94. A General Method for the Estimation of α-Keto-acids, and its Application to α-Keto-acid Metabolism in Pigeon Brain

By **Cyril Long**, *From the Department of Biochemistry, The Queen's University, Belfast*

(*Received 23 July 1942*)

The acceptance of the central position occupied by pyruvic acid in the intermediary metabolism of carbohydrate in animal tissues has recently led to a number of investigations on the behaviour of other α-keto-acids towards those enzymes which act upon pyruvic acid. The next higher homologues of pyruvic acid, namely, α-ketobutyric and α-ketovaleric acids, have received special attention [Green, Needham & Dewan, 1937; McGowan & Peters, 1937; Long & Peters, 1939; Cohen, 1939].

It has generally been observed that the effect produced by an enzyme on an α-keto-acid decreases progressively as the C-chain in the molecule is lengthened, i.e. the higher members of the series are less readily attacked than the lower members. An apparent exception, however, is the finding by Long & Peters [1939] that pyruvic and α-keto-butyric acids were equally rapidly utilized by a minced preparation of pigeon brain. Since there was not available, at that time, a satisfactory method of estimating small amounts of α-ketobutyric acid, their evidence was necessarily somewhat indirect. More recently, however, it has been felt that this claim should be re-examined by means of direct observations, and since it was intended to continue the investigation of α-keto-acid metabolism, both in brain and in other tissues, it was clearly desirable to develop a method for the accurate estimation of small amounts of α-keto-acids of the order 0·1–1·0 μmol.

For this purpose, the bisulphite-binding method of Clift & Cook [1932] was tested but proved to be unsatisfactory on account of the low recoveries obtained, even when using the pure Na salts of the α-keto-acids. The method finally adopted was based on that used by Lu [1939] for the determination of pyruvic acid in blood filtrates. By this means it was possible, with a high degree of accuracy, to estimate all the α-keto-acids studied. When the method was applied to the problem of the comparative utilization of pyruvic and α-ketobutyric acids in minced pigeon brain, the findings of Long & Peters [1939] were confirmed in detail.

Experimental

(1) *Reagents*

Na pyruvate. A gift from Prof. R. A. Peters; prepared from pure pyruvic acid [Peters, 1938].

Na α-ketobutyrate. Prepared according to Long & Peters [1939].

Na α-ketovalerate. Specimen used by Long & Peters [1939].

Na glyoxylate. Glyoxylic acid (1 g. B.D.H.) in conc. aqueous solution was carefully adjusted with NaOH (40 %) to *p*H 6, under ice-cold conditions. Addition of acetone caused precipitation of the Na salt. Recrystallized three times from aqueous acetone.

α-Ketoglutaric acid. Prepared according to Neuberg & Ringer [1915]. Since the free acid was a stable solid, it was not necessary to convert it into its Na salt.

(2) *The purity of the Na salts of the α-keto-acids*

Krebs [1938] showed that standard solutions of pure α-ketoglutaric acid, when treated with excess of acid $KMnO_4$ at room temperature, yielded the theoretical amount of CO_2 for oxidative decarboxylation, an equivalent of succinic acid being simultaneously produced. It is clear that the oxidative decarboxylation is dependent on the presence of the —CO.COOH group only, and must be given by all α-keto-acids.

$$R.CO.COOH + O \rightarrow R.COOH + CO_2.$$

The reaction has been used to determine the purity of the Na salts of those α-keto-acids listed above.

A solution containing 10–15μmol. of the Na salt of the α-keto-acid, dissolved in 5% H_2SO_4 (total vol. 2 ml.), was placed in the main chamber of a Dixon-Barcroft flask. $KMnO_4$ (approx. *N*; 0·2 ml.), contained in a Keilin cup, was added after temperature equilibration. The oxidation was conducted at 28° in air. CO_2 production was in all cases complete after 5 min. The CO_2 evolved in a control experiment in which 5% H_2SO_4 (2 ml.) was similarly treated, was deducted from the observed CO_2 production. This correction generally amounted to about 10μl. (about 3% of the total).

In Table 1 are shown the results obtained for the oxidation of the four Na salts of the α-keto-acids and for free α-ketoglutaric acid.

Table 1. *Evolution of CO_2 from α-keto-acids by acid $KMnO_4$, as an index of purity*

Each experiment represents the average of triplicate CO_2 productions, agreeing to ±1%. Recoveries calculated to nearest 0·5%.

