CCLXXI. STUDIES ON BRAIN METABOLISM
III. THE ANAEROBIC DISMUTATION OF α-KETO-ACIDS

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THE anaerobic dismutation of pyruvic acid has been demonstrated for various animal tissues [Krebs & Johnson, 1937, 1]. The present results which were arrived at independently[1] and with different methods confirm this reaction for pyruvic acid and show further an analogous dismutation of α-ketoglutaric acid.

In a previous paper [Weil-Malherbe, 1937, 1] the formation of succinic acid from pyruvic acid by brain tissue has been shown. The experiments to be described were undertaken to obtain a better insight into the mechanism of succinic acid formation, and also to decide if succinic acid is an intermediate in the main pathway of pyruvic acid oxidation. One way of approaching this problem is to analyse the fate of that part of pyruvic acid which is oxidized. If succinic acid lies in the main pathway the oxidized part of pyruvic acid should be entirely accounted for by succinic acid or by possible intermediates of succinic acid formation, whereas, if the succinic acid formation is only a side reaction, other products of oxidation should be found, or, failing this, a deficit should appear in the balance sheet. It is obvious that the oxidation of pyruvic acid, which proceeds rapidly to completion in the presence of oxygen, has to be slowed down, if it is intended to study balance sheets of this kind. This can be done satisfactorily by employing anaerobic conditions.

Analytical methods

(1) *Pyruvic acid.* Pyruvic acid was usually estimated by the carboxylase method with enzyme preparations made according to Axmacher & Bergstermann [1934]. In some cases bisulphite-binding capacity was also titrated; the results were always practically identical.

(2) *Ketoglutaric acid.* The analyses were done to determine the disappearance of added ketoglutaric acid only; titration of bisulphite-binding capacity was found to be quite adequate for this purpose. Although the method is unspecific no blank was ever found in the controls either at the beginning or at the end of the experiment. In some large-scale experiments ketoglutaric acid was estimated gravimetrically as 2:4-dinitrophenylhydrazone.

(3) *Lactic acid* was determined by the method of Friedemann *et al.* [1927].

(4) *Acetic acid.* The method previously described [Weil-Malherbe, 1937, 1] was used with some improvements. It is important that the volume of the solution be the same in all distillations and be kept constant during the distillation. If the solution to be analysed contains pyruvic acid, 1 ml. of 10% phenylhydrazine hydrochloride is added. It is not necessary to remove the precipitate,

[1] Preliminary accounts have been published elsewhere [Weil-Malherbe, 1936, 2, 3].

if any, since the phenylhydrazone is not decomposed under the mild conditions of the distillation. The solution is left for 30 min., made up to 7 ml. and transferred to the bulb of the Parnas apparatus; 1 ml. of 10 vol. % H_3PO_4 is added to make a total volume of 8 ml. Finally 5 g. NaH_2PO_4 are introduced, giving *p*H about 2. At an appreciably lower *p*H chlorides may interfere. Lactic acid is not volatile under the conditions described.

The recovery amounts to 85% in 25 ml. of the distillate; without addition of NaH_2PO_4 only 50% of acetic acid passes over in the same volume. The titration is carried out at room temperature with *N*/200 NaOH (CO_2-free) using phenolphthalein as indicator. Before the next estimation is started, the bulb of the apparatus is cleaned and 50 ml. more of distillate are collected to remove acid. This method was found to be both speedy and reliable. Owing to the comparatively small volume of the distillate 0·05 mg. of acetic acid can still be estimated with sufficient accuracy.

(5) *Succinic acid* was determined enzymically [Weil-Malherbe, 1937, 1]. The extracts were made from pig's heart by an improved method [Weil-Malherbe, 1937, 2]. In most cases freshly prepared enzyme solution was used. Rapid inactivation of the enzyme at 38° in the absence of substrate, as claimed by Elliott & Greig [1937], has never been observed, unless old extracts were used. The deterioration of the enzyme can be avoided to a certain extent by drying, although this involves a small loss of activity as compared with the fresh extract. In most experiments the analysis was performed on the original solution, but in some large-scale experiments a preliminary ether extraction was made.

(6) *Hydroxyglutaric acid.* (*a*) Polarimetric method: the rotation of hydroxyglutaric acid is increased in uranyl acetate solution [Mayer, 1931]. We find for sodium *l*(−)α-hydroxyglutarate $[\alpha]_D^{20°} = -8{\cdot}6°$ in water and $[\alpha]_D^{20°} = -47{\cdot}5°$ in 20% uranyl acetate, i.e. an increase of 550%. This increment is a constant which is characteristic of hydroxyglutaric acid and, when it is found, the presence of hydroxyglutaric acid may be inferred and its quantity assessed. The substance most likely to interfere with this estimation is malic acid; but the specific rotation of malic acid in neutral solution is increased from −3·0° to about −480° by the addition of uranyl acetate, i.e. by 16,000%. A small amount of malic acid will thus produce a relatively large increase of the increment calculated for hydroxyglutaric acid, but its quantity can be estimated from the theoretical values for hydroxyglutaric and malic acid respectively.

Owing to the low rotation of hydroxyglutaric acid this method is restricted to large-scale experiments. The polarimetric estimations were always done on ether extracts. These were neutralized with $Ba(OH)_2$; any precipitate was removed and 4 vol. of alcohol were added. The precipitate of Ba salts was decomposed with H_2SO_4, neutralized with NaOH and concentrated to 7 ml. The first polarimetric reading was now taken, using a 1 dm. tube. The solution was transferred to a small Erlenmeyer flask, made up to 10 ml. and 2 g. of uranyl acetate were added. After heating on the water-bath for 20 min. the solution was kept in the dark for 2 hr. It was then concentrated, filtered and made up to 7 ml., and again examined polarimetrically.

