LXXXVII. METABOLISM OF KETONIC ACIDS IN ANIMAL TISSUES

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In this paper experiments are described which show that ketonic acids can react in animal tissues according to the general scheme

$$\underset{\alpha\text{-ketonic acid I}}{R.CO.COOH} + \underset{\alpha\text{-ketonic acid II}}{R'.CO.COOH} + H_2O \rightarrow \underset{\text{carboxylic acid}}{R.COOH} + CO_2 + \underset{\alpha\text{-hydroxy-acid}}{R'.CH(OH).COOH} \quad \ldots\ldots(1)$$

or

$$\underset{\alpha\text{-ketonic acid}}{R.CO.COOH} + \underset{\beta\text{-ketonic acid}}{R'.CO.CH_2.COOH} + H_2O \rightarrow \underset{\text{carboxylic acid}}{R.COOH} + CO_2 + \underset{\beta\text{-hydroxy-acid}}{R'.CH(OH).CH_2.COOH} \quad \ldots\ldots(2).$$

Examples are given in which α-ketonic acid I as well as α-ketonic acid II in (1) are represented by pyruvic acid. In other cases the α-ketonic acid in (2) is pyruvic acid or α-ketoglutaric acid and the β-ketonic acid in (2) acetoacetic or oxaloacetic acid.

The reactions 1 and 2 elucidate a mechanism by which α-ketonic acids are broken down in the animal body. Although it has long been known, from the work of Embden, that α-ketonic acids undergo oxidation to the fatty acids which are shorter by one carbon atom, the question of the mechanism of this oxidation remained open. According to (1) and (2) the oxidation of α-ketonic acids is not brought about by molecular oxygen, but by a dismutation, that is to say by an intermolecular oxido-reduction. The oxidizing agent for the ketonic acid is a second molecule of ketonic acid which is reduced to the corresponding hydroxy-acid.

The reactions (1) and (2) appear to play a role in the course of the normal oxidative breakdown of carbohydrates, of fats and of the carbon skeleton of amino-acids. This will be discussed in full in subsequent papers.

I. General experimental methods

1. *Determination of α-ketonic acids*

(*a*) *Carboxylase method.* Pyruvic acid was usually determined by the carboxylase method [Warburg *et al.* 1930; Westerkamp, 1933]. Freshly pressed top yeast obtained from a local brewery was spread over filter-paper and dried at room temperature with the aid of a fan. The dried yeast, treated according to the directions of Westerkamp, yielded a powerful carboxylase. The addition of 1/5 vol. of 90 % glycerol stabilized the enzyme for about one week at 0° [see von Schoenebeck, 1935]. It is essential that the yeast used should be fresh and quickly dried [Wülfert, 1936].

(*b*) *Ceric sulphate method.* An alternative method applicable to other α-ketonic acids is based on the reaction

$$R.CO.COOH + 2Ce^{\cdot\cdot\cdot\cdot} + H_2O \rightarrow R.COOH + CO_2 + 2Ce^{\cdot\cdot\cdot} + 2H^{\cdot}$$

[Fromageot & Desnuelle, 1935]. The carbon dioxide formed is measured manometrically in the usual way.

The solution to be examined is acidified with 0·2 vol. of 1 : 1 aqueous H_2SO_4. The side-bulb of the cup is filled with 0·6 ml. of a saturated solution of ceric sulphate in $10N$ H_2SO_4. Sufficient ceric sulphate must be used to leave a yellow colour when the reaction is finished. The method is less specific than is the carboxylase method, since besides α-ketonic acids lactic, malic, acetoacetic and malonic acids may also yield CO_2. However, lactic and malic acids interfere only if present in relatively high concentration (above $M/100$) and this was not the case in our experiments. In the presence of malonic and acetoacetic acids the method is not applicable. Substances forming insoluble salts with ceric ions may also interfere, e.g. phosphate. Proteins or tissue extracts may complicate the determination by undergoing autoxidation in the presence of ceric ions. This reaction can be excluded by filling the manometric flasks with nitrogen.

An advantage of this method as compared with the carboxylase method is its applicability to those ketonic acids which are not readily split by carboxylase, (α-ketoglutaric acid, methylethylpyruvic acid).

2. *Tissue material*

The tissue slice technique was used in the ordinary way. Muscle tissue was minced according to Szent-Györgyi's directions and suspended in 3 vol. of saline. The saline, usually 3 ml. per flask, contained 0·025 M $NaHCO_3$ and was saturated with 5 % CO_2 unless stated otherwise. The temperature was 39–40°. Anaerobic conditions were obtained by placing yellow phosphorus in the centre chamber of the manometric flask, or by passing the gas mixture over heated copper. Rat tissues were used in most experiments.

3. *Metabolic quotients*

The rates of the metabolic changes are, as usual, expressed by quotients of the general formula

$$Q_{\text{Metabolite}} = \frac{x_{\text{Metabolite}}}{\text{mg. tissue} \times \text{hours}},$$

where $x_{\text{Metabolite}}$ (the change in the quantity of the metabolite) is expressed in μl. gas.

While the quotients are frequently constant for a period of 1 or 2 hours there is a rapid falling off in some reactions described in this paper (see e.g. Table V). It is essential in such cases to state the period for which the quotients are calculated.

II. Fate of pyruvic acid in animal tissues under anaerobic conditions

In this section evidence for the reaction

$$2 \text{ pyruvic acid} + H_2O \rightarrow \text{acetic acid} + CO_2 + \text{lactic acid} \qquad \text{.....(3)}$$

will be presented. It contains data concerning (1) the consumption of pyruvic acid, (2) the production of lactic acid, (3) the production of carbon dioxide, (4) the production of acetic acid. Furthermore, data concerning the formation of succinic and β-hydroxybutyric acids are presented. These two substances also arise anaerobically in the presence of pyruvic acid, probably by secondary reactions which follow reaction (3) under our experimental conditions.

