CCXXIII. PYRUVATE OXIDATION IN BRAIN
IV. THE OXIDATION PRODUCTS OF PYRUVIC ACID

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Detailed study of the biological oxidation of pyruvic acid is desirable owing to its important role as an intermediary metabolite in carbohydrate breakdown by tissue. A summary and discussion of some earlier work on pyruvic acid metabolism has been given in previous papers from this laboratory [Peters, 1936; 1937; McGowan, 1937].

Recently, several quantitative investigations have been made. Working on slices of rat brain, Weil-Malherbe [1937, 2] showed that under anaerobic conditions the amounts of lactic acid and CO_2 formed from pyruvic acid were quantitatively given by the Krebs dismutation [Krebs & Johnson, 1937, 1]:

$$2CH_3CO.COOH + H_2O \rightarrow CH_3CHOH.COOH + CH_3COOH + CO_2.$$

Only 80% of the acetic acid was found, but the deficit could be accounted for approximately by formation of succinic acid. With brei, the tendency was to form more succinic acid at the expense of acetic acid.

In aerobic studies, McGowan [1937] determined the O_2/pyruvic acid ratio for pigeon brain brei, obtaining a value of 450 μl./mg., the theoretical for complete combustion being 635. This evidence of incomplete oxidation was confirmed by the high value found for the respiratory quotient, 1·3, as compared with the calculated value of 1·2 for complete oxidation. It was also shown that under these conditions the formation of a small amount of lactic acid accompanied the respiration, but not sufficient to account for the low value of the O_2/pyruvate ratio, assuming the lactic acid to arise from the Krebs dismutation.

The object of the experiments to be described was to continue the above work, which involves determining the amounts of lactic and acetic acids formed during the respiration, and finally to obtain a complete balance sheet for the oxidation of pyruvic acid under these conditions.

Experimental method

The procedure differs in one important respect from that of McGowan, namely that unwashed tissue has been used in all cases. The reason for this is that the pyruvate oxidase system in brain is to some extent destroyed by washing, with consequent fall in respiration, thus making difficult the accurate determination of the small quantities of lactic and acetic acids involved. This difference in technique leads to slight corrections in the increased O_2 uptake due to pyruvate. The appropriate corrections to be applied are dealt with separately in the sections dealing with the calculation of lactic and acetic acid formation.

The brain was prepared in the following way. Three pigeons were killed by decapitation and the brains dissected out. The membranes and cerebellum were removed and the rest thoroughly minced with a bone spatula on a warm (38°) plate. The brei was then transferred, in roughly equal quantities (350 mg.), to the experimental bottles.

Na pyruvate[1] was used as substrate. A pure sample of this was kindly supplied by Prof. R. A. Peters. A fresh solution in $M/10$ phosphate Ringer solution, pH 7·3, was made up for each experiment, such that 0·2 ml. contained about 10 mg. This amount was added to each bottle containing a total of 2·8 ml. fluid; final concentration *c.* 0·03 M.

The respiration was measured as follows. The brain brei was introduced into the previously weighed bottles, each containing 1·5 ml. Ringer phosphate, pH 7·3, as used in previous experiments in this laboratory. After reweighing, the tissue was divided with a glass rod, 0·2 ml. of the Na pyruvate solution added and the total volume, including tissue, made up to 2·8 ml. with Ringer phosphate. In each experiment, four series of apparatus were used:

(1) Containing only tissue, stopped at the end of the equilibration period, i.e. just before the measurement of the respiration.

(2) Containing only tissue, not stopped until the end of the respiration.

(3) Containing tissue and pyruvate, stopped at the end of the equilibration period.

(4) Containing tissue and pyruvate, stopped at the end of the respiration.

Respiration was measured in O_2, using Barcroft-Dixon manometers. The usual technique of this laboratory was employed for the absorption of CO_2 (filter paper with 2N KOH). The rate of shaking was 108 oscillations per min. the bath being kept at 38°.

Determination of the lactate/pyruvate ratio

The difference in lactate content of bottles 3 and 4 gives the amount of lactate formed from pyruvate during the respiration period. The difference in O_2 uptake of apparatus 2 and 4 gives the uncorrected O_2 uptake associated with the formation of this amount of lactate. At the beginning of the respiration period, the amount of residual lactate is about 0·6 mg. per g. tissue, and this falls to about 0·2 mg./g. at the end of the respiration (Table I).

