N, 3.0; S, 6.8. $[C_{21}H_{33}O_4SO_4]^- [C_4H_{12}N_2]^{++}$, $2H_2O$ requires: C, 60.1; H, 9.0; N, 3.1; S, 7.1. $[C_{21}H_{33}O_4SO_4]^- [C_4H_{12}N_2]$ requires: C, 62.6; H, 8.9; N, 3.2; S, 7.3%. For comparison with piperazine X sulphate, see Table 1.

SUMMARY

*allo*Pregnan-3(β)-ol-20-one sulphate has been isolated from pregnant mares' urine and identified as its piperazine salt.

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Carbon Dioxide Fixation in Animal Tissues

By J. R. STERN (Fellow in the Medical Sciences, National Research Council, Washington),
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
University of Sheffield

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It is now firmly established that carbon dioxide can be incorporated into organic compounds by animal tissues. Two primary reactions are known, viz.

- (1) Pyruvate + $CO_2 \rightarrow$ oxaloacetate (Wood & Werkman, 1940).
- (2) α -Ketoglutarate + $CO_2 \rightarrow$ oxalosuccinate (Ochoa, 1945).

Previous work on CO_2 fixation was concerned mainly with establishing its existence and studying the enzymic mechanisms involved; information on the rates of reactions (1) and (2) in animal tissues has remained scanty. The work reported in this paper was undertaken in order to collect data on the rates of these two reactions in various animal tissues and to assess their quantitative significance in metabolism.

METHODS

Owing to the instability and reactivity of the initial products the measurement of the rates of reactions (1) and (2) offers great experimental difficulties. Direct determination of oxaloacetate or of oxalosuccinate would not indicate the extent of reactions (1) or (2) because these compounds

undergo rapid secondary changes. Oxaloacetate yields fumarate, malate, succinate, citrate and α -ketoglutarate; oxalosuccinate yields isocitrate, cisaconitate and citrate. These substances can arise under aerobic as well as anaerobic conditions.

Reaction (1) was examined in two ways. First, the sum of α -ketoglutarate and succinate formed on anaerobic incubation with pyruvate and CO_2 was determined. Data published by Krebs, Eggleston, Kleinzeller & Smyth (1940), on the anaerobic products formed from oxaloacetate in various tissues, showed that the sum of succinate plus α -ketoglutarate formed was fairly constant, amounting to 15–25% of the oxaloacetate utilized. The other products showed much greater variation. The sum of succinate plus α -ketoglutarate formed, therefore, may be taken to represent approximately one fifth of the CO_2 fixed by reaction (1).

A second indirect method of studying the rate of reaction (1), applicable in O_2 only, is based on the fact that the oxidation of succinate is largely prevented by the addition of malonate. The interruption of the tricarboxylic acid cycle at the stage of succinate causes the bulk of the di- and tricarboxylic acids to accumulate as succinate provided the rate of respiration is somewhat greater than that of the carboxylation of pyruvate. The amount of succinate found

on addition of malonate alone is thus an indicator of the total di- and tri-carboxylic acids in the tissue, and the increase found on addition of pyruvate may be ascribed to carboxylation.

For the study of reaction (2) it was decided to incubate tissues anaerobically with α -ketoglutarate and to measure the formation of citrate. If one assumes that all the oxalosuccinate formed through reaction (2) is reduced to isocitrate, then 89% of it would appear as citrate in the presence of aconitase. As it is possible that secondary reactions of citrate may occur, the rate of (2) may be greater than the amount of citrate formed indicates. On the other hand, citrate can be formed under some conditions by other mechanisms, but these were probably quantitatively insignificant except when oxaloacetate was present. Anaerobic conditions were chosen in order to exclude the formation of citrate by the oxidative reactions of the tricarboxylic acid cycle.

EXPERIMENTAL

The experiments were carried out on minced or homogenized material. Pigeon, rat and guinea pig brains were homogenized in a stainless-steel homogenizer similar to the glass apparatus of Potter & Elvehjem (1936). Pigeon liver was minced in the Fischer apparatus (Fischer, 1925), other tissues in the Latapie mill. The minced tissue was suspended in saline medium in the proportion 1 part of tissue plus 6.5 parts of medium, unless otherwise stated. The following media were used:

(1) Phosphate saline pH 7.4 (Krebs & Eggleston, 1940a), with N_2 or O_2 .

(2) Phosphate-saline-bicarbonate, prepared by adding 16 ml. of 1.3% $NaHCO_3$ to 100 ml. of medium (1). Gas: 5% CO_2 in N_2 or O_2 . The final bicarbonate concentration was 0.0214M, and when equilibrated with the gas mixture the pH of this medium was approximately 7.4.

To prepare a boiled yeast-extract a suspension of 1 g. of dried baker's yeast in 10 ml. of water was placed in a boiling water bath for 10 min. and then filtered. Boiled muscle extract was similarly prepared from a mixture of 1 part of minced pigeon-breast muscle and 1 part of phosphate saline.

