164. BIOLOGICAL SYNTHESIS OF OXALOACETIC ACID FROM PYRUVIC ACID AND CARBON DIOXIDE

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(Received 30 September 1940)

It was recently shown that pigeon liver synthesizes α -ketoglutarate from added pyruvate [Evans, 1940]. Experiments which were undertaken to elucidate the mechanism of this synthesis suggest that the primary step is a synthesis of oxaloacetate from pyruvate and carbon dioxide:

The oxaloacetate formed in this way is converted into citrate and α -ketoglutarate through the citric acid cycle, as previously described:

The net effect of 1, 2 and 3 is a synthesis of α -ketoglutarate (and malate) from pyruvate:

Experiments on tissues of vitamin B₁-deficient pigeons suggest that reaction 1 is the stage at which the vitamin enters the metabolism of pyruvate in animal tissues. This result removes one of the difficulties confronting the theory of the vitamin effect. From Peters' work it was known that the oxidation of pyruvate in animal tissues depends on the presence of vitamin B₁. In yeast the vitamin is required for the non-oxidative decarboxylation of pyruvate. It has been difficult to understand how one and the same catalyst could catalyse an oxidation in one cell and a non-oxidative process in another. It now appears that the vitamin is not directly concerned with the oxidation of pyruvate in animal tissues, but with a reaction preparatory to the oxidation, the "carboxylation" of pyruvate. This reaction is analogous to the decarboxylation of pyruvate in yeast (as can be demonstrated in model experiments) and there is no theoretical difficulty in attributing these two analogous reactions to the same catalyst.

The occurrence of reaction 1 has also been suggested by recent observations in the field of bacterial metabolism. Wood & Werkman [1938; 1940, 1], studying the metabolism of propionic acid bacteria, discovered that these organisms utilize inorganic CO₂, and form succinate in quantities equivalent to the CO₂ used. Elsdon [1938] showed that the formation of succinate in *B. coli* depends on the concentration of CO₂. Commenting on their observations, Wood & Werkman

¹ "Malate" stands for the equilibrium mixture of fumarate and l(-)malate.

wrote [1938] "Pyruvic acid... may be the point of entry for CO_2 " and later [1940, 1]: "A possible mechanism accounting for CO_2 utilization as well as succinic acid formation involves the addition of CO_2 to pyruvic acid to form oxaloacetic acid, followed... by the reduction to succinic acid." Experiments on *B. coli*, reported in this paper, fully support this hypothesis and the inference is that the synthesis of oxaloacetate from pyruvate and CO_2 is a general metabolic process which occurs in widely different organisms.

I. Experiments on pigeon liver

1. *Methods.* Pigeon liver was minced and suspended in saline media as described by Evans [1940] and Krebs & Eggleston [1940]. The suspensions were incubated under varying conditions at 40° . The following media were used:

(1) "Phosphate saline, pH 7.4", prepared as described by Krebs & Eggleston [1940].

(2) "Phosphate saline, pH 7.1", prepared in the same way as medium (1), except that the phosphate buffer used was made by diluting 33.3 ml. *N* HCl and 17.8 g. $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ to 1 l.

(3) "Phosphate-bicarbonate-saline, pH 7.4", prepared by addition of 20 ml. 1.3 % NaHCO_3 to 100 ml. medium (1); pH is approximately 7.4 when the medium is in equilibrium with a gas mixture containing 5 % CO_2 .

(4) "Phosphate-bicarbonate-saline, pH 7.1", prepared by addition of 10 ml. 1.3 % NaHCO_3 to 100 ml. medium (2); pH is approximately 7.1 when the medium is in equilibrium with a gas mixture containing 5 % CO_2 .

Since the tissue contains and produces considerable amounts of acid substances, pH of the tissue suspension is always lower than that of the medium used. The metabolic reactions under discussion are however little affected by a change of acidity between pH 6.8 and 7.4 and no attention was therefore paid to the accurate adjustment or determination of pH.

The analytical methods were the same as in the previous papers from this laboratory [see Krebs & Eggleston, 1940; Krebs, 1940; Krebs, Smyth & Evans, 1940; Krebs, Eggleston, Kleinzeller & Smyth, 1940].

