100. THE METABOLISM OF PYRUVATE IN PIGEON LIVER

By EARL ALISON EVANS, JR.¹

From the Department of Biochemistry, University of Sheffield

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It has been recently demonstrated [Krebs & Eggleston, 1940] that the oxidation of pyruvate by pigeon breast muscle occurs by way of the citric acid cycle. In certain other tissues, such as brain [Banga *et al.* 1939] and liver [Elliott *et al.* 1937], the available data suggest mechanisms for pyruvate removal different from that in muscle, and it seems desirable, therefore, to investigate in detail the differences in pyruvate oxidation in various animal tissues.

The present report deals with the utilization of pyruvate by minced pigeon liver. It was found that in this tissue the reactions comprising the citric acid cycle are present and that pyruvate is probably oxidized by way of the cycle when its concentration is low. In addition, two further reactions involving a rapid removal of pyruvate can occur in this tissue. The first of these, the formation of acetoacetate from pyruvate, has been known for some time [Embden & Oppenheimer, 1913; Annau, 1934; Edson, 1935]. The second reaction which has not been previously demonstrated in liver, is a synthesis of α -ketoglutarate from pyruvate. In contrast to muscle, the synthesis in liver does not require the addition of a C_4 dicarboxylic compound.

Procedure and Methods

In general, the procedure used in the present experiments were similar to those described in previous papers from this laboratory [see Krebs & Eggleston, 1940].

The liver from well-fed pigeons was removed immediately after death and minced in a small hand mincer of the "Fischer" type since the amount of tissue from a single pigeon was insufficient for the use of the "Latapie" mill available. The state of subdivision of the minced tissue is similar to that obtained with the latter apparatus. For each g. of wet tissue, 6.5 ml. of ice-cool, Ca-free phosphate saline [Krebs & Eggleston, 1940] were added. 3 ml. of the suspension were pipetted into conical Warburg vessels and further additions of substrates, water etc. arranged to give a final volume of 4 ml.; thus each ml. of the final suspension contained 100 mg. (wet wt.) of tissue. Preliminary experiments indicated that these conditions gave optimal respiration. Pyruvate is utilized by the suspensions at a rate equal to that of sliced pigeon liver. The use of the minced tissue in the present experiments was preferred since it permits the use of relatively larger quantities of tissue with consequent increased experimental effects.

In the aerobic experiments the vessels were filled with O_2 and NaOH was placed in the centre cups: in the anaerobic ones the gaseous phase was N_2 and the centre cups contained yellow phosphorus. The temperature of the bath in all experiments was 40°. Substrates were usually added from the side arm of the vessels after an equilibration period of 10 min. Malonate, however, was added directly to the liver suspension in order to eliminate the induction period preceding its inhibiting effect. Acidic substances were added as neutral Na salts.

¹ Fellow of the Rockefeller Foundation.

In those experiments in which α -ketoglutarate and succinate were determined, the vessels were removed from the bath at the end of the experimental period, 2 ml. sodium tungstate added and the solution washed into a measuring cylinder and quickly diluted to 20 ml. with water. 2 ml. 0.66*N* H_2SO_4 were immediately added and the protein precipitate was then filtered off. The determinations were carried out on an aliquot of the filtrate.

A similar procedure was followed when citrate was estimated, except that trichloroacetic acid (5 ml. 7% per 4 ml. tissue suspension) was used to deproteinize the solutions.

When pyruvate was to be measured, the reaction was stopped at the end of the experimental period by the addition of 1 ml. 3*M* acetate buffer of *pH* 5 for each 4 ml. of suspension. The contents of the vessel were filtered and an aliquot of the filtrate used for the determination. Pyruvic acid was determined by the carboxylase method. The respiratory quotient was measured by the method of Warburg & Yabusoe [1924]. Succinic acid was determined manometrically [Szent-Györgyi & Gözsy, 1935; Krebs, 1937] after ethereal extraction from the tungstate-deproteinized solution. Citric acid was determined by the method of Pucher *et al.* [1936]. Acetoacetic acid was estimated by the aniline reaction of Ostern [1933] as modified by Edson [1935]. Fumaric acid was reduced to succinate and determined as the latter; the details of this method will be published shortly.

