METABOLISM OF ACETOIN

IN MAMMALS

GABRIEL

METABOLISM OF ACETOIN IN MAMMALS

By

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Thus he whoever seaketh the pursuit of

Chemistry

wasteth his labor and

loseth his richess

IBN KHALDUN

MUQADDAMEH Chapter 33

A C K N O W L E D G M E N T

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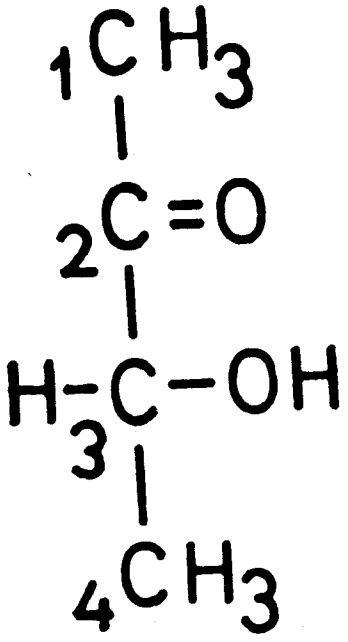
CHAPTER I

INTRODUCTION

Acetoin (acetyl methyl carbinol, 3 hydroxy 2 butanone) (fig. 1), has long been known to be a product of metabolism in living organisms. Widely distributed in nature, it is found in good amount in many foodstuffs of animal or plant origin. Alcoholic beverages, being products of fermentation contain appreciable quantities of acetoin. All wines contain amounts varying from 2 to 20 µg/l (1, 2). Butter, sour cream, and other milk products depending on storage conditions produce various amounts of acetoin due to bacterial fermentation (3). Acetoin is also considered as a natural constituent of wheat bread, the content depending on various factors, and on storage (4). In roasted coffee acetoin was detected in concentration of 15 µg/g. About one half of the diacetyl content is reduced to acetoin during staling, which may explain some loss of aroma (5). More generally, whenever fermentation occurs, we can detect the presence of acetoin.

A. Formation of acetoin

In animals pyruvic acid is metabolized through four major pathways, to lactate, acetyl CoA, alanine and four carbon dicarboxylic acids. Other pathways of



Acetoin

FIGURE 1. ACETOIN

metabolism of pyruvic acid do exist, one of which is acetoin formation: two pyruvates will condense giving acetoin and CO_2.

$$2CH_3CO\text{-}COOH \longrightarrow CH_3\text{-}CO\text{-}CHOH\text{-}CH_3 + 2CO_2$$

Experimentally the injection of pyruvate causes an increased amount of acetoin to appear in the blood.

The oxidation of alcohol, usually proceeds through the intermediate formation of acetaldehyde. Normally, acetaldehyde is further oxidized to acetate. However, as a minor pathway in acetaldehyde metabolism, it is converted to acetoin.

Experimentally, Berry and Stotz (6) found that the level of acetoin in the brain of intact male rats was elevated by the intraperitoneal injection of acetaldehyde. Again, more recently (7), it has been found that the formation of acetoin by brain mitochondria was proportional to the amount of acetaldehyde in the medium.

Mechanism of acetoin formation. The first step in pyruvic acid oxidation to Acetyl CoA is the well known carboxylase reaction. The enzyme has been isolated from pig heart and from pigeon breast muscle.

Carboxylase can act on pyruvic acid in the presence of thiamine pyrophosphate and divalent metallic ion (Mn^{++}) to give an "active acetaldehyde." The "active acetaldehyde"

was proved by enzymatic, and chemical procedures to be DL-α-hydroxyethyl 2 thiamine pyrophosphate (8). This aldehyde thiamine pyrophosphate complex can react in various ways to give active acetate, acetoin, and other products.

The formation of acetoin is postulated to involve the condensation of free acetaldehyde with the active acetaldehyde complex. The free acetaldehyde can be the result of dissociation of the complex (9) or can result from action of alcohol dehydrogenase on ethanol.

The mechanism of the condensation was studied by Juni (10) who used pyruvic-2-C^{14} and unlabelled acetaldehyde as substrates. He showed that pyruvic acid is decarboxylated giving CO_2 and forming carbon 1 and 2 of acetoin.

$$CH_3CO\text{-}COOH \xrightarrow{TPP} (CH_3CHO)\ TPP + CO_2$$

$$(CH_3CHO)\ TPP \xrightarrow{CH_3CHO} CH_3CO\text{-}\dot{C}HOH\text{-}\dot{C}H_3$$

Another mechanism of active acetaldehyde condensation also exists in living systems. The active acetaldehyde can react with pyruvic acid giving a condensation product α-acetolactic acid. This product was shown to occur when a large excess of pyruvic acid relative to acetaldehyde was present in pig heart preparations (11)

$$\text{"}CH_3CHO\text{"} + CH_3CO\text{-}COOH \longrightarrow CH_3\underset{\displaystyle COCH_3}{\underset{|}{C}}OH\text{-}COOH$$

In some bacteria, but not in yeast and pig heart preparations, decarboxylation of α-acetolactic acid is the major channel for the synthesis of acetoin (12-13).

$$CH_3\text{-}\underset{\underset{CO\text{-}CH_3}{|}}{C}O\text{-}COOH \longrightarrow CH_3CO\text{-}CHOH\text{-}CH_3 + CO_2$$

Bacterial extracts that do not decarboxylate α-acetolactic acid are incapable of acetoin formation (12). Whether α-acetolactic acid is capable of decarboxylation in mammals is unknown. It was not detected in pig heart preparation (11) but other tissues were not studied.

In 1947 Green and co-workers (14) were able to isolate an enzyme, diacetyl mutase, from pigeon breast muscle which catalyzes the reaction:

$$2CH_3CO\text{-}CO\text{-}CH_3 \longrightarrow CH_3CO\text{-}CHOH\text{-}CH_3 + CH_3COOH$$

Furthermore, minced preparations of pig heart containing diacetylmutase activity, were incubated with cocarboxylase and (Mn^{++}) using either acetaldehyde-1-2-C^{14} with unlabelled diacetyl as substrate. From the total yield and the total radioactivity of acetoin formed, it was concluded that the major reaction is a transcarboxylation (15).

$$CH_3CO\text{-}CO\text{-}CH_3 + CH_3\overset{\bullet}{C}H\overset{\bullet}{O} \longrightarrow CH_3COOH + CH_3CO\text{-}\overset{\bullet}{C}HOH\text{-}\overset{\bullet}{C}H_3$$

Later on Mizuha and Handler (16) confirmed the mechanism of transcarboxylation reaction. They found that a high concentration of thiamine in alkaline solution, non-enzymatically catalyzes several reactions of carbonyl compounds known to occur in biological systems. Among them, the formation of acetoin and acetate from acetaldehyde and diacetyl. The reaction was proved not to be an oxidation reduction reaction but one that involves the scission of diacetate into two moieties, one of which reacts with acetaldehyde to give acetoin.

Acetoin production was estimated in animals using two enzyme systems: a crude pig heart preparation and a purified pigeon breast muscle preparation both of which utilize pyruvate (17). In the first system the proportion of acetoin formed, to pyruvate utilized was 1.6% while in the second system 23% of the pyruvate ended up in acetoin. These studies suggest that acetoin is produced in appreciable amount in animals.