Tissue suspension (3 ml.) was pipetted into the main compartment of a Warburg cup. Further additions (substrates, inhibitors, buffer or water) amounting to 1 ml. were placed in the side arm, so that upon mixing the final suspension contained 1 part of tissue in 10 parts of suspension. In anaerobic experiments a stick of yellow phosphorus was placed in the centre well. The contents of the side arm were mixed with those of the main compartment immediately before placing the manometers in the water bath at 40°. At the end of the incubation period the manometers were removed from the bath, the cups detached, placed in iced water, and 1 ml. of 2N-HCl was added to the suspensions to stop the reaction.

For the determination of the sum of succinate and α -ketoglutarate, the acidified tissue suspension was deproteinized with tungstic acid. Succinate and the sum of succinate plus α -ketoglutarate were determined by the procedure of Krebs & Eggleston (1945) in the earlier experiments. Later, when it was realized that glutamate interfered, a modified method (Krebs & Eggleston, 1948) was used. Glutamic acid was determined according to Gale (1945). It represented the sum of glutamic acid plus glutamine. For

the determination of citrate the contents of the cup were washed with water into a 50 ml. measuring cylinder, diluted to 45 ml. and mixed with 5 ml. of 20% metaphosphoric acid. A measured part of the clear filtrate of this suspension was used for the citrate determinations. Citric acid was determined colorimetrically in the manner described by Krebs & Eggleston (1944) using a Spekker absorptiometer and a no. 6 filter.

For purposes of calculation the dry weight was taken to be 30% of the wet weight in the case of liver and 20% for other tissues.

RESULTS

Anaerobic formation of succinate and α -ketoglutarate in presence of pyruvate

Various tissues of the pigeon (liver, brain), rat (liver, kidney cortex, skeletal muscle), guinea pig (liver, brain), and sheep (liver, kidney cortex, heart, brain, spleen, pancreas and lung) were examined. Only in pigeon liver, which is already known to convert pyruvate into succinate and α -ketoglutarate, was an increased yield of these substances observed. As the experiments on other tissues gave essentially negative results they will only be summarily reported. In the first experiments all tissues appeared to contain initially some 'succinate plus α -ketoglutarate' (25–150 mg./100 g. wet wt., expressed as succinic acid). On anaerobic incubation without addition of pyruvate (40 min. at 40°), these dicarboxylic acids increased by 20–60 mg./100 g. in all tissues tested save rat muscle and sheep lung. Addition of pyruvate (0.02M) did not result in any further increase in these dicarboxylic acids, except in pigeon liver.

At this stage of the investigation it was realized that glutamic acid interfered with the determination of α -ketoglutarate and succinate by also yielding succinate on treatment with permanganate, and some of the above experiments were then repeated with the modified method avoiding this source of error (Krebs & Eggleston, 1948). With this method 400 mg. of fresh avian or mammalian tissue yielded no detectable amounts of succinate or α -ketoglutarate (i.e. less than 0.05 mg.) and Gale's (1945) method showed that they contained 0.4–1.3 mg. of glutamic acid. Further, on anaerobic incubation of mammalian tissues at 40° (pH 7.4; no substrate added; 40 min.) the amount of glutamic acid increased by 50% or more, possibly as a result of hydrolysis of proteins or peptides, whilst little or no succinic acid (< 0.05 mg.) and no α -ketoglutaric acid was formed. It must, therefore, be concluded that the succinic acid found in the earlier experiments had arisen from glutamic acid by oxidation with permanganate.

The above results do not contradict those of Moyle (1924) who found that succinic acid was present in muscle and increased on anaerobic incubation, for the amounts she reported would not be

measurable by the procedure employed in the present work.

In confirmation of previous work it was found that succinate and α -ketoglutarate were formed by pigeon liver on anaerobic incubation in the absence of pyruvate, and that addition of pyruvate (0.02–0.04M) increased the yields. In contrast to mammalian tissues, no change occurred in the initial glutamic acid concentration of pigeon liver on anaerobic incubation without pyruvate. In Table 1

Table 1. *Anaerobic synthesis of succinate plus α -ketoglutarate in pigeon liver*

(400 mg. fresh liver in 4 ml. suspension. $Q_{\text{succinate}}$ refers to the increase in the sum of succinate and α -ketoglutarate during incubation. The initial value of the sum was 76 $\mu\text{l./400 mg.}$)

Gas	Incubation time (min.)	$Q_{\text{succinate}}$	
		No pyruvate	0.04M pyruvate
5% CO_2 in N_2	20	2.1	3.8
	40	1.4	2.1
5% CO_2 in O_2	20	2.5	6.3
	40	1.8	5.0

the rates of formation of succinate plus α -ketoglutarate by pigeon liver are given in terms of the metabolic quotient Q ($\mu\text{l./mg. dry wt./hr.}$). These rates are comparable in magnitude to those reported earlier from this laboratory (Evans, 1940; Krebs & Eggleston, 1940b). On the assumption that anaerobically 20% of the total oxaloacetate formed by reaction (1) was converted to succinate plus α -ketoglutarate, the initial rate of reaction (1) was 19 in the presence of pyruvate and in N_2 . In all the other tissues examined, where no formation of succinate plus α -ketoglutarate was detectable on addition of pyruvate (i.e. less than 0.05 mg. was present in 400 mg. wet tissue after 40 min. incubation), the rate of reaction (1) in terms of $Q_{\text{oxaloacetate}}$ must have been below 1.