(*b*) *Manometric method.* Attempts to estimate hydroxyglutaric acid as succinic acid after oxidation with $KMnO_4$ and extraction with ether did not give satisfactory results, the yield of succinic acid being less than 40%. Use was therefore made of the fact that pyocyanine acts as a highly specific carrier for hydroxyglutaric dehydrogenase [Weil-Malherbe, 1937, 2]. In the arrangement used for the estimation of succinic acid, i.e. with low concentrations of enzyme and with brilliant cresyl blue as carrier, the oxidation of hydroxyglutaric acid is

so extremely slow as to be negligible. With a high enzyme concentration and pyocyanine as carrier succinic and hydroxyglutaric acids are oxidized simultaneously. The excess of the O_2 uptake in the "pyocyanine test" over that in the "brilliant cresyl blue test" is regarded as due to the oxidation of hydroxyglutaric acid, since this is the only substance, as far as we know, which is oxidized by the enzyme preparation only under the conditions of the first test, but not under the conditions of the second test.

The solution to be analysed is concentrated to about 2 ml. and the volume is measured. Two aliquots of 0·5 ml. each are measured into the side bulbs of two Warburg manometer vessels. One of these contains in the main part 1·5 ml. water, 0·5 ml. enzyme solution and 0·5 ml. 0·5% brilliant cresyl blue, the other contains 2 ml. enzyme and 0·5 ml. *M*/50 pyocyanine. Controls with 0·5 ml. of water in the side bulbs instead of solution are run simultaneously. At thermal equilibrium the contents of the side bulbs are tipped into the enzyme solution. The method gives theoretical results with a mixture of the pure acids. With solutions in which tissue had been incubated, however, a slight excess of the O_2 uptake in the "pyocyanine test" over that in the "brilliant cresyl blue test" was generally observed, even when the presence of hydroxyglutaric acid was not anticipated. Whether this was due to the presence of preformed hydroxyglutaric acid or of another substance, cannot be decided.

(7) *Acetone bodies* are estimated in two fractions, fraction I representing acetone + acetoacetic acid, fraction II β-hydroxybutyric acid. The solution is treated with basic lead acetate and copper-lime and, after centrifuging, transferred to a 50 ml. distilling flask fitted with a ground-in dropping funnel and so designed that a stream of gas can be led through the solution. The distillate is collected in a 25 ml. volumetric flask containing 1 ml. of 10% $NaHSO_3$ by means of a drawn-out adaptor, the end of which dips under the surface of the bisulphite solution. 1 ml. of 50% H_2SO_4 and about 20 ml. water are added to the solution in the distilling flask and the distillation is started, a slow stream of nitrogen being bubbled through to prevent sucking back of the distillate. Water is added from the dropping funnel to keep the volume more or less constant. After about 25 ml. of distillate have been collected, the distillation is discontinued and the receiver is replaced by a second flask also containing 1 ml. 10% $NaHSO_3$. 5 ml. of 66 vol. % H_2SO_4 are added to the solution in the distilling flask and the distillation is started again. As soon as the solution has started boiling, 10 ml. of 0·1% $K_2Cr_2O_7$ solution, or more if necessary, are added very slowly from the dropping funnel and 25 ml. of distillate are collected. The two distillates representing fractions I and II are now separately redistilled with addition of 2 ml. 5 *N* NaOH and about 0·5 g. of sodium peroxide, the distillates being again collected in 25 ml. volumetric flasks containing 1 ml. of 10% $NaHSO_3$. The acetone contained in the two final distillates is titrated according to Lindenberg [1936]. β-Hydroxybutyric acid yields 70–75% of the theoretical amount of acetone. The specificity of the method is quite sufficient. The method is applicable to quantities as small as 0·01 mg. of acetone and 0·025 mg. of β-hydroxybutyric acid.

Units. Quantities of metabolites are expressed as μl. gas (1 millimol = 22,400 μl.) or in *Q* values $\left(\frac{\mu l.}{\text{mg. dry weight} \times \text{hr.}}\right)$.

Results

I. *The anaerobic metabolism of pyruvic acid*

(1) *Results with brain slices.* By analysing aliquots of the same solution complete balance sheets of the anaerobic metabolism of pyruvic acid were obtained. For these experiments, which had to be done on a scale somewhat larger than usual, two Warburg manometer vessels of about 40 ml. vol. with side arm and centre well were used. The slices from four rat brains were divided into two equal portions, transferred to the vessels and there suspended in 10 ml. bicarbonate-saline. The gas space was filled with a mixture of 95% N_2 and 5% CO_2; the centre well contained 0·3 ml. of freshly prepared chromous chloride. At time t_0, after thermal equilibrium had been reached, 0·5 ml. of $M/5$ pyruvate was tipped from the side arm of one of the vessels. After 2 hr., during which period the pressure changes were recorded, the tissue was killed by the addition of 0·5 ml. 3N HCl, removed by centrifuging, dried and weighed. The solutions were put into a boiling water-bath for 5 min., then neutralized to litmus and cleared by centrifuging. 5 ml. of the solution were concentrated on the water-bath to 1 ml. and used for succinic acid estimation. Lactic acid estimation was done on 1 ml. of the original solution after copper-lime treatment, pyruvic acid estimation on 0·5 ml. and the remainder of the solution was used for estimation of acetic acid. CO_2 was calculated from the positive pressure observed. The bicarbonate content of the solutions remained constant, as has also been shown by Krebs & Johnson [1937, 1].