1. *Consumption of pyruvic acid*

It is well known that animal tissues are capable of metabolizing pyruvic acid, and two different mechanisms have been described whereby pyruvic acid can be removed: (1) oxidation in the presence of molecular oxygen, (2) reduction to lactic acid in the presence of certain hydrogen donators formed in the normal course of glycolysis (hexosediphosphate or triosephosphate).

In Table I data are recorded which demonstrate the rate at which pyruvic acid is removed anaerobically in various tissues. The highest rate is observed in testis; brain, liver, kidney follow next; the other tissues give smaller, but still significant figures.

Table I. *Anaerobic disappearance of pyruvic acid in animal tissues*

Tissue	mg.	Amount of pyruvate in the solution (μl.) Initial	Final	Amount of pyruvate used (μl.)	Duration of experiment (min.)	$Q_{Pyruvate}$
Liver (rat)	27·35	448	244	204	120	−3·74
,,	16·68	466	249	217	90	−8·77
,,	27·22	682	372	310	60	−11·4
Liver (pigeon)	17·87	530	254	276	140	−6·58
Brain (rat)	14·15	448	276	172	120	−6·08
,,	10·43	466	364	102	90	−6·52
,,	12·00	489	351	138	120	−5·72
,,	9·95	466	326	140	120	−7·04
Kidney (rat)	7·27	448	330	118	120	−8·1
,,	7·98	466	416	50	90	−4·17
Spleen (rat)	16·20	448	360	88	120	−2·72
,,	16·37	466	432	34	90	−1·39
Testis (rat)	9·20	466	312	154	90	−11·2
,,	11·20	489	292	197	120	−8·80
,,	16·63	555	293	262	120	−7·88
Pancreas (rat)	27·78	466	382	84	90	−2·02
Diaphragm (rat)	20·29	466	397	69	90	−2·27
Submaxillary gland (rat)	14·60	466	428	38	90	−1·73
Intestinal wall (rat)	44·53	488	262	226	120	−2·54
Jensen rat sarcoma	19·15	522	414	108	80	−4·23

In some tissues, such as brain or testis, the anaerobic disappearance of pyruvic acid is almost as high as the aerobic disappearance as shown in Table II.

Table II. *Disappearance of pyruvic acid in the absence and in the presence of oxygen*

Tissue	$Q^{O_2}_{Pyruvate}$	$Q^{N_2}_{Pyruvate}$
Brain (rat)	−9·28	−7·04
,,	−8·32	−6·08
Liver (rat)	−8·75	−3·74
,,	−8·54	−5·13
,,	−9·6	−7·54
Kidney (rat)	−16·8	−8·1
,,	−23·4	−6·6
Spleen (rat)	−5·45	−2·72
Intestinal wall (rat)	−2·94	−2·09
Testis (rat)	−14·2	−10·1
Jensen sarcoma	−6·1	−4·23

2. *Formation of lactic acid from pyruvic acid*

Reduction of pyruvic acid to lactic acid has been described by many authors, [e.g. Mayer, 1912; Embden & Oppenheimer, 1913; Khouvine *et al.* 1930; Haarmann, 1932; Elliott *et al.* 1935; Lawson, 1936], and according to the current view on glycolysis pyruvic acid is the immediate precursor of lactic acid. The reductant in the process is assumed to be some intermediate formed during glycolysis such as hexosediphosphate or a triosephosphate.

We find, however, that reduction of pyruvic acid occurs in tissues which contain very little or no carbohydrate, for instance in testis or brain. If these tissues are kept anaerobically in saline for 20–40 min. practically the whole of the stored carbohydrate has been glycolysed; yet pyruvic acid is still rapidly reduced.

Table III shows experiments in which the amount of pyruvic acid which disappeared was compared with the amount of lactic acid formed. Rat testis was used in the first instance (Table III) since the blank lactic acid formation in this tissue is small owing to the lack of stored carbohydrate. The tissue was

Table III. *Formation of lactic acid from pyruvic acid in rat testis*

(Determination of lactic acid by the method of Friedemann & Graeser [1933].)

Dry weight of testes (mg.)	Volume of medium (ml.)	Duration of exp. (min.)	Amount of pyruvic acid (μl.) in medium			Amount of lactic acid (μl.)		Ratio: lactic acid formed / pyruvic acid used
			Initial	Final	Change	Total	Increase due to pyruvic acid	
60	12	180	0	0	0	83	—	0·52
60	12	180	2710	1072	−1638	974	851	
70	12	180	0	0	—	135	—	—
70	12	180	2700	Not determined	—	1145	1010	
62	30	120	0	0	0	122	—	0·44
62	30	120	6690	4500	−2190	1085	963	
107	25	120	0	0	0	245	—	0·52
107	25	120	4830	2050	−2780	1730	1485	
126	6	140	0	0	0	94	—	0·64
126	6	140	1390	250	−1140	824	730	
129	4	140	0	0	0	201	—	0·49
129	4	140	1905	515	−1853	1110	910	

Table IV. *Reduction of pyruvic acid in various tissues*

(Initial concentration of pyruvic acid $M/50$.)

Tissue	mg.	Duration (min.)	Pyruvate used by tissue (μl.)	Extra CO_2 formed (μl.)		Extra lactic acid formed (μl.)	
				Total	In % of pyruvate used	Total	In % of pyruvate used
Brain (guinea-pig)	99·1	110	943	320	34	389	41
Kidney (guinea-pig)	47·8	160	622	242	39	228	37
Kidney (rat)	31·8	160	680	251	37	293	43
Intestine (rat)	70·7	160	1088	651	60	377	35
Liver (rat)	41·1	80	352	323	92	484	137
Liver (pigeon)	70·1	100	944	596	63	710	75
Lung (rat)	86	160	750	354	47	493	66
Liver (rat)	65·5	150	705	646	92	803	114
Muscle (pigeon)	2500	205	11400	4880	43	4780	42
,,	3750	190	17850	10280	58	6930	39
Heart (pig)	200	265	1350	347	26	840	62

shaken in saline containing pyruvate and after 2 hours' anaerobiosis, lactic and pyruvic acids were determined in aliquot parts of the solution. Two testes from one animal were used for each experiment, one testis serving as control. The ratio $\frac{\text{lactic acid formed}}{\text{pyruvic acid used}}$ lies between 0·44 and 0·64; the yield of lactic acid is thus about 50 % of the pyruvic acid, as would be expected from equation (3).