The malonate technique was applied to the same tissues. Addition of 0.02M-pyruvate to tissues suspended in a saline medium containing 0.01M-malonate did not increase the yield of succinate or of α -ketoglutarate beyond that obtained with malonate alone except in pigeon liver and sheep-kidney cortex. In the latter tissue $Q_{\text{succinate}}$ varied between 0 and 1.0 (average $Q = 0.6$ for five experiments) with malonate alone and was increased by addition of pyruvate to values between 0.6 and 3.0 (average $Q = 1.7$ for five experiments). In pigeon liver Evans (1940) found a $Q_{\text{succinate}}$ of 2.7 in the presence of 0.025M-malonate and of 8.7 with malonate and 0.03M-pyruvate. With the revised technique for determination of succinate and α -ketoglutarate, similar values were obtained. $Q_{\text{succinate}}^{\text{O}_2}$ was 4.7 with malonate and 7.4 with malonate and pyruvate (10 min. incubation). With regard to

the interpretation of these figures it is relevant that the aerobic conversion of oxaloacetate into succinate requires three oxygen atoms. As the Q_{O_2} of pigeon liver in the presence of pyruvate is about 18 the maximum value for $Q_{\text{succinate}}^{\text{O}_2}$ to be expected by aerobic reactions is about 12. Additional succinate and α -ketoglutarate, as already discussed, can be formed by anaerobic reactions, but it is clear that if the rate of carboxylation of pyruvate, in terms of $Q_{\text{oxaloacetate}}$, is about 12 or more, only part of the oxaloacetate will be converted into succinate. The yield of succinate in the malonate experiments is thus not incompatible with a $Q_{\text{oxaloacetate}}$ of 19 in pigeon liver, computed from other experiments.

Anaerobic formation of citrate in presence of α -ketoglutarate

(a) Pigeon liver.

1. *Citrate synthesis.* Table 2 shows the amounts of citrate formed by minced pigeon-liver suspensions upon anaerobic incubation with various substrates.

Table 2. *Anaerobic citrate synthesis from α -ketoglutarate and other substrates in pigeon liver*

(400 mg. fresh tissue in 4 ml. suspension. Incubation period 12 min. Phosphate-saline-bicarbonate (0.02M) medium; 5% CO_2 in N_2 .)

Exp.	Additions	Citrate formed ($\mu\text{l.}$)
1	None	~0
	0.04M- α -Ketoglutarate	91
2	None	~0
	0.04M- α -Ketoglutarate	92
	0.02M-Pyruvate	23
3	0.02M- α -Ketoglutarate	72
	0.02M-Oxaloacetate	124
	0.02M-Oxaloacetate,	206
	0.02M-pyruvate	

Pyruvate and oxaloacetate both alone and in combination yielded citrate, confirming the results of Krebs & Eggleston (1940b) and of Evans (1940). Amounts of citrate of similar magnitude were also formed on addition of α -ketoglutarate. In ten separate experiments Q_{citrate} varied from 2.4 to 4.1 (average 3.8) when the incubation period was 12 min. Other substrates such as L-lactate, L-glutamate, fumarate, succinate, acetoacetate and β -hydroxybutyrate (all 0.02M final concentration) did not form measurable amounts of citrate, i.e. less than 20 $\mu\text{l./120 mg. dry liver.}$

2. *Time course of citrate synthesis from α -ketoglutarate.* Table 3 demonstrates the time course of the citrate formation from added α -ketoglutarate. The initial rate was very rapid ($Q_{\text{citrate}} = 30$), but the reaction quickly came to a standstill; the maximum yield of citrate was obtained within 5 min.

Table 3. *Time course of citrate synthesis from α -ketoglutarate in pigeon liver*(0.02M- α -Ketoglutarate. Other conditions as in Table 2.)

Time (min.)	Citrate formed (μ l.)	Q_{citrate}
1	60	30
2	82	—
4	95	—
12	98	—

3. *Effect of α -ketoglutarate concentration.* The amount of citrate formed increased when the concentration of α -ketoglutarate was raised from 0.005 to 0.02M (Table 4). Increasing the concentration beyond 0.02M did not raise the yield of citrate.

Table 4. *Effect of α -ketoglutarate concentration on citrate synthesis in pigeon liver*

(Conditions as in Table 2.)

α -Ketoglutarate (M)	Citrate formed (μ l.)
0.005	33
0.01	57
0.02	92
0.04	94

Table 5. *Effect of bicarbonate (or carbon dioxide) concentration on citrate synthesis in pigeon liver*(Except for CO₂ and bicarbonate concentration, conditions as in Table 2.)

