

# XCVII. PYRUVATE OXIDATION IN BRAIN

## V. EVIDENCE DERIVED FROM THE METABOLISM OF $\alpha$ -KETOBUTYRIC ACID

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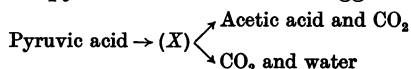
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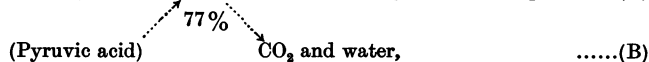
THE problem of the specificity of the pyruvate oxidation system in pigeon's brain tissue has been studied more fully by us, not only in the hope that it would throw fresh light upon the path of complete oxidation of pyruvic acid, but also in the belief that knowledge of the behaviour of other simple  $\alpha$ -keto-acids would contribute further to the elucidation of the metabolism of some of the oxidation products of the  $\alpha$ -amino-acids.

In particular it was hoped that some definite indication would be obtained as to whether the three reactions found to account quantitatively for the aerobic oxidation of pyruvic acid [Long, 1938] were entirely separate or were inter-related. This objective, we think, has been realized. We have now obtained evidence that the rate of oxidation ( $\mu$  mol./hr.) is the same for pyruvic acid and  $\alpha$ -ketobutyric acid; but whereas the latter gives as end-product  $\text{CO}_2$  and presumably the next lower fatty acid, a change requiring  $\frac{1}{2}\text{O}_2$  per mol., part of the pyruvic acid disappearing is much more fully oxidized. This suggests that there is a common path for the initial stage in the oxidation, intermediate compounds of similar type being formed from pyruvic and  $\alpha$ -ketobutyric acids, the sole difference being that in the case of pyruvic acid a further system intervenes capable of oxidizing this unstable intermediate to  $\text{CO}_2$  and water. The intermediate formed from  $\alpha$ -ketobutyric acid can only break down without  $\text{O}_2$  uptake to  $\text{CO}_2$  and, by analogy, propionic acid.

Hence the scheme for pyruvic acid oxidation suggested by Long [1938]



may be replaced by the more general scheme



with the special system for pyruvic acid. The figure 77% is given by

$$\frac{\text{Pyruvic acid giving } \text{CO}_2 \text{ and water}}{\text{Total pyruvic acid oxidized}} \times 100 = \frac{67}{67 + 19.6} \times 100 = 77,$$

no account being taken of the dismutation. This process has been shown to take place more rapidly under anaerobic conditions than in the presence of molecular  $\text{O}_2$ .

### HISTORICAL

Decarboxylation by yeast of  $\alpha$ -keto-acids, containing at least one  $\beta$ -hydrogen atom, was well established by Neuberg and his co-workers [Neuberg & Kerb, 1912; Neuberg & Peterson, 1914; also Hofmann, 1931]; it is also now known

that vitamin B<sub>1</sub> pyrophosphoric ester (cocarboxylase) is required in this system both for pyruvic acid [Lohmann & Schuster, 1937] and for  $\alpha$ -ketobutyric acid [Peters, 1937]. Contrary to its behaviour with yeast carboxylase,  $\alpha$ -ketobutyric acid showed no apparent reaction with the pyruvate oxidation system in unwashed brain and even appeared to inhibit pyruvate respiration. This provided no foothold for a belief in the similarity of the brain and yeast systems, nor did cocarboxylase prove nearly as efficient as vitamin B<sub>1</sub> in the catatorulin test [Peters, 1937]. At the same time McGowan & Peters [1937] had found no evidence for the oxidation of the  $\alpha$ -ketodicarboxylic acids,  $\alpha$ -keto-adipic and  $\alpha$ -ketoglutaric acids, by brain brei.

We have reinvestigated with yeast and brain the behaviour of  $\alpha$ -ketobutyric acid and also studied the homologue  $\alpha$ -ketovaleric acid.

## EXPERIMENTAL

### Reagents

*Pyruvic acid.* Pure Na pyruvate [Peters, 1938] was used. Before each experiment a small quantity was dissolved in Ringer phosphate, pH 7.3, so that 0.2 ml. when added to the respiration medium of total vol. 2.8 ml. gave a final concentration of c. 0.02 *M*.

*Succinic acid.* A "Kahlbaum" specimen of Na succinate was used, final concentration c. 0.04 *M*.

*$\alpha$ -Ketobutyric acid.* A sample of Na  $\alpha$ -ketobutyrate was prepared from the acid [Neuberg & Kerb, 1912] by the method of Peters [1937].

*$\alpha$ -Ketovaleric acid.* Two samples were prepared, one by Mr L. A. Stocken in this Department, by condensing together ethyl oxalate and ethyl *n*-butyrate, followed by the hydrolysis of the resulting ethyl ethyloxaloacetate with 5% H<sub>2</sub>SO<sub>4</sub>. B.P. 78°/14 mm. Low-melting colourless solid. Na salt prepared as for Na  $\alpha$ -ketobutyrate. Analysis: found: C, 43.5%; H, 5.2%. Calc. for C<sub>5</sub>H<sub>7</sub>O<sub>3</sub>Na: C, 43.5%; H, 5.1%. 2:4-Dinitrophenylhydrazone, m.p. 135–136° (corr.).

Unless otherwise stated, the concentration of  $\alpha$ -keto-acid was c. 0.02 *M*. This gave the maximal effect (cf. Tables IV, XIII).

*Vitamin B<sub>1</sub>.* Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

*Cocarboxylase.* A sample (50% purity) containing a little vitamin B<sub>1</sub> prepared by Mr H. W. Kinnersley in this Department by the method of Kinnersley & Peters [1938]. Also a synthetic specimen from Messrs Merck.

Values quoted in experiments are the average of duplicates or triplicates, the variation being not greater than  $\pm 1.5\%$ .