In Table IV experiments with other tissues are recorded and it will be seen that brain, kidney, lung, muscle and intestine all convert a similar proportion of pyruvic acid (about 40 %) into lactic acid. Elliott *et al.* [1935] give data for kidney and transplantable tumour which agree well with our results. In liver there is a higher yield of lactic acid. In this tissue reaction (3) is not the chief metabolic reaction induced by pyruvic acid; pyruvic acid in liver "activates" glycolysis, a reaction discussed in the following paragraph.

3. *Formation of carbon dioxide from pyruvic acid*

Mendel *et al.* [1931], using Warburg's manometric technique, studied the influence of pyruvic acid on lactic acid production in tissues and found that under certain conditions it increased the rate of lactic acid production from glucose. Rosenthal [1932] and Dickens & Greville [1933] confirmed Mendel's results. The effect is brought about by small concentrations ($10^{-3}M$ or less) of pyruvic acid and is probably connected with the role which pyruvic acid plays as intermediate in the anaerobic breakdown of carbohydrates. This is formulated by Meyerhof & Kiessling [1935] as:

Hexosediphosphate + 2 pyruvic acid = 2 phosphoglyceric acid + 2 lactic acid .....(4).

If conditions are such that the limiting factor for lactic acid formation is the amount of pyruvic acid present, pyruvic acid acts as an "activator" of lactic acid production.

The increased CO_2 output observed on adding pyruvic acid is, however, by no means always due to this effect. In some tissues and under certain conditions (absence of glucose, high concentration of pyruvic acid) the increased CO_2 output is chiefly due to reaction (3). In Table V some observations are recorded which

Table V. *Increase of anaerobic CO_2 production by pyruvic acid in animal tissues*

($M/50$ pyruvate.)

Tissue	Q_{CO_2} without pyruvate	Q_{CO_2} with pyruvate
Liver (rat) (first 40 min.)	4·82	13·4
Liver (rat) (second 40 min.)	1·51	3·14
Liver (cat) (first 40 min.)	5·86	7·62
Liver (cat) (second 40 min.)	2·27	3·39
Liver (pigeon) (first 40 min.)	3·96	7·92
Liver (pigeon) (second 40 min.)	2·55	4·53
Kidney (rat)	0·84	3·49
,,	2·12	5·90
Kidney (guinea-pig)	1·95	5·88
Brain cortex (rat)	0·37	3·34
Brain cortex (pigeon)	1·40	2·57
Intestine (rat)	4·85	6·72
Testis (rat)	1·04	5·06
,,	0·99	6·82
,,	0·43	5·45
Breast muscle (pigeon) (first 40 min.)	0·33	1·78
Heart (sheep) (first 40 min.)	0·1	1·3
Lung (rat)	4·72	7·75
Testis (guinea-pig)	0·87	4·56

show that there is a very marked increase in CO_2 formation in all tissues including those which have no significant store of carbohydrate. Similar data have been given previously for kidney by Elliott *et al.* [1935], but these authors did not ascertain whether the increased CO_2 formation was due to Mendel's "activation" of glycolysis or to other processes.

From the data presented in Table V it cannot be decided whether the increased CO_2 output is due to increased acid formation or to formation of CO_2 from pyruvic acid. This question can be settled by measuring the bicarbonate content of the solution at the beginning and at the end of the experiment. If the CO_2 output is derived from the bicarbonate, an equivalent decrease in bicarbonate would be expected. The following experiments show that the increased output of CO_2 is not accompanied by a fall in bicarbonate. The extra CO_2 must thus be due to a decomposition of the added pyruvate.

In order to make the measurement of the bicarbonate concentration accurate, comparatively low concentrations of bicarbonate must be used. The concentration used was about $M/200$ and the CO_2 pressure in the gas mixture was correspondingly low (1 %) so that *p*H was physiological (7·4). Four cups with side-arms were used for one experiment and they were filled in the following way:

	1	2	3	4
Main part	2 ml. saline; no tissue	2 ml. saline; tissue	2 ml. saline containing 0·01 *M* pyruvate; no tissue	2 ml. saline containing 0·01 *M* pyruvate; tissue
Side-arm	0·2 ml. 10 % H_2SO_4	0·2 ml. 10 % H_2SO_4	0·2 ml. 10 % H_2SO_4	0·2 ml. 10 % H_2SO_4

The cups were shaken for a certain period, usually for 2 hours, and the change of CO_2 pressure was read. Then the acid was tipped over. The cups 1 and 3 give, after addition of the acid, the initial amount of bicarbonate in the cups. Cups 2 and 4, during the period of shaking, measured the amount of CO_2 evolved owing to the activity of the tissues in the absence and in the presence of pyruvic acid. After the addition of the acid they furnished the final amount of bicarbonate. An experiment with rat testis gave the following figures:

No.	1	2	3	4
mg. tissue	0	15·88	0	15·63
Initial bicarbonate	264	—	264	—
CO_2 evolved during 120 min. before adding acid*	—	62·5	—	172
CO_2 evolved after adding acid	—	217	—	217
Total CO_2 present at the end	—	279·5	—	389

* Extrapolated for period of equilibration.

In the absence of pyruvic acid a slight increase in the total CO_2 is observed from 264 to 279·5 μl. This may be due to the bicarbonate introduced into the system with the tissue. In the presence of pyruvate, however, a large increase in the total CO_2 from 264 to 389 μl. is observed. This increase of $125-15·5=109·5$ μl. is equivalent to the extra CO_2 which causes the increased change of pressure before adding the acid, namely $172-62·5=109·5$ μl. These figures show that the increased CO_2 evolution in the presence of pyruvate is not due to an increased acid formation, but to decomposition of pyruvic acid. Experiments with brain, kidney and muscle gave similar results.