Bacteria can synthesize acetoin from sources other than pyruvic acid. It has been found that Aerobacter aceti can synthesize acetoin from lactic and succinic acids (18). Again, the same bacteria were able to produce acetoin in lesser quantities from fumaric and oxaloacetic acids (19).

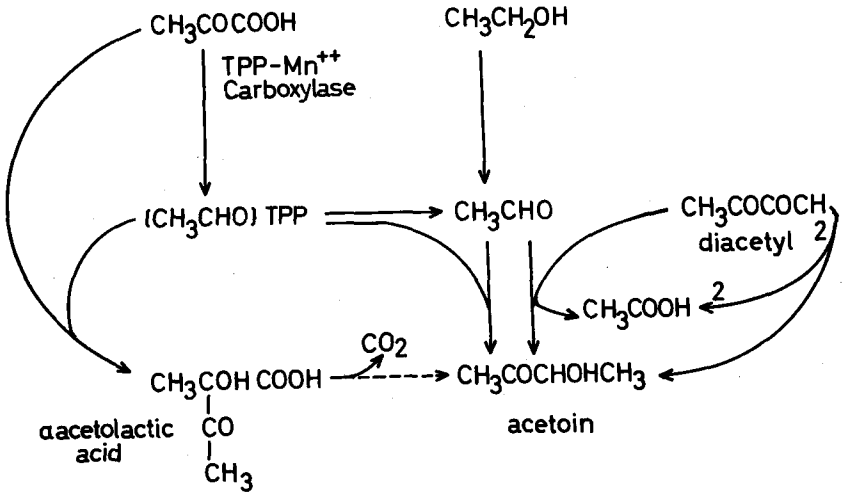
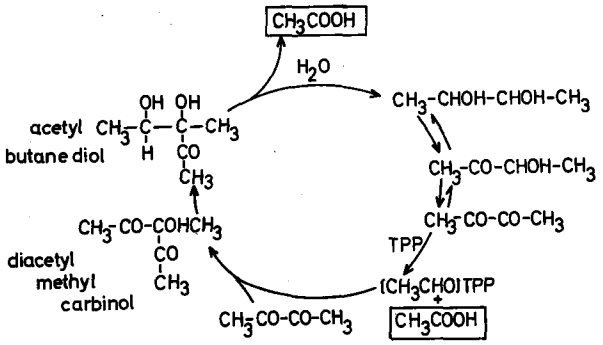


FIGURE 2. ACETOIN FORMATION IN MAMMALS. THE DECARBOXYLATION OF α-ACETOLACTIC ACID (BROKEN LINE) IS ONLY KNOWN IN BACTERIA.

B. Metabolism of acetoin

Acetoin is metabolized by many types of living cells through different pathways.

In bacteria: The metabolism of acetoin in different species has been investigated by different workers. Early studies showed that Bacillus megatherium can convert 2,3-butanediol to B-hydroxybutyric acid (20). Later on the same authors (21) showed that acetoin in some bacteria is not an intermediate in glycolysis but a product of a side reaction which may substitute as a source of carbon and energy. In 1953 Aubert and Gavard (22) demonstrated the existence of a dehydrogenase system which catalyzes the oxidation of 2,3-butane diol to acetoin in Neisseria winagradskyi. The most widely accepted mechanism is the one proposed by Juni and Heym in 1956 (23) who demonstrated that a number of bacteria are capable of using acetoin and its reduction product 2,3-butanediol as the sole source of carbon and energy. These bacteria develop a cycle pathway named the "2,3-butanediol pathway" capable of converting acetoin and butanediol to acetic acid (Fig. 3). These organisms possess an enzyme that can oxidize butane-diol to acetoin followed by another oxidation step to diacetyl. Diacetyl is then converted to diacetyl methyl carbinol, and acetate by a diphosphothiamine acyloin condensation reaction. The diacetyl methyl carbinol is further metabolized to acetyl butanediol which is



Butane 2,3 Diol Cycle

FIGURE 3. 2,3-BUTANEDIOL CYCLE.

hydrolyzed to 2,3-butanediol and acetic acid. All these reactions can occur anaerobically. The same authors studied the stoichiometry of the enzyme involved in the acyloin condensation, showing the specificity, the inhibitors and the co-factor for this enzyme (24). Another study (25) was done on the dehydrogenase involved in this cycle demonstrating that a single enzyme acted as 2,3-butanediol dehydrogenase and diacetyl reductase. Recently (26) the metabolism of acetoin in some bacteria was proved to be discontinuous. These bacteria were only capable of producing and metabolizing acetoin at certain stages of their growth.

Other living organisms like filaria Limnoides garnii (27) can synthesize acetoin from acetaldehyde and pyruvate and utilize the l form much faster than the other form of acetoin.

In mammals: In studying the elimination of ethyl alcohol and its metabolites, Greenberg (28) fed acetoin orally to a dog and followed its fate in blood and urine. Doses of 1 g/kg and 2 g/kg body weight were given. The concentrations in blood after one hour were 64-71 mg/100 ml; and 193-250 mg/100 ml respectively. After nine hours both levels were down to 1 mg/100 ml. The control blood before feeding contained no detectable acetoin. Acetoin appeared in the urine in a concentration of 1.2 that of the blood,

but the percentage loss of the administered dose in the urine was small. Also, acetoin was found to diffuse to the intracellular as well as the extracellular fluids.

In 1955 Järnefelt (29) studied the metabolism of acetoin and diacetyl in rat liver. He found that liver slices were able to metabolize both acetoin and diacetyl, while homogenates were unable to utilize acetoin, they could convert diacetyl to acetoin.

C. Pathologically

Variations in the metabolism of acetoin were observed in many pathological conditions: diabetes, panthothenic acid deficiency, liver damage, and various mental states.

Diabetes. Alloxan diabetic rats (30) were found to have an increase in urinary excretion of total acetoin (detected as the sum of acetoin and diacetyl). This elevation was later confirmed in diabetic patients. Also, following the administration of acetaldehyde to alloxan diabetic rats, blood acetaldehyde and acetoin levels were raised, and liver acetoin level decreased.

Experimental avitaminosis. Experimental avitaminosis produces a fluctuation in acetoin level. The amount of acetoin and diacetyl in the liver were markedly increased in mice by feeding a panthothenic acid deficient diet. The amounts of pyruvate and x-ketoglutarate also increased but

to a lesser degree. Administration of large amounts of fructose, succinate and isonicotinic acid hydrazide to man increased the concentration of acetoin and diacetyl in urine (31). This is expected, since panthotenic acid constitutes an integral part of CoA which is required for further oxidation of pyruvic acid through the tricarboxilic acid cycle.

Kidney and liver disease. In uremia and hepatic coma the blood levels of a acetoin and 2,3-butylene glycol were studied intensively by a group of Swiss workers (32-35). They found an increase in both acetoin and 2,3-butylene glycol in patients with kidney disease when psychic symptoms were present. In liver disease an elevation of both acetoin and butylene glycol was detected only in commatose states where values as high as 263 ug/100 ml for acetoin and 7480 ug/100 ml for butylene glycol were found. (Normal levels were estimated to be 14 ± 11 and 125 ± 57 ug/100 ml for acetoin and butylene glycol respectively) (36). Also, when consciousness was disturbed acetoin and 2,3-butylene glycol in blood increased, but there was no correlation between blood levels of these compounds, and the degree of impairment of consciousness.

