CITRIC ACID FORMATION FROM ACETOACETIC AND OXALACETIC ACIDS*

By F. EDMUND HUNTER and LUIS F. LELOIR

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

(Received for publication, April 9, 1945)

Breusch (1) reported the discovery of an enzyme, citrogenase, which catalyzes the formation of citric acid from oxalacetic acid and β-keto fatty acids, including acetoacetic acid. According to the proposed mechanism of reaction the β-keto acid and oxalacetic acid would condense to form an intermediate compound which would undergo hydrolysis to yield citric acid and a fatty acid shortened by the loss of 2 carbon atoms. The 2-carbon group removed from the fatty acid would appear in the citric acid and be metabolized to carbon dioxide and water by way of the "citric acid" or tricarboxylic acid cycle. Oxalacetic acid would be regenerated and the process could be repeated as often as β oxidation of the fatty acid occurred.

Independently Wieland and Rosenthal (2) published findings similar to those of Breusch with respect to acetoacetic acid, and Martius (3) has added partial confirmation of these reports. However, Weil-Malherbe (4) and Krebs and Eggleston (5) have reported that they found no evidence for the existence of a citrogenase for metabolizing acetoacetic acid. In the present paper the observations concerning citric acid formation from acetoacetic and oxalacetic acids by kidney tissue have been confirmed and the nature of this enzymatic reaction has been studied. Since the completion of this work Buchanan *et al.* (6) have reported that heavy carbon in acetoacetic acid added to homogenized kidney cortex appears in α-keto glutaric and fumaric acids, members of the tricarboxylic acid cycle.

Methods

Preparations—The following methods were used: oxalacetic acid, Krampitz and Werkman (7); acetoacetic acid, Ljunggren (8); acetyl phosphate, Lipmann and Tuttle (9); sodium pyruvate, Robertson (10).

Ketone Body Estimation—Acetoacetic acid was determined by the aniline method (Edson (11)) or by the method of Weichselbaum and Somogyi (12). In a number of experiments both methods were used. For the determination of acetoacetic acid by the aniline method in the presence of oxalacetic acid, use was made of the effect of cupric ions on the decomposition of the latter acid (Krebs (13)). The contents of the Warburg flasks (2 to 3 ml.)

* This work was supported in part by a grant from the Rockefeller Foundation.

were acidified with 0.3 ml. of glacial acetic acid and 0.3 ml. of 20 per cent copper sulfate was added. The oxalacetic acid was completely decarboxylated in 20 to 30 minutes at 25°, while the acetoacetic acid remained unaffected. Then 0.4 ml. of 40 per cent aniline in glacial acetic acid was tipped in from a side bulb in order to decarboxylate the acetoacetic acid.

β-Hydroxybutyric acid was determined according to the method of Weichselbaum and Somogyi (12).

Citric Acid Estimation—A modification of the pentabromoacetone method of Pucher, Sherman, and Vickery (14) was used. The preparation of the samples and the oxidation with permanganate were carried out as described by these authors. Then the mixture was decolorized with a slight excess of freshly prepared saturated sodium nitrite solution (about 1 ml.). 25 ml. of petroleum ether (Mallinckrodt, b.p. 35–60°) were added and the separatory funnel shaken vigorously for 1 minute. After complete separation of the petroleum ether the aqueous layer was discarded. The petroleum ether was washed twice with 10 ml. of water. Shaking for 10 seconds was sufficient. After the second wash water had been drained off, 5 ml. of 2.5 per cent sodium sulfide in 40 per cent ethylene glycol were added to the petroleum ether and the funnel was shaken about 15 seconds. All wash water was shaken out of the stem of the funnel. The yellow-colored layer was drained into a colorimeter tube graduated at 10 ml. The extraction was repeated twice with 2 ml. portions of the sulfide reagent. The extracts were combined in the colorimeter tube, made up to 10 ml. with the sulfide reagent, mixed, centrifuged for a short period, and read in a Klett-Summerson photoelectric colorimeter with Filter 42. At least one standard citric acid was run simultaneously with each group of determinations.

The described procedure has a number of advantages, with essentially the same accuracy as the original method. A great deal of time is saved. The use of sodium nitrite instead of ferrous sulfate for destroying excess permanganate and bromine does not interfere with the method and eliminates much of the tedious washing of the petroleum ether layer to get rid of the iron.

One petroleum ether extraction of the pentabromoacetone seems sufficient; determinations carried out with a second petroleum ether extract alone gave values equivalent to water blanks. Goldberg and Bernheim (15) have recently reported this same observation. With only one extraction fewer separatory funnels and manipulations are required.

