound, 126–126.5. The mixed m.p. of the two compounds was 126.5° C. Acetone 2.4-dinitrophenylhydrazone had a m.p. of 127.5; admixed with either the A.D.A. or the A.A. compound the m.p. was 126.5–127° C. Micro titrations of the 2.4-dinitrophenylhydrazones obtained from A.D.A. and A.A. using the technique of CLIFT AND COOK[5] gave the results shown in Table I:

TABLE I

MICRO TITRATIONS OF 2.4-DINITROPHENYLHYDRAZONES

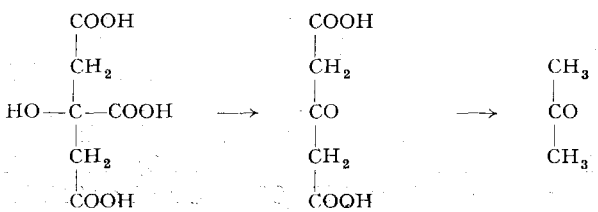
	Weight taken (mg)	*ml M/70 NaOH*		*Calc. as A.A.*
		Found	*Calc. as A.D.A.*	
A.D.A.	5.14	1.30	2.21	1.28
A.A.	4.28	1.02	—	1.06

Oxidation of citric acid, β-hydroxyglutaric acid and citramalic acid followed by the immediate condensation of the resulting keto acids with 2.4-dinitrophenylhydrazine. A few milligrams of each of these acids were heated with 0.5 ml of conc. H_2SO_4 in a small test tube until a yellow colour just appeared. The tube was then rapidly cooled in ice and 2 *N* NaOH added until the solution was just alkaline. The liquid was then transferred to a small separating funnel and 1 ml of a saturated solution of 2.4-dinitrophenylhydrazone in 2 *N* HCl added. The funnel was shaken and left to stand for 5 mins. The hydrazone formed was then extracted with ethyl acetate and the water layer run off. The ethyl acetate extract, containing the crude hydrazones, was then treated with sodium carbonate etc. according to the procedure of CLIFT AND COOK[5]. The final ethyl acetate extracts, which did not contain sufficient of the nitrophenylhydrazones for direct isolation, were used in subsequent paper-chromatographic and light absorption procedures (see later).

Light absorption measurements. The measurements of light absorption on the alkali-treated 2.4-dinitrophenylhydrazones were carried out in a Unicam S.P. 500 quartz spectrophotometer.

RESULTS

1. On oxidation, citric acid would be expected to yield acetone dicarboxylic acid and some acetone from dicarboxylation of the A.D.A. as follows:



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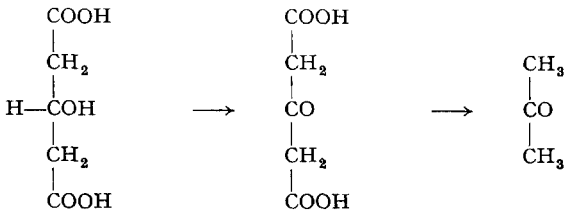
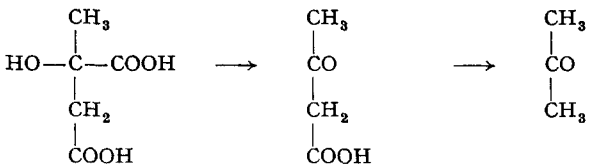
2. β-hydroxyglutaric acid should also be oxidised to A.D.A. and acetone:



3. On the other hand, citramalic acid, by analogy with citric acid, should undergo oxidative dicarboxylation to acetoacetic acid which also readily decomposes to give acetone:



The reactions involved in 1. and 2. should therefore, in the presence of 2.4-dinitrophenylhydrazine, yield the nitrophenylhydrazones of A.D.A. and acetone whereas the one involved in 3. should yield the nitrophenylhydrazones of A.A. and acetone. In fact, as will be seen from Fig. 1, all three reactions give essentially the same nitrophenylhydrazone chromatogram. Although chromatogram A (Fig. 1) does show a trace of a spot (spot 1) of a low R_F, spot 3 is undoubtedly the nitrophenylhydrazone of acetone. Spot 2 is in the position to be expected for the 2.4-dinitrophenylhydrazone of a monocarboxylic keto acid (see later and also EL HAWARY AND THOMPSON[2]). The 2.4-dinitrophenylhydrazone of a dicarboxylic keto acid would be expected to have a much lower R_F value. This is clear from Fig. 2 in which the 2.4-dinitrophenylhydrazone of α-ketoglutaric acid is included. EL HAWARY AND THOMPSON[2] showed that the R_F of the nitrophenylhydrazone of oxaloacetic acid (also a dicarboxylic keto acid) is very close to that of the α-ketoglutaric acid compound; the nitrophenylhydrazone which it will be suggested later is that of acetone dicarboxylic acid (Fig. 2) also has an R_F value close to that of the α-ketoglutaric acid compound. In other words, comparing the R_F values of the 2.4-dinitrophenylhydrazones of acetone, acetoacetic acid and acetone dicarboxylic acid, the carboxyl group is the functional group in the separation of the compounds on the chromatogram.

The explanation of the similarity of all the chromatograms of Fig. 1 lies in the study of the 2.4-dinitrophenylhydrazones obtained from pure acetone dicarboxylic acid and acetoacetic acid. Chromatograms for these compounds are shown in Fig. 3. In this figure chromatograms A and B are of the 2.4-dinitrophenylhydrazones prepared from A.D.A. and dissolved in phosphate buffer, pH 7.2. (EL HAWARY AND THOMPSON[2]) and in ethyl acetate respectively before "spotting". The material for C and D was obtained by running six parallel spots of A.D.A. (as chromatogram B in Fig. 2); three of the spots in position 1 were cut out and eluted with methanol, taken to a low volume under vacuum at 1° C and spotted to give chromatogram C; three of the spots in position 1 were cut out and eluted with 10% Na_2CO_3 and 1 ml 2 *N* NaOH added (as in

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Fig. 1

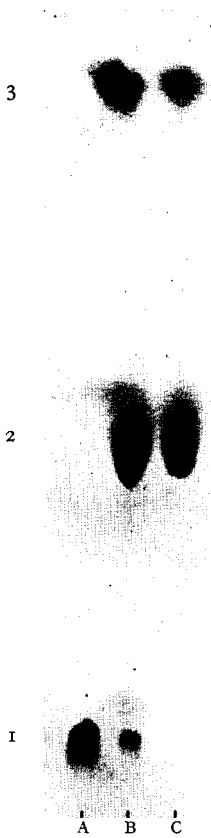

Fig. 2

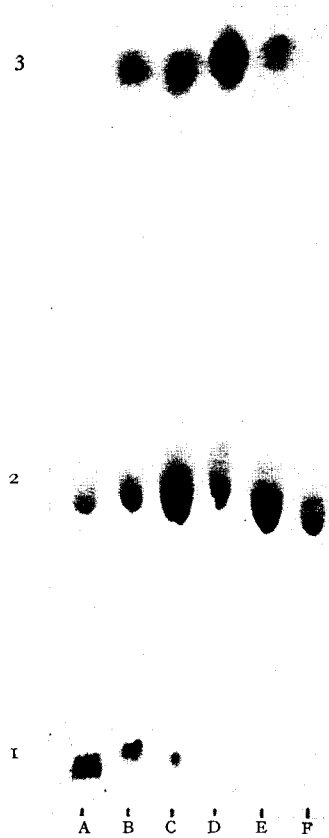

Fig. 3

Fig. 1. Chromatograms of 2.4-dinitrophenylhydrazones from oxidation products of
A. Citric acid B. β-OH-glutaric acid C. Citramalic acid

Fig. 2. 2.4-dinitrophenylhydrazone prepared from pure keto acids
A. α-ketoglutaric acid B. β-ketoglutaric acid (A.D.A.)
C. A.D.A. hydrazone after alkali treatment and reacidification

Fig. 3. 2.4-dinitrophenylhydrazones of A.D.A. and A.A. treated in various ways
A, A.D.A. spotted up in phosphate buffer, pH 7.2 B, A.D.A. in ethyl acetate
C, A.D.A. (spot 1 as chromatogram Fig. 2, B) eluted with methanol and respotted
D, as C but eluted by Na_2CO_3 technique etc. (see text) E, A.A. in ethyl acetate
F, A.A. spotted up in phosphate, pH 7.2

the procedure for measurement of absorption spectra—see later) reacidified with ice-cold 2 *N* HCl and the hydrazone extracted with a minimum of ethyl acetate and spotted to give chromatogram D. Chromatograms E and F were prepared from solid 2.4-dinitrophenylhydrazine dissolved in ethyl acetate (E) or phosphate buffer of pH 7.2 (F).