Table VI shows experiments in which the extra CO_2 output was compared with the amount of pyruvic acid removed. "Extra CO_2" is calculated from the increase in CO_2 pressure after addition of pyruvate. The last column shows that

the quantity of extra CO_2 amounts to 27–58 % of the pyruvic acid used, the average being about 40 %. Data for some other tissues will be found in Table IV, column 5.

Table VI.

Tissue	mg.	Duration (min.)	Extra CO_2 (μl.)	Pyruvic acid used (μl.)	Ratio $\frac{\text{extra } CO_2}{\text{pyruvic acid}}$
Testis (rat)	18·60	170	113	417	0·271
,,	28·25	170	234	615	0·381
,,	22·78	170	234	598	0·396
,,	16·63	100	85	262	0·32
,,	28·25	170	286	615	0·465
,,	22·78	170	223	598	0·374
,,	11·67	120	108	306	0·353
Liver (pigeon)	17·87	140	161	276	0·583
,,	20·28	100	78	224	0·358

4. *Formation of acetic acid*

(*a*) *Evidence for the formation of fixed acid.* In the preceding section it was shown that CO_2 is liberated from pyruvate in animal tissues. It is noteworthy that this CO_2 appears in the manometric experiment as free CO_2 and not as bicarbonate for this sheds light on the mechanism of the decarboxylation. If animal tissues decarboxylated pyruvic acid in the same way as yeast extract, i.e. by fission into aldehyde and CO_2, no change of pressure would have been observed in our experiments, since CO_2 remains in solution as bicarbonate ion at *p*H 7–8. The mechanism of the decarboxylation in animal tissues must therefore be different from that in yeast, since the pyruvic acid residue must be an acid which binds the sodium ion and prevents it from binding the CO_2. This residue was identified as acetic acid in the following way.

(*b*) *Identification of acetic acid.* Acetic acid is the most likely "fixed acid" to be formed from pyruvic acid by decarboxylation. In order to test this assumption we examined the solutions after the experiment for steam-volatile acids. Since pyruvic acid is slightly volatile with steam the experiments were arranged in such a way that most of the pyruvic acid added was used up during the experiment. Remaining traces were removed, together with proteins and other interfering substances by the Salkowski-Van Slyke copper-lime precipitation. The experiments were carried out on a larger scale with flasks described by Krebs [1933]. The steam-distillation was done at atmospheric pressure. The liquid was acidified with phosphoric acid and steam was passed through as long as the distillate contained acid, the volume of the liquid being kept constant at about 5 ml. The distillate was collected in *M*/100 alkali and the titration was carried out at 100° using phenol red as indicator. Lactic acid is not volatile under these conditions.

Most of the experiments were carried out with rat testis. The solution in which pyruvic acid was decomposed by the tissue under anaerobic conditions yielded regularly a steam-volatile acid in the expected quantity. For instance four testes (350 mg. dry) were shaken in 50 ml. saline containing pyruvate. The initial amount of pyruvic acid present was 11,850 μl. After 2 hours' anaerobiosis pyruvic acid was determined in an aliquot and 3480 μl. were found in the total volume. Therefore 8370 μl. pyruvate were used by the tissue. 15 ml. of the fluid were subjected to steam-distillation and acid equivalent to 4·90 ml. *N*/100 alkali

was found. The control experiment (testis without pyruvate), gave 0·41 ml. $N/100$ alkali. Expressed in μl. an additional 3820 μl. steam-volatile acid were thus formed in the pyruvic acid experiment. The ratio $\frac{\text{steam-volatile acid formed}}{\text{pyruvic acid used}}$ is $\frac{3820}{8370}=0{\cdot}46$. The amount of steam-volatile acid is thus of the same order as the amount of CO_2.

For the identification of acetic acid the lanthanum reaction of Krüger & Tschirsch [1929; 1930] was used which is highly specific for acetic and propionic acids. Since the latter can be excluded, the positive reaction may be regarded as conclusive evidence that the acid concerned is acetic acid. The combined neutral distillates (equivalent to a few ml. 0·01 N acid) were concentrated on a water-bath after the titration and the concentrated solution (15 ml.) was again acidified and steam-distilled until the steam was neutral. The distillate was neutralized, evaporated to a few ml. on a water-bath and mixed with the reagents for the lanthanum test (1 ml. 5% lanthanum nitrate, 1 ml. $M/50$ alcoholic iodine, a few drops N NH_3, warming in boiling water). The test was intensely positive (blue colour).

(*c*) *Quantity of steam-volatile acid formed in various tissues.* The determination of steam-volatile acid, if carried out under suitable conditions, gives an indication of the quantities of acetic acid formed. In Table VII data about the formation of

Table VII. *Anaerobic formation of steam-volatile acid in tissues in the presence of pyruvic acid*

Tissue	mg.	Duration (min.)	Pyruvic acid used (μl.)	Extra steam-volatile acid formed (μl.)	Volatile acid in % of pyruvic acid
Testis (rat)	120	120	3,470	1170	34
Testis (sheep)	8000	240	22,400	5970	27
Testis (guinea-pig)	1500	100	6,540	2842	43
Liver (rat)	100	85	425	78	18
,,	100	90	749	46	6
Kidney (rat)	100	130	1,680	335	20
Muscle (pigeon)	4500	110	20,100	4370	22
,,	2500	205	11,650	431	4
,,	3750	180	17,850	1455	8
Brain (guinea-pig)	100	248	1,250	369	29·5

steam-volatile acid from pyruvic acid are recorded for various tissues. It is of special interest to note that the yield varies in different tissues. It is of the expected order in testis, but is lower in other organs, especially in muscle.

