CII. ACETOPYRUVIC ACID ($\alpha\gamma$ -DIKETOVALERIC ACID) AS AN INTERMEDIATE METABOLITE IN ANIMAL TISSUES

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I. INTRODUCTION

IN a previous paper [1937] we have shown that muscle and other tissues are capable of converting pyruvic acid into β -hydroxybutyric acid. The present paper is concerned with the intermediate stages of this reaction.

That ketone bodies are formed from 2-carbon and 3-carbon compounds was first demonstrated in the case of liver tissue by the work of Embden and his school. Embden & Oppenheimer [1913] showed that perfusion of liver with pyruvic acid gives rise to an increased formation of acetoacetic acid. Annau [1934] and Edson [1935], working with chopped and sliced liver, confirmed this result. Loeb, in Embden's laboratory, showed [1912] that acetic acid, too, increases the yield of acetoacetic acid in liver, an effect which is pronounced, according to Friedmann [1913], in the liver of starved animals. Weil-Malherbe reported recently [1936] formation of acetoacetic acid from pyruvic acid in brain tissue.

There is thus no doubt that "synthetic" formation of ketone bodies takes place in animal tissues. It follows from our previous paper that the quantitative role of ketogenesis in intermediate metabolism is very considerable, since under some conditions almost the whole of the pyruvic acid which undergoes oxidation may be converted into β -hydroxybutyric acid.

Previous attempts [see Loeb, 1912; Friedmann, 1913; Annau, 1934] to explain the mechanism of ketogenesis from pyruvic and acetic acids were merely speculative. Our own experiments do not support any of the previous assumptions, as will be shown in section VII of this paper. On the other hand the experiments reported in this paper suggest that acetopyruvic acid ($\alpha\gamma$ -diketo-*n*-valeric acid) is an intermediate stage according to the following scheme (1):

The scheme suggests that the first step in ketogenesis is condensation of acetic and pyruvic acids giving rise to acetopyruvic acid. From acetopyruvic acid ketone bodies may arise in two different ways. Thus, by oxidative decarboxylation (reaction II) acetoacetic acid may be formed, or by an intramolecular dismutation (reaction III) β -hydroxybutyric acid may be produced.

It is certain, as will be shown in this paper, that both ketone bodies are formed from acetopyruvic acid, but it is not yet possible to decide whether acetopyruvic acid reacts according to (II), or to (III) or in both ways. Since acetoacetic and β -hydroxybutyric acids are readily interconvertible, as suggested by reaction (IV), the net effect of (III) can also be brought about by (II) + (IV), and that of (II) by (III) + (IV).

The evidence leading to scheme (1) is drawn from experiments in which the rate of ketogenesis was measured under varying conditions. The main new observation is the fact that in animal tissues acetopyruvic acid is rapidly converted into ketone bodies. Under aerobic conditions the main product is acetoacetic acid, under anaerobic conditions β -hydroxybutyric acid. The rate of reaction as compared with the rate of ketone body formation from pyruvic and acetic acids complies with the conditions expected from an intermediate.

II. METHODS

(1) *Acetopyruvic acid*. Sodium ethyl acetopyruvate was prepared according to Claisen & Stylos [1887], the free acid being obtained therefrom according to Mumm & Bergell [1912] and recrystallized from benzene. The m.p. was 98°. Acetopyruvic acid reacts readily with yeast carboxylase, the rate of decarboxylation being about a third of that of pyruvic acid. Since the yield of CO_2 is practically quantitative (see Table I), carboxylase may be used for the quantitative determination of acetopyruvic acid. Suitable experimental conditions are those described by Westerkamp [1933] and Krebs & Johnson [1937].

Table I. *Decarboxylation of acetopyruvic acid by yeast extract*

(Manometric experiment; 25°. The cup was filled with 1 ml. 0.00946 *M* acetopyruvate, 2 ml. H_2O and 0.2 ml. 3*M* acetate buffer (pH 5.0). The side-arm contained 0.8 yeast extract (Lebedev).)

