XIX. MICRO-DETERMINATION OF α-KETOGLUTARIC ACID

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α-KETOGLUTARIC acid is an intermediate metabolite in the oxidative breakdown of carbohydrate and of various amino-acids and its quantitative determination is therefore a matter of considerable interest. The method used in previous work for the identification and determination was the isolation of the 2:4-dinitrophenylhydrazone [Krebs, 1933; Weil-Malherbe & Krebs, 1935; Simola, 1936; Martius & Knoop, 1937]. This method is satisfactory if the identification is the main object, but it is not suitable for the quantitative determination of smaller quantities of α-ketoglutaric acid, where the solubility of the 2:4-dinitrophenylhydrazone (0·26 % according to Martius [1937]) is sufficiently great to give rise to low yields.

The method described in this paper is accurate, specific and applicable to quantities from 0·2 mg. upwards. The principle is as follows: α-ketoglutaric acid is first converted into its 2:4-dinitrophenylhydrazone. This is extracted with ether from the aqueous solution. The hydrazone is then freed from ether, dissolved in alkali and treated with permanganate in acid solution. Permanganate oxidizes the hydrazone quantitatively to succinic acid. The latter is determined manometrically by means of succinic dehydrogenase.

Oxidation of α-ketoglutaric 2:4-dinitrophenylhydrazone

The reaction on which the new method is based is the quantitative conversion of α-ketoglutaric 2:4-dinitrophenylhydrazone into succinic acid. I found that this reaction is brought about by oxidation with permanganate in acid medium. The best yields were obtained if the oxidation was carried out at room temperature in 5 % H_2SO_4. In the presence of an excess of $KMnO_4$, 7 mols. CO_2 and 1 mol. succinic acid are liberated from each mol. of α-ketoglutaric 2:4-dinitrophenylhydrazone. For instance, 0·99 mg. hydrazone gave 483 μl. CO_2 in a manometric experiment (calc. for 7 CO_2 : 480 μl.). At 20° more than 90 % of the CO_2 was evolved within 15 min. Table I shows that the theoretical amount of succinate is obtained under these conditions.

As will be seen, the results were satisfactory if 2 mg. or more of the hydrazone were used. With these quantities the error did not exceed 4·5 %. The percentage error naturally becomes greater when the quantities are smaller. Considerable variation of the concentration of the acid did not greatly affect the yield of succinic acid. In the presence of 20 % H_2SO_4 the yield was about 90 % and with 5 or 20 % acetic acid about 85–90 %. Incomplete yields were obtained when very concentrated solutions of the hydrazone were acidified and treated with permanganate. 1 ml. of the acidified solution should not contain more than 1 mg. hydrazone.

Temperature has no appreciable effect on the yields of succinic acid between 20 and 100°, provided that prolonged heating with acid permanganate is avoided.

Table I. *Oxidation of pure α-ketoglutaric 2:4-dinitrophenylhydrazone with $KMnO_4$*

A known amount of the hydrazone, dissolved in 10 ml. 0·01 *M* Na_2CO_3, was placed in a Kutscher-Steudel extractor. One-tenth vol. 50 % H_2SO_4 and 3 ml. 2 % $KMnO_4$ were added in succession. After 15 min. the mixture was extracted with ether. Succinic acid was determined as described previously [Krebs, 1937].

mg. hydrazone used	Succinic acid determination: μl. O_2 measured	μl. O_2 calculated	% recovery
10·58	366	364	99·4
10·58	367	364	100·5
5·29	181	182	99·4
5·29	178	182	97·8
3·82	127	131	97·0
3·82	126	131	96·3
3·82	129	131	98·5
1·98	70	68	102·9
1·98	69·5	68	102·2
0·85	31·9	29·2	109·0
0·85	29·1	29·2	100·0
0·425	12·0	14·6	82·0
0·425	17·8	14·6	122·0

Experimental details of the determination

Reagents required:

(1) 50 % sulphuric acid. (2) 15 % sodium tungstate. (3) Freshly distilled ether, free from peroxide. (4) 2:4-dinitrophenylhydrazine, 1 % dissolved in 10 % H_2SO_4. (5) $KMnO_4$, solid and 2 % aqueous solution. (6) Reagents for the determination of succinic acid (see Krebs [1937]).