Mental states. Acetoin levels in blood are said to be high in patients with manic depressive psychosis: In 96 non fasted patients with manic depressive psychosis the average concentration of acetoin in blood was higher and variation was greater than in 45 control persons. The acetoin

level was increased, and the concentration of 2,3-butanediol was decreased in normal persons by fasting. In fasted patients in the depressed phase acetoin increased with no significant change in 2,3-butanediol. In the manic phase acetoin decreased, and 2,3-butanediol increased (37, 38).

D. Scope of the investigation

The widespread existence of acetoin in living tissues, its appreciable rate of formation by muscles in mammals as well as its fluctuation, in many pathological states stimulated us to look for its fate in systems not investigated intensively in the past.

By the use of acetoin-1-C^{14} as a tracer, we tried to study the metabolism of acetoin in mammals as well as isolating and identifying its metabolites.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Acetoin-1-C^{14}. Acetoin-1-C^{14} was synthesized in our Laboratory by Ali and Al-Khalidi (39) according to the procedure of Brady, Rabinowitz, Van Baaler and Gurin (40).

2. Laboratory animals. Albino rats of the Wistar strain were obtained from the Animal House of the Medical School of the American University of Beirut. The guinea pigs were obtained from a local farm.

3. Chemicals. Chemicals used were B.P. or U.S.P. unless otherwise specified in the text.

B. Counting of radioactivity

Three methods of counting radioactivity were used:

1. 0.2 ml of the sample was placed on a stainless steel planchet, dried under an infra red lamp or in a desiccator under vacuum, and counted in a Baird Atomic gas flow counter with an efficiency of 25% and a background counting rate of 20 counts/minute. When necessary, these counts were corrected for selfabsorption using a standard curve.

2. For counting of solutions 0.1 ml of the water solution was introduced in a 20 ml scintillation glass vial. 3 ml of dioxane were mixed with the aquous solution to which 15 ml of scintillation mixture were added forming a very fine emulsion. The emulsion formed was counted immediately for five minutes in a Packard semi-automatic tri carb scintillation spectrometer model 3003. A gain of 6.5 and a window of 50 to 500 were found to be appropriate for C^{14} counts with a background of 12 counts per minute and an efficiency of 72%. Corrections for quenching were made for concentrated and colored solution by the internal standard method.

The same procedure was followed for counting ethanol or ether solutions, except that dioxane was omitted. The same gain and window was used resulting in a similar efficiency and background rate.

3. For counting on paper strips the sample solution was applied on a 1 x 1.5 inch 3 mm Whatman paper and then dried in a dessicator. The paper was put vertically in a 20 ml scintillation glass vial filled with 20 ml scintillation mixture. A gain of 6.5 and a window of 50 to 500 were found to be appropriate for C^{14} counting, with a background of 12 counts per minute.

Scintillation mixture. 50 mg of dimethyl POPOP /1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene (scintillation grade)/ and 4 grams of PPO /2,5-Diphenyloxazole (scin-

tillation Grade)/ were dissolved in one liter toluene laboratory reagent. The solution was kept in a dark bottle at room temperature.

C. Chemical and physical tests

1. Iodine value. The pyridine bromide method was performed (41) on a microscale, using a syringe micro burette (Micrometric Instrument Co., Cleveland, Ohio).

2. Potentiometric titration. The substance was introduced in a 5 ml beaker, having a micro bar magnet, and adapted over a magnetic stirrer. Two micro electrodes were dipped in the solution and a dry stream of CO_2 free nitrogen was blown on the surface. The solution was titrated against normal sodium hydroxide in a micro syringe burette. Potassium acid phthalate was used as a standard. Very dilute hydrochloric acid was used as a water blank.

3. Infra red and ultra violet spectrum. A Perkin Elmer infra red spectrometer model 237 was used. The I.R.s were run in ether solutions dried with sodium sulfate and in chloroform solutions. The ultra violet spectra were run in water solution on a Bausch and Lomb spectrometer Model 505.

4. Ascending paper chromatography. On a 3 mm Whatman paper three spots were applied: acetoin-1-C^{14}, the tested compound and a mixture of acetoin and the compound

to be tested. The solvent used was composed of n-propanol: water:diethylamine:85:15:1. The chromatogram was counted by cutting the paper 1 centimeter wide along the path of each spot. Each paper was counted by scintillation. The counts were reported on a graph each square representing one centimeter of the chromatogram.

5. Vacuum distillation. Vacuum distillation was performed by putting the substance with the reagent in a tube kept constantly at 40° C and connected to an other tube cooled at -80° C acting as a condenser and attached to a vacuum pump.

D. In vivo experiments

In vivo experiments and tracing experiments with labelled acetoin in the intact rat were performed in an apparatus consisting of a mason jar two liters capacity put horizontally. A wire gauze was placed inside the bottle at a height of two cm, in order to separate urine from feces. The bottle was connected to two tubes: an inlet tube going to the end of the jar and a short outlet one bubbling in four consecutive tubes, the first tube contained saturated 2,4-dinitrophenyl hydrazine in 3 normal sulphuric acid, and each of the 3 other tubes contained 40 ml normal NaOH.

1. Distribution of acetoin-1-C^{14} in liver and expired CO_2. 2 ml of a water solution containing 10^6 counts per minute acetoin-1-C^{14} and 0.5 gm glucose were injected intraperitoneally in a rat which was then introduced into the jar. The jar was checked for any leakage and a stream of air was pumped. After 3 hours the rat was anesthetized, the liver removed, sliced in three to four pieces and put in a 40 ml centrifuge tube containing 10 ml of a hot 30% KOH solution. The tube was kept in a boiling water bath until the solution became clear. The tube was then cooled to 45° C and 18 ml 95% ethanol were added. The temperature of the mixture was raised to 80° C for five minutes. The tube was cooled and centrifuged at 2000 R.P.M. for ten minutes. The supernatant was extracted with ether in a separatory funnel to remove the non-saponifiable lipids. An aliquot of the ether extract was counted on a planchet. The aquous layer was then acidified with five normal sulphuric acid, and extracted again with ether. An aliquot of the ether extract was counted on a planchet.

The precipitate after centrifugation was suspended in 15 ml of 10% TCA (trichloroacetic acid) solution. The suspension was centrifuged and to the supernatant containing glycogen 25 ml of 95% ethanol was added. The mixture was centrifuged again, the supernatant discarded. The glycogen residue was then purified by dissolving in water and

reprecipitation with alcohol. The glycogen was counted on a planchet. The sodium hydroxide solution containing the expired CO_2 was dried on a planchet and counted.

2. Tissue distribution of acetoin-1-C^{14} in the intact rat. An adult rat was injected intraperitoneally 6.6 um acetoin-1-C^{14} having a total of 1.2×10^6 counts per minute dissolved in 2 ml of 20% glucose solution, and placed in a jar arranged as above. At the end of the required time, the rat was anesthetized and dissected. The different organs were removed and analyzed in the following way: to 0.2 gm of the homogenized brain 0.5 ml of 10% TCA solution were added. The mixture was centrifuged and the supernate counted directly in a scintillation counter. The liver and kidneys were counted in the same way. The G.I. tract was removed, emptied from its contents by washing with saline. The contents were pooled with the feces homogenized, filtered and counted directly in the scintillation counter. The washed G.I. tract was homogenized. The homogenate was counted the same way as the preceding organs. The remaining viscera were homogenized and counted after protein precipitation with 10% TCA solution. After removing the skin, the body and carcass were ground and counted following protein precipitation with 10% TCA solution. The pooled NaOH solutions were counted directly in the scintillation counter. The radioactivity was checked to be in the CO_2

by putting 1 ml of the solution in an Erlenmeyer flask closed with a rubber stopper to which was attached a vial containing 1 ml of 5 N NaOH. 1 ml of 5 N sulphuric acid was injected through the rubber stopper. The flask was cooled at 4° C for 12 hours. At the end of the incubation the content of the suspended vial was counted directly in the scintillation counter. The urine was collected and counted directly in the scintillation counter.