In earlier experiments α -ketoglutarate was determined directly in the deproteinized solutions by the method of Krebs & Eggleston [1940] in which the acid is oxidized to succinate by treatment with KMnO_4 and MnSO_4 at 100° for 5 min. Since glutamic acid, if present, yields small but appreciable quantities of succinate under this treatment the procedure was checked by first extracting the acidified deproteinized solution with ether and subjecting the ether-soluble material to permanganate oxidation. The values thus obtained agreed satisfactorily with those from experiments in which the deproteinized solutions were treated directly with the oxidizing mixture. In latter experiments oxidation was performed at room temperature (20°) for a period of 1 hr. Under these conditions, the formation of succinic acid from sources other than α -ketoglutarate is negligible.

I. The utilization of pyruvate by pigeon liver

Aerobic utilization of pyruvate. The disappearance of added pyruvate from minced pigeon liver suspended in a Ca-free phosphate-saline medium is extremely rapid. The rate is indicated in the experiment in Table 1, in which 400 mg. wet

Table 1. *Pyruvate and O_2 utilization in minced pigeon liver*

4 ml. liver suspension; O_2 ; initial pyruvate, 2860 $\mu\text{l.}$ in 4 ml.

Period of incubation (min.)	O_2 used ($\mu\text{l.}$)	Pyruvate in medium ($\mu\text{l.}$)	Pyruvate used ($\mu\text{l.}$)
20	800	1300	1560
40	1590	440	2420
80	2925	207	2653
120	3765	185	2675

tissue (80 mg. dry wt.) are using pyruvate at the rate of 14 mg. per hr. The O_2 uptake of the liver suspensions is not usually increased by the addition of pyruvate but since pyruvate disappears at a much slower rate in the absence of O_2 (see Table 3) part of the O_2 consumed must be concerned with the removal

of pyruvate. It follows that the oxidation of those substrates responsible for the normal respiration of the minced tissue is inhibited by the addition of pyruvate. The ratio of O_2 consumed to pyruvate disappearing is 0.6, indicating incomplete oxidation of the pyruvate. This is likewise suggested by the R.Q. of the reaction being 1.4 (Table 2). Under similar conditions pigeon breast muscle completely oxidizes pyruvate to CO_2 and H_2O , the O_2 /pyruvate ratio being 2.5 and the R.Q. 1.2.

Table 2. *Respiratory quotient in the presence of pyruvate*

4 ml. liver suspension; O_2 ; 30 min.; method of Warburg & Yabusoe [1924]; initial pyruvate concentration, 0.03 *M*.

μ l. pyruvate used	1948
μ l. O_2 used	1183
μ l. CO_2 formed	1673
R.Q.	1.41
Ratio $\frac{O_2 \text{ used}}{\text{Pyruvate used}}$	0.60
Ratio $\frac{CO_2 \text{ formed}}{\text{Pyruvate used}}$	0.85

The effect of malonate. In pigeon muscle, the presence of *M*/400 malonate almost completely inhibits the utilization of pyruvate. Whilst in liver, high concentrations of malonate (*M*/40) cause a slight and possibly non-specific inhibition of pyruvate utilization, *M*/400 and *M*/4000 malonate are without appreciable effect either on the O_2 consumption or pyruvate utilization (Table 3).

Table 3. *Effect of malonate on O_2 and pyruvate utilization*

4 ml. liver suspension; pyruvate (initial concentration 0.03 *M*) added from side arm after 10 min.; malonate added directly to tissue; period of incubation 40 min.

Final concentration of malonate (<i>M</i>)	Gas	O_2 used (μ l.)	Pyruvate used (μ l.)
—	O_2	1309	1995
0.025	O_2	785	1548
0.0025	O_2	1259	2091
0.00025	O_2	1370	2019
—	N_2	—	376
0.025	N_2	—	225
0.0025	N_2	—	406
0.00025	N_2	—	396

If the inhibiting effect of malonate is due to its inhibition of succinic dehydrogenase, it follows that under the conditions of these experiments neither the utilization of pyruvate nor the uptake of O_2 by minced liver requires the activity of succinic dehydrogenase.