Ethylene glycol proved to be a better color stabilizer than pyridine. It has the additional advantage that it is immiscible with petroleum ether and therefore can be added during the extraction. The use of ethylene glycol in this manner facilitates making the time between the development of the color and the measurement with the colorimeter equal for all samples. A

linear relationship between color and citric acid was found between 0.2 and 1.0 mg. of citric acid, but not with smaller or larger amounts.

Enzyme Preparations—All experiments reported in this paper were carried out with enzyme preparations from kidney cortex, but the presence of the enzyme in heart muscle has also been confirmed. Breusch (1) states that he extracted citrogenase from tissue with 0.5 per cent sodium bicarbonate, while other workers have used slices, homogenates, or minced tissue. Preliminary experiments were carried out either with homogenates or with the cloudy extracts obtained from homogenates by centrifugation at 2500 R.P.M. for 10 to 15 minutes. In both cases 2 volumes of 0.5 per cent sodium bicarbonate were used with 1 volume of tissue. These preparations showed some activity. Experiments with the multispeed attachment for International centrifuges revealed that the enzyme was not truly extracted by sodium bicarbonate, but was associated with particles which were separated by high speed centrifugation (see Table I). The procedure finally adopted for preparing the enzyme system is described in the next paragraph.

The kidneys were removed from dogs anesthetized with ether, iced, and the cortex homogenized at 0° with 1 volume of distilled water in a stainless steel homogenizer of the type described by Potter and Elvehjem (16). The homogenate was strained through muslin and 0.05 volume of 10 per cent potassium chloride solution added with mixing. This preparation was centrifuged in the cold room for 15 minutes in the multispeed attachment of an International centrifuge at about 12,000 R.P.M. The supernatant fluid was discarded and the precipitate suspended in distilled water sufficient to restore the original volume. 10 per cent potassium chloride solution (one-tenth of the volume of the water) was added and the mixture thoroughly stirred. The tubes were again centrifuged at high speed. This washing of the tissue particles was repeated once more. Finally the precipitate was thoroughly suspended in enough 0.8 per cent sodium chloride to make about one-third of the original volume of homogenate. This yielded a thin yellowish paste which could be pipetted easily. All steps of the preparation were carried out in the cold. In a few cases kidney tissue from cats and rabbits was used, but attempts to use pig kidney obtained from the slaughter-house were abandoned because the material was inactive in about half of the trials. Acetone powders were completely inactive.

Experimental Procedure—Experiments were carried out in 25 ml. or 50 ml. Erlenmeyer flasks when citric acid only was estimated. Borate or bicarbonate buffer at pH 7.4 was used and all solutions were adjusted to this pH. Unless otherwise indicated 0.5 ml. of enzyme preparation was used and the final volume was made up to 1.1 ml. by various additions or by water. After the air above the liquid had been replaced with the appropriate gas, the flasks were stoppered and shaken horizontally during

the incubation period. Trichloroacetic acid filtrates were prepared for citric acid determination.

The balance experiments were carried out in Warburg flasks with 1 ml. of enzyme preparation and a final volume of 2.2 to 2.5 ml. Borate buffer was used when oxygen uptake was measured. For anaerobic experiments, bicarbonate buffer was used and the flasks were filled with nitrogen containing 5 per cent carbon dioxide. The gas evolution under these conditions is referred to as acid formation. At the end of the incubation period the contents of the flask were diluted with a measured amount of distilled water and an aliquot removed for estimation of ketone bodies according to the method of Weichselbaum and Somogyi. Acetoacetic acid estimation by the aniline method was carried out directly on the material remaining in the flask, without the removal of proteins. Then 5 ml. of 10 per cent trichloroacetic acid were added to the flask with thorough mixing. The filtrate was used for citric acid determinations. Aniline does not interfere in the citric acid estimation provided sufficient bromine is added and the precipitate formed is removed.

Results

Stability of Enzyme System—Whole kidney tissue, kidney homogenate, and the preparation of washed tissue particles lose little activity during several hours at 0°. Homogenates and washed preparations aged 16 to 20 hours at 0° still possessed about 40 per cent of the original activity. Dialysis of homogenates for 16 to 20 hours at 0° resulted in loss of most of the activity. In about half of these dialyzed preparations some activity could be restored by adding a boiled muscle or kidney extract or glutathione.

Additions Necessary for Citrate Formation—Addition of oxalacetate to homogenates caused some citrate formation. When acetoacetate was also added, there was a considerable increase in citrate formation, but when the tissue particles were washed several times, as described in the procedure for preparing the enzyme, the addition of acetoacetate caused little increase in citrate formation over that with oxalacetate alone. Some 50 to 60 per cent of the original extra citrate formation caused by acetoacetate could be restored by adding to the tissue particles the supernatant fluid from the first centrifugation. Heating the first supernatant fluid to 80° for 5 minutes did not destroy its ability to restore the activity of the system (see Table I).