2. *Effect of CO_2 on pyruvate utilization.* Two suspensions of the same liver were prepared, one in phosphate saline, and the other in phosphate-bicarbonate-saline of the same pH. Of each suspension 20 ml. were poured into a 50 ml. measuring cylinder and placed in a water bath at 40° . O_2 was passed at a rapid rate through the first suspension, O_2 with 5 % CO_2 through the second. The rate of gas flow was approximately 100 ml. per min. At the end of the incubation period 4 ml. 2*N* HCl were added to the suspensions whilst they were still in the water bath. After about 2 min. they were removed from the bath and pyruvate, citrate, α -ketoglutarate, succinate and fumarate + malate were determined in aliquots of the suspension. The results are shown in Table 1.

In the presence of bicarbonate and CO_2 the rate of pyruvate utilization is 37–94 % above that of the controls. There is also a large increase in the formation of α -ketoglutarate (115–170 %) of citrate (160–560 %) and of fumarate + malate (125–400 %).

These effects of CO_2 and bicarbonate, taken alone, are by no means definite proof of the participation of these substances in the chemical processes of the tissue. To explain the effects, it might be argued that CO_2 and bicarbonate are essential constituents of a "balanced medium" and that their action is similar to that of the other inorganic substances. Against this argument it can be pointed out that unlike the other inorganic substances, CO_2 and bicarbonate

Table 1. *Effect of CO₂ pressure on metabolism of pyruvate in pigeon liver*

All data refer to 10 ml. suspension; the quantities of metabolites are expressed in μ l.

Exp. no.	...	...	1		2		3	
Suspension used:			1 part liver + 13 parts medium		1 part liver + 13 parts medium		1 part liver + 9 parts medium	
Medium	...	...	Phosphate bicarbonate saline pH 7.1		Phosphate bicarbonate saline pH 7.1		Phosphate bicarbonate saline pH 7.4	
Gas	...	...	O ₂	5% CO ₂ in O ₂	O ₂	5% CO ₂ in O ₂	O ₂	5% CO ₂ in O ₂
Period of incubation (min.)			30	30	45	45	30	30
Initial pyruvate			9220	9220	8600	8600	8940	8940
Final pyruvate			7100	6320	6250	4050	7230	5660
Pyruvate used			2120	2900	2350	4550	1710	3280
Succinate formed			26	72	45	85	35	52
α -Ketoglutarate formed			277	680	435	938	285	763
Fumarate + l(-)malate formed			269	922	191	426	44	217
Citrate formed			46	120	46	276	40	263

have no general effect on metabolic processes in minced liver. As far as is known their effects on pyruvate utilization are specific; a specific chemical hypothesis, such as reaction 1, offers therefore a more satisfactory explanation.

3. *Anaerobic formation of α -ketoglutarate, citrate, fumarate and malate from pyruvate.* It was already mentioned by Evans [1940] that α -ketoglutarate can be formed from pyruvate under anaerobic conditions. In Evans's experiments phosphate saline was used as a medium and under these conditions the formation of α -ketoglutarate varied in magnitude and did not regularly occur. We find that the formation of α -ketoglutarate is larger and more consistent when "phosphate-bicarbonate-saline" is used as a medium, and that fumarate and malate are formed in considerable quantities together with α -ketoglutarate. Small amounts of citrate and of succinate are also formed (Table 2).

Table 2. *Formation of succinate, α -ketoglutarate, fumarate + malate and citrate from pyruvate under anaerobic conditions*

40°; 5% CO₂ in N₂; period of incubation 60 min.

	Exp. 1		Exp. 2	
Suspension used	1 part liver, 8 parts phosphate-bicarbonate saline, pH 7.4		1 part liver, 9 parts phosphate-bicarbonate saline, pH 7.4	
Quantity of suspension	3 ml.		4 ml.	
Substrate	(Control)	0.02 M pyruvate	(Control)	0.1 M pyruvate
μ l. Pyruvate used	—	1157	—	890
μ l. Succinate formed	0	52	20	45
μ l. α -Ketoglutarate	12	142	60	131
μ l. Fumarate + malate	26	216	100	334
μ l. Citrate	—	—	6	12

The following facts are relevant with regard to the explanation of the simultaneous formation of citrate, α -ketoglutarate, succinate, fumarate and malate:

(a) The substances enumerated are interconvertible, through the citric acid cycle, if the conditions are aerobic, but α -ketoglutarate and malate are not interconvertible if the conditions are anaerobic. Citrate can anaerobically be converted into α -ketoglutarate only if oxaloacetate is present [Krebs, 1940]. Succinate can arise anaerobically from both α -ketoglutarate and fumarate.