Gas (% CO ₂ in N ₂)	Bicarbonate (M)	pH (calc.)	Citrate formed with substrates (μ l.)	
			0.02M- α -Ketoglutarate	0.02M- α -Ketoglutarate + 0.02M-fumarate
0	0.0019	7.40	26	41
2.14	0.0105	7.42	37	113
4.52	0.0217	7.41	55	143
10	0.0591	7.45	87	162

4. *Effect of bicarbonate and carbon dioxide concentration.* In the following experiments the concentrations of bicarbonate and of carbon dioxide were varied in about the same proportions so that the pH remained approximately constant. Various amounts of 1.3% bicarbonate solution were added to a suspension of minced liver in phosphate-saline medium and the gas spaces of the vessels were filled with a corresponding mixture of carbon dioxide in nitrogen. The actual bicarbonate concentration of the final tissue suspension was determined experimentally in each instance on a duplicate sample. The amount of citrate formed rose as the concentration of bicarbonate increased from 0.002 to 0.06M (Table 5). The optimum concentration of bicarbonate appeared to be rather higher than 0.02M, the concentration employed routinely in other experiments because this is the approximate concentration of bicarbonate

in the blood plasma. Three experiments performed in phosphate-saline medium and in an atmosphere of nitrogen without carbon dioxide with alkali in the centre well to absorb carbon dioxide, yielded small, just measurable, amounts of citrate in each case ($30 \pm 5 \mu$ l.). It was impossible to exclude carbon dioxide rigorously under these conditions, because of its constant production from α -ketoglutarate by the tissue suspension.

5. *Effect of pH of medium.* The pH was varied by changing the carbon dioxide content of the gas mixtures. Thus, in addition to changes in pH, the concentration of free carbon dioxide varied, while that of bicarbonate remained constant. The data of Table 6 show that citrate formation increased when pH fell from 7.73 to 6.95. It was the same at pH 7.73 and 8.04.

6. *Effect of various substrates.* Representative effects of various substrates on the anaerobic citrate formation from α -ketoglutarate are shown in Table 7. Acetoacetate, β -hydroxybutyrate, and succinate (each in 0.02M concentration) inhibited citrate formation about 30%, 0.02M-L-lactate caused an inhibition of 60% and 0.02M-pyruvate was without effect.

Table 6. *Effect of pH on citrate synthesis in pigeon liver*

(0.02M-Bicarbonate. Other conditions as in Table 2.)

Gas (% CO ₂ in N ₂)	pH (calc.)	Citrate formed (μ l.)
1.05	8.04	61
2.14	7.73	60
4.52	7.40	98
12.8	6.95	112

Ammonium chloride (0.02M) completely abolished citrate formation. α -Ketoglutarate and ammonia are known to form glutamic acid under the conditions of the present experiments and this reaction can be coupled with the conversion of citrate into α -ketoglutarate (Adler, von Euler, Günther & Plass, 1939; Krebs, Eggleston & Hems, 1947). The effect

Table 7. *Effect of various substrates on citrate synthesis in pigeon liver*

(0.04M- α -Ketoglutarate present in each instance. Other conditions as in Table 2. Value in presence of β -hydroxybutyrate has been corrected for interference.)

Exp.	Additions	Citrate formed (μ L.)
1	None	91
	0.02M-L-Lactate	36
	0.02M- β -Hydroxybutyrate	67
	0.02M-NH ₄ Cl	~0
2	None	92
	0.02M-Pyruvate	94
	0.02M-Acetoacetate	64
	0.02M-Succinate	66
	0.02M-L-Glutamate	109

of ammonium chloride on the yield of citrate is probably connected with this dismutation. L-Glutamate had no appreciable effect on citrate formation. The small increase shown in Table 7 was not found regularly.

Fumarate (0.02M) caused an increased yield of citrate in the presence of α -ketoglutarate (Tables 5 and 8). The increase varied from 20 to 100% in different livers. As with α -ketoglutarate alone the maximum yield of citrate in the presence of both α -ketoglutarate and fumarate was reached within 12 min. The effect of fumarate depended on the bicarbonate and carbon dioxide concentrations (Table 5) and was greatest at suboptimal concentrations. Fumarate alone did not yield citrate. Malate was not examined since it may be taken for granted that it behaves like fumarate.

Oxaloacetate alone, as is well known, gives rise to citrate and in a short-term (12 min.) experiment (Table 8, Exp. 1) the effects of oxaloacetate and

α -ketoglutarate were roughly additive. In an experiment where the period of incubation was 30 min. (Table 8, Exp. 2) oxaloacetate and α -ketoglutarate together gave more citrate than could be accounted for by additive action only.

As Ochoa's (1945) mechanism of citrate formation involves the reduction of oxalosuccinate to isocitrate, it was thought that addition of a hydrogen donor might accelerate the formation of citrate. Hypo-sulphite which is known to regenerate the reduced form of the coenzymes (Warburg, Christian & Griese, 1935) was, therefore, added (0.036M), but it caused a considerable inhibition (Table 8, Exp. 1) which was not affected by simultaneous addition of lactate.