These experiments indicated that some factor necessary for citrate formation from acetoacetate was removed by washing of the tissue particles. This factor could be restored by addition of a boiled extract prepared from kidney or heart, or a water extract of dried yeast, but was not present in the ash. In the course of the search for the unknown factor the following substances were tested and found to be without effect: adenosine triphos-

phate, adenylic acid, cozymase, cocarboxylase, cytochrome *c*, cysteine, riboflavin, glycine, alanine, and pantothenic, ascorbic, *p*-aminobenzoic, succinic, fumaric, aspartic, and pyruvic acids.

The only substances found which could replace the heated tissue extract in restoring the activity of the system were glutathione, glutamic acid, and α-ketoglutaric acid. The effect of glutathione was discovered first and for a time it was believed to be the necessary substance. However, later experiments revealed that glutamic acid and α-ketoglutaric acid were more effective than glutathione and were able to restore activity to some preparations which were not affected by glutathione. This led to the conclusion that either glutamic acid or α-ketoglutaric acid was the essential substance.

TABLE I

Citric Acid Formation in Homogenates, Extracts, and Washed Particles Prepared from Kidney Tissue

Dog kidney cortex homogenized at 0° in 1.5 volumes of 0.5 per cent sodium bicarbonate; part centrifuged at 12,000 R.P.M. for 15 minutes in the cold room; precipitate resuspended in the original volume of bicarbonate solution or in its supernatant fluid; 3 ml. of tissue preparation + 100 micromoles of oxalacetate + 40 micromoles of acetoacetate; barium chloride 0.02 M; final volume 4.2 ml.; nitrogen with 5 per cent carbon dioxide as gas; pH 7.5; 1 hour at 37°. The results are given in micromoles.

Experiment No.	Tissue preparation	Citrate formed		
		With oxalacetate alone	With acetoacetate alone	With oxalacetate + acetoacetate
1	Homogenate	14.1	8.3	33
	Supernatant fluid	0.9	0.6	1.8
	Ppt. resuspended in bicarbonate	13.8	1.5	24
2	Homogenate	2.6		23
	Ppt. (washed 2 times)	1.4		2.8
	" + first supernatant fluid	3.6		15.9
	" + heated first supernatant fluid	2.4		13.8

These two substances seemed equally effective in equimolecular concentrations. The effect of glutathione may be due to glutamic acid released by hydrolysis (17). Table II gives the results of some of the experiments with different additions. The data clearly indicate that both oxalacetate and α-ketoglutarate (or glutamate) must be present if the addition of acetoacetate is to cause a large increase in citrate formation. As a result of the work of Buchanan *et al.* (6) it seems safe to assume that this represents a real conversion of acetoacetate to citrate.

Specificity of Oxalacetate As Substrate—Oxalacetate could not be replaced by pyruvate, fumarate, malate, or succinate in anaerobic experiments (Table III). Aerobically with added cytochrome *c* malate was slightly

active. Evidently it is converted to oxalacetate slowly by these preparations. In intact tissue any substance which can be converted to oxalacetate should be able to replace this substance.

TABLE II

Effect of Various Substances on Citrate Formation

Experiments with washed tissue particles suspended in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 50 micromoles of oxalacetate, 8 to 15 micromoles of acetoacetate, 10 micromoles of additions unless otherwise indicated; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in oxygen; 60 to 90 minutes at 28°. The results are given in micromoles.

Experiment No.	Additions	Citrate formed		
		With oxalacetate alone	With oxalacetate + acetoacetate	With acetoacetate alone
1	None	5.3	9.2	
	l-Glutamate	4.6	20.6	
	α-Ketoglutarate	4.0	19.2	
	Glutathione	4.4	11.7	
2	None	5.9	9.2	
	Glutathione, 5 micromoles	4.4	14.4	
	l-Glutamate	5.5	21.6	
	Glycine	5.7	8.9	
	l-Cysteine	7.3	11.2	
3	None	5.6	7.3	
	Glutathione	4.4	15.1	
	l-Cysteine	3.6	4.9	
4	None	5.4	8.6	
	l-Glutamate	3.9	13.4	1.4
	α-Ketoglutarate	3.3	10.4	1.3
	Glutathione	3.7	6.9	
	l-Aspartate	6.4	7.3	
	Succinate	3.9	5.2	
	dl-Alanine	4.6	5.8	
5	None	4.1	7.6	
	l-Glutamate, 2.5 micromoles	3.2	10.0	
	" 10 "	2.4	12.1	1.5
	" 20 "	2.2	8.4	
	α-Ketoglutarate, 2.5 micromoles	2.8	9.2	
	" 10 "	2.3	11.2	3.1
	" 20 "	2.1	10.5	