E. In vitro experiments on rat liver with labelled acetoin

1. Determination of the radioactive CO_2 evolved.

An adult rat was anesthetized, the liver was removed, and sliced in a micro latapie at 0° C. One gram of liver slices and 3 ml Krebs Ringer phosphate buffer at pH 7.4 were added to each of 4 125 ml Erlenmeyer flasks provided with a rubber stopper to which was attached a small vial containing 1 ml N NaOH solution. To two of these flasks 0.066 umole acetoin-1-C^{14} having a radioactivity of 1.2×10^4 counts per minute were introduced and incubated for two hours at 37° C. To the other two controls 2 ml of 10% TCA were added followed by 1.2×10^4 counts/minute acetoin-1-C^{14}. They were then incubated at 37° C for two hours. At the end of the incubation 2 ml of TCA solution were injected through the rubber stopper in the two experimental flasks. The four flasks were incubated again at 37° C for 30 minutes.

The content of each flask was centrifuged, extracted twice with ether and an qliquot of each aqueous liquid was plated on a planchet and counted. The sodium hydroxide solution in the vials were plated on a planchet and counted.

2. Time study on acetoin metabolism. The livers of two rats were sliced in a micro latapie at 0° C. The slices were introduced in a 250 ml Erlenmeyer flask and suspended in 3 volumes of Krebs Ringer phosphate buffer. 6.6 um of acetoin-1-C^{14} counting in a total of 1.2×10^6 counts per minute were added to each flask and incubated at 37° C. One ml of the suspension from each flask was pipetted at different time intervals: 0 time, 10 minutes, 30 minutes, 1 hour, 2 hours. To each 1 ml pipetted solution an equal volume of 10% TCA was added. The tubes were centrifuged. 0.2 ml of each supernatant were placed on 6 stainless steel planchets on which various reagents were added.

3. Homogenate studies. Two rat liver were homogenized in a waring blender at high speed at 0° C for 10 minutes in Krebs Ringer phosphate buffer. The suspended liver homogenates were centrifuged at 3000 R.P.M. at 0° C. The supernatant was separated from the cell debris by decantation. The cell debris were suspended in 3 times their volume of Krebs Ringer phosphate buffer. In 2 Erlenmeyer flasks 250 ml capacity, 5 ml of the soluble part were placed. In 2 similar flasks 5 ml of the suspended cell

debris were placed. In a third group a mixture of soluble part and the cell debris suspension was placed. To one flask of each of the 3 groups 3 ml of a 10% TCA solution were added, and these flasks were considered as control. To the 6 flasks 0.66 um acetoin-1-C^{14} with a total activity of 1.2×10^5 C counts per minute were added. The flasks were closed with a rubber stopper and shaken in an incubator for 3 hours at 37° C. At the end of the incubation time 3 ml of a 10% TCA solution was added to the 3 experimental flasks. The contents of the 6 flasks were centrifuged at high speed. The supernatant in each was separated from the precipitate by decantation. An aliquot of the clear solution was counted on a planchet after drying under infra red lamp

F. Purification of the metabolite

1. Sixteen healthy adult guinea pigs were anesthetized, the livers weighing approximately each 45 gm were removed, chopped at 0° C in a meat grinder using the medium knife. The homogenate from each liver was suspended in 150 ml Krebs Ringer phosphate buffer pH 7.4 contained in one litre Erlenmeyer flask. A total of 6 gm acetoin having a total activity of 1.2×10^6 counts per minute dissolved in 100 ml of Krebs Ringer, was dissolved among the flasks. The flasks were closed with a rubber stopper

and shaken in an incubator at 37° C for 4 hours.

2. At the end of incubation time, the content of the 16 flasks were pooled and 50% TCA solution in water were added slowly to the incubation mixture to an apparent complete protein precipitation, the mixture was then filtered through a Buchner funnel. The precipitate was washed twice with water and the washing added to the filtrate.

3. The pooled filtrate and washing were extracted 3 times in a separatory funnel with equal volumes of ether to remove lipids and emulsifying agents. The ether was washed with water, and the washing added to the aqueous solution. The ether was discarded.

4. The aqueous solution was neutralized to pH 4.5 with N NaOH; then passed through a celite charcoal column, 6 cm in diameter, 8 cm in length, made by suspending equal weight of celite and charcoal (Merck) in water and packed by gravitation. This step removes most of the aromatic compounds, sugars and large molecular substances.

5. The filtrate from the charcoal columns was neutralized with N NaOH to pH 8, and extracted exhaustively in a continuous ether extract apparatus for 72 hours. The ether layer contained most of counts, the aqueous portion was discarded.

6. The ether was evaporated down to about 100 ml at 4° C in an Erlenmeyer flask using a stream of nitrogen and extracted several times with small volumes of water to take out water soluble substances. The ether containing lipids was discarded.

7. Steps 5 and 6 were repeated once more. Several tests were run on this aqueous layer.

8. The water solution was vacuum distilled. The apparatus used was composed of a hard glass tube 20 cm height 3 cm diameter in which the water solution was introduced. It was attached to a vibrator to prevent bumping. The tube was connected through a trap made from a 3 x 20 tube cooled to -80° C to a vacuum pump. The temperature of the first tube was kept at 40° C in a water bath. The condensed part contained most of the count. The brown viscous residue was discarded.

9. The distillate was saturated with sodium bisulphite and transferred to a small continuous ether extract apparatus. The ether extraction was fractionated at 3 hours interval, the 0-6 and the 6-24 hours fractions were collected separately. The 6-24 hours fractions were concentrated by evaporation, then extracted with small portion of water. The water solution was saturated with sodium bisulphite and extracted continuously with ether for 4 hours. The ether was added to the 0-6 hours fractions. The aqueous solution was discarded.

10. The pooled ether was evaporated in a tube containing 2 ml water using a fine stream of nitrogen. The temperature was kept around 0° C. A safety trap at -80° C was attached to the tube.

11. The water solution having a volume of 4 ml was passed over a sephadex G-10 column made by soaking 50 gm of sephadex G-10 powder overnight in distilled water then packed by gravitation in a column 125 cm long and diameter 1 cm. The length of the packed material was 90 cm. The column was washed two days with bidistilled water before adding the solution. The column was eluted with bidistilled water at constant pressure using the Mariotte bottle. The rate of flow was 0.25 ml per minute. The temperature was kept at 20° C, the eluted solution was collected in 3 ml fractions using a fraction collector. 0.1 ml of each tube was counted in the scintillation counter.

12. Tubes 22, 23, 24, called Fraction X and tubes 25 to 32 called Fraction O were pooled separately. Each fraction was continuously extracted with ether for six hours. The water was discarded and the ether was dried with sodium sulphate.