	Exp.	Net CO_2 μl.	α-Keto-acid (μmol.) Calc.	α-Keto-acid (μmol.) Taken	% recovery
Na pyruvate	1	317	14·2	14·3	99·5
	2	378	16·9	16·8	100·5
	3	354	15·8	15·8	100
	4	392	17·5	17·5	100
	5	343	15·4	15·4	100
Na α-ketobutyrate	6	325	14·4	14·5	99·5
	7	329	14·7	14·5	101·5
	8	307	13·7	14·0	98
	9	304	13·6	13·6	100
Na α-ketovalerate	10	312	13·9	14·2	98
	11	351	15·7	15·7	100
	12	304	13·6	13·4	101·5
α-Ketoglutaric acid	13	334	14·9	14·7	101·5
	14	353	15·7	15·5	101·5
	15	380	17·0	16·9	100·5
Na glyoxylate	16	174	7·8	7·7	101
	17	368	16·4	16·3	100·5

From the values given in Table 1, it is seen that, within experimental error, all the α-keto-acids studied yielded the theoretical CO_2 production on treatment with acid $KMnO_4$. The samples must therefore be considered quite pure.

In the cases of α-ketoglutaric acid and the Na salts of pyruvic, α-ketobutyric and α-ketovaleric acids, the CO_2 production indicated in Table 1 is the amount actually observed, less the control. With Na glyoxylate, however, the evidence for absolute purity is not so direct, since formate which is produced by oxidative decarboxylation is itself slowly oxidized, with simultaneous CO_2 production, under the given conditions. Fig. 1 shows the extent of this oxidation at different time intervals. The graph connecting these two variables is a smooth curve, which on extrapolation to zero time gives the CO_2 evolution independent of formate oxidation. The extrapolated values are those quoted in Table 1.

It was unfortunate that this method could not be applied directly to the estimation of α-keto-acids in biological fluids, its two main disadvantages being lack of specificity and the fact that a relatively large amount of the α-keto-acid would be required. Thus in order to obtain a reasonably accurate estimation by this method, about 5 μmol. of the α-keto-acid would be needed, whereas in the protein-free filtrates from micro-metabolic processes it would be necessary to estimate 0·1 μmol. with the same degree of accuracy.

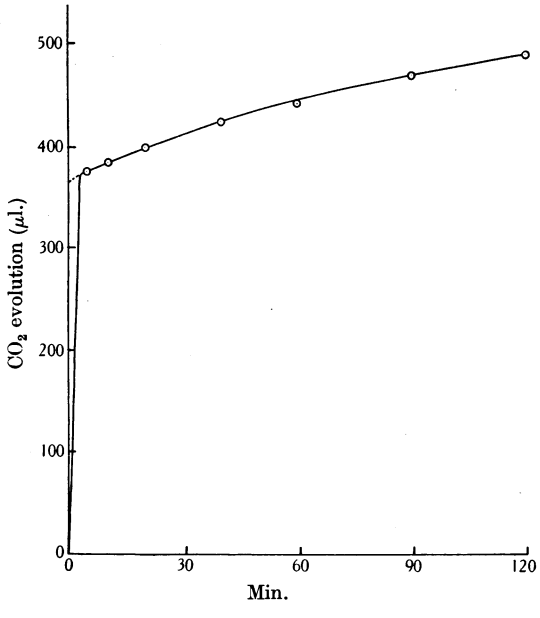

Fig. 1. The time course of CO_2 evolution from Na glyoxylate on treatment with acid $KMnO_4$ at 28°. Extrapolation to zero time is indicated by the discontinuous portion of the curve. Exp. 17.

(3) *The recovery of α-keto-acids by the bisulphite-binding method*

According to Clift & Cook [1932], standard solutions of pyruvic acid can be estimated by the bisulphite-binding technique with recoveries varying between 97 and 103 % (average value 100 %). Using freshly prepared solutions of Na pyruvate, with solid Na_2HPO_4 to liberate bound bisulphite [Lehnartz, 1928], it has not been possible in this work to obtain such a quantitative recovery, the average value being about 96·5 %. With the Na salts of other α-keto-acids and with free α-ketoglutaric acid, the recoveries have been even lower (Table 2).

Both first and second end-points of the titration with iodine were perfectly sharp, except in the case of α-ketoglutaric acid. Here, although the second end-point at alkaline reaction was quite definite, the first end-point at acid reaction showed considerable fading of colour. This variability in the case of α-ketoglutaric acid probably explains the lack of reproducibility observed in Exps. 15–18 (Table 2).