Specificity of Acetoacetate As Substrate—As may be seen from Table IV, some increases in citrate formation were found with β-hydroxybutyrate, crotonate, and butyrate, but the effect was smaller than with acetoacetate and these substances are known to be transformed into acetoacetate. Py-

ruvate gave some increase. Changes caused by acetate were probably not significant. Acetyl phosphate in different amounts was tested in several experiments, with and without glutamate, with negative results. Of the group of substances tested, acetoacetate is probably the only one which can serve as substrate. Whether the enzyme also acts on other β-keto fatty acids, as reported by Breusch, has not been determined.

Balance between Citrate Formation, Acid Formation, and Acetoacetate Disappearance—Tables V and VI show the results of experiments designed to determine the relation between acetoacetate disappearance and citrate formation.

The addition of oxalacetate to the kidney preparation results in the for-

TABLE III

Specificity of Oxalacetate

Experiments with washed tissue particles in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 10 micromoles of glutamate present in all samples; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in nitrogen; 60 minutes at 28°. The values are given in micromoles.

Experiment No.	Additions		Citrate formed	
			Without acetoacetate	With acetoacetate (9 micromoles)
1	Oxalacetate	50	4.0	13.0
	Fumarate	30	0.6	3.1
	l-Malate	30	1.8	4.4
	Succinate	30	1.3	1.4
	Pyruvate	30	0.7	1.9
2	None		0.8	1.4
	Oxalacetate	50	3.9	13.4
	Fumarate	100	1.2	2.4
	Pyruvate	75	1.1	2.1

mation of some citrate anaerobically as well as aerobically. When α-ketoglutarate or glutamate is added, the amount of citrate formed from oxalacetate is decreased. Martius (3) found that α-ketobutyrate decreased citrate formation from oxalacetate and pyruvate in heart muscle. The mechanism is probably the same in the two cases. These α-keto acids probably produce a competitive inhibition of citrate formation from pyruvate. In the present experiments pyruvate would result from decarboxylation of part of the oxalacetate. Although glutamate and α-ketoglutarate decrease the citrate formation with oxalacetate alone, they greatly increase the extra citrate formed upon the addition of acetoacetate. The differences in the amount of citrate found cannot be explained on the basis of

changes in citrate oxidation, for very little citrate was oxidized by the preparations used (Table VII).

Anaerobic Balances—The disappearance of acetoacetate (over and above that which is reduced to β-hydroxybutyrate) is small with oxalacetate alone, or with glutamate or α-ketoglutarate alone, and attains its maximum only when both oxalacetate and α-ketoglutarate or glutamate are present. As

TABLE IV

Specificity of Acetoacetate

Experiments with washed tissue particles in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 50 micromoles of oxalacetate, 5 micromoles of glutathione or 10 micromoles of glutamate, and 10 micromoles of additions; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in nitrogen; 60 to 90 minutes at 28°. The results are given in micromoles.

	Experiment No.	Additions	Citrate formed
Glutathione	1	None	4.7
		Acetoacetate	18.3
		Acetate	7.3
		Acetone	4.7
		Ethanol	4.6
		dl-β-Hydroxybutyrate	11.5
		Pyruvate	6.2
		Crotonate	7.2
		Butyrate	7.5
		Octanoate	5.3
Glutamate	2	None	5.6
		Acetoacetate	14.7
		Acetaldehyde	5.3
		Acetate	5.3
	3	None	6.7
		Acetoacetate	18.4
		Acetate	7.1
		Pyruvate	9.8
	4	None	4.0
		Acetoacetate	17.6
		Acetyl phosphate	4.9
		Pyruvate	5.0

nearly as could be determined, when acetoacetate was added to the otherwise complete system, 2 molecules of extra citrate[1] were formed for each

[1] According to Krebs and Eggleston (18) the amount of isocitrate and *cis*-aconitate in enzymatic equilibrium with the citrate is about 10 per cent, or much lower, 4 per cent, in the presence of magnesium ions. The citrate values reported have not been corrected for isocitrate and *cis*-aconitate, but this error is small, for with the washed kidney preparation and 0.02 M barium chloride only 8 per cent of added citrate disappeared aerobically. This figure represents the amount oxidized plus the amount converted into isocitrate and *cis*-aconitate.

molecule of acetoacetate which disappeared. For example, in Experiment 1 of Table V, 13.5 micromoles of citrate were formed with oxalacetate plus α-ketoglutarate. When acetoacetate also was added, completing the system, 30.2 micromoles of citrate were formed. Thus the extra citrate due to the addition of acetoacetate was 16.7 micromoles. The amount of citrate formed from oxalacetate alone is not used as a control value because

TABLE V

Anaerobic Balance

Warburg vessels containing 0.2 ml. of 0.4 M sodium bicarbonate + 0.1 ml. of 0.45 M barium chloride + 1.0 ml. of a suspension of washed tissue particles; 50 minutes at 30°; pH 7.3; gas, 5 per cent carbon dioxide in nitrogen; total volume 2.4 ml. All values are given in micromoles.