## DECARBOXYLATION BY YEAST

In the experiments of Neuberg and his co-workers,  $\alpha$ -ketobutyric acid [Neuberg & Kerb, 1912] and  $\alpha$ -ketovaleric acid [Hofmann, 1931] were easily decarboxylated by baker's yeast, washed with phosphate buffer, pH 6.2. Peters [1937] used yeast washed with alkaline phosphate by the method of Lohmann & Schuster [1937] to show the necessity for cocarboxylase in the decarboxylation of  $\alpha$ -ketobutyric acid. Ochoa [1938] demonstrated the activation of cocarboxylase by vitamin B<sub>1</sub> in the decarboxylation of pyruvic acid. These new developments have been extended to  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids.

Dry baker's yeast (supplied by the Distillers Co. Ltd.) was washed free from cocarboxylase [Ochoa & Peters, 1938, 1]. CO<sub>2</sub> production was measured in air

at 28° and pH 6.2, using Barcroft-Dixon manometers. Each bottle contained 1.0 ml. alkaline-washed yeast suspension, 0.10 ml. MgCl<sub>2</sub> (equivalent to 100 μg. Mg), cocarboxylase and vitamin B<sub>1</sub> where used. The pyruvic or α-keto-acid was contained in a Keilin cup, tipped in after 15 min. incubation period. Table I shows that the α-keto-acids are decarboxylated at about the same rate as pyruvic acid, and that the activation of cocarboxylase by vitamin B<sub>1</sub> takes place to approximately the same extent in all cases. The yeast decarboxylation system is therefore quite general for α-ketomonocarboxylic acids. If there is any significance in the individual values then α-ketovaleric acid and pyruvic acid are decarboxylated only 69 and 80 % as rapidly as α-ketobutyric acid.

Table I. *Decarboxylation of α-keto-acids by alkaline-washed yeast*

1.0 ml. washed yeast suspension; 0.10 ml. MgCl<sub>2</sub> (100 μg. Mg). Total vol. made up to 2.3 ml. with phosphate buffer, pH 6.2.

Exp. 211 (1 exp. out of 3 quoted)

	CO <sub>2</sub> production in 25 min. (μl.)	
	Nil	Cocarboxylase (2 μg.)
Residual	1	1
Pyruvate	19	316
α-Ketobutyrate	21	396
α-Ketovalerate	12	273

Exp. 217 (1 exp. out of 2 quoted)

	CO <sub>2</sub> production (μl.) in 30 min.			
	Nil	Vitamin B <sub>1</sub> (10 μg.)	Cocarboxylase (1 μg.)	Cocarboxylase (1 μg.) and V (10 μg.)
Residual	1	8	1	3
Pyruvate	27	31	181	465
α-Ketobutyrate	17	25	189	503

Exp. 212 (1 exp. out of 2 quoted)

Residual	1	6	1	1
α-Ketobutyrate	22	27	165	548
α-Ketovalerate	17	11	122	386

THE INTERACTION BETWEEN α-KETO-ACIDS AND THE PYRUVATE DEHYDROGENASE FROM BRAIN

The question now arises as to whether the pyruvate dehydrogenase from brain will react with other α-keto-acids, and if so, whether to the same degree as found for yeast. Lipmann [1937] showed that in the presence of methylene blue under anaerobic conditions, pyruvic acid was converted by the pyruvate dehydrogenase into acetic acid and CO<sub>2</sub>. It was also found that a simple washing would produce a preparation from pigeon's brain showing a reduction time with pyruvate and methylene blue only three times more rapid than in the absence of pyruvate. This finding has recently been confirmed [Peters & Wakelin, 1938]. Under such conditions the presence of residual substrates in the tissue was objectionable. By washing with 0.2 % KCl (see Appendix with R. W. Wakelin) the reduction time of the residue was very much increased. The technique finally used was as follows.

Normal pigeon cerebrum was finely minced ice-cold, washed three times with ice-cold Ringer phosphate pH 7.3, three times with 0.3 % KCl and once with 0.2 % KCl. During each washing the tissue was allowed to remain in contact

with the salt solution for about 15 min. so that diffusion of the substrates into the latter could take place. The bulky white suspension was shrunk by a final washing with Ringer phosphate. Observations were made *in vacuo* in Thunberg tubes at 38°, 50 mg. tissue and 0.2 ml. (40  $\mu$ g.) methylene blue being used. The keto-acids were tipped in from the hollow stopper after evacuation.

Table II. *Reactivity of  $\alpha$ -keto-acids with the pyruvate dehydrogenase from pigeon's brain*

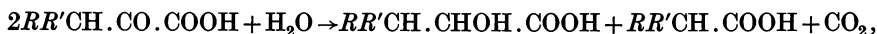
(2 expts. out of 3 quoted)

	Reduction times (min.)	
	Exp. 164	Exp. 170
Residual	167	164
$\alpha$ -Ketovaleate	104	60
Pyruvate	14	10½, 9½
$\alpha$ -Ketobutyrate	15	11½
Pyruvate + $\alpha$ -ketobutyrate	10	10½
Pyruvate + $\alpha$ -ketovaleate	—	10

The experiments in Table II show that pyruvate and  $\alpha$ -ketobutyrate react at the same rate with the pyruvate dehydrogenase system, strongly indicating that the active centre is the same for both.  $\alpha$ -Ketovaleate shows an almost negligible activity (15%), which is quite distinct from the slight reduction found for yeast. This inactivity cannot in any way be due to the effect of a poison, since the reduction time for pyruvate is not altered by the presence of  $\alpha$ -ketovaleate. This difference seems to suggest that some essential part of the system, perhaps the protein component, is different from the corresponding part of the yeast system.

#### *$\alpha$ -Keto-acids and the Krebs' dismutation*

If the pyruvate dehydrogenase plays a part in the Krebs' dismutation [Krebs & Johnson, 1937],



it might be expected that  $\alpha$ -ketovaleate would behave differently from pyruvate under these conditions. Weil-Malherbe [1937] showed that with slices of rat brain under anaerobic conditions, pyruvic acid was converted into the theoretical quantities of lactic acid and  $CO_2$ .