When the brain slices for these experiments were prepared, less care was taken than usually to avoid the white matter in order to save time and material. This explains the somewhat low Q values recorded in Table I. In the last column of this table the quantity of pyruvic acid disappearing is arbitrarily denoted as 200 and the quantities of the other metabolites are expressed in proportional units. The figure for succinic acid is doubled, since it arises from 2 equiv. of pyruvic acid.

It appears that the figures for lactic acid and CO_2 are as near to 100 as can be expected. For these two metabolites therefore the equation

$$2CH_3.CO.COOH + H_2O = CH_3.CHOH.COOH + CH_3.COOH + CO_2 \quad \text{.....(1)}$$

is realized by the experiment. On the other hand only 75–80% of the theoretical amount of acetic acid is found, but the remainder is almost entirely accounted for by succinic acid. From this the conclusion may be drawn that a certain part of the acetic acid, amounting to about 20%, has been further oxidized to succinic acid. The sum of acetic + succinic acids is approximately 100, so that there is no deficit left in the balance sheet. This shows that, under these conditions, there are no reactions following the oxidation of pyruvic acid other than formation of acetic and succinic acids.

The reductive equivalent which corresponds to oxidation of 20% of the acetic acid to succinic acid cannot be lactic acid. Were this so, the amount of lactic acid formed should be more than half the amount of the pyruvic acid used and, correspondingly, the amount of CO_2 should be less than this. It is not pyruvic acid at the expense of which this oxidation is accomplished. We know nothing at present about the nature of the hydrogen acceptor to which this oxidation is due.

In a series of experiments the relation of the evolution of CO_2 to the formation of acetic acid has been further studied. The increase of total CO_2 was determined

Table I. *Balance sheet of the anaerobic metabolism of pyruvic acid in rat brain slices*

	No substrate		$M/100$ pyruvate		Difference		Proportions (pyruvic acid disappearance = 200)
Exp. 1	μl.	Q value	μl.	Q value	μl.*	Q value	
Dry weight of slices (mg.)	164·8		175·8				
Pyruvic acid	0	0	−890	−2·53	−890	−2·53	200
CO_2	35·4	0·11	444	1·26	406	1·16	91
Lactic acid	129	0·39	570	1·62	432·5	1·23	97
Acetic acid	23	0·07	376	1·07	351	1·00	79
Succinic acid	0	0	43	0·12	43	0·12	19
Exp. 2							
Dry weight of slices (mg.)	190·0		168·5				
Pyruvic acid	0	0	−980	−2·90	−980	−2·90	200
CO_2	84·5	0·22	581	1·72	506	1·50	103
Lactic acid	126	0·33	600	1·78	488	1·45	100
Acetic acid	31	0·08	406	1·20	378·5	1·12	77
Succinic acid	0	0	38	0·11	38	0·11	15·5
Exp. 3							
Dry weight of slices (mg.)	178·0		182·0				
Pyruvic acid	0	0	−1070	−2·94	−1070	−2·94	200
CO_2	126	0·35	660	1·81	531·5	1·46	99
Lactic acid	144	0·40	700	1·92	553	1·52	103
Acetic acid	16	0·04	440	1·21	423·5	1·16	79
Succinic acid	0	0	47	0·13	47	0·13	17·5

* Corrected for weight.

in parallel experiments by acidification at the beginning of the experiment and after 2 hr. The tissue was suspended in 3 ml. of phosphate-saline, free from bicarbonate, or in 1·5 ml. of bicarbonate-saline containing $M/80$ $NaHCO_3$ and in equilibrium with 2·5% CO_2. Pyruvate was tipped at t_0 from one of the two side bulbs of the vessel. Acetic acid was determined in the solutions acidified at the beginning and at the end of the experiment.

The results for acetic acid (Table II) vary from 50 to 90% of the theoretical amount. The yield seems to be smaller with guinea-pig brain.

(2) *Results with minced brain.* Two guinea-pig brains were used for one experiment. The brains were finely chopped with scissors, ground with sand and 1 part of $M/100$ phosphate buffer *p*H 7·4. 2 ml. of the paste were measured (with the aid of a pipette with broken off point) into vessels provided with two side bulbs. Four vessels in all were used, two controls and two in which 0·2 ml. of $M/2$ pyruvate was tipped from one of the side bulbs at t_0. The gas space was filled with nitrogen, the centre well contained chromous chloride solution. At t_0 one of the controls and one of the pyruvate vessels were acidified by tipping in 0·3 ml. of $3N$ HCl. The remaining two vessels were acidified after 2 hr. When the pressure changes had ceased, the vessels were removed from the thermostat and the contents transferred to centrifuge tubes with about 8 ml. of wash water. After centrifuging the supernatant solution was heated in a boiling water-bath for 5 min. and neutralized. A further precipitate was centrifuged off. The precipitates were not washed, but the total volume, including the precipitate, was determined before and after every operation

Table II. *Formation of CO_2 and acetic acid during the anaerobic metabolism of pyruvic acid in brain slices*

The first of each bracketed pair was acidified at zero time and the second after 2 hr. (see text)

Species	Medium	mg. dry wt.	µl. total CO_2	Δ_1	Δ_2	µl. acetic acid	Proportion of acetic acid (CO_2 of decarboxylation = 100)
Rat	BS	10·49*	380			0	
		10·82	386	6		0	
	BS + *M*/100 pyruvate	10·39	410			0	
		8·56	463	53	48·3	35	72·5
Guinea-pig	BS	14·38	392			0	
		13·87	395	3		0	
	BS + *M*/100 pyruvate	18·91	405			0	
		17·48	473	68	64·2	42	65
Rat	PS	9·03	16·6			0	
		16·32	20·9	4·3		0	
	PS + *M*/100 pyruvate	16·97	23·5			0	
		13·06	68·7	45·2	41·8	38	90
Rat	PS	17·68	13·8			0	
		13·59	17·4	3·6		0	
	PS + *M*/100 pyruvate	21·37	20·0			0	
		23·06	98·0	78	72	54	75
Rat	PS	13·11	12·5			0	
		15·39	15·5	3		0	
	PS + *M*/100 pyruvate	15·83	15			0	
		21·52	75·5	60·5	56·3	38	67·5
Guinea-pig	PS	26·87	15·9			0	
		24·04	18·9	3		0	
	PS + *M*/100 pyruvate	34·64	19·9			0	
		39·93	112	92·1	87	44	50·5