The results of this series of chromatograms show that the 2.4-dinitrophenylhydrazone of A.D.A. readily breaks down, not directly to acetone, but through the intermediary of acetoacetic acid 2.4-dinitrophenylhydrazone. This breakdown is accelerated by the alkali extraction technique (Chromatogram D). Breakdown via the acetoacetic acid compound to the acetone compound must also occur during travel on the chromato-

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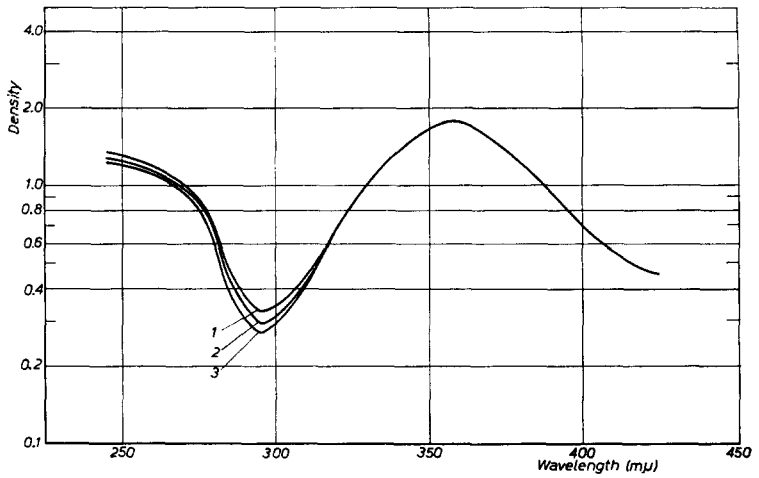

Fig. 4. Absorption spectra of the 2.4-dinitrophenylhydrazones in alcohol of:
1. pure acetone
2. Spot 3 from chromatogram of oxidised citramalic acid from the apple
3. Spot 3 from chromatogram of oxidised β-hydroxyglutaric acid (synthetic)

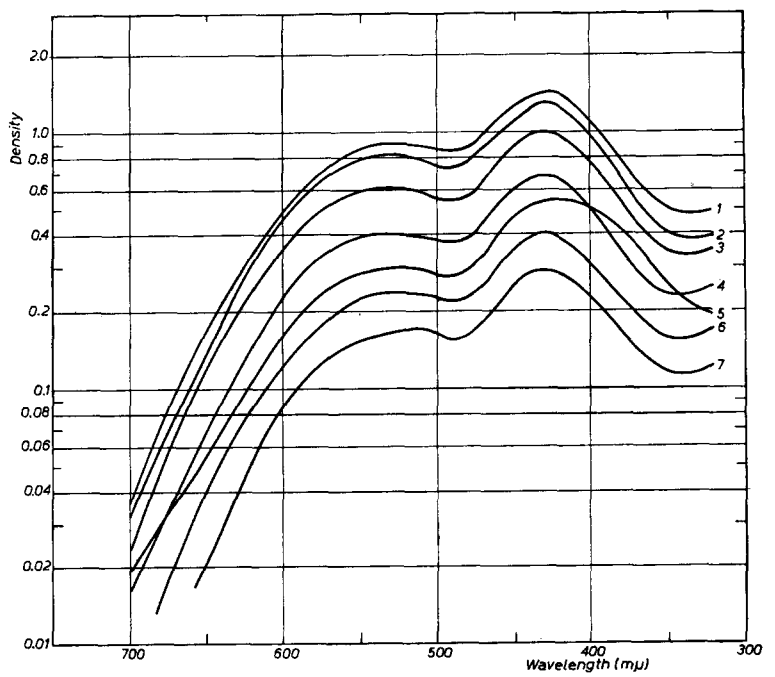

Fig. 5. Absorption spectra in alkali of the eluted spots of 2.4-dinitrophenylhydrazones:
1. of A.D.A. Fig. 3 B, Spot 1
2. of A.A. Fig. 3 E, Spot 2
3. of A.D.A. Fig. 3 B, Spot 2
4. of oxidation product of citric acid, Fig. 1 A, Spot 2
5. of α-ketoglutaric acid
6. of oxidation product of citramalic acid, Fig. 1 C, Spot 2
7. of oxidation product of β-hydroxyglutaric acid, Fig. 1 B, Spot 2

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gram since such breakdown is reduced by buffering the 2.4-dinitrophenylhydrazones of both A.D.A. and A.A. to pH 7.2.