Working with washed minced pigeon's brain, both in Krebs' bicarbonate and in Ringer phosphate saturated with  $CO_2$ , we have compared the  $CO_2$  evolution from pyruvic acid and the two  $\alpha$ -keto-acids (Table III). Since we have washed the tissue in many of the experiments to be described later, the method of washing may be given here [cf. McGowan, 1937]. The brains (cerebrum and optic lobes) of three or four pigeons were finely minced ice-cold, transferred to a centrifuge tube and washed three times with ice-cold Ringer phosphate (3  $\times$  30 ml.). After each centrifuging, the tissue was ground against the side of the tube with a glass rod. Finally, after being well mixed, it was transferred to the previously weighed experimental bottles in roughly equal quantities (270 mg.). Residual respiration was much reduced by this treatment, without impairing the activity of the system for metabolizing pyruvic acid.

The residual bicarbonate production (Exp. 168) is not increased by the presence of pyruvic or other  $\alpha$ -keto-acid, so that the acid production is an index of the extent to which the Krebs' dismutation proceeds. It will be

Table III. *The Krebs' dismutation of  $\alpha$ -keto-acids by washed pigeon brain brei*

(a) In Krebs' bicarbonate (pH 7.3). 1 exp. out of 4 quoted. Exp. 168. Duration 120 min.

	CO <sub>2</sub> production, $\mu$ l./g.	
	Acid production	Bicarbonate production
Residual	56	36
Pyruvate	172	33
$\alpha$ -Ketobutyrate	166	35
$\alpha$ -Ketovalerate	102	40

(b) In Ringer phosphate, saturated with CO<sub>2</sub>. pH 7.3. Exp. 229. Duration 210 min.

	Acid production
Residual	49
Pyruvate	198
$\alpha$ -Ketobutyrate	195
$\alpha$ -Ketovalerate	110

observed that with  $\alpha$ -ketobutyrate the dismutation takes place to exactly the same extent as with pyruvate, while the activity of  $\alpha$ -ketovalerate is only 40% of this. The difference between this value and the 15% activity of  $\alpha$ -ketovalerate towards the pyruvate dehydrogenase and methylene blue is very surprising. According to the accepted theory of the role of the pyruvate dehydrogenase in the Krebs' dismutation, identical values for  $\alpha$ -ketovalerate would be expected.

*Brain respiration in the presence of  $\alpha$ -keto-acids*

A comparison of the increased O<sub>2</sub> uptakes of respiring pigeon brain brei due to pyruvic and other  $\alpha$ -keto-acids is given in Table IV (Exp. 206). Since variable results of small magnitude were obtained with unwashed tissue, the figures refer to washed brain, using samples of Na salts of  $\alpha$ -keto-acids of the highest degree of purity. Less pure samples gave somewhat smaller effects.

Table IV.  *$\alpha$ -Keto-acids and brain respiration*

	Washed normal brain. pH 7.3				Av.
	O <sub>2</sub> uptake, $\mu$ l./g./hr.				
	0-1	1-2	2-3	Increase	
Exp. 206*					
Residual	299	161	94		
$\alpha$ -Ketovalerate	313	180	116	14, 19, 22	18
$\alpha$ -Ketobutyrate	466	332	268	167, 171, 174	171
Pyruvate	1038	843	761	739, 682, 667	696
Exp. 223					
Residual	349	177	100		
0.02 M $\alpha$ -ketovalerate	381	235	147	31, 58, 47	45
0.06 M $\alpha$ -ketovalerate	337	200	133	-12, 23, 33	15
Exp. 226	0-1½	1½-3	3-4½		
Residual	297	131	78		
0.02 M $\alpha$ -ketovalerate	328	161	97	31, 30, 19	27
0.06 M $\alpha$ -ketovalerate	301	166	105	4, 35, 27	22

\* 1 exp. out of 2 quoted.

The most striking point arising from these figures is the preservation of a constant metabolic rate for  $\alpha$ -ketobutyric acid during a period of 3 hr. Under

these conditions the oxidation of pyruvic acid falls off very little, the O<sub>2</sub> uptake being about 4 times that for  $\alpha$ -ketobutyric acid. As in previous cases,  $\alpha$ -ketovaleric acid seems to be rather inert in comparison. The O<sub>2</sub> uptake is only about 11% that of  $\alpha$ -ketobutyric acid, a figure approaching that found with the pyruvate dehydrogenase and methylene blue. Furthermore, increased concentration of  $\alpha$ -ketovalerate does not raise the respiration, a fall being observed in one case (Exp. 223).

We have attempted to discover the exact nature of the oxidation of  $\alpha$ -ketobutyric acid in brain by determining its R.Q. To this end, 6 manometers of the Dixon-Barcroft type, in duplicate, were used, containing

- (1) Tissue alone. O<sub>2</sub> uptake measured. CO<sub>2</sub> absorbed by KOH.
- (2) Tissue alone. Initial CO<sub>2</sub> in solution measured by tipping in acid at time of zero reading.
- (3) Tissue alone. The difference between O<sub>2</sub> uptake and CO<sub>2</sub> as acid production measured manometrically as respiration proceeded. Also CO<sub>2</sub>, formed during respiration as bicarbonate, measured by tipping in acid at end of respiration period.
- (4) Tissue and  $\alpha$ -ketobutyrate. O<sub>2</sub> uptake measured as in 1.
- (5) Tissue and  $\alpha$ -ketobutyrate. As for 2.
- (6) Tissue and  $\alpha$ -ketobutyrate. As for 3.

In bottles 2, 3, 5 and 6 CO<sub>2</sub> was not absorbed, but Keilin cups containing 0.2 ml. 20% H<sub>2</sub>SO<sub>4</sub> were provided.