(b) Anaerobically oxaloacetate yields citrate, α -ketoglutarate, succinate, fumarate and malate [Krebs, Eggleston, Kleinzeller & Smyth, 1940], i.e. the same substances which are formed from pyruvate in the presence of CO_2 and bicarbonate (Table 2). The proportions in which the substances appear are similar with oxaloacetate and with pyruvate; the rate of reaction is more rapid with oxaloacetate.

The results recorded in Table 2 may be explained in two different ways. Either it may be assumed that pyruvate is converted into oxaloacetate which in turn reacts to form the substances found as end-products. Or it may be assumed that there are three different and independent reactions leading from pyruvate: (1) to citrate, (2) to α -ketoglutarate, (3) to fumarate and malate. This second alternative is improbable and unsupported by experimental evidence. The first explanation, on the other hand, is supported by independent evidence.

4. *Aerobic formation of citrate, α -ketoglutarate, fumarate and malate from pyruvate.* Since citrate, α -ketoglutarate, fumarate and malate are aerobically interconvertible, as was pointed out in the previous paragraph, the simultaneous accumulation of all the substances is not unexpected when O_2 is present. Yet it can be shown by quantitative determination of all the reactants, including O_2 , that under certain conditions the end-products cannot be formed by oxidative interconversion through the citric acid cycle. The O_2 uptake is too small to account for this mechanism. This holds if the pyruvate concentration is high (0.1M) and the experimental period short (20 min.). Under such conditions reactions different from those forming the citric acid cycle prevail.

An experiment is shown in Table 3. Ten conical manometer cups were used, 5 of which had two side arms. The first 5 cups contained 3 ml. liver suspension in the main compartment and 0.5 ml. 2N HCl in the side arm. The remaining 5 cups had, in addition, 1 ml. 0.4M pyruvate in the second side arm. The gas space was filled with N_2 . The cups were shaken at 40° and after 10 min. the acid

Table 3. *Formation of citrate, α -ketoglutarate, succinate, fumarate and malate, and acetoacetate, from pyruvate under aerobic conditions*

Suspension: 1 part liver, 6.5 parts phosphate bicarbonate saline pH 7.4; 2.5% CO_2 in O_2 . The pigeon was starved for 48 hr. The data refer to 3 ml. suspension.

	Zero value $\mu\text{l.}$	After 20 min. incubation $\mu\text{l.}$	Change $\mu\text{l.}$
Pyruvate	8880	7150	- 1630
Citrate*	0	135	+ 135
α -Ketoglutarate	87	376	+ 289
Succinate	0	73	+ 73
Fumarate + malate	134	351	+ 217
Acetoacetate	7	52	+ 45
O_2 used	—	760	- 760
CO_2 formed (total)	—	877	+ 877

* Including isocitrate and *cis*-aconitate.

was added to the tissue in the first 5 cups to stop the metabolism. The determination in these cups provided the zero values. At the same time the pyruvate was added to the liver suspension in the other cups and shaking was continued for 20 min. The acid was then added from the second side arm. The contents of separate cups were used for the determination of succinate, α -ketoglutarate, fumarate + malate and of acetoacetate. The fifth cup was used for the determination of pyruvate and of citrate. In a parallel experiment the O_2 uptake and the CO_2 output were measured by the Dickens-Simer [1931] method. Since in this method no substrate can be mixed with the tissue after the closing of the manometer, pyruvate had to be added before the manometer was placed in the bath. Thus the period of measurement, beginning 15 min. later, did not exactly coincide with that of the first 10 cups. There are, however, good reasons for assuming that the rate of O_2 uptake is linear and that it is justifiable to extrapolate accordingly for the period preceding the measurements.

With the exception of acetoacetate the products found in this experiment are the same as those formed under anaerobic conditions. The quantities, however, are considerably larger, especially in the case of citrate. Moreover, these larger quantities are formed in a much shorter period of incubation (20 min., against 60 min. in the aerobic experiments) and it follows that the rates of reaction are much higher in the presence of O_2 .