Table I. *Residual lactic acid*

	mg. per g. tissue		
Exp.	Initial	Final	Difference
90	0·56	0·20	0·36
92	0·57	0·16	0·41
93	0·69	0·29	0·40
Average	0·61	0·22	0·39

This residual lactate disappearing is given by the difference between the lactate contents of bottles 1 and 2. The O_2 uptake associated with the oxidation of this 0·39 mg. lactic acid to pyruvic acid and beyond is 222 μl. In bottles 3 and 4, the presence of pyruvate inhibits this lactate oxidation [Green & Brosteaux, 1936] with its corresponding O_2 uptake. Hence the observed increased O_2 uptake, due to disappearance of pyruvate, must be corrected by adding 222 μl. per g. tissue. From this corrected O_2 uptake the amount of pyruvate disappearing during the respiration has been calculated, using the value for the ratio O_2 uptake/pyruvate disappearing = 450 found by McGowan [1937]. In these experiments, the respiration was usually measured in duplicate or triplicate. The initial values quoted in Table I for residual lactate must not be confused with those observed by Kinnersley & Peters [1929; 1930] for lactate

[1] We are greatly indebted to Dr E. Stedman for recommending the use of Na pyruvate.

formed in brain soon after death. Amounts of the order of 1 mg. lactate per g. brain were formed from carbohydrate within about 1 min. after decapitation. Those in Table I are found roughly 90 min. after mashing, the smaller values being due presumably to oxidation of lactate to pyruvate in this system.

The estimation of lactate in the Barcroft bottles was carried out in the following way. Each bottle was treated with 1 ml. 25% trichloroacetic acid to precipitate the proteins and prevent further respiration, and after ½ hr. the contents were filtered. The bottle was then washed out twice with 2 ml. 5% trichloroacetic acid. To the combined filtrates were added 5 ml. 10% $CuSO_4$ and 6 ml. of a 10% $Ca(OH)_2$ suspension. After ½ hr. the volume was made up to 25 ml. and the bulky blue precipitate centrifuged off. 15 ml. of the centrifugate were used for each lactate estimation, according to the method of Friedemann & Kendall [1929], using the improved absorption tower of R. B. Fisher (private communication) and Na_2HPO_4 for aldehyde liberation [Lehnartz, 1928]. The average recovery of added lactate, using pure Zn lactate, was 98%.

Table II summarizes the results of these experiments. In every case the value of the ratio lies between 4·9 and 5·4, the variations being within the experimental error of the method. The average value is 5·2 mol. lactic acid per 100 mol. pyruvic acid.

Table II. *Lactic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.) Uncorr.	Corr.	Pyruvic acid mg. (calc.)	Lactic acid mg.	L.A./P.A. molar %
91	210	404	1901	1990	4·42	0·25	5·4
92	180	366	1557	1635	3·63	0·20	5·4
		343	1452	1531	3·40	0·19	5·4
		339	1444	1517	3·37	0·18	5·1
93	200	355	1581	1660	3·69	0·19	4·9
		362	1611	1691	3·76	0·20	5·1
94	195	283	1203	1267	2·82	0·15	5·3
		307	1305	1373	3·05	0·16	5·2

Determination of the acetate/pyruvate ratio

As in the determination of lactate, the acetate production from respiration in pyruvate is equal to the difference in acetate content of bottles 3 and 4. There is a small amount of residual acetate formation, which is given by subtracting the acetate formed in 1 from that in 2. The net acetate production is therefore the difference between these two values. The small correction to be applied to the O_2 uptake is almost negligible, being only the O_2 absorbed in converting 0·39 mg. lactate into pyruvate, a value of about 50 μl. per g. tissue.

The estimation of acetate was based on the distillation method used by Weil-Malherbe [1937, 1, 2]. There are several substances which are volatile under the greatly reduced pressure employed. Free pyruvic acid, which is fairly volatile, is eliminated by conducting the distillation in the presence of phenylhydrazine [Weil-Malherbe, 1937, 2]: addition of pyruvic acid to the distilling flask did not increase the acidity of the distillate. Lactic acid is also slightly volatile under these conditions, but the amount distilled does not increase in proportion to its concentration [Virtanen & Pulkki, 1928]. In order to counteract any effect due to varying low concentrations, all distillations were carried out in the presence of a large excess of lactic acid, so that it was accounted for in the

blank estimation. The contents of the bulb of the Parnas apparatus were kept at about *p*H 2, at which reaction HCl is not liberated.