The effect of fumarate. The addition of fumarate together with pyruvate to minced pigeon liver has little effect on the O_2 uptake or on the utilization of pyruvate. In the presence of malonate a similar result is obtained (Table 4). These results are, again, in contrast to those obtained with muscle where the addition of fumarate to malonate-poisoned tissue causes the disappearance of an amount of pyruvate equivalent to the added fumarate and an increase in the consumption of O_2 of 2–3 molecules for each molecule of pyruvate disappearing.

Table 4. *Effect of fumarate on pyruvate utilization by pigeon liver*

4 ml. liver suspension; fumarate and pyruvate added from side arm after 10 min.; malonate added immediately to suspension; O_2 ; period of incubation, 40 min. after addition of pyruvate.

Exp.	Additions	O_2 used (μ l.)	Pyruvate used (μ l.)
1	0.03 <i>M</i> pyruvate	1617	2170
	{ 0.03 <i>M</i> pyruvate	1700	1948
	{ 0.01 <i>M</i> fumarate		
2	0.0025 <i>M</i> malonate	792	—
	{ 0.0025 <i>M</i> malonate	1122	1643
	{ 0.03 <i>M</i> pyruvate		
	{ 0.0025 <i>M</i> malonate	1169	1598
	{ 0.03 <i>M</i> pyruvate		
	{ 0.01 <i>M</i> fumarate		
	{ 0.0025 <i>M</i> malonate	1150	—
	{ 0.01 <i>M</i> fumarate		

The formation of acetoacetate and α -ketoglutarate. From the data already presented it is clear that the utilization of pyruvate by minced pigeon liver *under the conditions of the present experiments* is unlike the process that occurs in muscle. The question therefore arises as to the details of the process of pyruvate utilization in this tissue. Since during the initial stages of the reaction, the oxidation of pyruvate is incomplete (as indicated by the O_2 /pyruvate ratio of 0.6) it is to be expected that intermediate metabolites appear in the suspensions. In fact, two substances which must be considered as products of the incomplete oxidation of pyruvate were found to accumulate in pigeon liver suspensions under the conditions of these experiments: acetoacetate and α -ketoglutarate.

The formation of acetoacetate from pyruvate by liver has been previously described [Embden & Oppenheimer, 1913; Annau, 1934; Edson, 1935]. In the experiments with minced tissue $Q_{\text{acetoacetate}}$ is of the same order of magnitude as found with sliced tissue. In the presence of malonate (Table 7), there is an increase and on the addition of fumarate a decrease in the amount of acetoacetate present. This is in agreement with the earlier results of Annau [1935].

The synthesis of α -ketoglutarate from pyruvate by pigeon liver has not been previously described although the ability of this tissue to synthesize glutamine from ammonium pyruvate suggested that α -ketoglutarate was formed as an intermediate [Örström *et al.* 1939]. When pigeon liver is incubated aerobically with pyruvate, α -ketoglutarate rapidly appears as illustrated in Table 5 where

Table 5. *Synthesis of α -ketoglutaric acid in pigeon liver*

4 ml. suspension of liver; initial pyruvate concentration 0.03 *M*; O_2 .

Time (min.)	O_2 used (μ l.)	Pyruvate used (μ l.)	α -Ketoglutaric acid found (μ l.)
20	800	1560	227
40	1590	2420	284
80	2925	2653	287
120	3765	2675	203

$Q_{\text{ketoglutarate}}$ is +8.5 (calculated for the first 20 min. period). After 40 min. the quantity of α -ketoglutarate found remains approximately constant. That this may be interpreted as an "equilibrium" between the synthetic reaction and oxidative removal of the acid follows from the fact that the oxidation of α -ketoglutarate can occur at a rate ($Q_{\text{ketoglutarate}} = -10.5$, as calculated from

the data in Table 6) equivalent to its rate of synthesis. When pyruvate and α -ketoglutarate are added together to pigeon liver the utilization of both substrates is inhibited (Table 6), probably as a result of competition between the

Table 6. *α -Ketoglutarate synthesis and disappearance in pigeon liver*

4 ml. suspension liver; O_2 ; period of incubation 40 min.

