MAMMALIAN ACETATE METABOLISM

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CONTENTS

MAMMALIAN ACETATE METABOLISM

ABSTRACT

This work is a study of some aspects of the metabolism of free acetic acid in non-ruminant mammals. The metabolic significance of acetate is recognized in the ruminant and in certain micro-organisms where large amounts of acetate are utilized. The lack of a sufficiently sensitive method for acetate determination coupled with the low concentrations present in the tissues of non-ruminants has been a major impediment to studies on acetate metabolism in these animals in the past.

A method suitable for acetate determination in the tissues of non-ruminants has been developed. The study has been pursued by measurements of acetate in blood and tissues of the rat, and by investigating acetate production in *in vitro* preparations of rat liver and comparing it with that of ketone bodies.

Chapter 1. Introduction.

The reasons for undertaking this study are described: the presence of small amounts of acetate in animal tissues and its ready utilization by selective tissues suggest that acetate may have a role as a respiratory fuel analogous to that discussed by Krebs (1961a) for the ketone bodies.

The pathways of metabolism of acetate and the acetyl group are briefly outlined; and the possible precursors of free acetate and the

likely sites of its formation in the body are described.

Chapter 2. Development of a method for the measurement of acetic acid.

Many methods of acetate determination used by earlier workers have been investigated. Microdiffusion, (Serlin and Cotzias, 1955), even with various modifications, has not been found to be sufficiently sensitive for the present purpose; lactate and bicarbonate have been found to cause considerable interference in a micro-distillation method (Bartley, 1953). An attempt was made to obtain an active preparation of acetokinase (E. Coli), but since the K_m value of the enzyme is so high (0.3 M acetate, Rose, Grunberg-Manago, Korey and Ochoa, 1954), the amounts of enzyme required to measure low concentrations of acetate would be relatively large, so enzymic assays were not pursued.

Finally, a gas chromatographic method (Baumgardt, 1964) has been adopted. This system can take aqueous samples thus cutting out the need for cumbersome extraction procedures. However, the samples must first be purified by steam-distillation (Dr. E.F. Annison, personal communication). Isovalerate is used as a marker. The method is specific for acetic acid. The mean recovery of 1 - 10 µmoles of acetate added to blood, perfusion medium or tissue homogenate is between 93% and 105%, while the individual errors are ± 29% at worst. Amounts of acetate of 0.08 µmole and upwards can be determined.

Chapter 3. Acetate in rat blood and its origin.

The acetate level in rat blood, collected by cannulation of the aorta, remains at approximately 0.4 mM regardless of dietary alterations (feeding, fasting for 24 or 48 hr., high-roughage diet). But blood from the portal vein which drains i.a. the caecum, contains 4 times as much acetate, and small amounts of other volatile fatty acids, notably propionate. This difference in concentration in the blood from different parts of the body points to (i) an intestinal origin of acetate, and (ii) uptake of acetate from the blood by the liver and possibly the heart.

The caecum contents contain relatively large amounts of volatile fatty acids (approximately 50 μmoles/g.) of which about 50% is acetate. The volatile fatty acid composition is similar to that of sheep rumen contents (Annison, 1954b), although on a smaller scale. The concentration of acetate in caecum contents is only half that in the rumen, and relative to body weight, the rat caecum is only about 1% while the sheep rumen is approximately 10 - 20% (from Annison and Lewis, 1959; Bergman, Reid, Murray, Brockway and Whitelaw, 1965). Oral treatment of the rats with neomycin, an antibiotic, to reduce the bacterial activity of the gut to a minimum, decreases the acetate in the caecum contents to one-fifth and in the portal blood to one-third of their normal concentrations, the acetate in portal blood now being within the range found in aortic blood.

It is concluded that acetate in rat blood arises at least partly by bacterial activity in the intestine, and especially within the caecum.

A calculation based on the arterio-portal venous difference for acetate and on an approximation of portal blood flow rate (from Dobson and Jones, 1952) show that the acetate absorbed daily from the intestine, if completely oxidized, could account maximally for 10% of the basal caloric output in the rat, as compared with a corresponding value of 42% in the ruminant (Bergman et al. 1965).