In view of the low recoveries obtained in Table 2, it was decided that the bisulphite-binding technique was unsatisfactory for accurate work and another method had to be sought. It may be noted that a further disadvantage of the method is its limited application to studies of α-keto-acid metabolism since it cannot be used in the presence of arsenite, which is widely used as a specific inhibitor of α-keto-acid oxidations.

Table 2. *The recovery of α-keto-acids by the bisulphite-binding method*

Each experiment represents the average of closely agreeing triplicate titrations, except in the case of α-ketoglutaric acid (see text). Recoveries calculated to the nearest 0·5 %.

	Exp.	*N*/100 iodine ml.	α-Keto-acid (μmol.) Calc.	Taken	% recovery
Na pyruvate	1	1·776	8·88	9·18	96·5
	2	1·669	8·35	8·85	94·5
	3	0·908	4·54	4·68	97
	4	0·896	4·48	4·59	97·5
	5	0·905	4·53	4·77	95
	6	1·095	5·48	5·59	98
	7	1·003	5·02	5·27	95·5
				Average	96·5
Na α-ketobutyrate	8	0·814	4·07	4·72	86
	9	0·839	4·20	4·84	87
	10	0·804	4·02	4·64	86·5
	11	0·766	3·83	4·52	84·5
	12	0·779	3·90	4·60	85
				Average	86
Na α-ketovalerate	13	0·838	4·19	4·73	88·5
	14	0·920	4·60	5·22	88
				Average	88
α-Ketoglutaric acid	15	1·380	6·90	7·77	89
	16	0·860	4·30	5·63	76·5
	17	1·033	5·17	6·49	79·5
	18	0·946	4·73	5·67	82
				Average	82
Na glyoxylate	19	0·745	3·73	4·63	80·5
	20	1·328	6·64	8·13	81·5
				Average	81

(4) *General colorimetric method for the estimation of α-keto-acids*

Recently, Lu [1939] has applied the condensation in acid solution between 2:4-dinitrophenylhydrazine and pyruvic acid to the determination of the latter in blood filtrates by means of the intensity of the red-brown colour developed when the pyruvic acid 2:4-dinitrophenylhydrazone so formed is treated with excess NaOH. With certain modifications, this method has been used to estimate those α-keto-acids listed above. It is especially suitable for the accurate determination of 0·1–1·0 μmol. of the α-keto-acid.

A standard curve connecting concentration and colour intensity for each α-keto-acid was obtained as follows. Known amounts of the Na salt, viz. 0·9, 0·6, 0·3 and 0·15 μmol., dissolved in 5 % trichloroacetic acid (total vol. 2 ml.) together with a control consisting of 5 % trichloroacetic acid (2 ml.) were measured into five test-tubes. The following details apply to each of the five samples. The acid solution was treated with 0·2 % 2:4-dinitrophenylhydrazine hydrochloride in 2*N* HCl (1 ml.). After 15 min., during which all the α-keto-acid was converted into its 2:4-dinitrophenylhydrazone, ethyl acetate (2 ml.) was added, and the aqueous solution extracted using a teat-pipette. For this purpose, the liquid in the tube was rapidly drawn into and expelled from the teat-pipette 15 times, an operation requiring about 20 sec. The lower aqueous layer was transferred to another test-tube and extracted twice more with ethyl acetate (1 ml. each time). The three ethyl acetate extracts were combined and the aqueous layer discarded. This solution in ethyl acetate of the 2:4-dinitrophenylhydrazone together with unchanged 2:4-dinitrophenylhydrazine was then extracted with three successive 2 ml. portions of 10 % Na_2CO_3, and the combined Na_2CO_3 extracts washed once with ethyl acetate (1 ml.). After allowing at least 50 min. for the Na_2CO_3 extract to clear, this pale yellow solution

was quantitatively transferred to a 10 ml. measuring cylinder, treated with 3 ml. 2*N* NaOH, made up to 10 ml. with distilled water and thoroughly mixed. This treatment with alkali caused a deep red-brown colour to develop. The intensities of absorption of the five samples were compared in a Spekker Absorptiometer, using a purple colour filter. (The Hilger No. 7 filter from the H.455 set, which was used in the present investigation, absorbed completely between 520 and 680 mμ and transmitted maximally between 400 and 360 mμ.) The drum reading with the control was taken as the zero. In this manner the curves in Fig. 2 were obtained.