BS = bicarbonate-saline.
PS = phosphate-saline.
Δ_1 = CO_2 at $t_{2\ hr.}$ − CO_2 at t_0 = increase of total CO_2.
Δ_2 = Δ_1 (pyruvate) − Δ_1 (control) corrected for weight = CO_2 of decarboxylation of pyruvic acid.

which involved loss of volume, and the results were calculated on the basis of homogeneous solutions. Recovery experiments with minced brain have shown that the results with this procedure are far more satisfactory than when the precipitates are thoroughly washed. The error which is introduced by regarding the precipitate as part of the solution is apparently compensated by adsorption of dissolved substances on the surface of the precipitate.

The final clear solution was made up to 10 ml. and aliquots were taken for the different analyses in the manner described. Thus, balance sheets were obtained (Table III) which differed in a significant way from those obtained with brain slices. The relation, pyruvic acid used : CO_2 : lactic acid was again very close to 2 : 1 : 1, as in the experiments with brain slices. The fundamental reaction is therefore in this case, too, a dismutation of pyruvic acid. But the yield of acetic acid is very low, only about 10% of the theoretical amount. On the other hand, the formation of succinic acid is increased and accounts for about 60% of the oxidized pyruvic acid.

There remains a deficit of about 30%. Krebs & Johnson [1937, 1] have shown that in muscle 30% and more of the total pyruvate used (reduced + oxidized) is converted into β-hydroxybutyric acid anaerobically. Although it has been shown that, under different conditions, acetone bodies are formed from pyruvic acid in brain too [Weil-Malherbe, 1936, 3], only minute amounts,

Table III. *Balance sheet of the anaerobic metabolism of pyruvic acid in brei of guinea-pig brain*

	No substrate			Pyruvate				Proportions (pyruvic acid disappearance = 200)
Exp. 1	Initial μl.	Final μl.	Δ_1 μl.	Initial μl.	Final μl.	Δ_2 μl.	$\Delta_2 - \Delta_1$ μl.	
Pyruvic acid	0	0	0	2230	1610	−620	−620	200
CO_2	126	210	84	62·5	439	376·5	292·5	94
Lactic acid	255	297	42	243	539	296	254	82
Acetic acid	15·5	75	59·5	20	102	82	22	7
Succinic acid	—	—	—	—	—	—	—	—
Exp. 2								
Pyruvic acid	0	0	0	2175	1380	−795	−795	200
CO_2	163·5	239	75·5	95	569	474	398·5	100
Lactic acid	310	230	−80	290	620	330	410	103
Acetic acid	40	86	46	65	140	75	29	7
Succinic acid	71	75	4	108	235	127	123	62
Exp. 3								
Pyruvic acid	0	0	0	1930	1340	−590	−590	200
CO_2	75·5	167	91·5	108	495	387	275·5	93
Lactic acid	420	370	−50	385	600	215	265	90
Acetic acid	27	44	17	54	125	71	54	18
Succinic acid	60	70	10	68	162	94	84	57

if any at all, were formed from pyruvic acid anaerobically in minced brain (Table IV). This reaction therefore does not account for the deficit.

Table IV. *Formation of acetone bodies from pyruvic acid by brei of guinea-pig brain anaerobically*

3 ml. of brei per vessel. 2 hr.

	No substrate			Pyruvate			
	Initial μl.	Final μl.	Δ_1 μl.	Initial μl.	Final μl.	Δ_2 μl.	$\Delta_2 - \Delta_1$ μl.
Pyruvic acid	0	0	0	2180	1305	−875	−875
CO_2	156	325	169	180·5	808	627·5	458·5
Fraction I (acetone + acetoacetic acid)	0	0	0	0	0	0	0
Fraction II (β-hydroxybutyric acid)	0	0	0	0	10	10	10

The low yield of acetic acid in minced brain can be due to one of the following possibilities: (1) failure to detect acetic acid, (2) rapid removal of acetic acid, (3) no formation of acetic acid. The first two cases were excluded by experiment. Added acetic acid is quantitatively recovered immediately after addition as well as after 2 hr. anaerobic incubation, in presence and in absence of pyruvic acid (Table V). There is, of course, a fourth possible explanation: that an immediate

Table V. *Fate of acetic acid added to minced brain (horse) anaerobically.*

2 ml. brei per vessel. N_2

	Acetic acid		
Substrate	Initial μl.	After 2 hr. μl.	Δ μl.
0	26	96	70
0·15 ml. *M*/5 acetate	643	720	73
0·15 ml. *M*/2 pyruvate	33·5	134	100
0·15 ml. *M*/5 acetate + 0·15 ml. *M*/2 pyruvate	677	786	109

precursor of acetic acid, viz. nascent acetic acid, is far more reactive than the acetic acid which we add, and that this nascent acetic acid disappears rapidly in the minced brain but not in the slice. We can only avoid the improbable assumption of entirely different mechanisms in mince and slice, if we accept this last explanation.