Each bottle was treated with 0·2 ml. 10% H_2SO_4 to precipitate protein and prevent further respiration. There was evidence that the use of sulphuric acid for this purpose did not affect the results, since similar values were obtained, in another connexion, when further respiration was prevented merely by placing the bottles in cold-store. When precipitation was complete—the bottles were usually allowed to stand overnight in the refrigerator—the contents were centrifuged, 2 ml. of the clear liquid being used for each estimation. The total amount of acetate was calculated from the volume of fluid in each bottle, tissue being counted as fluid volume, since the amount of solid matter is only about 15% of the wet wt. of the brain.

To the sample were added 10 ml. of a solution containing 1 ml. 10% phenylhydrazine hydrochloride, 1 ml. syrupy phosphoric acid and 5 g. NaH_2PO_4. 0·2 ml. (10 mg.) lactic acid was added. The mixture was allowed to stand for at least ½ hr. before distillation, distilled water (2 ml.) being used to wash it completely into the bulb of the Parnas apparatus. Distillation in steam was carried out at 14–16 mm. pressure, an exact amount of distillate (55 ml.) being collected in each case. This was then titrated against freshly prepared *N*/200 CO_2-free NaOH, using bromothymol blue as indicator. The standard colour tint was a solution of sodium acetate to which a few drops of the indicator had been added. A clearly defined end-point at *p*H 7·6, sharper than with phenolphthalein, was obtained. Blank estimations were always performed, the usual value being about 0·25 ml. *N*/200 NaOH. Under these conditions, the recovery of added acetic acid, as shown in Table III, was 87·3%. Higher recoveries could be obtained by collecting more distillate, but this proved inconvenient.

Table III. *Recovery of added acetic acid*

Acetic acid mg.	*N*/200 NaOH (ml.) Calc.	Found	Recovery %
0·650	2·166	1·898	87·6
		1·884	87·0
0·325	1·083	0·979	90·4
		0·972	89·8
0·163	0·542	0·469	86·6
		0·457	84·4
0·065	0·217	0·185	85·3

The procedure therefore differs from that of Weil-Malherbe in the following respects. Enzymic oxidation of pyruvic acid was arrested by the use of 10% H_2SO_4. A stock solution of NaH_2PO_4, phosphoric acid and phenylhydrazine hydrochloride—"acetate mixture"—was employed for all distillations, and proved of great convenience. All estimations were carried out in the presence of a large excess of lactic acid. Finally bromothymol blue was used as indicator.

Table IV. *Acetic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.) Uncorr.	Corr.	P.A. mg. calc.	Acetic acid mg.	H.Ac./P.A. molar %
80	170	327	1131	1147	2·55	0·42	24·4
81	175	392	1432	1452	3·23	0·55	25·1
82	240	383	1895	1914	4·25	0·71	24·5
		368	1891	1908	4·24	0·74	25·5
86	185	356	1329	1346	2·99	0·53	25·9
		370	1367	1385	3·08	0·52	24·7
		377	1402	1421	3·16	0·51	23·8
87	185	348	1162	1180	2·62	0·44	24·6
		370	1217	1234	2·74	0·47	25·0

Acetic acid was identified by the method of Krüger & Tschirch [1929]. Four distillates were neutralized, evaporated to about 1 ml. and treated with 5 % lanthanum nitrate (0·5 ml.), $N/50$ I_2 in KI (0·5 ml.) and 1 drop of $4N$ NH_4OH. On warming gently at first and finally boiling, the solution turned green and then blue, a characteristic blue precipitate separating out on cooling. This identified either acetate or propionate. Since the formation of the latter was unlikely, acetate was inferred.

Table IV summarizes the results of the experiments. It will be seen that the % ratio lies between 23 and 26 in every case, with an average of 24·8.

Balance sheet of pyruvic acid disappearance

Since the amount of lactic acid formed is so very small, the fate of pyruvic acid cannot be accounted for merely by the summation of the amounts which are completely oxidized or which follow the anaerobic Krebs dismutation. It cannot be argued that the observed formation of lactic acid is too small owing to reoxidation to pyruvic acid. As previously stated, the presence of pyruvic acid should inhibit completely the lactic dehydrogenase. Moreover, the Krebs dismutation cannot account for such a large production of acetic acid. To the above two processes

$$\text{I. } CH_3CO.COOH + \tfrac{5}{2}O_2 \rightarrow 2H_2O + 3CO_2,$$
$$\text{II. } CH_3CO.COOH + \tfrac{1}{2}H_2O \rightarrow \tfrac{1}{2}CH_3CHOH.COOH + \tfrac{1}{2}CH_3COOH + \tfrac{1}{2}CO_2,$$

must be added a third

$$\text{III. } CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2,$$

which will account for the excess of acetic acid formed over and above that produced by the Krebs dismutation.