Time (min.)	$\mu\text{l. CO}_2$ formed after addition of the yeast
10	98
20	156
50	181
180	205*

* Calculated for complete decarboxylation 212 $\mu\text{l.}$

(2) *Determination of ketone bodies*. Acetoacetic acid was determined by the aniline method described previously [Ostern, 1933; Edson, 1936]. Controls showed that acetopyruvic acid does not react or interfere. β -Hydroxybutyric acid was determined by the Van Slyke-Denigès method as modified by Edson. The solution was boiled for one hour before the addition of dichromate and the precipitate formed was discarded. The filtrate gave small blanks when acetopyruvic acid was present. For 100 mol. of acetopyruvate present in the original solution, a precipitate was formed on addition of dichromate equivalent to 6–7 mol. β -hydroxybutyric acid. These blanks have been deducted.

(3) *The manometric technique and tissue slice method* were employed in the usual manner. The tissue was suspended in saline buffered by bicarbonate- CO_2

or phosphate. Controls were set up in all experiments to determine the non-enzymic reactions of acetopyruvic acid (see section VIII). The results are expressed in the usual notation.

III. FORMATION OF ACETOACETIC ACID FROM ACETOPYRUVIC ACID

If acetopyruvic acid is an intermediate as indicated by the scheme (1) it should yield acetoacetic acid under conditions in which acetic and pyruvic acids yield acetoacetic acid. That this is the case follows from Table II. Experiments are recorded in this table in which the formation of acetoacetic acid by

Table II. *Formation of acetoacetic acid in liver in the presence of acetopyruvic, pyruvic and acetic acids (1-6, rat liver; 7, pigeon liver)*

Final concentrations of the added substrates 0.01 M.			
No.	Medium	Substrates added	$Q_{\text{Acetoacetic acid}}$
1	Phosphate saline; 0.02 M NH_4Cl	—	0.37
		Pyruvate	1.20
		Acetopyruvate	1.63
2(a)	Phosphate saline	—	0.37
		Pyruvate	0.88
		Acetopyruvate	1.40
(b)	Phosphate saline; 0.02 M NH_4Cl	—	0.52
		Pyruvate	2.82
		Acetopyruvate	2.03
3	Phosphate saline; 0.02 M NH_4Cl	—	0.63
		Acetate	1.87
		Pyruvate	1.81
		Acetopyruvate	2.26
4	Phosphate saline; 0.02 M NH_4Cl	—	0.61
		Acetate	0.88
		Pyruvate	3.01
		Acetopyruvate	1.41
		Acetate + pyruvate	2.85
5	Bicarbonate saline; 0.02 M NH_4Cl	—	0.93
		Acetate	1.41
		Pyruvate	1.96
		Acetate + pyruvate	2.24
		Acetopyruvate	1.82
		Acetopyruvate + pyruvate	3.82
6	Bicarbonate saline; 0.02 M NH_4Cl	—	0.76
		Acetate	1.14
		Pyruvate	1.41
		Acetate + pyruvate	1.61
		Acetopyruvate	1.49
		Acetopyruvate + pyruvate	2.90
7	Bicarbonate saline; 0.02 M NH_4Cl	—	0.91
		Acetate	1.53
		Pyruvate	1.42
		Acetate + pyruvate	1.85
		Acetopyruvate	0.88
		Pyruvate + acetopyruvate	1.59

liver tissue in the presence of pyruvic, acetic and acetopyruvic acids was measured. The experimental details are given in Table II. Ammonium chloride was added to the medium because the formation of acetoacetic acid is more marked in the presence of this substance [Annau, 1934; Edson, 1935].

The results of the experiments may be summarized as follows:

(1) Pyruvic acid yields generally more acetoacetic acid than acetic acid. This is evidence against the scheme: $2 \text{ pyruvic acid} \rightarrow 2 \text{ acetic acid} \rightarrow \text{acetoacetic acid}$.