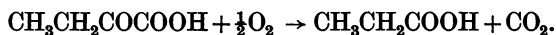
The net O<sub>2</sub> uptake was obtained by subtracting 1 from 4. In 3 and 6 the CO<sub>2</sub> formed by acid production was calculated from the observed reading and the corresponding O<sub>2</sub> uptake (1 and 4). To this was added the CO<sub>2</sub> produced in solution as bicarbonate. Initial CO<sub>2</sub> formed in 2 and 5 was then subtracted from these totals, the difference between the final values being the net CO<sub>2</sub> production due to oxidation of the  $\alpha$ -ketobutyrate. Experimental recordings are given in Table V.

Table V. R.Q. for brain respiration in presence of  $\alpha$ -ketobutyrate

Exp.	Duration min.	O <sub>2</sub> uptake $\mu$ l./g.			CO <sub>2</sub> production $\mu$ l./g.			Res. R.Q.	Net R.Q.
		Res.	$\alpha$ -Keto- butyrate	Net	Res.	$\alpha$ -Keto- butyrate	Net		
174	165	619	866	247	528	1138	610	0.85	2.46
175	180	593	877	284	505	1188	683	0.85	2.41
176	165	629	892	263	528	1138	610	0.84	2.31
179	180	548	960	412	484	1416	934	0.88	2.27
180	180	583	981	398	536	1507	971	0.92	2.44
207	180	624	1022	398	495	1510	1015	0.79	2.55
208	180	525	928	403	437	1383	946	0.83	2.36
209	220	641	1111	470	554	1636	1082	0.86	2.31
215	160	587	988	401	511	1493	982	0.87	2.45
216	160	660	1120	460	603	1648	1045	0.91	2.27
218	210	718	1212	494	675	1789	1114	0.94	2.25
Av.	180							0.87	2.37

The mean value is  $2.37 \pm 0.03$ . Agreement between individual values is not exceptionally good, but this was to be expected owing to the small differences in O<sub>2</sub> uptake and the fact that at least 8 separate measurements have to be recorded for the calculation of a single R.Q. In addition, there seems to be a dependence of the R.Q. on the duration of the experiment. Three experiments lasting only 120 min. gave R.Q.'s as high as 2.69, and are not included.

The figures are in striking contrast to the value  $1.30 \pm 0.04$  found by McGowan [1937] for pyruvic acid, and prove undoubtedly that  $\alpha$ -ketobutyric acid is much less completely oxidized. The suggestion is that  $\alpha$ -ketobutyric acid can only be oxidized as far as the next lower fatty acid and not completely to  $\text{CO}_2$  and water as in the case of pyruvic acid:



The fact that the R.Q. found is greater than 2.0 must be due, in some measure at least, to simultaneous anaerobic dismutation producing  $\text{CO}_2$ .

A most interesting and important fact now emerges from a study of the  $\text{O}_2$  uptakes for pyruvate and  $\alpha$ -ketobutyrate under the same conditions, and which is seen in Table VI. For these two acids, the same number of  $\mu$  mol. are oxidized in a given time; in the case of  $\alpha$ -ketobutyrate, simple oxidative decarboxylation alone takes place, but in the case of pyruvate, some of the acid disappearing is completely oxidized. However, the fact is that the same rates of oxidative removal of the two keto-acids are observed, independent of the subsequent path of oxidation.

Table VI. Comparison of the rate of oxidation of  $\alpha$ -ketobutyric and pyruvic acids

Exp.	Duration hr.	Total $\text{O}_2$ uptake, $\mu\text{l./g.}$			$\mu$ mol. oxidized	
		Res.	$\alpha$ -Keto- butyrate	Pyruvate	$\alpha$ -Keto- butyrate	Pyruvate
206	1	299	466	1038	14.9	16.2
	2	460	798	1881	30.2	31.1
	3	554	1066	2629	45.7	45.4
213	1	307	484	1086	15.8	17.0
	2	469	831	1987	32.3	33.1
214	1	330	507	1080	15.8	16.4
	2	508	864	1989	31.8	32.4
	3	614	1149	2772	47.8	47.3
	4	686	1383	3461	62.1	60.9

The method of calculating these quantities requires some explanation. The amount of pyruvic acid disappearing by oxidative processes is given by

$$\mu \text{ mol. pyruvic acid oxidized} = \frac{x_{\text{O}_2}}{450} \times \frac{86.6}{100} \times \frac{1}{88} \times 1000 = 0.0219 x_{\text{O}_2}.$$

$x_{\text{O}_2}$  is the  $\text{O}_2$  uptake ( $\mu\text{l.}$ ); 86.6 is the % pyruvic acid disappearing by oxidative processes giving  $\text{CO}_2$  and water, and  $\text{CO}_2$  and acetic acid [Long, 1938]; the factor 450 converts  $\text{O}_2$  uptake ( $\mu\text{l.}$ ) into pyruvic acid (mg.) [McGowan, 1937], and 88 is the mol. wt. of pyruvic acid. For  $\alpha$ -ketobutyric acid the calculation is much simpler, being

$$\mu \text{ mol. } \alpha\text{-ketobutyric acid oxidized} = x_{\text{O}_2} \times \frac{2 \times 1000}{22,400} = 0.0892 x_{\text{O}_2}.$$

These results suggest very strongly that both  $\alpha$ -ketobutyric acid and pyruvic acid undergo the same initial change under aerobic conditions. It would follow from this that each gives rise to an intermediate of similar type. Whereas the intermediate from  $\alpha$ -ketobutyric acid can only break down to  $\text{CO}_2$  and presumably propionic acid, part of the pyruvic acid intermediate can be completely oxidized to  $\text{CO}_2$  and water. The probability is that this special reaction is in some way connected with the simpler structure of this intermediate, the next higher homologue of which is unable to do this owing to the hydrocarbon chain.