Experiment No.	Additions			Ketone body recovery		Ketone body disappearance	Citrate formed	Acid formed
	Oxalacetate	Acetoacetate	α-Ketoglutarate	Acetoacetate	β-Hydroxybutyrate			
1	100			0.3	0.3		19.6	
	100	20.5		15.0	3.1	2.4	23.5	
	100	20.5	20	5.4	5.7	9.4	30.2	
		20.5	20	10.1	6.9	3.5	1.4	
		20.5		14.8	5.0	0.7	0.9	
	100		20	1.6	0.5		13.5	
2	100			0.2	0.6		25.1	26
	100	14		8.3	4.3	1.4	31.5	28
	100	14	20	1.0	2.7	10.3	36	33
		14	20	4.9	7.2	1.9	1.5	11.9
		14		9.9	4.0	0.1	2.0	3.7
	100		20	0.5	0.4		17.7	31
3	100			0.5	*		12.6	12.3
	100	16		14.3		1.7	16.0	15.5
	100	16	20	6.9		9.1	22	23
		16	20	12.4		3.6	3.3	5.0
		16		16.0		0	3.0	1.5
	100		20	0.3			6.3	18
			20	0.3			4.4	1.8
				1.0			2.7	0.6

* β-Hydroxybutyrate was not determined in Experiment 3.

α-ketoglutarate decreases this figure and this effect of α-ketoglutarate must still be present, at least in part, in the complete system. The ketone body disappearance in the complete system was 9.4 micromoles, as against 0.7 for the control with acetoacetate alone. Thus completion of the system increased the ketone body disappearance by 8.7 and the citrate formation by 16.7 micromoles. Small amounts of ketone bodies disappeared with

oxalacetate plus acetoacetate and with acetoacetate plus α-ketoglutarate. These changes were very probably due to the fact that small amounts of the substance necessary to complete the system were still present in the tissue

TABLE VI

Aerobic Balance

Warburg vessels containing 0.2 ml. of 0.5 M borate buffer of pH 7.5 + 0.5 mg. of cytochrome *c* preparation + 0.1 ml. of 0.45 M barium chloride + 1 ml. of a suspension of washed tissue particles; gas, oxygen; total volume 2.4 ml. Experiments 1 and 3, 60 minutes at 30°, Experiment 2, 100 minutes at 25°. All values are given in micromoles.

Experiment No.	Additions			Ketone body recovery		Ketone body disappearance	Citrate formed	Oxygen consumed
	Oxalacetate	Acetoacetate	α-Ketoglutarate	Acetoacetate	β-Hydroxybutyrate			
1	100			0.5	0.7		25	5.2
	100	16.5		12.9	0.7	2.9	30.3	7.1
	100	16.5	20	5.4	0.9	10.2	41	15.8
		16.5	20	3.6	2.0	10.9	13.5	25.2
		16.5		13.7	0.7	2.1	3.5	4.3
	100		20	0.5	0.4		14.3	7.5
2	100			0.4	*		23	11.1
	100	15		13.8		1.2	33	12.0
	100	15	20	5.7		9.3	39	22
		15	20	6.3		8.7	15.5	34
		15		14.3		0.7	10.9	11.8
	100		20	0.6			14.6	12.7
			20	0.3			5.4	26
				0			5.1	9.0
3†	100			1.5	*		22	6.3
	100	16		14.5		1.5	28	6.3
	100	16	20	2.8		13.2	46	23
		16	20	10.2		5.8	13	19.3
		16		15.0		1.0	5.1	4.3
	100		20	1.3			16	11.0
			20	0			6.0	18.1
				2.2			4.8	4.2

* β-Hydroxybutyrate was not determined in Experiments 2 and 3.

† With glutamate instead of α-ketoglutarate.

particles or were formed during the experiment. The data are in agreement with the equation proposed by Wieland and Rosenthal (2).