Chapter 4. Acetate in the tissues of normal and alloxan-diabetic rats.

The tissue levels of acetate are comparable with those found by an enzymic assay (Bergmeyer and Moellering, 1966), and are highest in liver at about 0.4 μmole/g. fr. wt., intermediate in kidney, and lowest in heart muscle which readily utilizes acetate (Williamson, 1962). Acute alloxan-diabetes causes the level to rise by 150% in liver and by 50% in kidney, while the concentration in blood and in heart muscle remain unchanged. Fasting (48 hr.) or bran-feeding have no effect on liver acetate level.

The factors which generate and dispose of free acetate are discussed, but the control of its level is not sufficiently understood to offer a satisfactory explanation for the altered levels in diabetes.

Chapter 5. Acetate and ketone body production by rat liver in vitro.

Preliminary experiments have shown that acetate is produced by rat liver slices, at a rate comparable to that of ketone body formation, and that it is increased by the addition of fatty acids. In the perfused

liver much higher rates of production of both acetate and ketone bodies are observed; the maximal endogenous rates are 45 μmoles acetate/g. fr. wt./hr., and 42 μmoles total ketone bodies/g. fr. wt./hr. (in the livers of 48 hr. fasted rats). This preparation is adopted for further investigation of the effects of dietary alterations and of the addition of various precursors of the acetyl group (fatty acids, pyruvate and ethanol).

The initial rate of acetate formation is constant, but it starts to decline more rapidly in the livers of fed rats (after 30 - 60 min.) than in those of 48 hr. fasted rats (after 60 - 90 min.). Ketogenesis is minimal when donor rats are fed and is markedly and progressively increased when the rats are fasted for 24 and 48 hr.

Addition of fatty acids with or without carnitine has no effect on acetate production, but greatly enhances that of ketone bodies. It is calculated from net metabolic changes, that 2 mM butyrate is quantitatively converted, and 0.5 mM oleate is 60% converted to ketone bodies. The fate of the remaining oleate has not been determined. It is probably partly converted to CO_2 and partly utilized by the biosynthetic reactions of acetyl CoA.

Added ethanol and pyruvate are strongly anti-ketogenic, and do not alter acetate formation when the donor rats are fasted. When the rats are fed, acetate production is enhanced by 5 mM ethanol (2 perfusions), probably related to the higher activities of liver alcohol and aldehyde dehydrogenases (EC 1.1.1.1., EC 1.2.1.3.) in that state (Büttner, 1965).

Such large-scale acetate production by rat liver preparations was not previously observed (Hochheuser, Weiss and Wieland, 1964; Hepp, Prüsse, Weiss and Wieland, 1966a,b). The evidence for the metabolic origin of the acetate appearing in the perfusion medium is summarised, and the likely endogenous precursors of this acetate are discussed.

Chapter 6. Additional comments.

On the basis of the present findings some possible approaches to the further study of acetate metabolism in the rat are suggested; and a modification to the method of acetate determination is tentatively proposed.

MATERIALS AND METHODS

I Materials.

[Many of the materials used specifically for acetate determinations and for specialized treatment of animals appear in the methods sections of Chapter 2, and Chapters 3 and 4, respectively. A Markham still was a gift of Dr. E.F. Annison, of Unilever.]

A. Standard laboratory reagents, of the purest analytical grade available, were usually obtained from British Drug Houses Ltd.

Isovaleric acid was a gift of Dr. D.S. Robinson.

Sodium heparin (powder), from Koch-Light & Co. Ltd., Slough, Bucks., was used as an anticoagulant in the collection of blood.

Heparin (1000 units/ml., for injection) was supplied by Boots Ltd., Nottingham. "Nembutal" veterinary pentobarbitone sodium B.P. was obtained from Abbott Laboratories Ltd., Queensborough, Kent.

B. Components of the perfusion medium and perfusion substrates.

Crystalline bovine serum albumin (Fraction V) was from Armour Laboratories Ltd., London, and was stored at 4° before dialysis. Visking dialysis tubing (36/32") was obtained from the Scientific Instruments Centre, London.