Additions	O_2 used (μ l.)	Pyruvate used (μ l.)	α -Ketoglutarate found (μ l.)	α -Ketoglutarate used (μ l.)
—	967	—	65	—
0.03 <i>M</i> pyruvate	1172	2187	211	—
0.03 <i>M</i> α -ketoglutarate	1116	—	782	562
{ 0.03 <i>M</i> pyruvate + 0.03 <i>M</i> α -ketoglutarate	973	1408	929	415

two substrates for the available oxidation mechanisms. This inhibition of ketoglutarate oxidation by pyruvate may be regarded as responsible for the accumulation of relatively large quantities of α -ketoglutarate under the conditions of these experiments.

Anaerobic formation of α -ketoglutarate. In some experiments a definite anaerobic synthesis of α -ketoglutarate in the presence of pyruvate was observed but the effect was small, varied in magnitude and did not regularly occur.

Formation of carbohydrate from pyruvate. The ability of liver tissue to synthesize carbohydrate from pyruvate is generally recognized. A conversion of pyruvate into carbohydrate occurring simultaneously with the complete oxidation of an additional quantity of pyruvate would account for the O_2 /pyruvate ratio found in the present experiments. However, measurements of the total carbohydrate content of the liver suspensions with and without added pyruvate showed no significant differences.

Formation of acetoacetate and α -ketoglutarate in the presence of malonate. The rapid oxidation of α -ketoglutarate makes it impossible to estimate the extent to which ketoglutarate formation accounts for the pyruvate which has disappeared. It is possible, however, to prevent the further oxidation of succinate, the primary product of ketoglutarate oxidation, by the addition of malonate. In these circumstances, measurement of the amount of ketoglutarate plus succinate formed serves as an indication of the quantitative importance of ketoglutarate synthesis in the utilization of pyruvate by pigeon liver. Such an experiment is presented in Table 7. Whilst malonate (*M*/400) has no effect on the quantities of pyruvate or O_2 consumed, increased quantities of both acetoacetate and of ketoglutarate plus succinate are found. On the assumption that each molecule of acetoacetate and of ketoglutarate and succinate require two molecules of pyruvate for their formation, the bulk of the pyruvate disappearing can be accounted for in terms of these two metabolites. In the experiment of Table 7, 63 % of the added pyruvate can be accounted for as ketoglutarate and succinate and an additional 28 % as acetoacetate. These calculations, it should be noted, are made on the assumption that an appreciable formation of ketoglutarate, succinate and acetoacetate does not occur from sources other than pyruvate when this substance is present in large excess.

Since the rate of pyruvate utilization by liver is not affected by malonate it is probable that the two reactions (α -ketoglutarate and acetoacetate synthesis) which can be demonstrated as accounting for the bulk of the pyruvate used by malonate-poisoned tissue, are of similar quantitative importance in the oxidation of pyruvate by liver tissue in the absence of malonate. It follows then that

Table 7. *Formation of acetoacetic acid and α -ketoglutaric acid in the presence of malonate*

4 ml. liver suspension; pyruvate added from side arm after 10 min., malonate added directly to tissue; O_2 ; period of incubation 30 min.

Additions	O_2 used (μ l.)	Pyruvate used (μ l.)	Acetoacetic acid formed (μ l.)	Ketoglutaric + succinic acid found (μ l.)
0.025 <i>M</i> malonate	580	—	34	164
0.03 <i>M</i> pyruvate	1087	1605	38	233
{ 0.025 <i>M</i> malonate 0.03 <i>M</i> pyruvate	952	1640	233	518

the observed differences between pyruvate utilization in muscle and in liver are due largely to the presence of these additional reactions in liver. Whilst muscle is incapable of synthesizing appreciable quantities of the dicarboxylic compounds used in its metabolism, the liver is capable of effecting such a synthesis.