(2) Acetic and pyruvic acids, if present simultaneously, frequently yield more acetoacetic acid than each of the single substrates (e.g. Nos. 6 and 8). This fact may be interpreted in two different ways. It could be assumed that ketone bodies arise from acetic and from pyruvic acid by entirely different independent reactions occurring simultaneously when the two substrates are present. We consider, however, another explanation to be more likely and assume that the two substrates act jointly in the way indicated by scheme (1).

(3) In the presence of acetopyruvic acid, more acetoacetic acid is usually formed than in the presence of acetic acid and about the same amount as in the presence of pyruvic acid. Acetopyruvic and pyruvic acids together yield more acetoacetic acid than the single substrates. This latter fact suggests that acetopyruvic acid, like other ketonic acids, is broken down by dismutation with pyruvic acid, according to the reaction: $\text{acetopyruvic acid} + \text{pyruvic acid} + \text{H}_2\text{O} = \text{acetoacetic acid} + \text{lactic acid} + \text{CO}_2$.

In summing up the conclusions drawn from Table II we may say that acetopyruvic acid causes formation of acetoacetic acid, and the velocity of this reaction complies with the scheme (1), according to which acetopyruvic acid is an intermediate stage in ketogenesis from acetic and pyruvic acids.

The evidence so far adduced is however not conclusive. It has to be borne in mind that the formation of acetoacetic acid in liver in the presence of an added substance does not necessarily prove conversion of the added substances into acetoacetic acid. Experiments with ammonium salts [Annau, 1934; Edson, 1935] or malonate [Momose, 1934; Szent-Györgyi, 1935; Jowett & Quastel, 1935] make this point clear. Ammonium salts or malonate, if added to liver, increase the formation of acetoacetic acid. Since malonic acid is not metabolized in liver it is evident here, as in the case of ammonium salts, that the increased yield of acetoacetic acid cannot be due to conversion of the added substance into acetoacetic acid.

It is, however, very unlikely that acetopyruvic acid causes a "toxic" ketogenesis like malonate, since acetopyruvic acid, unlike malonate, is actually metabolized. Parallel with ketone body formation there is disappearance of acetopyruvic acid and production of CO_2 . Moreover, benzoylpyruvic acid, which might be expected to have toxic effects if acetopyruvic acid were toxic, was found to be non-ketogenic in liver tissue.

IV. FORMATION OF β -HYDROXYBUTYRIC ACID FROM ACETOPYRUVIC ACID

If the conditions are anaerobic, acetoacetic acid undergoes reduction to β -hydroxybutyric acid in liver and other tissues. It would be expected, therefore, that if ketone bodies were formed anaerobically, β -hydroxybutyric acid would be the main product. Indeed, if pyruvic acid is added anaerobically, considerable quantities of β -hydroxybutyric acid are formed but only traces of acetoacetic acid. Data concerning the rates of formation of β -hydroxybutyric acid in various tissues have been published in the preceding paper of this series.

Acetopyruvic acid, if an intermediate in ketogenesis from pyruvic acid, should also yield β -hydroxybutyric acid in the absence of molecular oxygen. Experiments recorded in Table III show that β -hydroxybutyric acid is in fact formed in the presence of acetopyruvic acid.

Table III. *Formation of β -hydroxybutyric acid in the presence of acetic, pyruvic and acetopyruvic acids under anaerobic conditions*

Medium: bicarbonate saline

No.	Tissue	Substrates added (final concentration 0.01 M)	$Q_{\beta\text{-Hydroxybutyric acid}}$
1	Rat liver	—	0.01
		Acetate	0.01
		Pyruvate	1.25
		Acetopyruvate	1.64
		Acetate + pyruvate	1.44
2	Rat liver	—	0.16
		Pyruvate	1.22
		Acetopyruvate	1.30
3	Rat liver	—	0.13
		Acetopyruvate	0.54
4	Pigeon breast muscle	—	0.03
		Acetopyruvate	0.64
5	Pigeon breast muscle	—	0.02
		Pyruvate	0.15
		Acetopyruvate	0.42

Furthermore, the following conclusions may be drawn from the experiments:

(1) Acetic acid does not yield β -hydroxybutyric acid under anaerobic conditions. The fact that oxygen is necessary for ketogenesis in presence of acetic acid indicates again that the scheme: 2 acetic acid $\rightarrow$ acetoacetic acid $\rightarrow$ β -hydroxybutyric acid is not valid. Oxygen is necessary, in our view, because it enables the cells to produce pyruvic acid from carbohydrate or lactic acid.