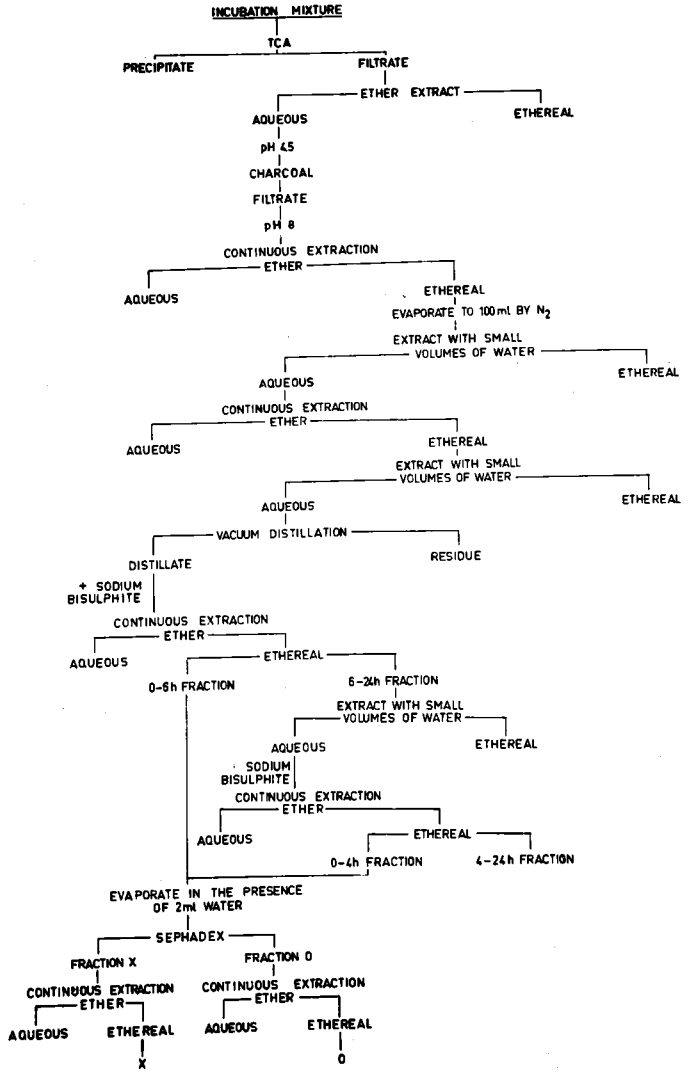


FIGURE 4. DIAGRAMMATIC SCHEME FOR THE PURIFICATION OF ACETOIN METABOLITE.

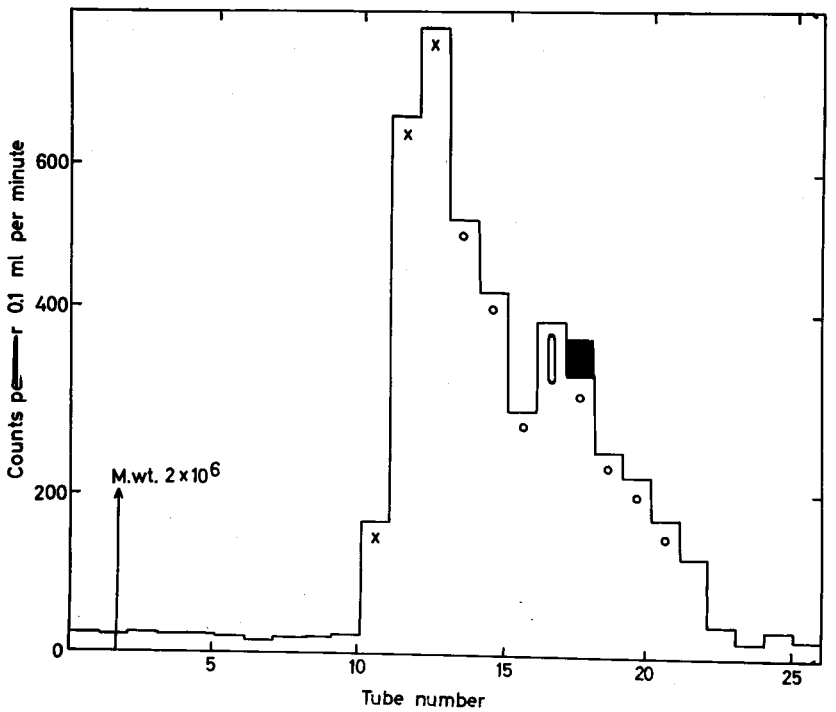


FIGURE 5. ELUTION PROFILE OF THE METABOLITE ON SEPHADEX G-10.

CHAPTER III

RESULTS

A. Metabolic studies

1. Distribution of acetoin-1-C^{14} in liver and expired CO_2. The object of this experiment was to study the route by which acetoin is metabolized. By injecting acetoin-1-C^{14} intraperitoneally in a rat and analyzing the content of the liver for radioactivity in the glycogen, saponifiable and non-saponifiable ether extracts we tried to see if acetoin goes through a classical metabolic pathway. The CO_2 expired collected in NaOH tubes was also counted. The procedure and the apparatus are described under Methods (p. 18). The results are shown in Table 1. We can observe that after 3 hours incubation only 2.5% of the counts were found in the CO_2. Lesser amounts were detected among the non-saponifiable, the saponifiable ether extracts, and the glycogen. The 2,4-dinitro-phenylhydrazine tube contained no counts.

2. Tissue distribution of acetoin-1-C^{14} in the intact rat. The object of this experiment was to study the possibility of accumulation of acetoin or its metabolites in one or more organs and to know the way by which acetoin or its metabolites can be excreted. We obtained the following results (see Table 2) by injecting acetoin-1-C^{14}

TABLE 1

Distribution of Radioactivity in Liver and Expired CO_2 3 Hours After the Injection of Acetoin-1-C^{14} Intraperitoneally in the Rat

Fraction	Total Counts per minute
Amount injected	1 x 10^6 C.P.M.*
Expired CO_2 collected	25,820 C.P.M.**
Non-saponifiable ether extract	900 C.P.M.**
Saponifiable ether extract	107 C.P.M.**
Glycogen	2,000 C.P.M.**
2,4-dinitrophenylhydrazine	No counts

* The acetoin was counted as the 2,4-dinitrophenyl-hydrazone in a gas flow counter.

** Counted on a gas flow counter after drying under infra red lamp.

TABLE 2

Tissue and Excreta Distribution of Radioactivity After the Injection of Acetoin-1-C^{14} Intraperitoneally in the Rat

	Experiment I	Experiment II	Experiment III
Total hours of incubation	12	15	24
Total C.P.M./organ*			
Kidneys	3,360	4,200	28,800
Viscera	1,800	1,810	800
G.I. tract	3,600	20,140	70,800
Brain	470	1,600	2,200
Liver	7,200	8,200	51,800
Total C.P.M./Excreta*			
Urine	160,000	162,000	278,000
Intestinal content	70,600	22,820	33,800
Expired CO_2	155,000	166,000	180,600
Total counts injected*	1.2×10^6	1.2×10^6	1.2×10^6

* Counted directly in a scintillation counter.

intraperitoneally, and analyzing the different organs and excreta for radioactivity following the procedure described under Methods (p. 19). High number of counts were found in urine and expired CO_2, while the counts in other organs were comparatively low.

3. Determination of radioactive CO_2 evolved. To determine how much the carbon one of acetoin was completely oxidized to CO_2 by the liver in vitro, rat liver slices were incubated with acetoin-1-C^{14}. The CO_2 evolved was collected and counted according to the procedure described under Methods. The results are summarized in Table 3. No counts were detected in the CO_2. Higher fixed counts were found in the experiment flasks.