5. *Formation of succinic acid*

The quantities of lactic acid, CO_2 and acetic acid formed in testis and brain account for the bulk of the metabolized pyruvic acid. In other tissues, however, the low yield of acetic acid leaves a considerable fraction of pyruvic acid the fate of which remains to be explained. It follows from the quantities of lactic acid and CO_2 that the missing fraction arises from pyruvic acid by oxidative decarboxylation and the product must therefore be closely related to acetic acid. In a search for possible substances we found two more products appearing if pyruvic acid is metabolized anaerobically viz. succinic and β-hydroxybutyric acids. Succinic acid is formed in small amounts, β-hydroxybutyric acid in very considerable amounts in some tissues such as muscle and in smaller quantities in other tissues such as testis.

The formation of succinic acid from pyruvic acid in muscle was first made probable by Toenniessen & Brinkmann [1927]; it was recently postulated for kidney by Elliott *et al.* [1935] and experimentally shown for brain by Weil-Malherbe [1936]. The new feature in our experiments is the demonstration that the conversion of pyruvic acid into succinic acid proceeds in the absence of molecular oxygen.

We do not propose to discuss the mechanism of the succinic acid formation in this paper, but confine ourselves to presenting a few data which show the magnitude of the succinic acid production in various tissues in the presence of pyruvic acid (Table VIII). Succinic acid was determined by a modification of Szent-Györgyi & Gözsy's method [1935] which will be described later.

Table VIII. *Formation of succinic acid in the presence of pyruvic acid under anaerobic conditions*

(118 mg. succinic acid are equivalent to 22,400 μl. gas.)

Tissue	mg.	Duration (min.)	Succinic acid produced in absence of pyruvic acid (μl.)	Pyruvate used (μl.)	Succinic acid formed in the presence of pyruvic acid (μl.)
Testis (guinea-pig)	1500	100	280	6,540	752
Testis (sheep)	8000	180	685	17,590	4220
Testis (rat)	350	120	226	8,370	500
Liver (rat)	59	120	35	—	73
,,	80	120	18	—	60
,,	100	120	30	—	90
,,	60	120	44	—	78
Brain (rat)	30	120	14	—	16
Brain (rabbit)	60	60	45	—	50
Kidney (rat)	30	120	0	—	20
Kidney (rabbit)	150	60	0	—	89
Kidney (rat)	60	60	25	—	91
,,	50	60	5·5	—	70
Muscle (pigeon)	250	60	117	—	164
,,	200	60	59	—	84

6. *Formation of β-hydroxybutyric acid*

β-Hydroxybutyric acid was determined by Edson's [1935] modification of Van Slyke's method. The method is not applicable to acetoacetic acid if pyruvic acid is present, but it yields reliable figures for β-hydroxybutyric acid. The specificity of the test is, however, not absolute and the evidence for the presence of β-hydroxybutyric acid cannot therefore yet be considered as conclusive. The data are collected in Table IX. The yield of β-hydroxybutyric acid is highest in muscle, amounting to 25–32 % of the pyruvic acid used.

The production of comparatively large quantities of this "ketone body" from pyruvic acid is a fact of general interest. There are some data in the literature which bear on the problem of the mechanism of the reaction. Embden & Oppenheimer [1912], Annau [1934] and Edson [1935] recorded a synthesis of acetoacetic acid from pyruvic acid in liver under aerobic conditions and Weil-Malherbe [1936] recently found acetoacetic acid arising from pyruvic acid in brain. It should further be mentioned that acetic acid, under aerobic conditions, gives rise to the production of acetoacetic acid in liver [Loeb, 1912; Jowett & Quastel, 1935; Edson, 1935]. Since acetoacetic and β-hydroxybutyric acids are readily interconvertible, it remains to be shown which substance arises primarily in the presence of pyruvic and acetic acids.

Table IX. *Formation of "β-hydroxybutyric acid" in the presence of pyruvic acid under anaerobic conditions*

Tissue	mg.	Time (min.)	Pyruvic acid used (μl.)	Extra β-hydroxy-butyric acid formed (μl.)	β-Hydroxy-butyric in % of pyruvic acid
Muscle (pigeon)	3300	205	11,650	3720	32
,,	5000	190	17,850	5790	32
,,	330	175	1,120	280	25
Heart (pig)	265	265	1,344	264	20
Liver (rat)	100	90	749	72	10
,,	26·6	110	—	52	—
,,	23·5	120	—	116	—
,,	28·6	120	—	81·5	—
,,	50·8	100	606	98	16
Kidney (rat)	66·3	80	581	78·5	13
Testis (rat)	60	120	—	50	—

7. *Balance sheet of the anaerobic metabolism of pyruvic acid*

Five substances, lactic, acetic, succinic, β-hydroxybutyric acids and CO_2 have now been identified as products of the anaerobic metabolism of pyruvic acid. The quantities formed indicate clearly that there are several reactions concerned with the removal of pyruvic acid. It is not possible to express the anaerobic fate of pyruvic acid by one formula. However, in testis and in brain reaction (3) accounts for the removal of up to 80 % of the pyruvic acid; this reaction is an intermolecular oxido-reduction or dismutation in which 50 % of the pyruvic acid is reduced to lactic acid, while the remaining 50 % is oxidized to acetic acid and CO_2.

In order to explain the data obtained in other tissues where the yield of acetic acid is low (muscle), we may assume that the acetic acid undergoes secondary changes resulting in the formation of β-hydroxybutyric acid. The net effect of these secondary reactions may be thus:

$$2 \text{ acetic acid} \rightarrow \text{acetoacetic acid} + H_2O \quad \text{.....(5),}$$

$$\text{Acetoacetic acid} + \text{pyruvic acid} + H_2O \rightarrow \beta\text{-hydroxybutyric acid} + CO_2 + \text{acetic acid} \quad \text{.....(6)}$$

(5) and (6), it should be expressly stated, are not intended to present the mechanism of the formation of β-hydroxybutyric acid. It is indeed unlikely that 2 mol. of acetic acid condense and much more probable that acetic acid and pyruvic acid combine primarily.