Special apparatus required:

(1) Continuous extractors (Kutscher-Steudel, preferably with interchangeable joints). (2) Warburg-Barcroft manometers with conical flasks provided with centre chamber and sidearm. The sidearm should hold at least 1 ml. liquid. (3) Separating funnels, 50 ml. and larger sizes.

Removal of frothing substances. The ethereal extraction of aqueous tissue extracts or of urine is frequently interfered with by the presence of frothing substances which can be removed by treatment with tungstic acid. To 20 ml. aqueous solution (urine, tissue extract, dilute blood or serum) are added 1 ml. 15 % tungstate and, drop by drop, with stirring, 1 ml. 50 % H_2SO_4. The precipitate is filtered off after at least 30 min. and an aliquot of the filtrate is used for the determination. Precipitates which may appear in the filtrate on standing do not interfere with the further procedure.

Formation of the 2:4-dinitrophenylhydrazone. The amount of 2:4-dinitrophenylhydrazine which has to be added depends on the quantity of ketonic and aldehydic compounds present in the solution. It will usually suffice to add 1 ml. 1 % solution to 5 ml. filtrate. A yellow colour of the aqueous phase after the first ethereal extraction indicates an excess of 2:4-dinitrophenylhydrazine.

The solution is allowed to stand for 30 min. before the extraction. The formation of the 2:4-dinitrophenylhydrazone is then complete.

Extraction. I have investigated the partition of α-ketoglutaric 2:4-dinitrophenylhydrazone between ether and 5 % H_2SO_4 and find that the hydrazone is completely extracted if the solution is treated twice in a separating funnel with 1/5 vol. ether.

Treatment of the ethereal extract. The ethereal solution is evaporated on a steam bath and 2–5 ml. 2*N* NaOH are added to dissolve the residue containing hydrazones and hydrazine. The solution is quantitatively washed into a graduated measuring cylinder. The solution (about 20 ml.) is acidified with 50% H_2SO_4 so as to bring the acidity to about *N*. 3 ml. 2% $KMnO_4$ are then added. The solution is allowed to stand for 15 min. and, if the purple colour of the permanganate disappears, fresh oxidizing agent, either solid or in solution, must be added. The volume of the liquid is then read and the MnO_2 formed is filtered off. Succinic acid is extracted from an aliquot of the filtrate in the continuous extractor and determined as described before [Krebs, 1937].

Specificity. Substances which may interfere with the determination of α-ketoglutaric acid are succinic acid and such substances as may yield succinic acid on treatment with permanganate. To the latter group belong glutamic acid, α-hydroxyglutaric acid, arginine, ornithine, butyric acid, valeric acid, *n*-hexanoic acid, suberic acid. Table II shows the yields of succinic acid obtained from these substances under various conditions.

Table II. *Formation of succinic acid on oxidation of various substances by $KMnO_4$ in acid solution*

Room temperature, 5% H_2SO_4, 0·5% $KMnO_4$. The following substances yielded no succinic acid: acetic acid, citric acid, *iso*citric acid, *cis*- and *trans*-aconitic acids, *n*-octanoic acid, stearic acid, sebacic acid, proline, tyrosine, pyruvic 2:4-dinitrophenylhydrazone.

Substance	mg. used	Duration of treatment with $KMnO_4$	Amount of succinic found (μl. O_2 measured)	Yield %
Glutamic acid	17·3	15 min.	13	1
Glutamic acid	17·3	15 hr.	26	2
α-Hydroxyglutaric acid	7·4	15 min.	494	88
α-Hydroxyglutaric acid	7·4	15 hr.	515	92
Arginine monohydrochloride	12·1	15 min.	28	4
Arginine monohydrochloride	12·1	15 hr.	242	27
Ornithine monohydrochloride	81	15 min.	7·2	0·1
Ornithine monohydrochloride	81	15 hr.	188	3·3
n-Butyric acid	80	15 min.	24·5	0·5
n-Butyric acid	80	15 hr.	125	2·6
n-Valeric acid	100	15 hr.	16·3	0·15
n-Hexanoic acid	100	15 hr.	77	0·8
Suberic acid	87	15 hr.	37·5	0·3

A simple control experiment, however, is sufficient to determine the amount of succinic acid which is not derived from α-ketoglutaric acid. An aliquot of the solution in which α-ketoglutaric acid is to be determined is extracted with ether in the absence of 2·4-dinitrophenylhydrazine and the extract is otherwise treated in the same way as it is in the determination of α-ketoglutaric acid. This ethereal extract contains all the interfering substances, but only traces of α-ketoglutaric acid. If the succinic acid found in this control is deducted, the method determines only those ketonic or aldehydic compounds which yield succinic acid on oxidation with permanganate. α-Ketoglutaric acid is the only compound of this type known to occur in biological material, and the method is thus highly specific.