4. Time study on acetoin metabolism. To prove that acetoin is continuously metabolized, as well as to study the chemical nature of the metabolites (in a time range chosen) a time study was run. From an incubation mixture of acetoin-1-C^{14} with rat liver slices, samples were taken, dried under an infra red lamp in the presence of different reagents, for different time intervals. See Methods (p. 21). The results obtained are summed up in Table 4. Fig. 6 shows the increase in fixed counts with time for the sample dried in a dessicator. It was observed that the 30 minute sample in experiment two was low in general probably due to experimental errors.

TABLE 3

Determination of Radioactive CO_2 Evolved on Incubation of Acetoin-1-C^{14} with Liver Slices

	Radioactive CO_2*	Total fixed counts after incubation*
Experiment 1	No counts	8150 C.P.M.
Experiment 2	No counts	7755 C.P.M.
Control 1	No counts	2000 C.P.M.
Control 2	No counts	850 C.P.M.

* Counted on a planchet after drying under infra red lamp.

TABLE 4

Time Study on Acetoin Metabolism

0.2 ml of Sample I +	Counts per minute				
	0 time	10'	30'	1 hr	2 hr
0.2 ml N/10 H_2SO_4*	0	109	91	130	185
0.2 ml N NaOH*	240	250	207	180	200
0.2 ml H_2O*	0	21	51	65	253
0.2 ml saturated hydroxylamine solution 0.2 ml N NaOH*	160	35	35	87	67
0.2 ml 2,4-dinitrophenyl hydrazine reagent (42)*	120	151	197	253	238
0.2 ml H_2O**	60	128	173	305	472
0.2 ml of Sample II +					
0.2 ml N/10 H_2SO_4*	0	3	30	77	207
0.2 ml N NaOH*	123	105	157	191	185
0.2 ml H_2O*	61	9	17	47	113
0.2 ml saturated hydroxylamine solution 0.2 ml N NaOH*	0	0	27	30	71
0.2 ml 2,4-dinitrophenyl hydrazine reagent (42)*	37	47	59	123	209
0.2 ml H_2O**	50	110	85	203	263

* Dried under an infra red lamp and counted in a gas flow counter.

** Dried in a dessicator and counted in a gas flow counter.

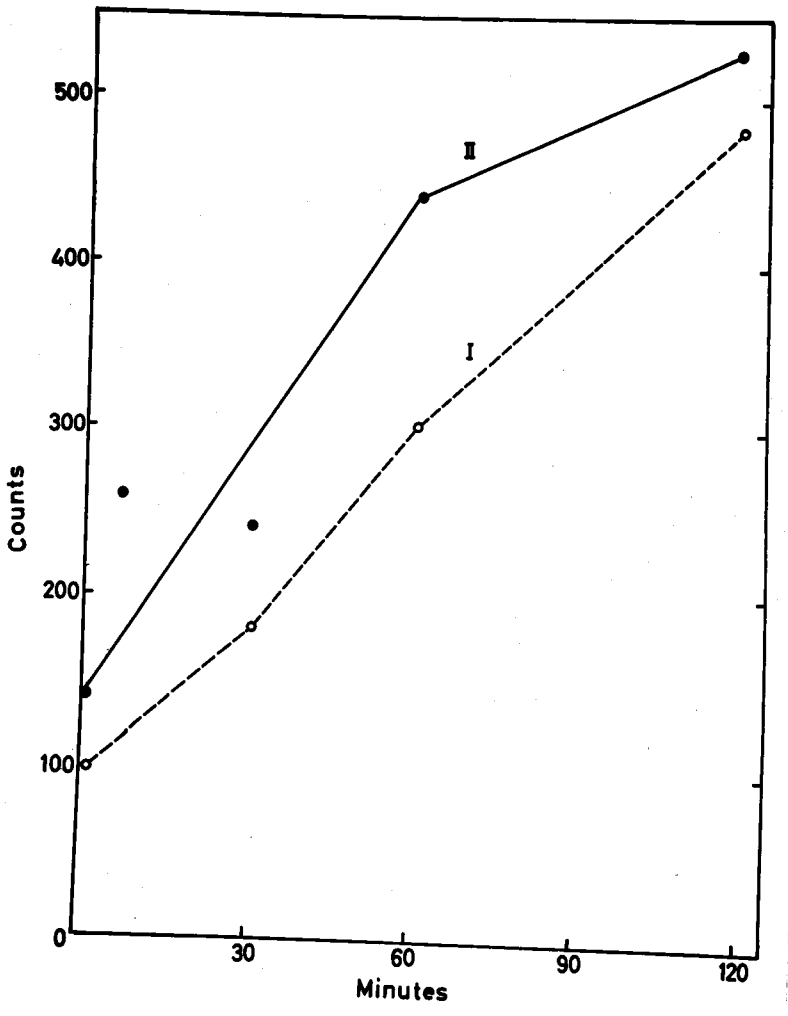


FIGURE 6. INCREASE IN FIXED COUNTS WITH TIME ON DRYING THE METABOLITES IN A DESSICATOR UNDER VACUUM

5. Homogenate studies. To investigate which part of the liver cells contained the enzymes responsible for the metabolism of acetoin, rat livers were homogenized and centrifuged separating the cell debris and the soluble part. Each part alone and a third portion containing a mixture of the soluble part, and the cell debris were incubated separately with acetoin-1-C^{14}. The experimental details are summarized under Methods (p. 21). It appeared from the results of this experiment that none of these separate three portions were able to give any fixed counts.

B. Identification of accumulated product of acetoin metabolism

1. Comparative vacuum distillation between the metabolite and acetoin. Vacuum distillation was comparatively performed on the Step 7 of the purification procedure and acetoin-1-C^{14} (See Methods, p. 17). Two vacuum distillation sets were attached in parallel to a vacuum pump. The first distillation compared the volatility of the compound to that of acetoin. The distillate, and residue were counted. In view of the results shown in Table 5, acetoin appeared to be more volatile than the compound. Another comparative distillation was run between acetoin and the same fraction in the presence of an excess of sodium bisulphite to fix any aldehydic or methyl ketone

TABLE 5

Comparative Vacuum Distillation of the Metabolite and Acetoin

	Total counts per minute*	
	Metabolite	Acetoin
Distillation alone		
Distillate	250	670
Residue	588	60
Distillation in the presence of an excess sodium bisulphite		
First distillate	240	54
Second distillate	160	54
Third distillate	240	200
Residue	350	800

* Counted directly in a scintillation counter.

group. Each sample was distilled nearly to dryness, the residue redissolved in water, and redistilled. The operation was repeated twice. The results are summarized in Table 5.

2. Iodine value. Iodine value was performed on the ether solution of the compound. The value was found to be zero.

3. Potentiometric titration. Potentiometric titration of the compound was carried out on a 5 mg sample (see Methods, p. 16). A pK value of 4.65 to 4.7 was found after subtracting the water blank value. A comparative titration was run with acetic acid (see Fig. 7).

4. Comparison between acetoin and the metabolite by ascending paper chromatography. Fraction 7 of the purification was compared to acetoin in order to prove that the radioactivity of our fraction is not acetoin. Three spots were applied on a paper: the compound, acetoin-1-C^{14}, and a mixture of the compound and acetoin. The chromatogram obtained by ascending chromatography was cut, counted, and plotted graphically (See Methods, p. 16). From the results obtained (see Fig. 8) it can be clearly observed that a net difference in the R_f of acetoin, and that of the compound do exist. Also the acetoin in the solvent used appears to have a higher R_f than that of its metabolite indicating a higher polarity.