Before discussing the interpretation of these results it is necessary to consider the assumptions which may be made to explain the utilization of pyruvate. If it is assumed that a condensation of 2 molecules of pyruvate of the type discussed by Toeniessen & Brinkmann [1930; see also Krebs, 1936] occurs, the formation of 1 molecule of α -ketoglutarate requires 2 molecules of pyruvate and 1 molecule of O_2 . If succinate, fumarate, malate and citrate arise from α -ketoglutarate by way of the citric acid cycle, the relations between the products formed and pyruvate and O_2 usages are as shown in the following list:

Substance formed from pyruvate	Molecules of pyruvate required	Molecules of O_2 required
α -Ketoglutarate	2	1
Succinate	2	$1\frac{1}{2}$
Fumarate + malate	2	2
Citrate	3	3
Acetoacetate	2	1

This hypothesis, applied to the experiment of Table 3, would postulate an O_2 uptake of 1283 μ l., but only 760 μ l. were absorbed. The hypothesis must therefore be discarded. Other schemes involving the primary formation of succinate from acetate [see Weil-Malherbe, 1937] are still less satisfactory. An alternative explanation which is in accordance with the facts is offered by reactions 1, 2 and 3. According to this scheme no O_2 is required for the formation of citrate, α -ketoglutarate and fumarate and the anaerobic formation of these substances (Table 2) demonstrates that O_2 is in fact not essential. If O_2 increases the rate of reaction—as it does—its effect on reaction 1 must be indirect, for instance through the supply of energy. Reaction 1 being endothermic requires energy; the supply of energy is comparatively small under anaerobic conditions and is increased when O_2 is available.

5. *Utilization of CO_2 .* According to 4, CO_2 disappears when reactions 1, 2 and 3 occur. It has not been possible, however, to demonstrate the disappearance of CO_2 in the presence of pyruvate and this is due to the fact that 4 is not the only reaction occurring under the conditions of the experiments. At least two other

reactions in which CO_2 is formed take place, viz. the formation of acetoacetic acid and the dismutation of pyruvate:

The disappearance of CO_2 in 4 is counterbalanced by the production of CO_2 in 5 and 6. At present there seems to be little prospect of separating 4 from other reactions; perhaps it will be possible to demonstrate the utilization of CO_2 conclusively by the use of carbon isotopes.¹

6. *The role of vitamin B₁*. It is known from the work of Peters and his school [Peters & Sinclair, 1933; Peters & Thompson, 1934; Thompson, 1934; Peters, 1936, 1, 2; McGowan, 1937; McGowan & Peters, 1937; Peters, 1937; Ochoa & Peters, 1938; Banga *et al.* 1939; Long & Peters, 1939; Peters, 1940, 1, 2] that vitamin B₁ is concerned with the utilization of pyruvate in pigeon brain and in pigeon kidney. The nature of the chemical reaction catalysed by the vitamin in animal tissues has been much discussed [see Peters and his school; Lipmann, 1937, 1, 2; Quastel & Webley, 1939; Weil-Malherbe, 1940], but the problem must still be regarded as unsolved. On the other hand, the reaction effected by the vitamin in yeast is known [Lohmann & Schuster, 1937]: the vitamin, in the form of its pyrophosphate ester, catalyses the conversion of pyruvic acid into acetaldehyde:

This reaction does not occur in animal tissues. Hitherto no reaction which can be regarded as analogous to 7 was known to take place in animal tissues and the vitamin effects in yeast and in animal tissues therefore appeared to be due to two different mechanisms, one oxidative and the other non-oxidative. In view of the high degree of specificity generally shown by biological catalysts the assumption of such a dual function is somewhat unsatisfactory.

The new reaction 1 resembles 7 more closely than any other reaction known to occur in animal tissues, and in view of this it seemed probable to us that reaction 1 might be the reaction catalysed by the vitamin in animal tissues.

To test this hypothesis pigeons were fed on polished rice until the symptoms of severe beriberi appeared (head retraction, paralysis or weakness of legs and wings). The removal of pyruvate from liver suspensions was then measured under conditions in which reaction 1 is assumed to be the chief metabolic reaction responsible for the removal of pyruvate, i.e. at high concentrations of pyruvate and of CO_2 , and in the presence of O_2 . Further details of the conditions are given in Table 4.

Under the conditions of these experiments, the rate of pyruvate removal was about 2000–3000 $\mu\text{l. per hr.}$ (Q_{pyruvate} about -35) when normal pigeons were used. The rate was found to be greatly reduced in vitamin-deficient animals and the degree of the reduction was greater the severer the symptoms. In Exp. 1, where the symptoms were severest, the rate was reduced to 675 $\mu\text{l. per hr.}$, and in Exp. 2 where symptoms of deficiency were less pronounced, the rate was 1790 $\mu\text{l. per hr.}$ Addition of small quantities of vitamin restore the rate to normal level. The effect of the vitamin is very striking: in Exp. 1, 0.6 $\mu\text{g. per ml.}$ liver suspension increased the pyruvate usage from 675 to 2100 $\mu\text{l.}$, i.e. more than 300%. The quantities of vitamin required are similar to those in brain.