The high value recorded for the R.Q. of  $\alpha$ -ketobutyric acid offers a certain difficulty. This problem can now be examined more closely in the light of the

last-mentioned conclusions. For pyruvic acid, Long [1938] showed that 86.6% disappeared aerobically by oxidative reactions, and 10.4% simultaneously by the Krebs' dismutation. Since identical amounts of pyruvic acid and  $\alpha$ -ketobutyric acid disappear oxidatively, the analogy might be taken further, i.e. it seems reasonable to suppose that the extent of dismutation is also the same in the two cases, especially in view of the equal rates found under anaerobic conditions (Table III). In that case the theoretical R.Q. would be:

$$\frac{86.6 + 5.2}{0.5 (86.6)} = \frac{91.8}{43.3} = 2.12.$$

It is difficult to account for the experimental value of 2.37, but two facts may be mentioned which would lead to higher values than the theoretical:

(1) Experiments on the R.Q. of pyruvic acid gave values of *c.* 1.47 in cases where the tissue had not been thoroughly washed.

(2) Samples of  $\alpha$ -ketobutyric acid not of the highest degree of purity gave values of 3.59 and 3.56 (Exps. 146 and 154).

Although the effect due to 2 is probably absent from Table V, nevertheless incomplete washing of the tissue might well account for the anomaly. Lastly, judging from the influence of duration on the value of the R.Q., the calculated result might be achieved by neglecting the first  $\frac{1}{2}$  hr. of the respiration.

Some experiments have been carried out to compare the extent of Krebs' dismutation taking place under aerobic and anaerobic conditions in pyruvate solutions. The amount of dismutation proceeding aerobically was calculated from the observed O<sub>2</sub> uptake (10.4% of the pyruvic acid disappears by dismutation). It was found that the amount of anaerobic dismutation decreased with time from about 23% during the 1st hour to 17% after 3 hr., the % referring to the anaerobic pyruvate disappearance compared with the pyruvate metabolized aerobically. Experiments have thus been continued for 3-4 hr., since this was the period in which 10.4% dismutation was found to take place aerobically [Long, 1938], Table VII; cf. also Barron & Lyman [1939].

Table VII. *Dismutation of pyruvic acid under aerobic and anaerobic conditions*

Ringer phosphate, pH 7.3; for anaerobic experiments this was saturated with CO<sub>2</sub>.

Exp.	Duration min.	Anaerobic		Aerobic	
		Net CO <sub>2</sub> $\mu$ l./g.	Pyruvate $\mu$ mol.	Net O <sub>2</sub> $\mu$ l./g.	Pyruvate $\mu$ mol.
226	180	99	8.8	1985	5.2
227	180	81	7.2	1816	4.8
230	200	111	9.9	2183	5.7

Thus there seems to be little doubt that more dismutation of pyruvate takes place under anaerobic conditions than in the presence of O<sub>2</sub>. It was for this reason that we could not assume identical rates of dismutation of  $\alpha$ -ketobutyric acid in the presence and absence of O<sub>2</sub> and hence calculate the "aerobic R.Q." directly.

#### *$\alpha$ -Ketobutyric acid and the catatorulin effect with avitaminous brain*

With washed avitaminous brain, small but definite catatorulin effects were observed on adding vitamin B<sub>1</sub> to the tissue respiring in solutions of  $\alpha$ -ketobutyrate. With  $\alpha$ -ketovalerate the effect was negligible. No catatorulin effects



were observed with the washed avitaminous brain alone. Table VIII also shows the lowered level of respiration of the avitaminous brain in the presence of  $\alpha$ -ketobutyric acid, a phenomenon so far unexplained.

Table VIII. *Catatorulin effects with  $\alpha$ -ketobutyric acid*

pH 7.3, 38°. 4  $\mu$ g. vitamin B<sub>1</sub> used

Exp.	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Catatorulin effects (last 2 hr.)
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-3	
Exp. 199						
Residual	434	322	253	206	141	
Residual + vitamin	417	335	249	189	142	-4, -17, 1
$\alpha$ -Ketobutyrate	293	219	187	144	123	
Same + vitamin	312	261	222	181	164	35, 37, 41
Exp. 147						
Residual	387	232	124	69		
Residual + vitamin	400	234	124	71		2, 0, 2
$\alpha$ -Ketobutyrate	225	136	79	50		
Same + vitamin	268	172	109	71		36, 30, 21

The catatorulin effects are seen to be very small. However, mainly owing to the increased water content of the tissue due to the conditions of washing, pyruvic acid has a catatorulin effect of only 160-200  $\mu$ l./g./hr. (see Table X).

*Effect of  $\alpha$ -keto-acids on pyruvate respiration in brain*

Working with the unwashed brain brei from avitaminous pigeons, at pH 7.3 and 38°, Peters [1937] obtained evidence that  $\alpha$ -ketobutyric acid inhibited the catatorulin effect. We have continued this work and have studied in detail the action of this acid and of  $\alpha$ -ketovaleric acid on pyruvate respiration in normal and avitaminous brain.

Figures are quoted for the normal brain in Table IX. The effects are due to pure samples of  $\alpha$ -keto-acids and are of the same order for both washed and unwashed tissue.

Table IX. *The inhibition of pyruvate respiration in brain by  $\alpha$ -keto-acids. Normal brain*

I. Unwashed tissue

	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net pyruvate oxidation (last hr.)	Av.
	0- $\frac{1}{4}$	$\frac{1}{4}$ - $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2		
(a) $\alpha$ -Ketobutyric acid. 1 exp. out of 4 quoted.							
Exp. 196							
Residual	1366	1120	883	677	436		
Pyruvate	2553	2353	2173	1977	1845	1300, 1409	1355
$\alpha$ -Ketobutyrate	1411	1272	1049	897	744		
Pyruvate + $\alpha$ -ketobutyrate	2522	2290	2128	1991	1783	1094, 1039	1067
(b) $\alpha$ -Ketovaleric acid. 1 exp. out of 5 quoted.							
Exp. 197							
Residual	1414	1178	898	719	490		
Pyruvate	2833	2580	2330	2075	1904	1356, 1414	1385
$\alpha$ -Ketovalerate	1206	1054	832	674	500		
Pyruvate + $\alpha$ -ketovalerate	2733	2475	2220	1913	1779	1239, 1279	1259

Table IX (cont.)