(1) 2 oxalacetate + 1 acetoacetate → 2 citrate

Additional information concerning the reactions taking place is furnished by the acid formation, as measured by carbon dioxide evolution from the

bicarbonate buffer. The acid formation by the system with oxalacetate alone represents approximately 1 molecule of acid per molecule of citrate formed. (The decarboxylation of oxalacetate to pyruvate, which takes place rapidly in these experiments, occurs without any acid formation.) This relationship is what would be expected on the basis of either of the following equations proposed by Krebs *et al.* (19), in which one extra acid group is produced for each citrate formed.

(2) 3 oxalacetate → 1 citrate + 1 malate + 2 bicarbonate

(3) 2 oxalacetate + 1 pyruvate → 1 citrate + 1 malate + 1 bicarbonate

When glutamate or α-ketoglutarate is added to the washed tissue plus oxalacetate, there is a large acid formation not accounted for by the citrate

TABLE VII

Citric Acid Oxidation by Washed Kidney Tissue Particles

1.0 ml. of suspension of washed tissue particles + 0.5 mg. of cytochrome *c* preparation + 0.2 ml. of 0.5 M borate buffer at pH 7.4 + 1.2 ml. of total additions; 0.02 M NaCl or $BaCl_2$, 0.01 M phosphate, 0.004 M $MgCl_2$, 1 mg. of adenylic acid, and 18.5 micromoles of citrate when indicated; gas, oxygen; 60 minutes at 30°. All values are given in micromoles.

Additions to tissue	Oxygen consumption	Citrate after incubation
NaCl	0.8	0.6
" + citrate	1.4	17.3
$BaCl_2$	1.0	0.8
" + citrate	2.4	17.8
NaCl + phosphate, $MgCl_2$, adenylic acid	1.4	1.0
" + " " " " + citrate	1.6	18.5
$BaCl_2$ + " " " "	2.1	0.7
" + " " " " + citrate	3.6	17.7

formed. This is most probably the result of oxidation of α-ketoglutarate to succinate by the oxalacetate.

(4) 1 α-ketoglutarate + 1 oxalacetate → 1 malate + 1 succinate + 1 bicarbonate

Glutamate, α-ketoglutarate, and acetoacetate, when added separately, cause very little acid formation. When acetoacetate is added along with α-ketoglutarate, there is some acid formation, indicating that acetoacetate can oxidize α-ketoglutarate to succinate.

The acid formation, acetoacetate disappearance, and extra citrate formation of the complete system are in general agreement with the following equation, in which the acid formation to citrate formation ratio is 1 and the

citrate formation to ketone body disappearance ratio is 2. Equation 5 represents the sum of Equations 1 and 4.

(5) 3 oxalacetate + 1 acetoacetate + 1 α-ketoglutarate → 2 citrate + 1 malate + 1 succinate + 1 bicarbonate

Aerobic Balances—Cytochrome *c* was added in the experiments presented in Table VI. Glutamate and α-ketoglutarate are the only substances which cause a marked increase in the oxygen uptake of the system. When oxalacetate is also present, the oxygen uptake is lower, probably because oxalacetate can replace oxygen as an oxidant.

Aerobically the relation of acetoacetate disappearance to citrate formation in the complete system is the same as in the anaerobic experiments, but with α-ketoglutarate alone large amounts of acetoacetate disappear, yet do not appear as β-hydroxybutyrate or as citrate. This fact suggests that the primary reaction is the conversion of acetoacetate to an intermediate compound which condenses with oxalacetate, if present, to form citrate.

Influence of Oxygen—Wieland and Rosenthal (2) reported that oxygen was necessary for citrate formation from oxalacetate and acetoacetate. Weil-Malherbe (4) found no citrate formation from acetoacetate with tissue slices anaerobically. The fact that the reaction occurred anaerobically in our experiments is probably due to the excess oxalacetate used, which acted as an oxidant. In some of our experiments with small amounts of oxalacetate and crude homogenates very little citrate was formed anaerobically. These results can be explained by the fact that a large amount of oxalacetate is removed by reduction and by decarboxylation, for we have found that if enough oxalacetate is added the reaction proceeds well anaerobically.

Aerobically, considerable amounts of acetoacetate disappear in all samples which have α-ketoglutarate added; *i.e.*, in all samples in which α-ketoglutarate oxidation is taking place. The disappearance may not always equal that in the complete system, but it is much greater than the controls with acetoacetate alone or with acetoacetate plus oxalacetate. Anaerobically, acetoacetate disappears (over and above that which is reduced) only when oxalacetate and α-ketoglutarate are both present and not with addition of α-ketoglutarate alone. This is because anaerobically α-ketoglutarate is oxidized to a significant extent only in the samples containing the excess oxalacetate. It seems clear that the removal of acetoacetate is dependent on α-ketoglutarate oxidation. If molecular oxygen is not available, oxalacetate can act as the oxidant in this reaction.