When the same experiment was carried out with muscle tissue of vitamin-deficient pigeons, no significant vitamin effect was observed (Table 5). Both

¹ See note at the end of this paper.

Table 4. *Effect of vitamin B₁ on pyruvate utilization in pigeon liver*

Minced pigeon liver suspended in 6.5 parts phosphate-bicarbonate saline, pH 7.4; 3 ml. of this suspension mixed with 1 ml. approx. 0.2 *M* pyruvate; 5% CO₂ in O₂; period of incubation 60 min.; duplicates in several experiments.

No.	State of pigeon	Quantity of aneurin (Roche Products) added per ml. suspension μ g.	μ l. initial pyruvate in suspension (4 ml.)	μ l. final pyruvate	μ l. pyruvate utilized	Increase due to vitamin (average) %
1	Fed 22 days on polished rice. Paralysis of legs and wings, head retraction	0	4360	3690	670	—
		0	4360	3680	680	
		0.625	4360	2250	2110	211
		0.625	4360	2270	2090	
		6.25	4360	2365	1995	178
		6.25	4360	2430	1930	
		62.5	4360	2285	1975	198
		62.5	4360	2315	2045	
2	Fed 25 days on polished rice, weakness of legs and wings, no head retraction	0	4440	2650	1790	
		0.2	4440	2370	2070	16
		1.0	4440	2065	2375	33
3	Fed 24 days on polished rice. Severe paralysis of wings and legs, no head retractions, no convulsions	0	4350	2865	1485	
		0	4350	3210	1140	
		0.8	4350	1620	2730	99
		0.8	4350	1865	2475	
4	Fed 35 days on polished rice. Severe paralysis of legs and wings, no head retraction, no convulsions	0	4300	3255	1045	164
		0.8	4300	1540	2760	
5	Normal, well fed	0	4440	1955	2395	
6	Normal (starved 48 hr.)	0	4440	2685	1755	
		20	4440	2780	1660	
7	Normal, well fed	0	4440	980	3460	
8	Normal, well fed	0	4500	830	3670	

Table 5. *Effect of vitamin B₁ in muscle and liver of the same pigeon*

Experimental conditions as stated in Table 4

Tissue used	Quantity of vitamin added (μ g. to 4 ml.)	μ l. pyruvate used
Breast muscle	0	440
"	0.8	470
Liver	0	1540
"	0.8	2760

liver and muscle can metabolize pyruvate through the citric acid cycle [Krebs & Eggleston, 1940; Evans, 1940], and if the vitamin played a part in one of the reactions of this cycle an effect would be expected in both tissues. The absence of the effect from muscle suggests that the vitamin takes part in a reaction of pyruvate which occurs in liver, but is of no major importance in muscle. So far two reactions to which this applies are known: reaction 1 and the synthesis of ketone bodies (reaction 5), and since there is no reason to assume that other reactions are of major importance for pyruvate removal in pigeon liver, we come to the conclusion that one of the reactions 1 and 5—or both—require the vitamin.

To examine this question experimentally, the effect of the vitamin on acetoacetate formation was measured in vitamin-deficient liver (Table 6). The acetoacetate formation was found to be somewhat reduced in the B₁-deficient

Table 6. *Effect of vitamin B₁ on the acetoacetate production in pigeon liver*

Experimental conditions as stated in Table 4; acetoacetate was determined according to Edson [1935].

No.	Condition of pigeon	Quantity of vitamin added	μ l. pyruvate added	μ l. pyruvate used	μ l. acetoacetate found
		μ g. per ml. suspension			
1	Fed 24 days on rice. Severe paralysis of legs and wings	0	4350	1140	63
		0.8	4350	2475	224
2	Fed 35 days on rice. Severe paralysis of legs and wings	0	4300	1045	27
		0.8	4300	2760	58
3	Normal, well fed	0	4500	3670	210
		25	4500	3520	246
4	"	0	4440	3460	87
5	"	0	4440	2395	128

animals (average 45 μ l. against 142 μ l. in normal liver). Addition of the vitamin restored the acetoacetate formation to the normal level. Compared with the absolute increase in pyruvate usage, however, the increase in acetoacetate formation is small. In Exp. 1 the acetoacetate formation accounts for 24 %, in Exp. 2 for only 3.6 % of the pyruvate usage caused by the vitamin (calculated on the basis of scheme 5).