		O <sub>2</sub> uptake, $\mu\text{l./g./hr.}$				Net pyruvate oxidation (last 1½ hr.)	Av.
		0-¼	¼-½	½-1	1-2		
(a) $\alpha$ -Ketobutyrate. 1 exp. out of 3 quoted.							
Exp. 198							
Residual		483	337	276	178		
Pyruvate		1115	1026	921	761	645, 583	614
$\alpha$ -Ketobutyrate		609	522	463	371		
Pyruvate + $\alpha$ -ketobutyrate		1126	1020	920	802	457, 431	444
		O <sub>2</sub> uptake, $\mu\text{l./g./hr.}$				Net pyruvate oxidation (last 2 hr.)	Av.
		0-¼	¼-1	1-2	2-3		
(b) $\alpha$ -Ketovalerate. 1 exp. out of 2 quoted.							
Exp. 221							
Residual		421	274	186	102		
Pyruvate		1092	990	871	743	685, 641	663
$\alpha$ -Ketovalerate		424	300	207	133		
Pyruvate + $\alpha$ -ketovalerate		1089	962	801	628	594, 495	545
(c) Comparison of $\alpha$ -ketobutyrate and $\alpha$ -ketovalerate.							
Exp. 223							
Residual		416	281	177	100		
Pyruvate		1139	1027	915	792	738, 692	715
$\alpha$ -Ketovalerate		435	326	236	148		
Pyruvate + $\alpha$ -ketovalerate		1175	1011	812	607	576, 459	518
$\alpha$ -Ketobutyrate		615	476	378	311		
Pyruvate + $\alpha$ -ketobutyrate		1139	1006	850	703	472, 392	432

One conclusion which may be drawn from these figures is that  $\alpha$ -ketobutyrate inhibits pyruvate respiration more than does  $\alpha$ -ketovalerate. For  $\alpha$ -ketobutyrate the inhibition with unwashed brain is *c.* 21% and with washed brain *c.* 38%; in the case of  $\alpha$ -ketovalerate the corresponding figures are 9 and 23%, at concentrations of *c.* 0.02 *M* in both cases. This difference would be expected from the mechanism of the inhibition (see later). It might be mentioned that in earlier experiments, in which  $\alpha$ -keto-acids of doubtful purity were used, similar net inhibitions were observed; in addition, however, the residual respiration level in the unwashed brain was lowered by  $\alpha$ -ketobutyrate, also, the extent of such inhibition decreasing with time.

Turning to the avitaminous brain, we have observed inhibitions of the catatorulin effect in pyruvate solutions by the  $\alpha$ -keto-acid homologues. The impure acids gave inhibitions of *c.* 30% with unwashed brain, similar to those found by Peters [1937]. The figures quoted in Table X are for washed avitaminous brain using purest samples of the  $\alpha$ -keto-acids.

Here again the inhibition of the catatorulin effect is greater with  $\alpha$ -ketobutyrate (37%) than with  $\alpha$ -ketovalerate (27%). Incidentally, the real inhibition of the catatorulin effect is slightly greater than that given in Table X, owing to the fact that the catatorulin effect of the  $\alpha$ -ketobutyric acid alone has not been taken into account. The inhibiting effects produced by  $\alpha$ -ketobutyrate would then be increased by about 36  $\mu\text{l./g./hr.}$  In the case of  $\alpha$ -ketovalerate, the correction on this account would be negligible.

As to the nature of the inhibition, two alternatives seemed possible; either the  $\alpha$ -keto-acids were exerting a general effect on the respiration by inhibiting the action of the indophenol oxidase system; or the effect was peculiar to pyruvate respiration, *i.e.* competitive inhibition, such as has been observed in

Table X. *Inhibition of the catatorulin effect in washed avitaminous brain by  $\alpha$ -keto-acids*

4  $\mu$ g. vitamin B<sub>1</sub> used. pH 7.3

	O <sub>2</sub> uptake $\mu$ l./g./hr.			Catatorulin effects (last 2 hr.)	Av.
	0-1	1-2	2-3		
(a) $\alpha$ -Ketobutyric acid. 1 exp. out of 2 quoted.					
Pyruvate	582	405	310		
Pyruvate + vitamin	745	603	513	198, 203	201
Pyruvate + $\alpha$ -ketobutyrate	496	390	247		
Same + vitamin	618	520	389	130, 142	136
(b) $\alpha$ -Ketovaleric acid.					
Exp. 238					
Pyruvate	655	479	374		
Pyruvate + vitamin	813	677	566	198, 192	195
Pyruvate + $\alpha$ -ketovalerate	681	474	334		
Same + vitamin	798	622	472	148, 138	143

brain with lactate [Jowett & Quastel, 1937] and succinate [Quastel & Wheatley, 1931] in the presence of hydroxymalonic and malonic acids respectively. The effect on unwashed brain brei respiring in succinate seemed to favour the first view since an inhibition of the succinodehydrogenase system was observed comparable in magnitude with that found for the pyruvate oxidase system (Table XI).

However, when washed tissue was used, the inhibition of the succinodehydrogenase system was considerably reduced. In one exp. (186, Table XI) no inhibition was observed at all.

Table XI. *Effect of  $\alpha$ -keto-acids on succinate respiration in normal brain. S = succinate*

I. Unwashed tissue

(a)  $\alpha$ -Ketobutyric acid. 1 exp. out of 3 quoted.