The sum of (5) and (6) is:

$$\text{Acetic acid} + \text{pyruvic acid} \rightarrow \beta\text{-hydroxybutyric acid} + CO_2 \quad \text{.....(7).}$$

If we eliminate acetic acid in (3) by means of reaction (7) we obtain:

$$3 \text{ pyruvic} + H_2O \rightarrow \beta\text{-hydroxybutyric acid} + \text{lactic acid} + 2CO_2 \quad \text{.....(8).}$$

Reaction (8) is derived under the supposition that the total acetic acid is replaced by β-hydroxybutyric acid. In actual fact there is always a certain amount of acetic acid found (see Table VII) and a better approximation to the experimental conditions in muscle is obtained if only part of the acetic acid in (3) is replaced by β-hydroxybutyric acid. An example is scheme (9) which is derived from (3) and (8) by replacing 4 out of 5 mol. acetic acid.

$$14 \text{ pyruvic acid} + 5H_2O \rightarrow 5 \text{ lactic acid} + 4\ \beta\text{-hydroxybutyric acid} + 9CO_2 + \text{acetic acid} \quad \text{.....(9).}$$

Scheme (9) is in good agreement with the experimental facts obtained in pigeon muscle. Table X gives an instance in which all the substances concerned were determined in one experiment (see further data in Tables IV, VII and IX). The agreement between facts and theory is as close as can be expected, seeing that the formation of succinic acid has been neglected in the scheme and that there may be other side reactions.

Table X. *Anaerobic pyruvic acid metabolism in pigeon breast muscle*

Substrate	Quantity metabolized (− = used, + = formed) (all figures corrected for blanks) (μl.)	Substance formed in % of pyruvate used	
		Experimentally	Calculated from (9)
Pyruvic acid	−17,850	—	—
β-Hydroxybutyric acid	+ 5,790	32·5	28·6
Lactic acid	+ 6,930	38·8	35·8
Steam-volatile acid (acetic acid)	+ 1,455	8·2	7·1
Total CO_2*	+10,290	57·7	64·0
Free CO_2	+ 8,490	47·6	53·0
Bicarbonate	+ 1,800	11·5	11·0

* If reaction (9) occurs at *p*H 7·4, 4 of the 9 CO_2 molecules formed should be found as bicarbonate. The actual *p*H in our muscle experiments was lower, since the acid formed by the muscle decomposed a large fraction of the bicarbonate of our saline. The *p*H during the experiment which began usually after a 20 min. period of equilibration was between 6·4 and 6·7, calculated on the grounds of determinations of bicarbonate and the CO_2 content of the gas mixture. If a known amount of bicarbonate was added to the muscle suspension, 60% was decomposed by the weak acids present in the muscle and appeared as free CO_2. The amount of bicarbonate expected is therefore less than 4/9 of the total. The figures given as "calculated" in the last column of Table X have been worked out under the assumption that 60% of the bicarbonate formed originally was converted into free CO_2; the data agree well with this expectation. The determination of free and bound CO_2 was carried out according to the principle described in section II, 3.

The data obtained with other tissues may also be explained on the assumption that a certain amount of acetic acid, varying from tissue to tissue, reacts according to (7). An exception is liver tissue where the increase in lactic acid formation exceeds the consumption of pyruvic acid. This fact can be explained as a catalytic effect of pyruvic acid on glycolysis as described by Mendel *et al.* [1931].

III. Method of measuring the rate of the pyruvic acid dismutation

The rate of reaction (3) can be determined manometrically by measuring the formation of free CO_2; with this technique the effects of inhibitors and of environment can be easily studied. In this section some results obtained with rat testis will be briefly summarized.

The rate of CO_2 formation from pyruvic acid rises slightly (from $Q_{CO_2}=5{\cdot}3$ to $Q_{CO_2}=8{\cdot}3$) if the *p*H rises from 6·8 to 8·1. The concentration of pyruvic acid affects the reaction within $M/500$ and $M/50$, but further increase does not increase the value of Q_{CO_2}.

Inhibitors of the dismutation are octyl alcohol (saturated), arsenious oxide ($M/100$) and iodoacetate. $M/1000$ iodoacetate, however, reduced the dismutation of pyruvic acid by only 20%, while the glycolysis was reduced by 80% under the same conditions.

No effect was observed on addition of the following substances which were tested because an inhibiting or stimulating effect might have been expected:

SH-glutathione ($M/200$), insulin, glucose ($M/100$), *dl*-lactate ($M/50$), acetate ($M/50$), fumarate ($M/50$), glutamate ($M/50$), malonate ($M/50$), tartronate ($M/50$), ammonium chloride ($M/50$).

IV. Dismutations of other ketonic acids

In this section experiments will be described which demonstrate that other ketonic acids may react similarly to pyruvic acid. In some tissues, especially in liver and kidney, reaction (3) may be modified and the reduction of pyruvic acid to lactic acid may be replaced by the reduction of acetoacetic acid to β-hydroxybutyric acid, according to the equation:

$$\text{Pyruvic acid} + \text{acetoacetic acid} + H_2O \rightarrow \text{acetic acid} + CO_2 + \beta\text{-hydroxybutyric acid} \quad \text{.....(10)}$$

or oxaloacetic acid may take the place of acetoacetic in (10):

$$\text{Pyruvic acid} + \text{oxaloacetic acid} + H_2O \rightarrow \text{acetic acid} + CO_2 + \text{malic acid} \quad \text{.....(11).}$$

Another modification is the reaction:

$$\alpha\text{-Ketoglutaric acid} + \text{acetoacetic acid} + H_2O \rightarrow \text{succinic acid} + CO_2 + \beta\text{-hydroxybutyric acid} \quad \text{.....(12).}$$

1. *Pyruvic and acetoacetic acids*

Jowett & Quastel [1935] have already demonstrated that addition of pyruvic acid accelerates the rate of anaerobic reduction of acetoacetic acid to β-hydroxy-

Table XI. *Anaerobic metabolism of acetoacetic and pyruvic acids in liver and kidney*