This provides an explanation of the fact that on oxidation citric acid, β-hydroxyglutaric acid *and* citramalic acid yield acetoacetic acid (and subsequently, acetone). That A.D.A. has at least a fleeting existence in the oxidation of citric and β-hydroxyglutaric acids is clear from the trace of spot 1 in chromatogram A of Fig. 1.

Light absorption curves of the various 2.4-dinitrophenylhydrazone preparations support the chromatographic evidence. The absorption curves shown in Fig. 4 confirm that spot 3 in the chromatograms is due to the 2.4-dinitrophenylhydrazone of acetone. The curves in Fig. 5 do not appear superimposed on one another owing to the different concentrations (and consequently the number of absorbent moleculars in the path of the light beam) of the various preparations. This difference is inevitable because the degree of oxidation in the various preparations cannot be controlled, neither can the amount of decomposition to the dinitrophenylhydrazone of acetone. Since, however, the optical density is plotted as the logarithm and the concentration factor is thereby eliminated (BRADFIELD AND FLOOD[6]), the virtual identity of all the curves, except that for α-ketoglutaric, is clear, for by suitable vertical transposition, they do superimpose on one another. Identical curves to those shown were obtained by using the FRIEDEMANN AND HAUGEN[7] method directly on the oxidation mixtures and on the prepared samples of the 2.4-dinitrophenylhydrazones of A.D.A. and A.A. Once again it is evident that acetoacetic acid alone is responsible for spot 2 and is the "end" keto acid in the oxidation of citric, β-hydroxyglutaric and citramalic acids. The 2.4-dinitrophenylhydrazone of α-ketoglutaric acid shows a shift to a lower wavelength for the second peak. Acetone would not be a contaminant of the alkali solution used for the measurement of light absorption since its hydrazone is insoluble in alkali. Even the eluted spot of "pure" A.D.A. hydrazone (spot 1)—Fig. 5, curve—has, during the elution and preparation of the coloured sodium compound, decomposed to the hydrazone of acetoacetic acid (spot 2) as it does during the running of the chromatogram.

DISCUSSION

The results of the analysis of the hydrazones of acetone dicarboxylic acid and acetoacetic acid show that, although carefully prepared with a view to minimising decomposition, the nitrogen content of the acetone dicarboxylic acid compound is much too high, while that for acetoacetic acid is very close to the theoretical value. The high nitrogen value for acetone dicarboxylic acid hydrazone must be due to partial decomposition to the hydrazone of acetoacetic acid. This emphasis the great instability of the acetone dicarboxylic acid which when dissolved in alkali and back-titrated goes *completely* to acetoacetic acid (Table I). The decomposition of the acetoacetic acid to acetone clearly takes place much more slowly. The data presented provide a complete explanation of the otherwise puzzling fact that on oxidation both β-hydroxyglutaric acid and citramalic acid, compounds differing considerably in their structure, yield the same keto acid, namely acetoacetic acid.

These results also throw light on the fact that quinic and citric acids (quinic acid → citric acid on oxidation) yield, on oxidation, small amounts of malonic acid. Oxidation of citric (and consequently, quinic acid) leads to the formation of acetone dicarboxylic acid and it has been suggested (HULME AND ARTHINGTON[8]), that this acid breaks down

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directly to malonic acid. The ease with which acetone dicarboxylic acid loses one molecule of CO_2 to give acetoacetic acid suggests that this latter acid might be an intermediate in the oxidation of acetone dicarboxylic acid. It follows also that oxidation of citramalic acid should yield, via acetoacetic acid, some malonic acid. In fact, paper chromatographic evidence shows that on oxidation of citramalic acid acetoacetic acid by hydrogen peroxide in the presence of Fe^{+2} (Fenton's reagent) malonic acid is produced (HULME[9],). The readiness with which acetoacetic acid breaks down to acetone may be an added explanation of the low yields of malonic acid in such reactions *in vitro* (HULME AND ARTHINGTON[8]).