Exp. 192	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net succinate oxidation (last hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2		
Residual	1194	1010	776	599	494		
Succinate	2755	2262	1833	1290	1146	691, 652	672
$\alpha$ -Ketobutyrate	1290	1100	896	760	676		
S + $\alpha$ -ketobutyrate	2538	2110	1776	1373	1067	613, 391	502

(b)  $\alpha$ -Ketovaleric acid. 1 out of 3 quoted.

Exp. 195	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net succinate oxidation (last hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2		
Residual	1430	1059	867	627	490		
Succinate	2872	2354	1833	1380	1117	753, 627	690
$\alpha$ -Ketovalerate	1127	956	780	600	483		
S + $\alpha$ -ketovalerate	2188	1990	1520	1137	957	537, 474	506

Table XI (cont.)

## II. Washed tissue

(a)  $\alpha$ -Ketobutyric acid.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net succinate oxidation (last hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2		
Exp. 186						
Residual	384	266	190	—		
Succinate	782	607	489	—	341, 299	320
$\alpha$ -Ketobutyrate	592	457	382	—		
S + $\alpha$ -ketobutyrate	936	798	692	—	341, 310	325
Exp. 239						
Residual	460	293	206	172		
Succinate	880	718	587	490	381, 318	350
$\alpha$ -Ketobutyrate	611	466	383	338		
S + $\alpha$ -ketobutyrate	1041	825	689	566	306, 228	267

(b)  $\alpha$ -Ketovaleric acid. 1 exp. out of 2 quoted.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net succinate oxidation (last 2 hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-2	2-3		
Residual	409	279	181	102		
Succinate	784	650	506	348	325, 246	286
$\alpha$ -Ketovalerate	452	354	237	148		
S + $\alpha$ -ketovalerate	789	657	469	321	232, 173	203

The absence of any general effect on the indophenol oxidase system was conclusively proved by our finding that  $\alpha$ -ketobutyric acid had no action on the respiration of pig's heart muscle extract in the presence of cytochrome *c* and quinol (Table XII).

Table XII.  $\alpha$ -Ketobutyric acid and the indophenol oxidase

## Exp. 183. 1 exp. out of 2 quoted.

Quinol mg.	Indophenol oxidase ml.	Cytochrome <i>c</i> ml.	$\alpha$ -Keto-butyrate mg.	$\mu$ l./g./hr.	
				0- $\frac{1}{2}$	$\frac{1}{2}$ -1
5	—	—	—	101	76
5	—	—	5	73	85
5	0.20	—	—	632	327
5	0.20	—	5	656	338
5	0.20	0.10	—	782	177
5	0.20	0.10	5	784	153

Finally, it was observed that the inhibition of pyruvate respiration in washed normal brain was increased on tripling the concentration of the  $\alpha$ -ketobutyric acid (Table XIII).

Table XIII. Effect of increasing the concentration of  $\alpha$ -ketobutyric acid on pyruvate respiration in washed normal brain

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net pyruvate oxidation (last 1 $\frac{1}{2}$ hr.)	Av.
	0- $\frac{1}{4}$	$\frac{1}{4}$ - $\frac{1}{2}$	$\frac{1}{2}$ -1	1-2		
Exp. 198						
Residual	483	337	276	178		
Pyruvate	1115	1026	921	761	645, 583	614
0.02 <i>M</i> $\alpha$ -ketobutyrate	609	522	463	371		
Same + pyruvate	1126	1020	920	802	457, 431	444
0.06 <i>M</i> $\alpha$ -ketobutyrate	602	526	475	371		
Same + pyruvate	1037	963	855	680	380, 309	345

On increasing the concentration of  $\alpha$ -ketobutyric acid from 0.02 to 0.06 *M*, the inhibition of pyruvate respiration is raised from 28 to 44 %.

Although there has been no attempt to work out the kinetics in detail, there can be little doubt that competitive inhibition is taking place. It has already been shown that the initial stage in  $\alpha$ -keto-acid oxidation is the same for pyruvic acid and  $\alpha$ -ketobutyric acid. If the two are allowed to compete for an enzyme, they will do so in proportion to their individual concentrations. In brain tissue under aerobic conditions, the amounts of the corresponding intermediates formed will be in this same ratio. Since the breakdown of the  $\alpha$ -ketobutyric acid intermediate is unaccompanied by an uptake of  $O_2$ , an inhibition of pyruvate respiration will be observed the magnitude of which will depend on the relative concentrations.

In the avitaminous brain, inhibition is also observed, and this can only mean that both  $\alpha$ -ketobutyrate and pyruvate are competing for the centres activated by vitamin  $B_1$ . Taken separately they both show catatorulin effects, that due to  $\alpha$ -ketobutyrate being smaller than in the case of pyruvate. We are thus led to the conclusion that vitamin  $B_1$  is essential for the initial stage in the oxidation. Support for this proposed mechanism of the catatorulin effect is to be found in the fact that the % inhibition of pyruvate respiration in normal brain and the catatorulin effect with pyruvate in the avitaminous brain by  $\alpha$ -ketobutyrate are approximately the same (c. 35 %).

The case of  $\alpha$ -ketovalerate can be provisionally explained. This substance is oxidized to a small extent only by the normal and avitaminous brain, which suggests that its rate of forming the corresponding intermediate is low. Thus one would expect the competition with pyruvate to be smaller, and this is what has been found. Such a view, however, is not quite consistent with the finding that 0.02 *M*  $\alpha$ -ketovalerate saturates the system (Table IV), an excess not increasing the rate of formation of the intermediate. Owing to the small increase in  $O_2$  uptake observed, it has not been possible to determine the saturation concentration of  $\alpha$ -ketovaleric acid in the brain system, but by analogy this is probably considerably below 0.02 *M*.