It might be concluded from these experiments that the vitamin takes part in both the synthesis of oxaloacetate and the synthesis of acetoacetic acid and such a hypothesis is not unattractive considering that both reactions are probably analogous in their mechanism: the most likely mechanism of acetoacetate formation involves the synthesis of acetopyruvate [Krebs & Johnson, 1937]:

The analogy with reaction 1 is obvious if the latter is written in the following form:

However, the effect of the vitamin on acetoacetate formation, as shown in Table 6, is not necessarily a direct one. The synthesis of ketone bodies may be linked up with other processes and indirectly dependent on reaction 1. It must therefore be left open whether the vitamin takes part in reaction 1 only, or in both 1 and 8.

The conclusions reached here are in accordance with the view of McGowan & Peters [1937] that in animal tissues vitamin B₁ is concerned specifically with pyruvic acid, and not with α -ketonic acids in general. They also agree with the statement of Sober *et al.* [1940] that the vitamin "is essential for the synthesis of endogenous citric acid from its precursors", since reaction 1 is an essential step in the synthesis of citric acid.

The new conception has the great advantage of offering analogous explanations for the actions of the vitamin in yeast and in animal tissues. In yeast, its active form catalyses the "decarboxylation" of pyruvate (reaction 7); in

animal tissues it catalyses the "carboxylation" of pyruvate (reaction 1). The close analogy of the two reactions can be strikingly demonstrated by a simple model experiment: aniline is well known to catalyse the decarboxylation of oxaloacetate (i.e. the reversal of reaction 1); we find that the same catalyst can decarboxylate pyruvate according to reaction 7. The rate of the second reaction is very low when the pyruvate concentration is below 0.01 *M* and the reaction was therefore previously overlooked. The rate is rapid at high concentrations of pyruvate as shown in Table 7. Thus one and the same catalyst, aniline, can decarboxylate α -ketonic acids as well as β -ketonic acids. The fact that reaction 1 is reversed in the model hardly detracts from its instructive value.

Table 7. *Decarboxylation of pyruvic acid in the presence of aniline*

25°. 4 ml. pyruvic acid solution, 0.5 ml. 50% citric acid, 0.5 ml. aniline citrate [see Edson, 1935].

Final con- centration of pyruvic acid (<i>M</i>)	μ l. CO ₂ formed		
	10 min.	20 min.	30 min.
0.8	136	219	301
0.2	40	52	63
0.05	10	12	13
0.0125	0	0	0

II. *Synthesis of oxaloacetate in bacteria*

The recent literature on bacterial metabolism contains a number of observations which suggest that reaction 1 may also occur in propionic acid bacteria, *Bacterium coli commune*, and other bacteria.

Wood & Werkman showed that propionic acid bacteria utilize CO₂ and produce succinic acid in quantities equivalent to the amount of CO₂ used. This equivalence can be interpreted in two ways. In their first paper [1936] Wood & Werkman [see also Krebs, 1937] suggested that CO₂ might act as a hydrogen acceptor in one of the reactions leading to the formation of succinate. The equivalence would be accounted for, if the following reaction took place:

Later Wood & Werkman [1940, 1] discussed a mechanism involving reaction 1 and a reduction of oxaloacetate to succinate:

The experiments reported so far in the literature do not decide between these two alternatives.

For *B. coli* Elsdon [1938] showed that the formation of succinate from pyruvate depends on the pressure of CO₂. This observation, again, can be explained by scheme 9 or scheme 10.

Studying the succinate formation from pyruvate in *B. coli* we have made a new observation which supports scheme 10. It was found that, together with succinate, fumarate and malate arise from pyruvate (Table 8). Since added succinate is not converted into fumarate under the conditions of the experiments (which were anaerobic), fumarate and malate cannot be formed through succinate and scheme 9 is thus ruled out as far as the formation of fumarate and malate is concerned. Although (theoretically) the formation of succinate through scheme 9 is not impossible, this scheme is now superfluous and we think it

Table 8. *Formation of succinate, fumarate and malate from pyruvate in B. coli*

15–20 mg. washed cells (dry weight) grown on broth agar [Krebs, 1937] with 2% glycerol, suspended in 4 ml. saline containing 0.05–0.1 M NaHCO₃; gas phase, CO₂; 40°. The CO₂ evolved comprises CO₂ liberated by acid from NaHCO₃ and CO₂ formed from pyruvate. The CO₂ output gives an indication of the extent to which the added pyruvate has been decomposed. One mol. of pyruvate yields approximately one mol. of CO₂.