(2) Acetopyruvic acid yields more β -hydroxybutyric acid than pyruvic acid.

V. FORMATION OF CARBON DIOXIDE FROM ACETOPYRUVIC ACID

The formation of CO_2 from acetopyruvic acid is best demonstrated in anaerobic experiments. Aerobically, there is a large production of respiratory CO_2 and the increment expected from decarboxylation of acetopyruvic acid is only 10–20% of the "blank". Anaerobically, the "blank" CO_2 production is almost nil; and the increment can therefore be measured accurately. In

Table IV. *Formation of CO_2 from acetopyruvic acid under anaerobic conditions*

Q_{CO_2} represents the new formation of CO_2 from organic substances, not CO_2 liberated from bicarbonate

	Tissue (wt. in mg.)	Substrate added	Time (min.)	CO_2 formed ($\mu\text{l.}$)	Q_{CO_2}
1	Pigeon breast muscle	—	70	+ 50	0.3
		Acetopyruvate	70	+ 208	1.25
2	Pigeon breast muscle	—	121	+ 11	0.05
		Acetopyruvate	121	+ 163	0.49
3	Rat liver	—	80	— 1	—
		Acetopyruvate	80	+ 68	0.75
4	Rat liver	—	90	— 6.5	0
		Acetopyruvate	90	+ 99.5	1.20
5	Rat liver	—	90	— 0.5	0
		Acetopyruvate	90	+ 292	3.54
6	Rat kidney	—	90	+ 10	0.24
		Acetopyruvate	90	+ 54	0.58

arranging the experiments we have to bear in mind that it is not sufficient to measure the CO_2 output manometrically in the usual way because the routine method if applied in the presence of bicarbonate does not distinguish between the new formation of CO_2 and the liberation of CO_2 from bicarbonate. It is necessary, therefore, to determine the evolution of free CO_2 together with the change in bicarbonate. A suitable experimental arrangement was described in the preceding paper [Krebs & Johnson, 1937]. Data obtained with this method are given in Table IV. In the absence of acetopyruvic acid there is practically no CO_2 production; the small figures obtained are, with the exception of the first experiment, within the limits of error. With acetopyruvic acid, on the other hand, considerable quantities of CO_2 appear and the quantities are of the order expected from the data on ketogenesis.

VI. DISAPPEARANCE OF ACETOPYRUVIC ACID FROM ANIMAL TISSUES

Using the carboxylase method we determined the disappearance of acetopyruvic acid in the presence of various tissues. Table V shows that the quantities which disappear exceed the amounts of ketone bodies formed. This indicates that there are other reactions, in addition to conversion into ketone bodies by which acetopyruvic acid disappears. About the same quantities are utilized aerobically and anaerobically. Addition of glucose slightly increases the rate of disappearance in some tissues.

Table V. *Disappearance of acetopyruvic acid from tissues*

40°. Bicarbonate saline		
Tissue	Experimental conditions	$Q_{\text{Acetopyruvate}}$
Rat liver	Anaerobic	-2.80
"	Anaerobic; 0.02 <i>M</i> glucose	-2.50
Rat testis	Anaerobic	-2.30
"	Anaerobic; 0.02 <i>M</i> glucose	-2.56
Rat kidney	Anaerobic	-4.61
"	Anaerobic; 0.02 <i>M</i> glucose	-4.15
Pigeon brain	Aerobic	-2.56
"	Aerobic; 0.02 <i>M</i> glucose	-3.10
"	Anaerobic	-2.10
"	Anaerobic; 0.02 <i>M</i> glucose	-2.87
Rat liver	Anaerobic	-3.00
"	Aerobic	-2.93

VII. ALTERNATIVE SCHEMES OF KETONE BODY SYNTHESIS

Loeb [1912] suggested that 2 mol. of acetic acid may condense to form acetoacetic acid. Evidence against this assumption is mentioned in sections III and IV of this paper.