In order that the method described may be used with the greatest accuracy and reliability, a number of experimental details need stressing. First, it is essential that the same reagent specimens be used for determining the standard curve and for the estimation of unknown quantities of the given α-keto-acid. It has been found that the ethyl acetate specimen, especially, must not be changed. It is equally important that the 0·2% solution of 2:4-dinitrophenylhydrazine hydrochloride in 2*N* HCl should be fairly fresh and must remain clear. The curves shown in Fig. 2 are those obtained using a given set of reagents. With other reagents, and especially when different samples of ethyl acetate have been employed, the colour intensity has varied quite considerably (±20%). However, when the reagents are unchanged, the same standard curve is always obtained. This is shown in the case of pyruvic and α-ketobutyric acids in curves *A* and *D*, Fig. 2. Another point of practical importance is that sufficient time (15 min.) should be allowed for the α-keto-acid to react with the 2:4-dinitrophenylhydrazine in acid solution, before the first extraction with ethyl acetate. With shorter time intervals, the reaction is incomplete, and low values are obtained when the absorption intensity is ultimately determined. Finally, a sufficient quantity of alkali must be used for the colour development. Lu [1939] recommended the use of 4 ml. *N* NaOH. When this amount was used, it was found that the absorption intensity diminished quite rapidly during the determination. However, when the quantity was increased to 3 ml. 2*N* NaOH, the intensity became stabilized over at least 90 min. [cf. also Bueding & Wortis, 1940].

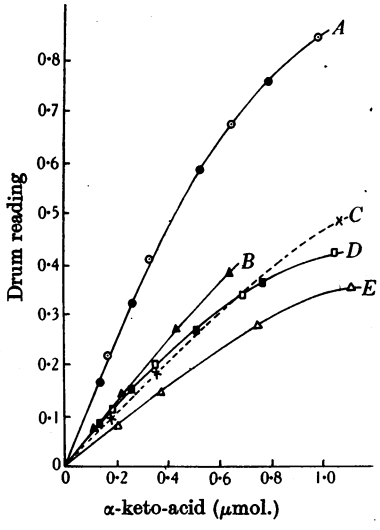

Fig. 2. The absorption intensity (drum reading) of the 2:4-dinitrophenylhydrazones of α-keto-acids in excess NaOH as a function of concentration. Purple filter, No. 7 Hilger (H.455). Curve *A*, pyruvic acid; curve *B*, α-ketovaleric acid; curve *C*, glyoxylic acid; curve *D*, α-ketobutyric acid; curve *E*, α-ketoglutaric acid. Curves *A* and *D* each show two experiments.

From the curves in Fig. 2 it will be seen that at any given molecular concentration, the colour complex from pyruvic acid possesses a greater intensity of absorption than that from any other member of the series. Its next higher homologue, α-ketobutyric acid, absorbs less than half as strongly, but the following member of the series, α-ketovaleric acid, absorbs more strongly than α-ketobutyric acid. There appears to be no progressive diminution of the intensity of absorption with increasing length of the carbon chain. It is interesting to note that the introduction of an additional —COOH group into α-ketobutyric acid, to give α-ketoglutaric acid, is accompanied by a diminution of the intensity of absorption. In order to produce the same absorption intensity as one molecular equivalent of pyruvic acid, about four equivalents of α-ketoglutaric acid are required. Lu [1939] reported a rather similar figure, 4·5. Table 3 shows the molecular quantities of the different α-keto-acids required to produce the same absorption intensity as 0·3 μmol. pyruvic acid.

Table 3. *The relative molecular concentrations of α-keto-acids required to produce a given absorption intensity*

	Drum reading	α-Keto-acid (μmol.)	Ratio		Drum reading	α-Keto-acid (μmol.)	Ratio
Pyruvic acid	0·360	0·300	1·0	α-Ketovaleric acid	0·360	0·615	2·1
α-Ketobutyric acid	0·360	0·781	2·6	α-Ketoglutaric acid	0·360	1·210	4·0

After having obtained the standard curves for the different α-keto-acids, it was then necessary to determine the recovery from deproteinized tissue extracts under the conditions to be observed experimentally.