II. *The citric acid cycle in pigeon liver*

Oxidation and synthesis of citrate. In the preceding section it has been shown that α -ketoglutarate can arise directly from pyruvate in pigeon liver. The further oxidation of α -ketoglutarate would lead to oxaloacetate and this substance may possibly be further metabolized, as in muscle, by condensing with additional pyruvate to form citrate, i.e. by the citric acid cycle. The presence of the citric acid cycle in pigeon liver would require that the tissue should be capable of oxidizing citrate at a rate at least one-sixth that at which O_2 was being consumed and also that citrate could be synthesized from pyruvate and oxaloacetate. That pigeon liver satisfies both these criteria is demonstrated in Tables 8 and 9. In Table 8 Q_{citrate} is -14.3 as compared to Q_{O_2} of -16.2 : the oxidation of citrate

Table 8. *Oxidation of citrate in pigeon liver*

4 ml. liver suspension; O_2 .

Exp.	Additions	Time (min.)	O_2 used (μ l.)	Citrate found (μ l.)	Citrate used (μ l.)
1	Citrate (1344 μ l.)	30	648	700	644
2	Citrate (1344 μ l.)	60	1290	200	1144

Table 9. *Anaerobic formation of citrate in pigeon liver*

4 ml. liver suspension; N_2 ; period of incubation 60 min.

Additions	Pyruvate found (μ l.)	Pyruvate change (μ l.)	Succinate found (μ l.)	Ketoglutarate found (μ l.)	Citrate found (μ l.)
2604 μ l. pyruvate	2095	- 509	106	75	4
{ 2604 μ l. pyruvate 4480 μ l. oxaloacetate	3840	+ 1236	121	349	373
{ 2604 μ l. pyruvate 2240 μ l. oxaloacetate	2560	- 44	97	343	126

occurs at a rate far more rapid than that required by theory. Table 9 illustrates the anaerobic synthesis of citrate in pigeon liver from oxaloacetate and pyruvate. Q_{citrate} , which in the presence of pyruvate alone is very small, is increased to about 7 in the presence of $M/20$ oxaloacetate and to about half this amount when the oxaloacetate concentration is $M/40$. Together with citrate, α -ketoglutarate

is formed under these conditions in relatively large amounts. From analogy with muscle, one may conclude that this arises from the oxidation of citrate to α -ketoglutarate by oxaloacetate. The rate of citrate synthesis, as calculated in these experiments from the quantity of citrate found, is therefore minimal and a more precise estimation of the rate of citrate synthesis should be based on the amounts of citrate plus α -ketoglutarate formed.

In muscle, the synthesis of citrate from oxaloacetate and pyruvate as well as the oxidation of citrate to ketoglutarate is coupled with the reductive formation of one mole of malate [Krebs, 1940, 1, 2]. If a similar reaction occurred in liver, therefore, one would expect to find a quantity of malate and fumarate formed, equal to one mole for every mole of citrate produced plus two moles of malate for each mole of ketoglutarate. In Table 10 an experiment in which oxaloacetate alone was added indicates that malate and fumarate are formed in quantities of the magnitude expected. In this experiment the quantity of oxaloacetate added was small and little citrate was found, the bulk of the citrate having been further oxidized to ketoglutarate. The quantity of malate plus fumarate necessary to account for the citrate and ketoglutarate formed should be, as calculated from the above considerations, 405 μ l., whilst 580 μ l. were found.

Table 10. *The fate of oxaloacetic acid in pigeon liver*

4 ml. liver suspension; N₂; period of incubation 40 min.

	μ l.	
Oxaloacetate added	1344	= 0.240 milliequiv. C added
CO ₂ and HCO ₃ ' formed*	1285	
Ketoglutarate found	197	} 580 = 0.234 milliequiv. C recovered
Pyruvate found	103	
Fumarate found	121	
Malate (calculated)†	459	
Succinate found	61	
Citrate found	11	

* Method of Warburg & Yabusoe [1923]. Corrected for CO₂ evolved from tissue alone and for HCO₃' from oxaloacetate.