Action of Inhibitors—The extra citrate formation caused by the addition of acetoacetate to the otherwise complete system was not influenced by 0.01 M malonate and cyanide or 0.02 M fluoride. Azide in 0.01 M concentration and 0.001 M iodoacetate produced 20 to 25 per cent inhibition. The

system was completely inhibited by 0.01 M iodoacetate, selenite, and arsenite.

Action of Barium Ions—Barium ions were added in nearly all experiments because Wieland and Rosenthal suggested that they decrease citrate oxidation. However, later experiments indicated that very little citrate was oxidized by the washed kidney preparations with or without barium, as was ascertained by measuring citrate disappearance and oxygen uptake with added cytochrome *c* (Table VII). The barium ions did tend to stimulate acetoacetate disappearance and citrate formation in some of our experiments. Further work will be necessary to determine the mechanism of this effect.

DISCUSSION

Although citrate formation has been followed in most studies of the enzyme citrogenase, it is very probable that an intermediate analogous to *cis*-aconitic acid is the first substance formed from oxalacetic and acetoacetic acids. That *cis*-aconitate or isocitrate and not citrate must be formed first has been demonstrated in the case of pyruvate by Wood *et al.* (20) and indicated in the case of acetoacetate by the work of Buchanan *et al.* (6). *Cis*-aconitate would give rise to isocitrate and the remainder of the tricarboxylic acid cycle would follow from this point (Krebs (21)). The citrate is formed from *cis*-aconitate in a side reaction, especially when the cycle is stopped at isocitrate. Krebs and Eggleston (18) have shown that the equilibrium isocitrate $\rightleftarrows$ *cis*-aconitate $\rightleftarrows$ citrate is such that from 89 to 96 per cent of the material is citrate under different circumstances. For this reason it is possible to use citrate formation to follow what is really *cis*-aconitate formation. Since very little citrate is oxidized by our preparations, the cycle must be blocked at the isocitrate step, presumably because of absence or inactivity of the necessary enzymes.

The experiments show that very little acetoacetate disappears and very little extra citrate is formed when acetoacetate is incubated with washed kidney preparation and oxalacetate. When α-ketoglutarate, glutamate, or glutathione is also added, acetoacetate disappears and extra citrate is formed. Reduction to β-hydroxybutyrate accounts for only a small part of the acetoacetate disappearance.

The increase in oxygen uptake on the addition of α-ketoglutarate indicates that this substance is oxidized by our preparations. Anaerobically in the presence of oxalacetate it produces considerable acid formation which can be explained by oxidative decarboxylation. In this case oxalacetate would be the oxidant. It seems therefore likely that the oxidation of α-ketoglutarate is necessary for the transformation of acetoacetate into citrate in our preparations. The activity of glutamate is probably due to

conversion to α-ketoglutarate by oxidation or by transamination with some of the oxalacetate. Such reactions proceed very rapidly in kidney. Glutathione is less active than glutamate. It is known (Woodward and Reinhart (17)) that glutathione can be split into cysteine, glycine, and glutamic acid by kidney extracts. Further work is necessary to determine whether the oxidation of other substances can replace α-ketoglutarate oxidation. Buchanan *et al.* (6) reported that a number of substances stimulated the disappearance of acetoacetate, but did not determine whether this effect was due to their oxidation or to their conversion to oxalacetate, as was possible with all the substances used.

It appears that citrate can be formed by two mechanisms: (*a*) from oxalacetate and pyruvate, (*b*) from oxalacetate and acetoacetate with the simultaneous oxidation of α-ketoglutarate. Either two entirely different mechanisms operate or a common substance formed from pyruvate and from acetoacetate condenses with oxalacetate to form the precursor of citrate. Krebs (21) has suggested and Martius (3) has supported the idea that a reactive 2-carbon compound formed by the oxidation of pyruvate, and not pyruvate itself, condenses with the oxalacetate. It seemed possible that acetyl phosphate might be the reactive 2-carbon compound, since it can be formed in pyruvate oxidation (Lipmann (22)) and might arise from acetoacetate in a reaction coupled with α-ketoglutarate oxidation. Ochoa (23) has shown that this oxidation can produce phosphate esterification. Acetyl phosphate was tested, but it did not produce any citrate formation. Addition of phosphate or adenosine triphosphate produced no increase in citrate formation; yet the intervention of phosphate in the reaction has not been entirely ruled out, for the amount of phosphate in the system was 3 to 4 $\times$ 10^{-3} M, sufficient for α-ketoglutarate oxidation to be 75 per cent of its maximum (23).