A detailed study of the inhibition by L-lactate showed that the inhibition was greater the higher the lactate concentration (Table 9, Exp. 1), and that it was of the same order in the presence of added fumarate or oxaloacetate as with α -ketoglutarate alone. Lactate also inhibited citrate formation from oxaloacetate alone, and from oxaloacetate plus pyruvate (Table 9, Exp. 2). Pyruvate (0.02M) did not affect lactate inhibition. In some experiments L-glutamate (0.02M) partly or wholly reversed the inhibition by lactate (Table 9, Exps. 2 and 3). The anaerobic or oxidative removal of citrate was not affected by 0.04M-L-lactate.

7. *Aerobic citrate synthesis.* Aerobically the yields of citrate in the presence of α -ketoglutarate were about 40% higher than anaerobically (Table 10). The effects of various substances in the presence of oxygen were similar to those observed in anaerobic experiments. Since the interpretation of aerobic experiments is complicated by the fact that citrate may arise by other reactions (i.e. through the tricarboxylic acid cycle) these experiments were not pursued.

Table 8. *The effect of oxaloacetate, fumarate, lactate and hyposulphite (Na₂S₂O₄) on citrate synthesis in pigeon liver*

(Incubation period 12 min. for Exp. 1 and 30 min. for Exp. 2. Other conditions as in Table 2.)

Exp.	Additions	Citrate formed (μ L.)
1	0.02M- α -Ketoglutarate	61
	0.02M- α -Ketoglutarate, 0.02M-oxaloacetate	165
	0.02M-Oxaloacetate	121
	0.036M-Hyposulphite	~0
	0.036M-Hyposulphite, 0.02M- α -ketoglutarate	29
	0.036M-Hyposulphite, 0.02M- α -ketoglutarate, 0.02M-lactate	31
2	0.02M- α -Ketoglutarate	98
	0.02M- α -Ketoglutarate, 0.02M-oxaloacetate	182
	0.02M-Oxaloacetate	36
	0.02M-Oxaloacetate, 0.02M-fumarate	88
	0.02M-Oxaloacetate, 0.02M-fumarate, 0.02M- α -ketoglutarate	243
	0.02M- α -Ketoglutarate, 0.02M-fumarate	172
	0.02M- α -Ketoglutarate, 0.02M-fumarate, 0.02M-lactate	53
	0.02M- α -Ketoglutarate, 0.02M-lactate	31
	0.02M-Oxaloacetate, 0.02M-lactate	~0
	0.02M- α -Ketoglutarate, 0.02M-oxaloacetate, 0.02M-lactate	60

Table 9. *The effect of lactate on citrate synthesis under various conditions in pigeon liver*

(Conditions as in Table 2.)			Citrate formed (μ l.)
Exp.	Additions		
1	0.02 M- α -Ketoglutarate		98
	0.02 M- α -Ketoglutarate, 0.02 M-fumarate		151
	0.02 M- α -Ketoglutarate, 0.005 M-lactate		69
	0.02 M- α -Ketoglutarate, 0.005 M-lactate, 0.02 M-fumarate		83
	0.02 M- α -Ketoglutarate, 0.01 M-lactate		50
	0.02 M- α -Ketoglutarate, 0.01 M-lactate, 0.01 M-fumarate		80
	0.02 M- α -Ketoglutarate, 0.04 M-lactate		36
2	0.02 M- α -Ketoglutarate		72
	0.02 M- α -Ketoglutarate, 0.02 M-pyruvate		71
	0.02 M- α -Ketoglutarate, 0.02 M-pyruvate, 0.02 M-lactate		39
	0.02 M- α -Ketoglutarate, 0.02 M-lactate, 0.02 M-L-glutamate		76
	0.02 M-Oxaloacetate		124
	0.02 M-Oxaloacetate, 0.02 M-lactate		60
	0.02 M-Oxaloacetate, 0.02 M-pyruvate		206
3	0.02 M-Oxaloacetate, 0.02 M-pyruvate, 0.02 M-lactate		84
	0.02 M- α -Ketoglutarate		100
	0.02 M- α -Ketoglutarate, 0.01 M-lactate		49
	0.02 M- α -Ketoglutarate, 0.02 M-L-glutamate		112
	0.02 M- α -Ketoglutarate, 0.02 M-L-glutamate, 0.01 M-lactate		81

Table 10. *Effect of oxygen on citrate synthesis in pigeon liver*(5% CO₂ in O₂. Other conditions as in Table 2.)