No.	Tissue	Substrates added M		$Q_{\text{Acetoacetate}}$	Q_{Pyruvate}	$Q_{\beta\text{-Hydroxybutyrate}}$	$Q^{N_2}_{CO_2}$
		Aceto-acetate	Pyruvate				
1	Liver (rat)	0·01	—	−1·17	—	—	—
		—	0·01	—	− 5·13	—	—
		0·01	0·01	−4·83	− 9·28	—	—
2	,,	—	—	—	—	—	2·13
		0·01	—	−5·72	—	—	2·64
		—	0·01	—	−11·4	—	18·7
		0·01	0·01	−9·24	−13·9	—	19·7
3	,,	—	—	—	—	+0·57	2·20
		0·01	—	−4·20	—	+2·02	2·41
		—	0·01	—	− 5·42	+1·58	3·77
		0·01	0·01	5·72	− 8·70	+4·73	6·25
4	,,	—	—	—	—	—	2·79
		0·01	—	—	—	—	4·9
		—	0·01	—	—	—	11·9
		0·01	0·01	—	—	—	25·5
5	,,	—	—	—	—	—	2·93
		0·01	—	—	—	—	1·81
		—	0·01	−2·97	—	—	7·68
		0·01	0·01	−9·56	—	—	15·2
6	Kidney (rat)	—	—	—	—	—	3·23
		0·01	—	−2·37	—	—	3·31
		—	0·01	—	2·7	—	4·94
		0·01	0·01	−3·35	3·35	—	6·22
7	,,	—	—	—	—	0·14	2·36
		0·01	—	−2·52	—	1·89	3·14
		—	0·01	—	− 6·55	0·89	4·66
		0·01	0·01	−3·41	− 7·74	4·22	6·48

butyric acid in various tissues. They did not, however, discuss the mechanism of this effect and the question of the oxidative equivalent of the reduction.

We determined the change in the concentrations of acetoacetic and pyruvic acids in the presence of liver and other tissues. Addition of pyruvic acid increases the rate of disappearance of acetoacetic acid and increases the rate of pyruvic acid disappearance, as shown in Table IX. The total quantities of pyruvic and acetoacetic acids which disappear are usually not stoichiometrical, since (10) is not the only reaction by which the acids are removed.

The increased disappearance of acetoacetic acid leads to an increased β-hydroxybutyric acid formation, as shown before by Jowett & Quastel. There is also an additional CO_2 formation as would be expected if acetoacetic and pyruvic acids react according to (10). The extra CO_2 is roughly equivalent to the extra acetoacetic acid.

2. *Ketoglutaric and acetoacetic acids*

Evidence for reaction (12) is presented in Table XII. It will be seen that acetoacetate increases the yield of succinic acid formed from α-ketoglutaric acid.

Parallel with the increase in succinic acid there is an increased rate of disappearance of acetoacetic acid and of production of CO_2. The changes are of the order expected from (12).

Similar results were obtained in rat kidney and liver.

Table XII. *Anaerobic formation of succinic acid from ketoglutaric acid in rabbit kidney*

Substrates (final concentration)	$Q_{Succinate}$	$Q_{Acetoacetate}$	Q_{CO_2}
—	0·25	—	1·41
M/50 ketoglutarate	1·13	—	2·64
M/50 acetoacetate	0·47	−4·32	2·34
M/50 ketoglutarate + *M*/50 acetoacetate	2·16	−5·26	4·46

Additional evidence for (12) is contained in Table XIII. An experiment is recorded in this table in which the effects of acetoacetate and of α-ketoglutarate on the respiration of pigeon's brain were studied. Both α-ketoglutarate and acetoacetate if added singly increase the rate of respiration, but if added together the increase exceeds by far the sum of the single increments. This may be explained by the assumption that reaction (12) is a primary step in the breakdown of α-ketoglutarate.

Table XIII. *Oxidation of α-ketoglutaric and acetoacetic acids in sliced pigeon's brain cortex*

(Phosphate saline 40°.)

Substrates (final concentration)		...	*M*/100 α-keto-glutarate	*M*/100 acetoacetate	*M*/100 α-keto-glutarate *M*/100 acetoacetate
Oxygen (μl.) absorbed per mg. tissue	20 min.	4·3	4·8	5·5	7·5
	40 min.	7·5	8·9	10·6	14·6
	60 min.	10·0	12·3	15·3	21·1
	80 min.	12·1	15·3	19·6	27·4
	100 min.	14·0	18·0	23·8	33·8

3. *Pyruvic acid and oxaloacetic acid*

The experimental proof for the reaction (11) offers difficulties owing partly to the spontaneous decomposition of oxaloacetic acid into pyruvic acid and CO_2, and partly to side reactions by which malic acid disappears. Szent-Györgyi & Straub [1936] found that 7 % of the added oxaloacetate was decarboxylated in 10 min. under conditions similar to those in our experiments. We have, therefore, not been able to prove reaction (11) conclusively, but its reality is suggested by the fact that the simultaneous addition of oxaloacetic and pyruvic acids to tissues causes a large increase of the anaerobic CO_2 production. Some data are given in Table XIV. Since the effect is observed in tissues which do not contain

Table XIV. *Anaerobic CO_2 production in presence of oxaloacetic and pyruvic acids*

	$Q_{CO_2}^{N_2}$			
Tissue		Pyruvate *M*/100	Oxaloacetate *M*/100	Oxaloacetate *M*/100 + pyruvate *M*/100
Testis (rat)	2·36	5·75	5·28	6·72
Brain (rat)	1·14	3·87	—	6·00
Spleen (rat)	2·97	3·84	—	4·05
Testis (rat)	—	6·54	—	8·31
,,	1·62	5·92	4·46	6·00
,,	1·30	4·38	—	7·55
Brain (rat)	—	3·15	—	4·98
Muscle (pigeon)	—	6·2	—	8·0
Kidney (rat)	—	4·5	—	6·8

any significant carbohydrate store such as testis and brain, the CO_2 production cannot be due to some "activating" effect on glycolysis and equation (11) is the simplest explanation for the observed CO_2 production. It will be seen that addition of oxaloacetic acid alone causes a considerable increase in the CO_2 evolution, and it may be assumed that the effect is also due to reaction (11), since part of the added oxaloacetic acid rapidly yields pyruvic acid by decomposition. The CO_2 is not formed from bicarbonate since the bicarbonate concentration remains constant, nor is it formed by decarboxylation of oxaloacetic acid, since at *p*H 7·4 this reaction would not yield free CO_2, but bicarbonate.