Strain of <i>B. coli</i> *	μl. pyruvate added	Period of incubation (min.)	μl. CO ₂ evolved	μl. succinate	μl. fumarate + malate†
No. 3	4480	260	3500	398	650
No. 4705	4480	250	4720	350	575
No. 4705	2240	140	2210	187	870
No. 4833	2240	230	2250	127	325
No. 4854	2240	370	2195	158	86

* The strains were isolated from faeces and urine by Dr M. S. Spink and Dr D. Lewis in the Department of Bacteriology of this University.

† The ratio of fumarate/malate in the equilibrium mixture was found to be the same as in animal tissues [see Krebs, Smyth & Evans, 1940].

should therefore be discarded. For it seems highly improbable to us that there are in one cell two different mechanisms leading to the formation of a 4-carbon chain from pyruvate.

Scheme 10 postulates that oxaloacetate and fumarate are converted into succinate at least as rapidly as pyruvate. Data showing that this is the case have already been presented [Krebs, 1937].¹

DISCUSSION

1. *Appraisal of the evidence for reaction 1.* The evidence for reaction 1 is necessarily indirect, since the secondary reaction of oxaloacetate makes it impossible to demonstrate directly the disappearance of CO₂ and the formation of oxaloacetate. In animal tissues the secondary reactions are known to yield fumarate, malate, α-ketoglutarate, citrate, succinate and CO₂ [Krebs, Eggleston, Kleinzeller & Smyth, 1940] and these substances are therefore expected to be formed instead of oxaloacetate. This is the case, and since the proportions in which the various substances appear are of the expected order, the evidence for the synthesis of oxaloacetate may be regarded as satisfactory.

In *B. coli* the secondary reactions of oxaloacetate are somewhat different in detail though the underlying principle is the same. The common principle is the reduction of one part of the oxaloacetate at the expense of the oxidation of another part. The difference is in the nature of the oxidative and reductive reactions. In animal tissues the reduction leads mainly to malate and fumarate. In *B. coli* it proceeds further to the stage of succinate. The oxidative reactions in animal tissues are those formulated by the citric acid cycle. There is no citric acid cycle in *B. coli* and the details of the oxidative processes in this organism are not yet clear [see Krebs, 1937]. The main end-product of the oxidation is CO₂. Thus in the case of *B. coli* reaction 1 will result in an accumulation of succinate; conversely, the synthesis of succinate can be taken as evidence for reaction 1.

The failure to show directly the disappearance of CO₂ in animal tissues and in *B. coli* [see Krebs, 1937] is satisfactorily explained. On the other hand the

¹ Experiments on propionic acid bacteria supporting scheme 10 will be reported in a separate paper.

effect of CO_2 on pyruvate usage and on the formation of α -ketoglutarate, succinate and related compounds is direct evidence for the participation of CO_2 in the synthesis of these substances.

Although no single experiment represents conclusive evidence for reaction 1, we think that the force of the cumulative evidence leaves little doubt about the occurrence of the reaction.

2. *Analogous reactions in vitro.* It is of interest to note that the reaction of Grignard compounds with CO_2 is analogous to reaction 1, e.g.:

In both cases CO_2 is added to the carbon chain and a carboxylic acid is formed. It may not be fortuitous that reaction 1, like the reaction of the Grignard compounds, depends on the presence of magnesium.

3. *Occurrence of reaction 1 in other tissues and organisms.* Reaction 1 appears to be of minor importance in skeletal and cardiac muscle, but it is very probable that it plays an important part in brain and in several other tissues. This remains to be investigated. It is also highly probable that the reaction occurs in those plants, moulds and bacteria [see Wood & Werkman, 1940, 2] in which oxaloacetate [see Virtanen & Laine, 1938], malate, succinate or citrate are formed.

4. *The role of vitamin B₁ in bacteria.* In *Staphylococci* and *Lactobacillus delbrueckii* vitamin B₁ has been shown to catalyse the oxidative decarboxylation of pyruvate [Hills, 1938; Barron & Lyman, 1939; Lipmann, 1937, 2].

In *Streptococcus haemolyticus* it appears to take part in the following reaction [Barron & Lyman, 1939].

On first impressions it may be thought that the mechanism of the vitamin effect in these cases must be different from that proposed earlier in this paper. This is, however, not necessarily the case. There are several observations which suggest that oxaloacetate acts as a hydrogen carrier in the oxidation of pyruvate:

If this is true reaction 1 must occur in these organisms and the vitamin effect can be explained on the same basis as in animal tissues. Scheme 11 implies that the Szent-Györgyi cycle occurs in bacteria. Evidence in favour of this will be discussed in a subsequent paper.