DISCUSSION

In order to clarify the various points concerning the effect of  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids on the respiration of pyruvate and succinate in washed and unwashed brain, the following Table XIV is appended. Effects due to samples not of the highest degree of purity are listed in brackets whenever their behaviour differs from that of the purest specimens.

Table XIV. *Net effects of  $\alpha$ -keto-acids on the respiration of pigeon brain brei*

	Normal brain						Avitaminous brain			
	Residual		Succinate		Pyruvate		Cat. effect with pyr.		Residual	
	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.
$\alpha$ -Ketobutyrate	+ (-)	+	-	0?	-	-	-	-	-	-
$\alpha$ -Ketovalerate	-	+	-	-	-	-	-	-	-	-

+ indicates increased respiration; - indicates an inhibition.

The most significant points arising out of the work described are (a) that the rate of decarboxylation by the yeast carboxylase system is practically the same for three  $\alpha$ -keto-acids, and (b) that the rate of oxidation by brain tissue is the same for two of them, pyruvic acid and  $\alpha$ -ketobutyric acid; (c) the difference

between these two lies in the further metabolism of pyruvic acid, so that part of the initial oxidation product is completely oxidized to  $\text{CO}_2$  and water. Since cocarboxylase is essential for decarboxylation by yeast, the inference from (a) and (b) is that it is also responsible for the initial change in brain. In the avitaminous brain this change is accelerated by the addition of vitamin  $\text{B}_1$ ; and in view of the recent finding by Ochoa & Peters [1938, 2] that a limited though definite synthesis of cocarboxylase from vitamin  $\text{B}_1$  takes place in surviving brain tissue, this suggestion is at least plausible. Further support for cocarboxylase as an essential constituent of the pyruvate oxidase system is to be found in the statements of Ochoa & Peters [1938, 1] and Westenbrink & Goudsmit [1938] that normal brain tissue contains *c.* 4  $\mu\text{g./g.}$  cocarboxylase, while the free vitamin  $\text{B}_1$  content seems to be negligible. The main difference between the yeast and brain systems is that in the latter case oxidation accompanies decarboxylation [cf. Lipmann, 1937]. Lastly the oxidative decarboxylation is specific,  $\alpha$ -ketovalerate being hardly affected by the pyruvate dehydrogenase in brain. From this fact, the inference must be drawn that the protein part of the enzyme is different in yeast and brain.

In regard to the methylene blue experiments, it is to be noted that pyruvic acid and  $\alpha$ -ketobutyric acid cause decoloration at practically the same rate; hence this does not follow the total  $\text{O}_2$  uptake, but only that produced by the postulated system A. Thus, support is obtained for the view that during the intensive washing with hypotonic salt solutions, the components of system B have been lost.

#### SUMMARY

1. Cocarboxylase is essential for the decarboxylation by yeast of  $\alpha$ -ketovaleric acid as well as for pyruvic and  $\alpha$ -ketobutyric acids. Vitamin  $\text{B}_1$  increases the rate of decarboxylation of  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids only in the presence of cocarboxylase.  $\text{CO}_2$  evolution with  $\alpha$ -ketovaleric acid is slightly less than with the others.

2. Pyruvic and  $\alpha$ -ketobutyric acids are equally reactive with the pyruvate dehydrogenase system in brain under anaerobic conditions in the presence of methylene blue.  $\alpha$ -Ketovaleric acid is much less reactive.

3. The Krebs' dismutation proceeds to the same extent with  $\alpha$ -ketobutyric acid and pyruvic acid; again  $\alpha$ -ketovaleric acid is less affected.

4. Washed brain tissue causes oxidative decarboxylation of  $\alpha$ -ketobutyric acid giving presumably propionic acid (next lower fatty acid).  $\alpha$ -Ketovaleric acid is oxidized to only a very slight extent. The purity of the acids used was of the greatest importance, high values for the R.Q. of  $\alpha$ -ketobutyric acid being obtained in the presence of a slight impurity.

5.  $\alpha$ -Ketobutyric acid and  $\alpha$ -ketovaleric acids enter into competitive inhibition with pyruvic acid both in the normal and avitaminous brains respiring *in vitro*. There is no inhibition of the indophenol oxidase system.

6. Pyruvic and  $\alpha$ -ketobutyric acids are oxidized at exactly the same rate under identical conditions. In the former case, however, most of the initial oxidation product undergoes complete combustion to  $\text{CO}_2$  and water. This indicates that there is an initial common path (A) for oxidative decarboxylation; in the case of pyruvic acid a further system (B) causes complete oxidation of part of the intermediate.

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## APPENDIX

WITH R. W. WAKELIN

Lipmann [1937] showed that thoroughly extracted rat brain catalysed the reduction of methylene blue in the presence of pyruvate; the control without pyruvate showed practically no residual reduction. Pigeon brain, on the other hand, would only yield preparations which gave about a three-fold quicker reduction time with pyruvate than the control. We have found that if the pigeon's brain tissue is washed first with Ringer phosphate, *pH* 7.3, and then with either 0.2 or 0.3 % KCl, a hypotonic solution, residual substrates are almost completely removed. After this washing, it is best to shrink the preparation in Ringer phosphate solution, as the hypotonic solutions lead to marked swelling of the tissue. For exact details of this preparation, see the text, p. 761. Variations of this procedure such as preliminary extraction with distilled water do not give satisfactory results. Not only substrates, but also the capacity for giving appreciable O<sub>2</sub> uptakes with pyruvate, are removed by our treatment; however, all the necessary components to give aerobic oxidation of succinic acid are present, though much reduced as compared with unwashed tissue. The pyruvate dehydrogenase present, unlike the complete oxidase system, is moderately resistant to freezing. Since O<sub>2</sub> uptake is here abolished without loss of the capacity for dehydrogenation, it is system B which is eliminated. It is interesting to note that we found in some experiments an inhibition of O<sub>2</sub> uptake with pyruvate due to methylene blue, in amounts which would give excellent results in the anaerobic experiments.

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