The method of calculating the percentages of the above processes is as follows. 5·2 % lactic acid can only be derived from the Krebs dismutation; hence process II takes place to the extent of 10·4 %. Of the acetic acid formed, 5·2 % comes from the Krebs dismutation, so that the remaining 19·6 % is formed in process III. The amount of I can be deduced from a consideration of the O_2/pyruvate ratio.

450 μl. O_2 are required to oxidize 1 mg. pyruvic acid under these *in vitro* conditions. Process III oxidizes 1 mg. pyruvic acid with an accompanying 127 μl. O_2. Then, since this process takes place to the extent of only 19·6 %,

$$19{\cdot}6/100 \times 127 = 25\,\mu\text{l.}$$

of O_2 are absorbed. All the remaining O_2 is used up by process I. For complete oxidation, 1 mg. pyruvic acid requires 635 μl. O_2; it follows, then, that the amount undergoing complete oxidation is

$$425/635 \times 100 = 67\,\%.$$

The disappearance of pyruvic acid can thus be accounted for quantitatively by an appropriate summation of the three following equations. The extent to which each process takes place is represented by the accompanying percentages.

$CH_3CO.COOH + \tfrac{5}{2}O_2 \rightarrow 3CO_2 + 2H_2O$	67 %
$CH_3CO.COOH + \tfrac{1}{2}H_2O \rightarrow \tfrac{1}{2}CO_2 + \tfrac{1}{2}CH_3CHOH.COOH + \tfrac{1}{2}CH_3COOH$	10·4 %
$CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow CO_2 + CH_3COOH$	19·6 %

97 % of the pyruvic acid is accounted for.

From the above data, the respiratory quotient can be calculated:

$$\text{R.Q.} = \frac{5{\cdot}2 + 19{\cdot}6 + 3\,(67)}{\frac{1}{2}\,(19{\cdot}6) + \frac{5}{2}\,(67)} = \frac{225{\cdot}8}{177{\cdot}3} = 1{\cdot}28.$$

This agrees well with the experimental value of 1·3 found by McGowan [1937]; in fact if his one high value is excluded his average R.Q. is 1·285.

Discussion

The preceding section provides us with a detailed knowledge of the ultimate fate of pyruvic acid when enzymically oxidized by pigeon brain. The importance of this knowledge is that we now have quantitative data which any theory of aerobic pyruvic acid oxidation must take into account. The results must, however, be interpreted with care.

The fact that a balance sheet can be drawn up by combining three equations in an appropriate manner does not necessarily mean that all three processes can take place independently of one another, or even that, as such, they take place at all. It may well be that the formation of lactic acid by the Krebs dismutation is independent of the oxidative processes. Nevertheless there is no direct evidence that lactic acid is formed in this manner in the system. It may possibly arise by some other process, so far unknown.

An "explosive" oxidation of pyruvic acid giving CO_2 and water, i.e. reaction I without any intermediary, seems most improbable. Several plausible theories of pyruvic acid oxidation have been proposed involving a series of oxidation products usually in the form of a cycle, as in the Toenniessen-Brinkmann [1930] scheme and the Krebs & Johnson [1937, 2] citric acid cycle. If there were no evidence to the contrary [McGowan & Peters, 1937], these might appear possible for aerobic oxidation in brain.

The possibility of acetic acid being an intermediate stage of pyruvic acid metabolism is very unlikely, since it has no noticeable effect on brain respiration, either in the presence or absence of pyruvate [McGowan & Peters, 1937; Elliott *et al.* 1937]. If the formation of acetic acid is not independent of other oxidative mechanisms, then it may conceivably arise from the breakdown of some initial stage of an unknown cycle. It is suggested that the following scheme might show the relationship between acetic acid formation and some general mechanism of pyruvate oxidation:

Pyruvic acid → (X) → Acetic acid

(X) → Cycle regenerating some pyruvic acid, CO_2 and water being eliminated.

Hence a cyclical theory must involve some unknown stage (X).

It is clear from the balance sheet that if there is any formation of citric acid [Simola & Alapeuso, 1938], then it could only arise from about 3 % of the pyruvic acid apparently unaccounted for. Of course, the above remarks apply only to aerobic pyruvate oxidation in brain, and do not in any way embrace anaerobic oxidations such as are brought about by methylene blue.

Summary

Lactic and acetic acids have been found in definite amounts when pigeon brain brei has respired in pyruvic acid. The quantities determined account for 30 % of the pyruvate disappearing, the remainder being oxidized completely to CO_2 and water, thus enabling a complete balance sheet to be drawn up.

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