† *L*-Malate/fumarate ratio = 3.8 [see Krebs, 1940, 2]

About 97 % of the carbon added in the form of oxaloacetate can be accounted for by the products listed in Table 10, which thus represents a fairly complete balance sheet.

It is clear then, that liver tissue is capable of removing pyruvate by the citric acid cycle when oxaloacetate is present. It follows that α -ketoglutarate can arise in liver tissue in two ways, as a result of the oxidation of citrate or by formation from pyruvate without added C₄ dicarboxylic compounds. There are, therefore, two cyclic processes in liver, the citric acid cycle, and another cyclic series of reactions similar to that discussed by Toeniessen & Brinkmann [1930] and by Elliott *et al.* [1937]

According to the latter scheme oxaloacetate reacts by way of decarboxylation whilst in the citric acid cycle it condenses with pyruvate to yield citrate. Data in reference to the relative importance of the two cycles have already been presented in Table 9. If oxaloacetate according to scheme (1) was entirely decarboxylated to pyruvate then the effect of oxaloacetate should be simulated by the addition of an appropriate quantity of pyruvate. As illustrated in Table 9, however, the anaerobic formation of ketoglutarate and citrate from oxaloacetate is much larger than that found in the presence of pyruvate alone, in this

particular experiment more than four times. It can only be concluded that the greater part of the added oxaloacetate (in this experiment at least 80%) is metabolized by way of the citric acid cycle rather than by way of decarboxylation to pyruvate.

DISCUSSION

The mechanism by which ketoglutarate is formed from pyruvate alone is unknown. The original scheme of Toeniessen & Brinkmann [1930] for succinate synthesis from pyruvate in muscle:

can be eliminated in the case of pigeon liver since this tissue is incapable of metabolizing formate at an appreciable rate. Also the possibility that the ketoglutarate is formed by transamination between the added pyruvate and glutamic acid present in the tissue can be excluded because the free glutamic acid content of pigeon liver is too low (about 45 mg. per 100 g. wet wt. of tissue) to account for the quantities of ketoglutarate formed.

Pigeon muscle is apparently incapable of synthesizing appreciable quantities of the dicarboxylic acids which are catalytic agents in its oxidation of carbohydrate. The ability of the pigeon liver to synthesize α -ketoglutaric acid, therefore, may be of general metabolic significance, the liver acting as a source of these compounds (possibly transported in some other form) for those tissues incapable of their direct formation.

SUMMARY

1. Pyruvate is rapidly oxidized by minced pigeon liver in a calcium-free phosphate saline medium, the O_2 /pyruvate being about 0.6 and the R.Q. about 1.4.
2. The utilization of pyruvate and O_2 in pigeon liver, unlike the similar reaction in muscle, is not inhibited by malonate. The addition of fumarate to malonate-poisoned liver is likewise without effect.
3. The reactions by which pyruvate is removed when present in high concentrations (0.02–0.04 *M*) result in an accumulation of α -ketoglutarate. In the presence of malonate, acetoacetate and succinate accumulate in addition to α -ketoglutarate, and under these conditions the pyruvate utilized can be completely accounted for in terms of these three substances. The formation of α -ketoglutarate in liver does not require the addition of a C_4 -dicarboxylic acid as does the similar reaction in muscle.
4. Oxaloacetate and pyruvate react in liver, as in muscle, to form citrate and α -ketoglutarate. Added citrate is rapidly oxidized by liver. These facts indicate that the citric acid cycle can occur in pigeon liver.
5. The relative importance of the citric acid cycle and the formation of α -ketoglutarate from pyruvate may be assessed from the relative rates of the two reactions. α -Ketoglutarate arises from oxaloacetate and pyruvate at least 4–5 times as rapidly as from pyruvate alone, and it is probable that at low physiological concentrations, pyruvate is metabolized largely by way of the citric acid cycle. The direct synthesis of α -ketoglutarate is the main reaction of pyruvate removal only when the concentration of pyruvate is high, or when no oxaloacetate is available, e.g. in malonate-poisoned tissue where the citric acid cycle is interrupted.

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