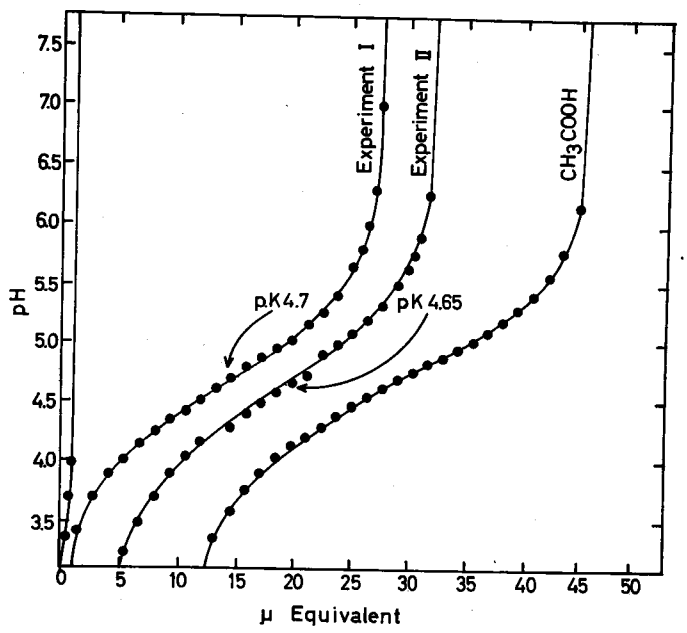


FIGURE 7. TITRATION CURVE OF THE COMPOUND AND ACETIC ACID.

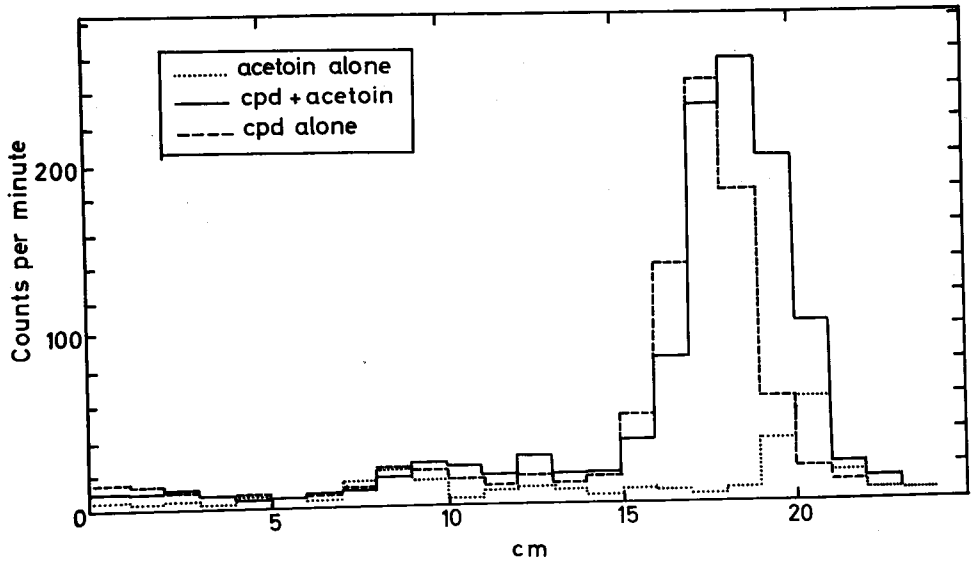


FIGURE 8. COMPARISON OF ACETOIN AND THE METABOLITE BY PAPER CHROMATOGRAPHY.

5. Infra red and ultra violet spectra of fraction O and X. The infra red spectrum of the X fraction in ether solution having an activity of 1000 counts per minute per ml was run. From this, a similarity of the finger print region of the spectrum to an ether solution of acetoin was demonstrated (Fig. 9). The infra red spectrum of the O fraction in ether solution having an activity of 2000 counts per minute per ml was also run (Fig. 10). The spectrum suggested clearly the presence of a small chain carboxylic acid. An other spectrum of the same fraction, dried with sodium sulphate and evaporated under a dry stream of nitrogen, was run in chloroform solution and compared to many acids dissolved in the same solvent. Similarity to propionic acid was clearly shown (Fig. 10). The ultra violet spectra did not show any absorption above 200 mu.

6. Physical properties of the metabolite. It is a volatile and extremely hygroscopic acid with an acetic acid odor.

7. Microanalysis. The elementary analysis for carbon, hydrogen and the C-methyl were performed on a liquid sample obtained by the evaporation of an ether extract. Alfred Bernhart, Mikroanalytisches Laboratorium, 433 Mülheim, Germany, performed the carbon hydrogen and Dr. Franz Pascher, Mikroanalytisches Laboratorium, Bonn,

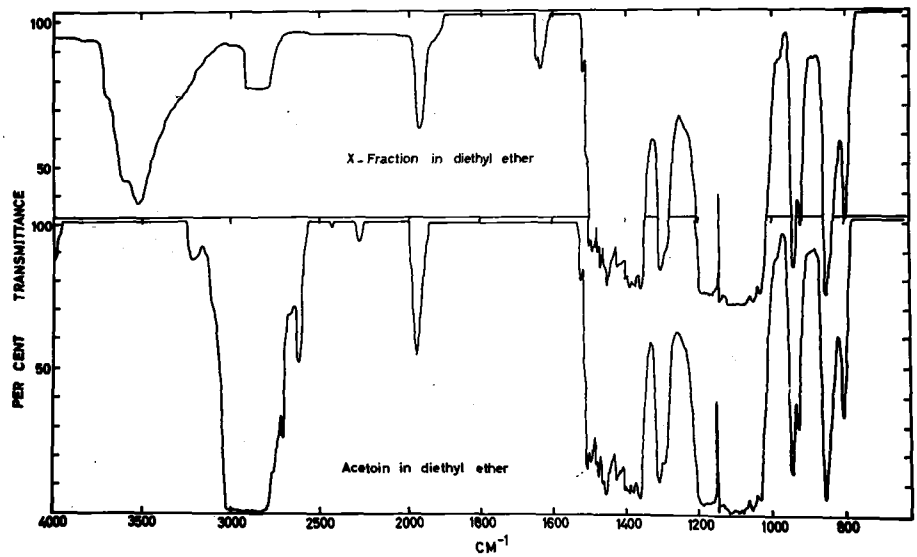


FIGURE 9. INFRA RED SPECTRA OF FRACTION X AND ACETOIN.