Additions	Citrate formed (μ l.)
0.04 M- α -Ketoglutarate	164
0.02 M- α -Ketoglutarate	142
0.02 M- α -Ketoglutarate, 0.02 M-fumarate	192
0.02 M-Fumarate	39
0.02 M- α -Ketoglutarate, 0.02 M-pyruvate	133
0.02 M- α -Ketoglutarate, 0.02 M-L-lactate	69
0.02 M- α -Ketoglutarate, 0.01 M-acetoacetate	109
0.02 M- α -Ketoglutarate, 0.01 M- β -hydroxybutyrate	117

8. *Effect of inhibitors.* The addition of 0.02 M-malonate caused 32 % inhibition of citrate formation in nitrogen and 65 % inhibition in oxygen (Table 11). The inhibition in oxygen was partly reversed by

addition of fumarate. Sodium fluoride, 0.02 M, inhibited citrate formation in nitrogen by 47 % (Table 11). Adenosinetriphosphate (0.002 M), co-enzyme (5 μ g./ml.) or 0.001 M-MnCl₂ had no effect on citrate formation from α -ketoglutarate or on the inhibition by lactate. Thus none of these substances were limiting factors under the conditions of these experiments.

(b) Other tissues

The formation of citrate in the presence of α -ketoglutarate was examined in other tissues. It occurred to a considerable extent in pigeon-breast muscle, guinea pig and rat liver, and to a less extent in sheep liver, heart and kidney cortex (Table 12). No citrate formation was detected in guinea pig, rat and sheep brain, sheep spleen, and rat and sheep testis, though the latter tissue contained large amounts of citrate (see Schersten, 1936; Dickens, 1941).

Table 11. *The effect of malonate and fluoride on citrate synthesis in pigeon liver*

(Incubation period 12 min. in Exps. 1-3; 30 min. in Exp. 4. Other conditions as in Table 2.)

Exp.	Gas	Additions	Citrate formed (μ l.)	Inhibition (%)
1	5% CO ₂ in O ₂	0.04 M- α -Ketoglutarate	136	—
		0.04 M- α -Ketoglutarate, 0.02 M-malonate	42	69
		0.04 M- α -Ketoglutarate, 0.002 M-malonate	86	37
2	5% CO ₂ in O ₂	0.02 M- α -Ketoglutarate	142	—
		0.02 M- α -Ketoglutarate, 0.02 M-fumarate	192	+35
		0.02 M- α -Ketoglutarate, 0.02 M-malonate	50	65
		0.02 M- α -Ketoglutarate, 0.02 M-malonate, 0.02 M-fumarate	123	36
3	5% CO ₂ in N ₂	0.02 M- α -Ketoglutarate	61	—
		0.02 M- α -Ketoglutarate, 0.02 M-malonate	41	32
		0.02 M- α -Ketoglutarate, 0.02 M-Na fluoride	32	47
4	5% CO ₂ in N ₂	0.02 M- α -Ketoglutarate	158	—
		0.02 M- α -Ketoglutarate, 0.02 M-malonate	110	31
		0.02 M- α -Ketoglutarate, 0.005 M-malonate	135	14

Table 12. *Citrate synthesis by various tissues in the presence of α -ketoglutarate*

(400 mg. fresh tissue in 4 ml. suspension except with sheep testis (800 mg.) and rat testis (250 mg.). Incubation period 20 min. except with pigeon-breast muscle (12 min.). 5% CO_2 in N_2 or O_2 .)

Tissue	Gas	Additions	Citrate formed ($\mu\text{l.}$)
Pigeon-breast muscle	N_2	0.02M- α -Ketoglutarate	47
		0.02M- α -Ketoglutarate	30
		None	~ 0
Guinea-pig liver	N_2	0.04M- α -Ketoglutarate	69
		0.04M- α -Ketoglutarate, 0.02M-fumarate	123
	O_2	0.04M- α -Ketoglutarate	53
		0.04M- α -Ketoglutarate, 0.02M-fumarate	180
		None	~ 0
Rat liver	N_2	0.04M- α -Ketoglutarate	48
		0.04M- α -Ketoglutarate, 0.02M-fumarate	66
		0.04M- α -Ketoglutarate, 0.02M-lactate	~ 0
		None	~ 0
	O_2	0.04M- α -Ketoglutarate 0.04M- α -Ketoglutarate, 0.02M-fumarate	69 133
Sheep liver	N_2	0.04M- α -Ketoglutarate	25
		0.04M- α -Ketoglutarate, 0.02M-fumarate	32
		0.04M- α -Ketoglutarate, 0.02M-lactate	~ 0
Sheep heart	N_2	0.04M- α -Ketoglutarate	35
		0.04M- α -Ketoglutarate, 0.02M-fumarate	55
		0.04M- α -Ketoglutarate, 0.02M-L-glutamate	26
Sheep kidney	O_2	0.04M- α -Ketoglutarate	25
		0.04M- α -Ketoglutarate, 0.02M-fumarate	38
	N_2	0.04M- α -Ketoglutarate, 0.02M-fumarate	26
Sheep testis	N_2	None	24
		0.04M- α -Ketoglutarate	25
		0.04M- α -Ketoglutarate, 0.02M-fumarate	37
Rat testis	N_2	None	32
		0.04M- α -Ketoglutarate	41
		0.04M- α -Ketoglutarate, 0.02M-fumarate	39