5. *The mechanism of the biological succinic acid formation.* It is generally accepted that succinic acid can be synthesized by animal tissues, bacteria and moulds from compounds containing less than 4 carbon atoms. The mechanism of this synthesis has been discussed by many authors. The earliest hypothesis [Thunberg, 1920] suggested that the synthesis was brought about by condensation, coupled with dehydrogenation, of 2 molecules of acetic acid:

Experiments designed to demonstrate a synthesis of succinate from added acetate failed and at the same time made it clear that pyruvate is a precursor

of succinate in animal tissues and in bacteria [Toeniessen & Brinkmann, 1930; Krebs & Johnson, 1937; Krebs, 1937; Weil-Malherbe, 1937; Elsdén, 1938]. The above scheme was therefore modified. Toeniessen & Brinkmann [1930] assumed that 2 molecules of pyruvate condense to form $\alpha\alpha'$ -diketo-adipic acid which in turn was believed to form succinate and 2 molecules of formate:

Krebs [1936] suggested that pyruvate and acetate may condense to form α -keto-glutarate and Weil-Malherbe [1937] thought that acetate may react according to (12) in the nascent state. These attempts to explain the fact that pyruvate can be converted into succinate were not supported by direct experimental evidence. It is now very probable that they are incorrect and that reaction (1) is the primary step by which the 4 carbon chain is synthesized from a shorter chain.

It is significant that the succinate formation from hexoses in *B. coli* also depends on the CO_2 pressure [Elsdén, 1938]. We regard this as evidence in favour of the view that reaction 1 is a stage in the succinate formation not only from pyruvate but also from hexoses. This hypothesis postulates that the succinate formation from pyruvate, oxaloacetate, malate and fumarate should be at least as rapid as the succinate formation from the other succinate-forming substrates. Data in the papers of Elsdén [1938] and Krebs [1937] fulfil this postulate.

Whilst it is by no means impossible that there is a second mechanism of succinate synthesis from carbohydrate in animal tissues or in *B. coli*, there is nothing, as far as we can see, to be said in favour of such an alternative. The facts can all be explained by the mechanism proposed in this paper.

SUMMARY

1. Suspensions of minced pigeon liver form the same substances from added pyruvate and from added oxaloacetate, viz. α -ketoglutarate, fumarate, malate, succinate and citrate. Various explanations are discussed. The only satisfactory explanation is the assumption that a formation of oxaloacetate from pyruvate and CO_2 (reaction 1) takes place.

2. The rate of pyruvate usage in pigeon liver depends on the concentrations of CO_2 and bicarbonate. This supports the view that CO_2 takes part in pyruvate removal as an active metabolite, as indicated by reaction 1.

3. In vitamin B_1 -deficient pigeons the rate of pyruvate usage of liver suspension is greatly reduced. The normal rate is restored by the addition of pure vitamin B_1 . Under the same conditions pyruvate usage in muscle suspensions, i.e. pyruvate usage through the citric acid cycle, is not increased by the vitamin. It follows that the vitamin takes part in a reaction present in liver, but absent from muscle, and by the method of elimination the conclusion is reached that the vitamin catalyses reaction 1, the "carboxylation" of pyruvate. It is not excluded that in addition the vitamin takes part in the synthesis of acetoacetate from pyruvate (see § I, 6).

4. In *B. coli* fumarate and malate are formed (together with succinate) when pyruvate is anaerobically fermented. This supports the view, previously expressed by Wood & Werkman, that reaction 1 occurs in bacteria. The CO_2 fixation and the equivalence between CO_2 fixation and succinate formation in

propionic acid bacteria (Wood & Werkman) and the effect of CO_2 on the succinate formation in *B. coli* (Elsden) are satisfactorily explained by this assumption.

This work was aided by grants from the Rockefeller Foundation and the Medical Research Council.

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Note added on 31 October 1940. With the help of radio-active carbon (C_{11}) Ruben & Kamen (*Proc. Nat. Acad. Sciences*, **26**, 418, 1940) have recently succeeded in demonstrating the "assimilation" of CO_2 by rat liver and other heterotrophic cells. Attempts to identify the organic molecules containing the radio-active carbon atoms were unsuccessful. It is to be expected that active carbon will be found in the organic acids formed in the course of the citric acid cycle.