A standard amount of the Na salt of the given α-keto-acid, contained in 1 ml. aqueous solution in a Dixon-Barcroft flask, and a water control were treated with 1 ml. 25 % trichloroacetic acid. 1 ml. of a brain suspension, equivalent to 300 mg. fresh brain, was then added, and after allowing 15 min. for complete precipitation of protein, the samples were filtered through 5·5 cm. filter papers (Whatman No. 42) into 10 ml. measuring cylinders. Each flask was rinsed with 2 ml. 5 % trichloroacetic acid three times, and the fluid used for washing the protein precipitate on the filter paper. After allowing to drain overnight, the volumes were made up to 10 ml. with distilled water. A 2 ml. aliquot was taken for estimating the amount of α-keto-acid by the method already described. At the same time, an equivalent amount of the Na salt of the α-keto-acid, not subjected to this extraction process, was estimated by the same method. A comparison of the absorption intensities of the two against the control gave a direct figure for the recovery of the α-keto-acid from the protein extract.

Table 4 shows three experiments, using pyruvic and α-ketobutyric acids, in which the recovery was determined. The average recovery is 97–99 %, a figure sufficiently near to the theoretical to make corrections unnecessary.

Table 4. *Recovery of pyruvic and α-ketobutyric acids from tissue extracts*

	Drum reading		
	Standard solution	Tissue extract	% recovery
Na pyruvate	0·572	0·555	97·0
Na α-ketobutyrate	0·386	0·375	97·2
	0·232	0·230	99·1

Returning to the curves in Fig. 2, it will be seen that those for pyruvic, α-ketobutyric, α-ketovaleric and α-ketoglutaric acids are of the same type. In all four cases the intensity of absorption is proportional to the concentration in very dilute solution; at higher concentrations, however, the absorption intensity increases less rapidly than would correspond with proportionality. The cause of this deviation from linearity is to be found in the fact that at higher concentrations the solution is deep red in colour, whereas at lower concentrations the tint is red-brown. It would therefore appear that on increasing the concentration, the maximum of the absorption band is moved towards the higher wave-lengths, so that the absorption intensity would no longer be expected to be directly proportional to the concentration. In order to show that this is probably the case, and that the cause is not associated with incomplete extraction of more concentrated solutions, the following experiment was designed. A solution of α-ketobutyric acid 2:4-dinitrophenylhydrazone in 10 % Na_2CO_3 was prepared as described. 3, 2 and 1 ml. of this extract were treated with 3 ml. 2*N* NaOH to develop the colour, and the volumes made up to 10 ml. The absorption intensities were compared against a control, and, as was to be expected, the relationship between concentration and absorption intensity was not linear. However, when the absorption intensities were converted into absolute amounts of α-ketobutyric acid, using the standard curve, they were seen to be almost in the exact ratio 3:2:1. The same was true using pyruvic acid 2:4-dinitrophenylhydrazone. The details of these experiments are given in Table 5.

Glyoxylic acid (curve *C*, Fig. 2) behaves differently from the other members of the α-keto-acid series, in that the relationship between concentration and absorption in-

Table 5. *The non-linear relation between concentration of α-keto-acid 2:4-dinitrophenylhydrazone and intensity of absorption*

	α-Keto-acid 2:4-dinitro-phenyl-hydrazone (ml.)	Drum reading	Ratio	From standard curve. α-Keto-acid (μmol.)	Ratio
Na pyruvate	1	0·228	1·00	0·187	1·00
	2	0·442	1·93	0·383	2·05
	3	0·608	2·67	0·575	3·07
Na α-Ketobutyrate	1	0·108	1·00	0·170	1·00
	2	0·189	1·75	0·329	1·94
	3	0·268	2·48	0·519	3·05

tensity does not fall very far short of linearity, even at relatively high concentrations. This fact is also to be explained in terms of the wave-length of maximum absorption. Glyoxylic acid 2:4-dinitrophenylhydrazone gives with excess NaOH a bright orange-coloured solution, quite distinct from the red colours obtained with the other α-keto-acids. Moreover, the tint does not appear to vary appreciably with changes of concentration, so that an approximately linear relationship would be expected. It may be noted that glyoxylic acid differs in one further respect from its higher homologues, in that the colour complex of its 2:4-dinitrophenylhydrazone in excess alkali is rather unstable, the intensity of absorption falling by about 15 % in an hour. This instability has been taken into account when plotting curve *C*, Fig. 2.