DISCUSSION

Rate of carbon dioxide fixation by pyruvate in minced and homogenized animal tissues. In pigeon liver the computed rate for the carboxylation of pyruvate ($Q_{\text{oxaloacetate}} = 19$) was of the same order of magnitude as the rate of respiration. In sheep-kidney cortex the rate as determined by the malonate technique was, on average, 1.7. In all other tissues examined (rat liver, kidney cortex and muscle, pigeon brain, guinea pig liver and brain, and sheep liver, heart, brain, spleen, pancreas, and lung) the rate of carboxylation must have been less than 1 since no formation of succinate or α -ketoglutarate could be measured by the methods used. It should be emphasized that the non-detection of the reaction only indicates that the rate was below 1 under the given experimental conditions. Experiments published by Solomon, Vennesland, Klemperer, Buchanan & Hastings (1941), Wood, Lifson & Lorber (1945) and Anfinsen, Beloff, Hastings & Solomon (1947), in fact, make it very probable that the carboxylation of pyruvate occurs in mammalian

liver. It can be calculated from the data given by Buchanan, Hastings & Nesbitt (1942) that the rate at which carbon dioxide was incorporated in glycogen in rabbit-liver slices under their experimental conditions was, in terms of $Q_{\text{CO}_2}^{\text{glycogen}}$, between 0.14 and 0.48 in four experiments (average 0.26). As aspartic and glutamic acids containing carbon dioxide were synthesized at the same time (Anfinsen *et al.* 1947) these figures are the minimum rates. They are compatible with the conclusion reached from the present experiments that the rate of the carboxylation of pyruvate is below 1 in mammalian liver.

Mechanism of citrate formation from α -ketoglutarate. The dependence of citrate formation upon the concentration of α -ketoglutarate and of bicarbonate and carbon dioxide in these experiments is in accordance with the conception, already firmly established by Ochoa (1945), Grisolia & Vennesland (1947), and Floyd, Medes & Weinhouse (1947) that the primary step is the carboxylation of α -ketoglutarate and formation of oxalosuccinate, followed by its reduction to isocitrate and rearrangement to citrate. These three stages involve at least three enzyme

systems: oxalosuccinic carboxylase, isocitric dehydrogenase and aconitase. The stimulating effect of fumarate, and the inhibitory action of lactate which can be partially reversed by glutamate, are presumably connected with the second stage, but no explanation for the cause of the effects can be offered. It is of interest that lactate also inhibits the formation of citrate from oxaloacetate and pyruvate which is an oxidative process.

Rate of carbon dioxide fixation by α -ketoglutarate.

The formation of citrate on addition of α -ketoglutarate was demonstrable in pigeon liver, pigeon-breast muscle, guinea pig, rat and sheep liver, sheep heart and sheep-kidney cortex, whilst negative findings were obtained with guinea pig, rat and sheep brain, sheep spleen and rat and sheep testis. In pigeon liver the rate was very high immediately after addition of the α -ketoglutarate but fell to zero within a few minutes. Q_{citrate} was 30 during the first minute. The total amounts of citrate formed were independent of the concentration of α -ketoglutarate at the higher concentrations, and the stoppage of the synthesis, therefore, cannot be ascribed to a simple equilibrium effect.

In view of the rapid change of rate, Q values calculated from data referring to experimental periods of more than a few minutes indicate only a minimum value for the initial velocity. In the experiments given in Table 12 the period of incubation was 12 or 20 min. and the average Q values for the period of incubation reached 2.9 in pigeon-breast muscle, 4.6 in guinea-pig liver, 3.3 in rat liver, 0.8 in sheep liver, and 2.1 in sheep heart. The initial Q values were in all probability much higher.

Significance of the carbon dioxide fixation by α -ketoglutarate. Grisolia & Vennesland (1947) have already pointed out that the conversion of citrate into α -ketoglutarate is the only metabolic process which citrate, once formed, is definitely known to undergo. Hence it would appear that the carboxylation of α -ketoglutarate cannot result in the incorporation of assimilated carbon in any compounds other than the four tricarboxylic acids. These acids can be readily formed in animal tissues through the oxidative reactions of the tricarboxylic acid cycle, and as long as no further reactions of citrate are known, it is difficult to visualize the specific biological significance of the carboxylation of α -ketoglutarate. It has

been suggested (Dr H. McIlwain, personal communication) that the formation of citrate from α -ketoglutarate, which is an endergonic reaction, might serve as a temporary storage of energy. If the idea of Lipton & Barron (1946) that citrate might undergo fission to oxaloacetate and acetate should prove correct, then it would be possible to offer an explanation of the occurrence of the carboxylation of α -ketoglutarate.

SUMMARY

1. The rate of carbon dioxide fixation by pyruvate ($Q_{\text{oxaloacetate}}$) was investigated in minced tissues by determining the accumulation of succinate and α -ketoglutarate (a) anaerobically, and (b) aerobically in the presence of malonate.