Although the trials with acetyl phosphate were negative, there is still a strong suggestion that α-ketoglutarate oxidation may be coupled with changes in the acetoacetate which prepare it for condensation with oxalacetate. In aerobic experiments without oxalacetate, α-ketoglutarate was oxidized and acetoacetate disappeared, but it did not appear either as β-hydroxybutyrate or as citrate. Under such conditions an unstable intermediate 2-carbon compound might be hydrolyzed to acetate.

Acetate is rapidly metabolized by kidney tissue and it has been suggested that it enters the tricarboxylic acid cycle (21). Rittenberg and Bloch (24) have recently provided additional evidence in favor of this idea. Acetate might enter the cycle directly or it might be synthesized to acetoacetate before conversion to citrate. Medes *et al.* (25) have recently demonstrated the synthesis of acetoacetate from acetate in kidney tissue. Acetate did not increase citrate formation in our preparations, but this could have been due to inactivity of the necessary enzymes.

SUMMARY

The formation of citric acid from acetoacetic acid and oxalacetic acid in kidney tissue has been studied. The necessary enzymes were found in the insoluble particles from homogenized kidney cortex. Citric acid was not oxidized by these preparations.

When oxalacetic acid and acetoacetic acid were added to the washed tissue particles, very little acetoacetic acid was removed and very little extra citric acid was formed unless α-ketoglutaric acid, glutamic acid, or glutathione was also added. The activity of glutamic acid is believed due to conversion to α-ketoglutaric acid. The activity of glutathione is believed due to hydrolysis to yield glutamic acid.

The evidence indicates that α-ketoglutaric acid was oxidized in both the aerobic and in the anaerobic experiments. In the first case oxygen was the ultimate oxidant; in the second case excess oxalacetic acid acted as the oxidant.

Since the conversion of acetoacetic acid to citric acid occurred only in experiments in which α-ketoglutaric acid (or glutamic acid) was being oxidized, this conversion must be linked to α-ketoglutaric acid oxidation in the preparations used.

Balance experiments with determinations of citric acid, acetoacetic acid, β-hydroxybutyric acid, oxygen consumption aerobically, and acid formation anaerobically were carried out. The results were essentially the same aerobically and anaerobically. About 2 molecules of extra citric acid were formed for each molecule of acetoacetic acid which disappeared. Reduction to β-hydroxybutyric acid did not account for the disappearance of acetoacetic acid. Approximately one extra acidic group was formed for each molecule of citric acid formed from pyruvic acid or from acetoacetic acid.

In aerobic experiments without oxalacetic acid there is still a removal of ketone bodies which is dependent on α-ketoglutaric acid oxidation, but very little citric acid is formed. It is suggested that the primary reaction may be the conversion of acetoacetic acid to an intermediate compound which condenses with oxalacetic acid, if present, to form the precursor of citric acid.

Modified methods are described for the estimation of citric acid and for acetoacetic acid in the presence of oxalacetic acid.

The authors are greatly indebted to Professor Carl F. Cori and Dr. S. P. Colowick for many valuable suggestions and criticisms.

BIBLIOGRAPHY

1. Breusch, F. L., *Science*, **97**, 490 (1943).
2. Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).

3. Martius, C., *Z. physiol. Chem.*, **279,** 96 (1943).
4. Weil-Malherbe, H., *Nature*, **153,** 435 (1944).
5. Krebs, H. A., and Eggleston, L. V., *Nature*, **154,** 209 (1944).
6. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **157,** 747 (1945).
7. Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, **35,** 595 (1941).
8. Ljunggren, G., *Biochem. Z.*, **145,** 422 (1924).
9. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153,** 571 (1944).
10. Robertson, W. B., *Science*, **96,** 93 (1942).
11. Edson, N. L., *Biochem. J.*, **29,** 2082 (1935).
12. Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, **140,** 5 (1941).
13. Krebs, H. A., *Biochem. J.*, **36,** 303 (1942).
14. Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, **113,** 235 (1936).
15. Goldberg, A. S., and Bernheim, A. R., *J. Biol. Chem.*, **156,** 33 (1944).
16. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114,** 495 (1936).
17. Woodward, G. E., and Reinhart, F. E., *J. Biol. Chem.*, **145,** 471 (1942).
18. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **37,** 334 (1943).
19. Krebs, H. A., Eggleston, L. V., Kleinzeller, A., and Smyth, D. H., *Biochem. J.*, **34,** 1234 (1940).
20. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139,** 483 (1941).
21. Krebs, H. A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3,** 191 (1943).
22. Lipmann, F., *Federation Proc.*, **1,** pt. 2, 122 (1942).
23. Ochoa, S., *J. Biol. Chem.*, **155,** 87 (1944).
24. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **157,** 749 (1945).
25. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157,** 751 (1945).