Friedmann observed that aldehyde ammonia causes acetoacetic acid formation in liver and assumed [1913] that acetaldehyde may play a part in ketone body synthesis according to the scheme:

Crotonic acid formed first according to this scheme is known to be readily converted into acetoacetic acid in liver.

We find, however, that pure acetaldehyde is not ketogenic; the ketogenic effect of aldehyde ammonia must be ascribed to the ammonia [Edson, 1935].

Moreover, aldehyde does not seem to be formed in any significant amounts in animal tissues. There is thus no evidence left supporting scheme (2).

Annau [1934] assumed that *parapyruvic acid* [Wolff, 1899] may act as intermediate in ketogenesis. We tested this view by adding *parapyruvate*; prepared according to Wolff, to liver slices. The substance does not yield acetoacetic acid; therefore it cannot be an intermediate.

VIII. NON-ENZYMIC REACTIONS OF ACETOPYRUVIC ACID

Experiments with acetopyruvic acid are complicated by the fact that this diketonic acid is a very reactive substance which reacts in neutral solutions and at blood temperature with various substances occurring in cells or in the saline medium. We have examined some of these reactions in so far as they may interfere with the manometric methods employed.

(1) *Reactions with inorganic ions.* If a neutral solution of sodium acetopyruvate is added to a physiological salt solution, or to pure solutions of NaHCO_3 , a considerable quantity of CO_2 appears on mixing, while the concentration of bicarbonate decreases. The quantity of CO_2 evolved is greater if the solution contains, in addition to Na, bivalent cations, Ca or Mg. The evolution of CO_2 ceases after a few minutes. Table VI gives a few data showing the magnitude of this effect.

Table VI. CO_2 evolved on mixing a neutralized solution of sodium acetopyruvate with bicarbonate-containing solutions

Manometric experiments: the solutions were in equilibrium with 5% CO_2 in N_2 . 40°

Solution (3 ml.)	Acetopyruvate added	CO_2 evolved ($\mu\text{l.}$)
0.04 M NaHCO_3	0.1 ml. 0.2 M	20
0.13 M MgSO_4 ; 0.04 M NaHCO_3	0.1 ml. 0.1 M	162
Physiological saline with 0.04 M NaHCO_3	0.3 ml. 0.1 M	167

The reaction is probably due to the formation of complex compounds of the type described for other diketones [see Sidgwick, 1929]:

In this reaction basic equivalents are bound and a result is an increase in acidity. The determination of the carbon dioxide may be used as a measure of the extent to which basic equivalents are converted into a complex form.

(2) *Reactions with nitrogenous substances.* If sodium acetopyruvate is added to solutions which contain NaHCO_3 (0.03 M) and glutamic acid (0.5 M) an evolution of CO_2 ensues which continues over a long period of time. Parallel with the CO_2 evolution there is an equivalent decrease in bicarbonate. We have not yet studied this reaction in detail. These non-enzymic reactions of acetopyruvic acid whatever be their mechanism, have to be taken into account in manometric experiments on the intermediary metabolism of the substance.

SUMMARY

1. Acetopyruvic acid ($\alpha\gamma$ -diketo-*n*-valeric acid) is metabolized in animal tissues (liver, muscle, kidney, testis, brain).
2. Acetoacetic acid is shown to be formed in liver, if the conditions are aerobic.
3. β -Hydroxybutyric acid and carbon dioxide are shown to be formed if the conditions are anaerobic.
4. Ketone bodies are formed more rapidly from acetopyruvic acid than from acetic and pyruvic acids. It is therefore justifiable to assume that aceto-pyruvic acid is an intermediate stage in the synthesis of ketone bodies from acetic and pyruvic acids as suggested by scheme (1).

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