Only negligible amounts of succinic acid were found in the control determination when normal urine or tissue extracts were analysed so that the control is often unnecessary. It is not essential if even considerable quantities of glutamic acid, arginine or ornithine are present, since these substances are practically

insoluble in ether. A control must be carried out, however, if significant quantities of succinic acid or large amounts of lower fatty acids or of α-hydroxyglutaric acid are present.

Recovery experiments. Known quantities of α-ketoglutaric acid added to urine were recovered satisfactorily as shown in Table III.

Table III. *Recovery of α-ketoglutaric acid from human urine*

α-Ketoglutaric acid found in 100 ml. urine mg.	α-Ketoglutaric acid added to 100 ml. urine mg.	α-Ketoglutaric acid recovery	
		Found mg.	Calculated mg.
2·59	0·93	3·51	3·52
3·73	1·86	5·30	5·59
1·49	1·86	3·51	3·35

α-Ketoglutaric acid in human urine

All urines that were tested contained α-ketoglutaric acid, the quantities excreted varying between 10 and 40 mg. per day (Table IV). 100 ml. urine thus contain enough α-ketoglutaric acid for accurate determination.

Table IV. *α-Ketoglutaric acid in human urine*

Initials	Sex	Age	Clinical diagnosis	Quantity of urine 24 hr. ml.	α-Keto-glutaric acid excreted in 24 hr. mg.
C. W.	♂	24	Normal	625	15·5
J. W.	♂	44	Chronic nephritis	965	21·4
W. C.	♂	18	Normal	484	14·5
A. G.	♀	53	Diabetes mellitus, controlled with insulin	1120	10·2
E. S.	♀	50	Acute heart failure	1450	37·5
E. J.	♀	39	Diabetes mellitus, controlled with insulin	1600	39·2
M. S.	♀	54	Cardiac failure	480	12·0
I. S.	♂	59	Normal	690	25·7
J. H.	♂	17	Diabetes mellitus, untreated	790	11·8

α-Ketoglutaric acid in human blood serum

Two specimens of blood were examined. The serum (100 ml.) was deproteinized according to the Folin-Wu method (at *p*H 4·5) and the determination was made on the filtrate. The first specimen, from a case of polycythemia vera, contained 1·05 mg. α-ketoglutaric acid in 100 ml., the second, from a case of cardiac decompensation, 0·75 mg. in 100 ml.

Shortened procedure

If α-ketoglutaric acid is present in a solution free from succinic acid or those substances listed in Table II, the procedure may be shortened. The oxidation to succinic acid can be carried out in this case directly by addition of $KMnO_4$ after acidification of the solution. The free acid like its 2:4-dinitrophenylhydrazone yields succinic acid quantitatively. For instance 6·09 mg. α-ketoglutaric acid gave 930 μl. CO_2 and 4·86 mg. succinic acid (calc. 930 μl. CO_2, 4·91 mg. succinic acid). The oxidation is complete within 20 min. (20°, 5% H_2SO_4, excess of $KMnO_4$).

Succinic and α-ketoglutaric acids, if present together in moderate quantities, may be determined in the following way. An aliquot is directly extracted with ether and succinic acid is determined as described previously. Another aliquot is treated with $KMnO_4$; the additional succinic acid found in this fraction is equivalent to the α-ketoglutaric acid present, provided that interfering substances (see Table II) are absent.

Summary

A method for the quantitative determination of α-ketoglutaric acid in aqueous solutions is described. α-Ketoglutaric acid is first converted into the 2:4-dinitrophenylhydrazone which is then extracted with ether and oxidized quantitatively to succinic acid with permanganate; the succinic acid is determined manometrically by means of succinic dehydrogenase. The method is applicable to quantities from 0·2 mg. upwards. Ten human urines contained between 10 and 40 mg. α-ketoglutaric acid per 24 hr. specimen.

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