The ease with which citramalic acid oxidises to acetoacetic acid is of interest in considering its position in the metabolism of apple peel tissue. *In vivo* this reaction may be reversible. Acetoacetic acid is concerned in the metabolism of fats and citramalic acid has only been found in the fat-containing peel tissue and not in the pulp which is practically free from oils and fats. Furthermore, the apple is known to produce appreciable quantities of acetone (HUELIN[10], MEIGH[11]) which may be a result of the decarboxylation of acetoacetic acid. Further investigation of the metabolism of citramalic acid in the peel of apples is being carried out.

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SUMMARY

1. A study is made of the oxidation products of an acid isolated from the peel of apples, synthetic citramalic and β-hydroxyglutaric acids, coupling the oxidation products with 2.4-dinitrophenylhydrazine for this purpose.

2. As a result of this study, it is confirmed that the acid from the peel is citramalic acid and an explanation is provided for the observation that both citramalic acid and β-hydroxyglutaric acid yield acetoacetic acid on oxidation.

3. The data presented also explain why quinic acid and citric acid yield, on oxidation, malonic acid and suggest that there is an intermediate formation of acetoacetic acid.

4. In the light of these results the possible function of citramalic acid in the apple peel is discussed and it is suggested that the acetone known to be given off by apples might ultimately derive from this acid.

RÉSUMÉ

1. L'auteur a étudié les produits d'oxydation d'un acide isolé du péricarpe de la pomme, de l'acide citramalique synthétique et des acides β-hydroxyglutariques en les couplant avec la 2,4-dinitrophénylhydrazine.

2. Les résultats de cette étude confirment que l'acide isolé du péricarpe est l'acide citramalique, et l'auteurs propose une hypothèse qui rend compte de la production d'acide acétoacétique par oxydation des deux acides citramalique et β-hydroxyglutarique.

3. Les résultats obtenus expliquent également pourquoi l'acide quinique et l'acide citrique fournissent, par oxydation, de l'acide malonique et suggèrent la formation intermédiaire d'acide acétoacétique.

4. A la lumière de ces résultats, le rôle possible de l'acide citramalique dans le péricarpe de la pomme est discuté et l'auteurs suggère que l'acétone à laquelle les pommes donnent naissance pourrait en dernière analyse provenir de cet acide.

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ZUSAMMENFASSUNG

1. Es wird eine Untersuchung der Oxydationsprodukte einer aus den Schalen von Äpfeln isolierten Säure, synthetischer Citraapfelsäure und von β-Oxyglutarsäure durch Kupplung der Oxydationsprodukte mit 2.4-Dinitrophenylhydrazin ausgeführt.

2. Das Ergebnis der Untersuchung bestätigte, dass die Säure aus den Schalen Citraapfelsäure ist und es wird eine Erklärung für die Beobachtung gegeben, dass sowohl Citraapfelsäure wie β-Oxyglutarsäure bei der Oxydation Acetessigsäure ergeben.

3. Die vorliegenden Daten erklären ebenfalls, weshalb Chinasäure und Citronensäure bei der Oxydation Malonsäure geben und lassen die intermediäre Bildung von Acetessigsäure vermuten.

4. Im Hinblick auf die Ergebnisse wird die mögliche Funktion der Citraapfelsäure in der Apfelschale besprochen und die Vermutung ausgesprochen, dass das — wie bekannt — von Äpfeln abgegebene Aceton letzten Endes von dieser Säure stammen könnte.

REFERENCES

[1] A. C. HULME, *Biochim. Biophys. Acta*, 14 (1954) 36.
[2] M. F. S. EL HAWARY AND R. H. S. THOMPSON, *Biochem. J.*, 53 (1953) 340.
[3] H. v. PECHMANN, *Ann.*, 261 (1890) 155.
[4] R. H. A. PLIMMER, *Practical Organic Biochemistry*, Longmans, Green and Co., London, 1918, p. 119.
[5] F. P. CLIFT AND R. P. COOK, *Biochem. J.*, 26 (1932) 1800.
[6] A. E. BRADFIELD AND A. E. FLOOD, *J. Chem. Soc.*, (1952) 4740.
[7] T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
[8] A. C. HULME AND W. ARTHINGTON, *J. Exp. Bot.*, 4 (1953) 129.
[9] A. C. HULME, 1953. Unpublished Results.
[10] F. E. HUELIN, Aust, *J. Sci. Res.*, B, 5 (1952) 328.
[11] D. F. MEIGH, 1953. Private Communication.

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