2. $Q_{\text{oxaloacetate}}$ was computed for the conditions employed to be 19 in pigeon liver and, on the average, 1.7 in sheep-kidney cortex. In pigeon brain, rat liver, kidney cortex and muscle, guinea pig liver and brain, and sheep liver, heart, brain, spleen, pancreas and lung, no carbon dioxide fixation by pyruvate could be detected by the above methods, and $Q_{\text{oxaloacetate}}$ therefore, must have been less than 1 in these tissues.

3. The rate of carbon dioxide fixation by α -ketoglutarate (Q_{citrate}) was studied by measuring the formation of citrate under anaerobic conditions.

4. In pigeon liver citrate formation was rapid immediately after addition of α -ketoglutarate but ceased within a few minutes. During the first minute Q_{citrate} was 30. Pigeon-breast muscle, guinea pig, rat and sheep livers, and sheep heart and kidney cortex all formed citrate from α -ketoglutarate. No detectable amounts of citrate were formed by guinea pig, rat or sheep brain, sheep spleen, or rat and sheep testis.

5. Citrate formation was found to be dependent upon the concentration of α -ketoglutarate and of bicarbonate and carbon dioxide. It was enhanced by fumarate and inhibited by L-lactate or ammonium chloride. The inhibition by lactate was partly reversed by L-glutamate. Lactate also inhibited citrate formation from oxaloacetate and pyruvate.

6. The significance of the findings is discussed.

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The Copper-retaining Powers of Different Cacao Soils

By H. LEES, *Department of Chemistry and Soils, Imperial College of Tropical Agriculture, St Augustine, Trinidad, B.W.I.*

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Brun (1945) has shown that soil organic matter (SOM) holds copper in a complex, non-leachable form; the work of Hasler (1943) suggests that most metallic trace elements are so retained in soil, the order of their retention being roughly parallel to their power of forming metallo-organic complexes (Mellor & Maley, 1948). From such complexes between trace elements and SOM (Bremner, Mann, Heintze & Lees, 1946) the trace elements presumably become slowly available to plants growing in the soil. In view of the fact that Trinidad cacao soils have been exploitatively cropped for 150 years, a depletion of trace elements in these soils may, in part, be responsible for the general drop in cacao yields apparent over the last 25 years. The present work was therefore begun in order to discover whether 'good' cacao soils (i.e. those on which the decline has been least marked) had a greater power than 'bad' soils to retain a trace element against leaching, and to establish a relation between the percentage of SOM in a soil and the power of the soil to retain a trace element. The trace element chosen was copper, in which cacao is rich (Lindow, Elvehjem & Peterson, 1929).

EXPERIMENTAL

Copper was determined by the method of Sherman & McHargue (1942). Soil organic matter was estimated by the method of Hardy (1929). Because the upper layers of a cacao soil probably play the dominant part in cacao nutrition (McCreary, McDonald, Mulloon & Hardy, 1943), the top 3 in. of soil, air dried and ground to a powder, have been used in all experiments. The soils were divided into 'good' soils and 'bad' soils on the basis of planters' estimates of the cacao yields to be expected from them. Good soils were those estimated to yield 7 or more bags of cacao

per 1000 picquets, bad soils were those estimated to yield less.

The Cu-retaining powers of different soils (Fig. 1) were determined as follows. A 5 g. sample of soil was shaken, over a period of 30 min., with 15 ml. 0.5M-Na₂SO₄ containing 1.0 mg. Cu as CuSO₄ after which the soil was centrifuged down, and the supernatant liquid poured off. The soil was then again shaken with 15 ml. 0.5M-Na₂SO₄ containing no Cu and, after 30 min., again centrifuged down, the supernatant liquid being added to the initial one. After 7 such washings, all exchangeable Cu was presumed to have been removed from the soil. The amount so removed was determined by analysis of the bulked supernatant liquids and the amount retained determined by difference.

The data on which Fig. 3 is based were obtained in two different ways on each soil:

(a) Either 1, 2, 4, 8, or 16 g. of soil were mixed with 50 ml. 0.33M-Na₂SO₄ containing, as CuSO₄, either 1.0 mg. Cu (for the bad soil) or 5.0 mg. Cu (for the good soil). After intermittent shaking over a period of 1 hr. the soil was centrifuged down, and the equilibrium concentration of Cu in the supernatant liquid determined.

(b) Either 1, 2, 4, 6, 8, or 10 mg. Cu as CuSO₄, contained in 30 ml. 0.33M-Na₂SO₄, were mixed with 5 g. soil. Procedure then followed that described in (a).

Preliminary tests showed that equilibration of Cu between soil and solution was attained in less than 5 min. The longer equilibration periods used were adopted as a safety measure.

RESULTS

The relation between the estimated percentage of SOM and the copper-retaining power of the soil is shown for thirty-two different cacao soils in Fig. 1. Some 2.5% of the estimated SOM of the soils is apparently inactive in retaining copper; moreover, the percentage of the active SOM (total SOM % - 2.5) is not linearly related to copper retention. In fact the curve of Fig. 1 closely follows the relation