The rate of the reaction (11) is higher than that of the analogous reactions (3) and (10) in most tissues (brain, testis, kidney, muscle) and in view of the fact that under physiological conditions oxaloacetic acid is available in tissues, it is likely that (11) is the preferential way by which pyruvic acid is oxidized.

V. Discussion

1. *Decarboxylation of α-ketonic acids.* The reactions (1) and (2) indicate the manner in which animal tissues decarboxylate α-ketonic acids. Animal tissues do not possess a "carboxylase" of the type occurring in yeast. The decarboxylation in animal tissues is an oxidation in which another molecule of ketonic acid acts as oxidizing agent.

2. *Aerobic and anaerobic metabolism of pyruvic acid.* The question arises whether anaerobic reactions are the only reactions by which pyruvic acid is metabolized in tissues. If this be so it would be expected that the rate of disappearance of pyruvic acid should be independent of the presence of oxygen. In

some tissues, such as brain and testis the effect of oxygen on the pyruvic acid consumption is indeed small and it may be, therefore, that in these tissues the primary step in pyruvic acid oxidation is an anaerobic process. In some other tissues, however, especially in kidney, the difference between the anaerobic and aerobic rates of metabolism of pyruvic acid is considerable and the anaerobic dismutations cannot account for the whole pyruvic acid breakdown.

3. *Respiratory carbon dioxide.* The reactions (1) and (2) represent a mechanism by which CO_2, one of the end products of respiration, is formed and it is of interest to discuss the question of what fraction of the total respiratory CO_2 arises by these reactions. The maximum amount of CO_2 formed from anaerobic dismutation is about 60–80 % of the CO_2 formed in respiration. In some tissues, as for example in kidney, it is less (20–60 %).

It is not yet clear by what process the rest of the respiratory CO_2 is formed. The decarboxylation of oxaloacetic acid is another possibility, but its quantitative significance cannot yet be assessed.

4. *Ketonic acids as carriers of oxygen.* The hydroxy-acids resulting from the dismutation, i.e. lactic, *l*(-)malic or β-hydroxybutyric acid, can be re-oxidized in tissues by specific mechanisms in which the oxidizing agent is eventually molecular oxygen. The ketonic acid formed by re-oxidation may subsequently oxidize another molecule of ketonic acid, and in this way, ketonic acids may act as oxygen carriers. Szent-Györgyi [1935; 1936] was the first to suggest that a ketonic acid, oxaloacetic acid, is an oxygen carrier in tissues. The work reported in this paper shows that Szent-Györgyi's view holds for the special case of the oxidation of α-ketonic acids.

5. *Ketone bodies as intermediates in carbohydrate metabolism.* In muscle, and to a less extent in other tissues, β-hydroxybutyric acid is the chief end-product of the anaerobic oxidation of pyruvic acid. The ketone bodies are thus not only intermediates in fat but also in carbohydrate metabolism. In relation to this conclusion, it appears of special interest to note that the majority of tissues, although unable to oxidize butyric and higher fatty acids, oxidize readily β-hydroxybutyric acid (Dakin & Wakeman [1910], Friedmann & Maase [1910], Snapper & Grünbaum [1927], Jowett & Quastel [1935], Edson & Leloir [1936]). This fact seems explicable if we consider the ketone bodies as intermediates in carbohydrate breakdown.

6. *Carrier-linked oxido-reductions.* Quastel & Wooldridge [1928] suggested that intermolecular oxido-reductions require, in addition to the "dehydrogenase", an intermediate carrier for the hydrogen transport. Green *et al.* [1934] verified this view experimentally by *in vitro* experiments on isolated systems. There is little doubt that reactions (1) and (2) belong to the group of "carrier-linked" reactions. The nature of the intracellular carrier is, however, obscure.

7. *Role of vitamin B_1.* Peters [1936] has shown that the oxidation of pyruvic acid in pigeon brain requires the presence of vitamin B_1. If the oxidation, as suggested by the present paper, is brought about by an anaerobic dismutation, a vitamin effect would be expected to occur anaerobically in the presence of pyruvic acid. In preliminary experiments an effect on the dismutation of pyruvic acid was indeed shown by crude commercial preparations of vitamin B_1. Crystalline vitamin, however, failed to act.

VI. Summary

1. Pyruvic acid is metabolized in animal tissues under anaerobic conditions. The following substances are found as end products of the anaerobic metabolism of pyruvic acid (1) lactic acid, (2) acetic acid, (3) carbon dioxide, (4) succinic

acid, (5) β-hydroxybutyric acid. The evidence for the formation of the first four substances may be considered conclusive. The evidence for the formation of β-hydroxybutyric acid is based on the Van Slyke-Denigès mercuric sulphate reaction.

2. The quantities of the products formed suggest that the primary reaction is a dismutation according to reaction (3). This reaction represents the main anaerobic reaction of pyruvic acid in testis or brain.

3. The data obtained in other tissues, especially muscle suggest that acetic acid disappears by secondary reactions in which β-hydroxybutyric acid is the main end-product, according to the scheme (7).

4. Evidence is given for the occurrence of reactions analogous to (3) in which α-ketoglutaric acid, oxaloacetic acid and acetoacetic acid take part (reactions (10), (11) and (12)).

5. The schemes (1) and (2) represent a mechanism by which α-ketonic acids are oxidized and decarboxylated in animal tissues.

6. The reactions (7) and (8) indicate that ketone bodies are not only intermediates in fat but also in carbohydrate metabolism.

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