II. *The anaerobic metabolism of α-ketoglutaric acid*

Under anaerobic conditions dismutation of ketoglutaric acid takes place according to the following equation:

$$2CO_2H.CO.(CH_2)_2.CO_2H + H_2O = (CH_2.COOH)_2 + CO_2H.CHOH.(CH_2)_2.CO_2H + CO_2 \quad \ldots\ldots(2).$$

The relation of 2 : 1 : 1 for the disappearance of ketoglutaric acid : the formation of succinic acid : the evolution of CO_2 is well realized by the experiment. No differences are found whether brain slices or minced brain are used (Tables VI and VII).

Table VI. *Balance sheet of the anaerobic metabolism of ketoglutaric acid in rat brain slices*

Bicarbonate-saline. 95% N_2 + 5% CO_2. Duration: 3 hr.

	No substrate		*M*/100 ketoglutarate		Difference		Proportions (ketoglutaric acid disappearance =200)
Dry wt. of slices (mg.)	145·3		111·8				
	μl.	*Q* value	μl.	*Q* value	μl.*	*Q* value	
Ketoglutaric acid	0	0	−384	−1·15	−384	−1·15	200
CO_2	127	0·29	276·5	0·83	179	0·54	93
Succinic acid	19·4	0·04	186	0·55	171	0·51	89
Hydroxyglutaric acid	0	0	231	0·69	231	0·69	120

* Corrected for weight.

Table VII. *Balance sheet of the anaerobic metabolism of ketoglutaric acid in brei of guinea-pig brain*

	No substrate			Ketoglutarate				Proportions (ketoglutaric acid disappearance =200)
	Initial μl.	Final μl.	Δ_1 μl.	Initial μl.	Final μl.	Δ_2 μl.	$\Delta_2 - \Delta_1$ μl.	
Exp. 1. 2 ml. brei, 2 hr.:								
Ketoglutaric acid	0	0	0	1850	1460	−390	−390	200
CO_2	104	168	64	66·5	318	251·5	187·5	96
Succinic acid	37	40	3	48	240	192	189	97
Exp. 2. 3 ml. brei, 3 hr.:								
Ketoglutaric acid	0	0	0	1870	1260	−610	−610	200
CO_2	173	461	288	182	790	608	320	105
Succinic acid	35	90	55	32	398	366	311	102
Hydroxyglutaric acid	0	0	0	0	289	289	289	95

The figures for hydroxyglutaric acid, which were obtained by the manometric method, must be regarded as rough estimates owing to difficulties of the estimation. They were calculated by subtracting the value of "hydroxyglutaric acid" observed in the control, which was not regarded as real, from the value observed in the solution containing ketoglutaric acid. It appears, however, that there is increased formation of a substance which is oxidized by an extract

under conditions characteristic for the oxidation of $l(-)\alpha$-hydroxyglutaric acid in a quantity close to the theoretical.

The evidence for the formation of hydroxyglutaric acid could be confirmed (1) by demonstrating that after anaerobic incubation of brain with ketoglutaric acid a substance is formed with optical properties which agree fairly well with those of $l(-)\alpha$-hydroxyglutaric acid (2) by isolation of the zinc salt of hydroxyglutaric acid.

From a number of similar experiments the following may be quoted as an example: 100 g. of minced horse brain were placed in each of two flasks, one containing 100 ml. $M/50$ phosphate buffer pH 7·4 and the other containing 100 ml. $M/20$ ketoglutarate in $M/50$ phosphate buffer. The flasks were filled with nitrogen and incubated for 3 hr. Then 100 ml. of 90% alcohol containing 10% HCl were added, the tissue removed by centrifuging and washed twice with 50% alcohol. After concentration *in vacuo* to about 100 ml. the solutions were deproteinized with trichloroacetic acid and the unchanged ketoglutaric acid was precipitated with 2:4-dinitrophenylhydrazine. The amount of ketoglutaric acid thus recovered corresponded to a disappearance of 11,000 μl. during the experiment. The solutions were again concentrated to about 20 ml. and continuously extracted with ether for 48 hr. The ether extracts were further treated as indicated in the description of the polarimetric method. Estimations of succinic acid on aliquots of the extracts showed 388 μl. in the control and 4320 μl. in the solution which contained ketoglutaric acid, i.e. formation of 3932 μl. of succinic acid. Polarimetric examination of the control solution showed before addition of uranyl acetate a rotation of $\pm 0{\cdot}00°$, after addition $+0{\cdot}03°$. The rotation of the other solution was before addition of uranyl acetate $-0{\cdot}04°$, after addition $-0{\cdot}40°$, i.e. an increase of 1000%. Assuming that this increase is due to the presence of a mixture of hydroxyglutaric and malic acids it can be calculated that the mixture consists of about 90% hydroxyglutaric acid and 10% malic acid. If 10% of the succinic acid has been further oxidized to malic acid, about 400 μl. of malic acid have been formed. This would have a rotation of $-0{\cdot}16°$. If this value is deducted from the observed value, the rotation of $-0{\cdot}24°$ corresponds to 4200 μl. of hydroxyglutaric acid, which agrees very well with the theoretical amount.