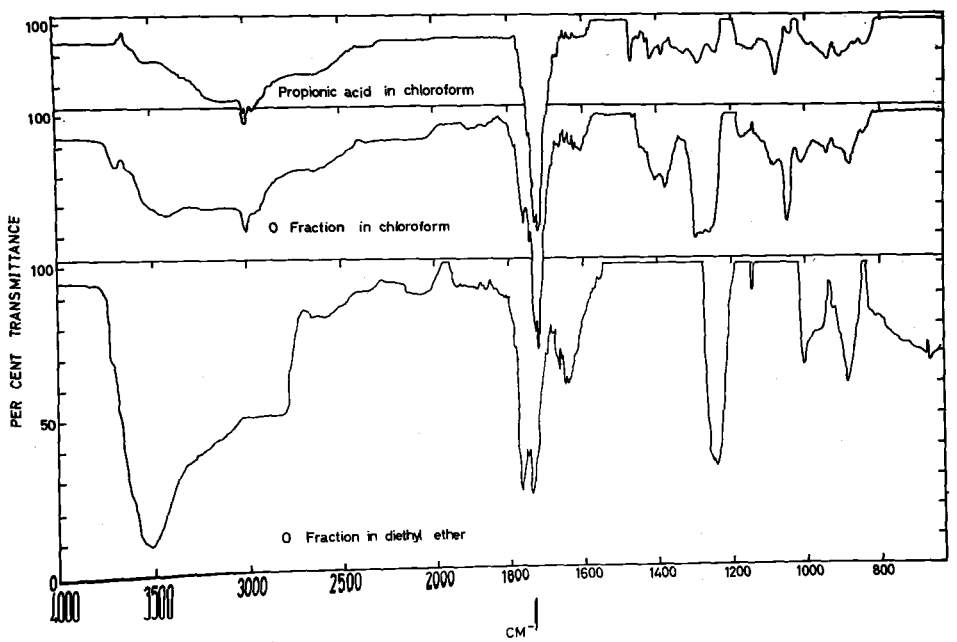


FIGURE 10. INFRA RED SPECTRA OF FRACTION O IN ETHER, FRACTION O IN CHLOROFORM, AND PROPIONIC ACID IN CHLOROFORM.

Buschstrasse 54, Germany, performed the C-methyl determination. The results and the expected values for propionic acid containing water contaminent are reported in Table 6.

8. Molecular weight. Using sephadex separation the compound came just after acetoin (molecular weight 88), but just before acetic acid (molecular weight 60), indicating a molecular weight between these true values. Another molecular weight calculated on titration equivalent gave an approximate value of 80.

9. Functional groups. The pK determination, as well as the infra red spectrum indicated the presence of a carboxylic group. Other functional groups tested chemically were found to be absent.

TABLE 6

The Elemental and C-Methyl Analysis of the Compound

	Found	Calculated for $C_3H_6O_2 \cdot 1.8H_2O$	Calculated for $C_3H_6O_2 \cdot 2H_2O$
H	10	9.1	9.1
C	32.4	34	32.7
O*	57.6	57.5	58
C-Methyl**	15.57	14.7	14.2

* By difference.

** Run on another sample.

CHAPTER IV

DISCUSSION

The existence of acetoin as a product of metabolism in living organism has been known for a long time. It was detected in lower living organism; bacteria, fungi, etc. as well as in higher animals. The metabolism of acetoin in microorganism was intensively studied by many scientists. In higher animals specially mammals, acetoin was found to be produced in appreciable quantities. Its overall metabolism was studied superficially by people who were interested principally in the fate of ingested ethyl alcohol. However, little is known about the intermediate steps of its metabolism.

In this report we tried to investigate more intensively the metabolism of acetoin by mammals. The first part was concerned mainly to prove that acetoin is a substance metabolized by mammals. By injecting acetoin-1-C^{14} intraperitoneally to rats we were able to isolate after 24 hr collection 15% of the radioactivity in the expired CO_2, indicating a partial oxidation of the injected acetoin. The remaining counts were found in urine and distributed among various tissues. In studying the metabolism of acetoin *in vitro* in rat liver, we were able to confirm

Järnefelt's work (29) that only the intact liver cells were able to metabolise acetoin to less volatile compounds; liver homogenates were incapable of metabolizing acetoin. By running a control experiment where proteins were precipitated before incubation with acetoin, we showed that this is not a polymerization but an enzymatic reaction. Again, in the time study experiment in vitro the increase fixed counts was proportional to the incubation time. Finally, by comparing the metabolite and acetoin on paper chromatography two spots with different R_fs were obtained suggesting the production of a radioactive compound different from acetoin (Fig. 8). From these experiments we can definitely conclude that acetoin is a compound metabolized by normal tissues.

As regard the route through which acetoin is metabolized we were unable to demonstrate a CO_2 fixation using $C^{14}O_2$ and unlabeled acetoin. Also, we cannot eliminate the possibility of this fixation; the metabolism of acetoin is probably a minor pathway and the CO_2 fixed by other metabolites will be higher. Furthermore, by looking back on the result of the in vivo experiment (Table 1) we can see that the carbon one of acetoin did not go to glycogen, saponifiable or non-saponifiable lipids, indicating that acetoin is not metabolized through any of the main pathways of metabolism.

The oxidation of acetoin *in vivo* was very slow (Table 1 and 2), only 2.5% of the counts were detected in the expired CO_2 3 hours after intraperitoneal injection of acetoin-1-C^{14} and only 15% were found after 24 hours indicating that transformations leading to the accumulation of a slowly oxidized substrate.

In vitro liver slices were incapable of oxidizing carbon one of acetoin to CO_2 but a substance different from acetoin accumulated. This might be due to the absence of some enzymes needed to the complete oxidation of acetoin to CO_2, or it is possible that the liver is actually capable of the complete oxidation of acetoin *in vivo* but failed to do so *in vitro* due to unknown factors introduced in the preparation of the slices. These experiments show that acetoin is metabolized in a non-classical way, and that its metabolism in liver slices leads to the accumulation of an intermediate which is not further oxidized in these slices. We, therefore, attempted to isolate this metabolite . The compound we isolated was identified as propionic acid. This is shown by its odor, volatility, identity in I.R. spectra, agreement of the carbon, hydrogen, C-methyl group and carboxyl group analysis with theoretical values, as well as the agreement of the determined pK value of the carboxylic group and the rough molecular weight determination to their expected respective values.

The radioactivity in the propionic acid fraction could not be due to contamination with acetoin as a radioactivity of 1000 counts per minute per ml was found in the X Fraction, the I.R. spectra of which was identical to that of acetoin (Fig. 9), while in the O Fraction isolated simultaneously and containing 2000 counts per minute per ml gave an I.R. spectra identical to that of propionic acid with no evidence of contamination (Fig. 10).

Propionic acid is probably not the original compound accumulated by liver slices but rather a non-biological breakdown product of it. In the early steps of the purification the counts were found in a non-volatile fraction even when plated from TCA filtrate. Furthermore, it was extracted with ether from a water solution adjusted to pH 8 (Fig. 4). In addition, the propionic acid is known to be readily converted to succinic acid and oxidized to CO_2 readily in liver (43). The nature of the original derivative of propionic acid to which acetoin is converted and the metabolic pathway involved remain unknown. This derivative must have been somewhat chemically labile as the mild conditions employed in the purification were enough to cause its breakdown.

SUMMARY

This research program was designed to study the metabolism of acetoin in mammals and to isolate its metabolites. The study was done on guinea pigs and albino rats *in vivo* and *in vitro*. By injecting acetoin-1-C^{14} intraperitoneally to a rat we found that it was partially oxidized to CO_2 in 24 hours. No radioactivity was found in glycogen, saponifiable and non-saponifiable lipids but counts were found in urine. *In vitro* experiments using rat liver slices we showed that acetoin is metabolized giving an accumulation product derivative of propionic acid labile chemically. Moreover, liver homogenates were unable to metabolize acetoin. A purification procedure for the accumulated metabolite is described in which propionic acid was isolated and identified.

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