(5) *The utilization of pyruvic and α-ketobutyric acids by washed preparations of minced pigeon brain*

The primary object in attempting to develop a suitable method for the estimation of small amounts of α-keto-acids was to test directly the indications obtained by Long & Peters [1939] that pyruvic and α-ketobutyric acids were equally well utilized by a minced preparation of pigeon brain, a conclusion which had not been expected in view of previous work on α-keto-acid metabolism. The evidence put forward by Long & Peters was briefly as follows. The ultimate fate of pyruvic acid, when utilized by respiring minced pigeon brain, was known with some accuracy [Long, 1938]. A balance sheet accounting for 97 % of the pyruvic acid disappearing showed that all previous observations could be explained on the assumption that three reactions were taking place:

(1) $CH_3CO.COOH + 2\frac{1}{2}O_2 \rightarrow 3CO_2 + 2H_2O$ ... ... 67·0 %

(2) $CH_3CO.COOH + \frac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$ ... ... 19·6 %

(3) $CH_3CO.COOH + \frac{1}{2}H_2O \rightarrow \frac{1}{2}CH_3COOH + \frac{1}{2}CH_3CHOHCOOH + \frac{1}{2}CO_2$ 10·4 %

Reaction (3) is an anaerobic process (dismutation), whereas (1) and (2) require the presence of oxygen. From the values quoted above, the calculated R.Q. was found to be in complete agreement with that determined experimentally by McGowan [1937]. Using the percentages given for the two aerobic reactions, the amount of pyruvic acid utilized by oxidative processes only was readily calculated from the observed net O_2 uptake.

The experimentally determined R.Q. for α-ketobutyric acid under the same conditions was found to be somewhat greater than 2·0, indicating that reaction (1) could not be taking part. In the same manner, the amount of α-ketobutyric acid utilized by oxidative decarboxylation only (reaction (2)) was calculated from the corresponding O_2 uptake. When identical samples of washed minced brain tissue were allowed to respire in pyruvate and α-ketobutyrate, side by side, and the separate net O_2 uptakes measured, it was calculated that the same amount of these two α-keto-acids was utilized by oxidative processes in a given period. Under anaerobic conditions it had been found that pyruvic and α-ketobutyric acids evolved the same volume of CO_2 in a given time, so that similarly

there was no doubt that they were also being utilized anaerobically at the same rate. This being so, it naturally followed that the total utilization of pyruvic and α-ketobutyric acids, whether due to aerobic or anaerobic processes, should also be identical. Since, however, a suitable method of estimating minute quantities of α-ketobutyric acid was not then available, it was not possible to test this point directly. Using the general method for α-keto-acid estimation, described above, this has now been done.

The experimental details for the preparation of the washed tissue mince and for the conduct of the respiration were exactly as described by Long & Peters [1939]. After mincing the cerebrum and optic lobes of three pigeons under ice-cold conditions, the brei was washed with ice-cold Ringer phosphate, *p*H 7·3, three times. Weighed portions were transferred to Dixon-Barcroft flasks containing *M*/50 pyruvate or α-ketobutyrate, and allowed to respire at 28 or 38°. The amount of each acid was determined in control flasks at the beginning of the respiration period, and also in the experimental flasks at the end of this period. The results of four experiments are shown in Table 6.

Table 6. *The utilization of pyruvic and α-ketobutyric acids by washed preparations of respiring minced pigeon brain*

Exp.	Temp.	Duration min.	Utilization μmol./g. tissue/hr. α-Ketobutyrate	Pyruvate
1	38	160	20·9	22·0
2	38	220	21·9	22·9
3	38	180	27·0	27·1
4	28	180	8·5	8·7

From Table 6 it is seen that within experimental error pyruvic and α-ketobutyric acids are equally rapidly utilized by washed preparations of minced pigeon brain. Thus the conclusions of Long & Peters [1939] are confirmed in detail.

Summary

1. Pyruvic, α-ketobutyric, α-ketovaleric, α-ketoglutaric and glyoxylic acids were found to give low values when estimated by the bisulphite-binding method, although acid $KMnO_4$ oxidation showed the samples to be quite pure.

2. A colorimetric method of estimating these α-keto-acids, based on the intensity of the red-brown colour obtained by the action of excess NaOH on the corresponding 2:4-dinitrophenylhydrazones, has been developed and found to be especially suitable for quantities of the order 0·1–1·0 μmol.

3. Using this method of estimation, it has been shown that pyruvic and α-ketobutyric acids are equally well utilized by a washed preparation of minced pigeon brain.

The author is greatly indebted to Prof. D. C. Harrison for much helpful criticism and advice.

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