Isolation of zinc hydroxyglutarate. Several attempts which were made with minced horse brain yielded products which were undoubtedly mainly zinc hydroxyglutarate, but analysis showed that they were not quite pure. A successful experiment was carried out with minced pig's heart, in which the dismutation of ketoglutaric acid is more vigorous than in brain. 100 g. of minced pig's heart were incubated in 200 ml. $M/20$ bicarbonate and $M/20$ ketoglutarate solution for 3 hr. in an atmosphere of 95% N_2 + 5% CO_2. The operations up to the ether extraction were the same as described in the preceding paragraph. The greatest difficulty in this isolation was the separation of succinic and hydroxyglutaric acids, the salts of which have very similar solubilities. Zinc hydroxyglutarate does not crystallize from a mixture with zinc succinate. A preliminary fractionation could be effected by taking advantage of the higher ether-solubility and dissociation constant of succinic acid. The solution was adjusted to pH 4·0 with phosphoric acid and Na_2HPO_4 and was extracted with ether for 4 hr. Most of the succinic acid was extracted, together with very little of the hydroxyglutaric acid. The solution was now acidified with H_2SO_4 and the extraction continued for 48 hr. Extracts of four experiments were thus combined in two fractions. Both fractions were worked up separately in the following way: the residue of the ether extract was taken up in water and

neutralized with baryta. The precipitate was removed and the solution added to 4 vol. of alcohol. The precipitate was separated, decomposed with H_2SO_4 and again precipitated by the addition of basic lead acetate. A further precipitate was obtained from the mother-liquors by the addition of 1 vol. of alcohol. The combined precipitates were decomposed with H_2S and the solutions of the free acids neutralized with NaOH and concentrated to 7 ml. At this stage the succinic acid fraction showed the following rotations: before addition of uranyl acetate $+0{\cdot}03°$, after addition $-0{\cdot}08°$. There was therefore very little hydroxyglutaric acid present. The addition of uranyl acetate produced a heavy precipitate in the succinic acid fraction, which was filtered off before the polarimetric examination and again combined with the solution afterwards. The hydroxyglutaric fraction showed the following rotations: Before addition of uranyl acetate $-0{\cdot}30°$, after addition $-2{\cdot}14°$. The original solution contained 290 mg. of solid. $[\alpha]_D$ was therefore increased from $-7{\cdot}3°$ to $-50{\cdot}1°$ by about 700%. This shows that nearly pure hydroxyglutaric acid was present. The hydroxyglutaric acid fraction was now treated with an equal vol. of alcohol and the precipitate which appeared was added to the succinic acid fraction. Both fractions were now acidified with H_2SO_4 and extracted separately with ether for 48 hr. The ethereal solutions were dried with Na_2SO_4 together with a little $BaCO_3$ to remove traces of H_2SO_4. On evaporation of the ether white crystals appeared in both flasks. Those in the extract from the hydroxyglutaric acid fraction were small needles. The ether was not completely evaporated, but the last drop was left and decanted from the crystals. It contained brown impurities. The residue was taken up in water and neutralized with zinc carbonate. The solution of the zinc salt was concentrated to about 5 ml. and after 2 days a small amount of the typical warty crystals of zinc hydroxyglutarate appeared. Yield after reprecipitation: 45 mg. The mother-liquors still contained some of the zinc salt, but it could not be crystallized. (Found (Weiler): loss of weight at 80° *in vacuo*: 20·0%. C, 28·33; H, 2·94; Zn, 31·2%. Calc. for $C_5H_6O_5Zn+3H_2O$ 20·28% H_2O; calc. for $C_5H_6O_5Zn$ C, 28·38; H, 2·86; Zn, 30·92%.)

The residue from the succinic acid fraction was twice recrystallized from ether and yielded 112 mg. of crystals. M.P. 182°. Mixed melting-point with an authentic specimen of succinic acid: 182°.

Discussion of sections I and II

(1) *The oxidative mechanism of decarboxylation.* The results which have been reported show that the decarboxylation of a molecule of keto-acid under anaerobic conditions is only accomplished at the expense of another molecule of the keto-acid which is reduced to the hydroxy-acid. It is therefore not necessary to assume a carboxylase action for the anaerobic decarboxylation of keto-acids, as has been suggested [Simola & Puutula, 1937]. The limiting factor for this dismutation is apparently the velocity with which the keto-acid can be reduced. The dismutation is not inhibited by the accumulation of the hydroxy-acid, as could be shown by experiment. The decarboxylation is greatly accelerated, if other, more suitable hydrogen acceptors, such as dyes or oxygen, are offered (see section III).

(2) *The mechanism of the conversion of pyruvic into succinic acid.* The following facts require explanation: (*a*) the relation (I) CO_2 evolution : lactic acid formation : pyruvic acid disappearance = 1 : 1 : 2 in both slices and brei of brain. (*b*) Almost the theoretical amount of acetic acid and only a small proportion of succinic acid are formed with brain slices. (*c*) Almost no acetic acid and a considerable proportion of succinic acid are formed in the brei.

The relation (I) shows that, when succinic acid is formed, a second hydrogen acceptor (X) must enter the reaction according to the equation:

$$4CH_3.CO.COOH + X + 2H_2O = (CH_2.COOH)_2 + 2CH_3.CHOH.COOH + 2CO_2 + XH_2 \quad \ldots\ldots(3).$$

The fact that this reaction is small in the slice and large in the mince suggests that only a small amount of the hydrogen acceptor X is available in the slice, but that it is liberated when the structure of the cell is destroyed.

Equation (3) shows that 2 mol. CO_2 appear per mol. of succinic acid formed. Now, if α-ketoglutaric acid were an intermediate, as we have previously suggested, it would itself undergo a dismutation and a certain proportion of hydroxyglutaric acid would appear according to the equation:

$$8CH_3.CO.COOH + 3H_2O = (CH_2.COOH)_2 + CO_2H.CHOH.(CH_2)_2.CO_2H + 4CH_3.CHOH.COOH + 3CO_2 \quad \ldots\ldots(4).$$

This reaction would be a simple dismutation and it would be difficult to explain why it does not occur equally well in the slice as in the brei. In addition it would alter relation (I) to 0·75 : 1 : 2. In experiments with a small proportion of succinic acid formation this would not affect the observed values greatly, but it would be expected to become noticeable when the succinic acid formation accounted for a large proportion of the pyruvic acid oxidized as in the experiments with minced brain. It appears further that, if equation (4) is realized, the amount of succinic acid formed cannot account for more than 50 % of the oxidized pyruvic acid. But we have observed up to 62 % without accounting for some acetic acid which was found too. Krebs & Johnson [1937, 1] even report an experiment with sheep's testis where the amount of succinic acid formed accounts for 80 % of the oxidized pyruvic acid.

From this we conclude that the suggestion that ketoglutaric acid is an intermediate of the formation of succinic acid from pyruvic acid can no longer be maintained.

An alternative suggestion is that succinic acid is formed by the oxidative condensation of 2 mol. of acetic acid *in statu nascendi*. In the minced brain where hydrogen acceptors are apparently readily available for this reaction a large amount of succinic acid is formed. In the slice nascent acetic acid cannot be dealt with and accumulates. It is necessary to assume the nascent state of acetic acid as a participant of the reaction, since added acetic acid is completely inert. The difficulties implied in the assumption of the collision of 2 mol. as short-lived as nascent acetic acid on the active surface are however fully realized.

It seems that in minced brain part of the nascent acetic acid disappears in other reactions. There is a deficit of about 30 %. Since in experiments dealing with the dismutation of ketoglutaric acid almost the theoretical amount of succinic acid is found, this deficit cannot be due to a further oxidation of succinic acid.

(3) *Differences of the aerobic and anaerobic metabolisms of pyruvic acid.* McGowan & Peters [1937] first pointed out possible differences between the aerobic and anaerobic paths of breakdown of pyruvic acid. They observed that the extra O_2 uptake of B_1-avitaminous brain catalysed by vitamin B_1 is the same in presence of pyruvate as in the presence of pyruvate + succinate. They argued that, if succinic acid is an intermediate of pyruvic acid oxidation, the saturation of succinic dehydrogenase should diminish the extra O_2 uptake due to the vitamin. Our own unpublished experiments show a similar phenomenon: the O_2 uptake of slices of normal rat brain in presence of pyruvic acid + succinic

acid is almost an arithmetical summation of the O_2 uptakes observed with either of the substrates alone. This has even been observed with mixtures of glutamic and succinic acids [cf. Weil-Malherbe, 1936, 1, Table IV, last exp.] and of ketoglutaric and succinic acids. Now the character of succinic acid as an intermediate in the oxidative breakdown of glutamic and ketoglutaric acids cannot be disputed and yet saturation of succinic dehydrogenase by added succinic acid does not diminish the oxidation of its precursor. Although this is difficult to understand, the argument of McGowan & Peters loses much of its force in view of these observations.

The ingenious conception of the "citric acid cycle" [Krebs & Johnson, 1937, 2] has, however, shown a path of pyruvic acid breakdown which is probably not accessible anaerobically, since it depends on a continuous supply of oxaloacetic acid and any oxaloacetic acid present would be rapidly reduced anaerobically. This, and the great number of oxidative reactions involved, makes it impossible that the succinic acid which is formed anaerobically from pyruvic acid is formed via citric acid. On the other hand, the evidence which has previously been presented [Weil-Malherbe, 1936, 1; 1937, 1] in support of the hypothesis that ketoglutaric acid is an intermediate of succinic acid formation can be satisfactorily explained by the "citric acid cycle".

There is, of course, no reason why the mechanism of succinic acid formation which operates anaerobically should not also occur aerobically. The large amount of succinic acid arising from pyruvic acid anaerobically in minced brain certainly does not suggest that this reaction is only an unimportant bypath of the oxidation. The conditions under which synthesis of citric acid occurs in preference to the direct synthesis of succinic acid are still a problem for future research.

III. *The anaerobic metabolism of α-keto-acids in presence of brilliant cresyl blue*

It has already been briefly reported [Weil-Malherbe, 1936, 2] that the CO_2 evolution from α-keto-acids under anaerobic conditions can be greatly accelerated by the addition of brilliant cresyl blue. Brilliant cresyl blue was chosen because it is less toxic to brain tissue than other dyes [Dickens, 1936], and secondly, because there is hardly any formation of acid upon its reduction. Brilliant cresyl blue is itself slightly acid. It was titrated by tipping a certain quantity of the solution of the dye into bicarbonate solution and measuring the CO_2 liberated manometrically. The dye was then neutralized by the addition of the calculated amount of bicarbonate, so that no pressure changes were observed when the neutralized dye was tipped into bicarbonate saline. The reduction of 112 μl. of neutralized dye by cysteine in bicarbonate solution at *p*H 7·4 set free 8·5 μl. of CO_2. This correction has been applied to all results. The experiments were carried out with brain slices in bicarbonate-saline in an atmosphere of 95% N_2 and 5% CO_2 with chromous chloride in the centre well. The dye was tipped in from the side bulb after the absorption of oxygen had ceased.

The catalytic effect of brilliant cresyl blue disappears as soon as it is reduced, and is therefore due to its capacity as hydrogen acceptor. In this respect it can replace oxygen to a certain extent. From the time in which a certain quantity of dye is reduced by the tissue a Q value can be calculated. If this Q value is compared with the quantity of CO_2 evolved in the same period it appears that $Q_{dye} : Q_{CO_2} = 2 : 1$. Since 2 mol of dye correspond to 1 mol. of O_2 in their capacity as hydrogen acceptors the relation $Q_{CO_2} : Q_{dye/2}$ corresponds to the R.Q. The values of this "dye-R.Q." agree very well with the aerobic R.Q. (Table VIII).

Table VIII. *Decarboxylation of keto-acids in presence of brilliant cresyl blue*

Guinea-pig brain slices in bicarbonate-saline. The slices were used immediately for Exp. 2 and after 1 hr. shaking in O_2 for Exp. 1.

Substrate	mg.	µl. dye	Time of decolor. min.	µl. CO_2	$Q_{dye/2}$	Q_{CO_2}	R.Q.	R.Q. aerobically (av. values)
Exp. 1:								
0	19·39	70	90	34·5	1·20	1·19	0·99	1·00
M/100 pyruvate	12·90	70	65	46·4	2·50	3·32	1·33	1·28
M/100 ketoglutarate	19·30	70	60	36·9	1·81	1·91	1·06	1·10
0	10·86	0	—	—	—	0·65	—	—
M/100 pyruvate	16·07	0	—	—	—	1·72	—	—
M/100 ketoglutarate	12·95	0	—	—	—	0·95	—	—
Exp. 2:								
0	14·33	0	—	—	—	1·00	—	—
	13·93	65	60	32·5	2·33	2·33	1·00	—
	32·93	130	50	64	2·37	2·33	1·02	—
M/100 ketoglutarate	13·29	0	—	—	—	1·21	—	—
	16·56	65	32	34·6	3·68	3·91	1·06	—
	26·30	130	40	64·6	3·70	3·68	0·99	—

These results do not agree with those of Lipmann [1937], who, studying the oxidation of pyruvic acid by a washed brei of rat brains in presence of methylene blue, finds 1 mol. of CO_2 per mol. of dye reduced. This would correspond to R.Q. 2, i.e. the product of oxidation of pyruvic acid would not be dehydrogenated by the dye. This is the more surprising, as we have found that in minced brain a considerable proportion of succinic acid is formed even in absence of a dye and this would certainly reduce part of the dye without giving rise to CO_2 production.

The disagreement between our results and those of Lipmann becomes still larger, if, as in Lipmann's results, the CO_2 which is formed in absence of the dye is subtracted from the value observed in presence of the dye. When we do this, we find only 1 mol. CO_2 per 3–4 mol. of dye, a proportion which would be difficult to interpret. The proportion we expect, if the dye acts as a substitute for O_2, is that of 2 mol. dye to 1 mol. CO_2, and this we do in fact observe with remarkable accuracy if we do not take into account the CO_2 formed in absence of the dye. We conclude from this that the processes which underly the CO_2 evolution in absence of the dye are not simply accelerated in presence of the dye, but that they are stopped and replaced by other processes. Only thus can the absence of an additional effect of the dye be explained. As we have seen, the processes underlying the decarboxylation of α-keto-acids under strictly anaerobic conditions are dismutations. The conditions in presence of a dye approach those prevailing in presence of O_2, and it seems that, under these conditions, dismutations are suspended and replaced by direct dehydrogenation. Lipmann [1937] has come to the same conclusion by different reasoning.

The opposite view is put forward by Krebs & Johnson [1937, 1] who incline to the theory that a dismutation is an obligatory mechanism preceding the decarboxylation even under aerobic conditions and point out that the disappearance of pyruvic acid is, in some cases, almost as large anaerobically as aerobically. But, if the same dismutation were to precede the aerobic and the anaerobic oxidations, the rate of anaerobic disappearance should be twice that of the aerobic disappearance, since anaerobically the reduced equivalents accumulate, whereas aerobically they are largely reoxidized. Two metabolic figures which can more justly be compared are the anaerobic and aerobic

CO_2 evolutions. We find that the anaerobic CO_2 evolution by brain slices is, in presence of pyruvic acid, at least three times smaller and, in presence of ketoglutaric acid, at least five times smaller than the aerobic CO_2 evolution.

Summary

The anaerobic metabolism of pyruvic and α-ketoglutaric acids in sliced and minced brain has been studied.

The following facts have been observed:

(1) The relation, pyruvic acid disappearance : lactic acid formation : CO_2 evolution = 2 : 1 : 1 in both slices and mince, indicating a dismutation of pyruvic acid.

(2) With brain slices about 80 % of the theoretical amount of acetic acid is found. The remaining 20 % is accounted for by succinic acid.

(3) With minced brain only about 10 % of the theoretical amount of acetic acid is found. The yield of succinic acid, on the other hand, is increased and accounts for about 60 % of the oxidized pyruvic acid. The deficit of 0 % not accounted for by β-hydroxybutyric acid.

(4) The relation, ketoglutaric acid disappearance : succinic acid formation hydroxyglutaric acid formation : CO_2 evolution = 2 : 1 : 1 : 1, in both slices and mince, indicating a dismutation of ketoglutaric acid.

The evidence for the formation of *l*(−)α-hydroxyglutaric acid consists (*a*) in the formation of a substance which is oxidized by an enzyme extract under conditions characteristic for *l*(−)α-hydroxyglutaric acid, (*b*) in the formation of a substance the optical properties of which agree with those of *l*(−)α-hydroxyglutaric acid, (*c*) in the isolation of zinc hydroxyglutarate.

(5) The anaerobic decarboxylation of α-keto-acids is accelerated by the addition of brilliant cresyl blue. The relation of dye reduced : CO_2 evolved = 2 : 1, if the CO_2 evolution in absence of the dye is not subtracted.

The following conclusions are drawn:

(1) The decarboxylation of α-keto-acids is an oxidative process.

(2) The conversion of pyruvic acid into succinic acid is brought about by an oxidative condensation of 2 mol. of nascent acetic acid. The hydrogen acceptor for this reaction is not pyruvic acid and is probably available in small amounts in the slice and liberated upon destruction of the cell.

α-Ketoglutaric acid is probably not an intermediate in this reaction.

(3) Dismutations of α-keto-acids occur only under strictly anaerobic conditions. They are replaced by dehydrogenations in presence of oxygen or of a dye.

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