Aged human blood stored 4-5 weeks at 4°, and no longer suitable for transfusion, was provided by the United Oxford Hospital Blood Transfusion Centre. This still possessed the full O_2-carrying capacity, but did not

glycolyse. Washed red blood cells were obtained from this by the method of Hems, Ross, Berry and Krebs (1966).

Pyruvic acid was obtained as the sodium salt from C.F. Boehringer & Son Ltd. Ethanol (99 - 100%, v/v), sodium butyrate and oleic acid (98%, a colourless preparation) were from British Drug Houses Ltd. Stearic, cis-linoleic and octanoic acids were obtained from Sigma London Chemical Co., Ltd. DL-carnitine HCl, from Koch-Light & Co. Ltd., was stored as a 0.1 M solution at -20° until required.

C. Requirements for enzymic assays.

Nicotinamide-adenine dinucleotide (NAD^+) and reduced nicotinamide-adenine dinucleotide ($NADH.Na_2$) were supplied by Boehringer & Son. Ltd. Sodium DL-3-hydroxybutyrate was from British Drug Houses Ltd. L-lactic acid as a 95% solution from Boehringer & Son Ltd. was purified by the method of Krebs (1961b) to give a crystalline powder containing 99% of the L-form. Acetoacetate was prepared by Mr. R. Hems from acetoacetic ester by the method of Krebs and Eggleston (1945).

Lactic dehydrogenase was obtained from Boehringer & Son Ltd. D-3-hydroxybutyrate dehydrogenase was preapred by Dr. D.H. Williamson by the method of Bergmeyer, Gawehn, Klotzsch, Krebs and Williamson (1967).

D. Materials for the isotope recovery experiment.

Sodium [2-^{14}C]acetate (0.1 mc, 0.82 mg.), 97% pure by dilution analysis, was supplied by The Radiochemical Centre, Amersham, Bucks. Butyl PBD [2-(4'-t-Butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxdiazole] was from CIBA (A.R.L.) Ltd., Duxford, Cambridge. Scintillation fluid

consisted of 8 g. butyl PBD and 80 g. Naphthalene dissolved and mixed in 600 ml. toluene and 400 ml. 2-methoxy ethanol.

E. Animals.

Wistar rats fed a standard 'rat-cube' diet were used. Male animals, 250 - 400 g. were employed unless stated otherwise.

II Methods.

The following metabolites were assayed enzymically:

Acetoacetate and D-3-hydroxybutyrate were assayed according to the method of Williamson, Mellanby and Krebs (1962) employing D-3-hydroxybutyrate dehydrogenase. L-lactate was assayed by the method of Hohorst (1963) and pyruvate by that of Bücher, Czok, Lamprecht, and Latzko (1963) both utilizing lactic dehydrogenase.

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
P_i	Inorganic orthophosphate
PP_i	Inorganic pyrophosphate
NAD^+	Nicotinamide-adenine dinucleotide, oxidized
NADH	Nicotinamide-adenine dinucleotide, reduced
CoA	Coenzyme A
β-hydroxybutyrate	D-3-hydroxybutyrate

ENZYME NOMENCLATURE

[Trivial names of enzymes adopted throughout the text for brevity are listed below, together with their formal nomenclature.]

Acetokinase - ATP:acetate phosphotransferase (EC 2.7.2.1).

Acetyl CoA synthetase - Acetate:CoA ligase (AMP) (EC 6.2.1.1).

Alcohol dehydrogenase - Alcohol:NAD oxidoreductase (EC 1.1.1.1).

Aldehyde dehydrogenase - Aldehyde:NAD oxidoreductase (EC 1.2.1.3).

Carnitine acetyl transferase - Acetyl CoA:carnitine O-acetyltransferase (EC 2.3.1.7).

Deacylase - Acetyl CoA hydrolase (EC 3.1.2.1).

Phosphotransacetylase - Acetyl CoA:orthophosphate acetyltransferase (EC 2.3.1.8).

Pyruvate decarboxylase - 2-oxo-acid carboxy-lyase (EC 4.1.1.1).

Pyruvate dehydrogenase - Pyruvate:lipoate oxidoreductase (acceptor-acetylating) (EC 1.2.4.1).

CHAPTER 1. INTRODUCTION.

CHAPTER 1

INTRODUCTION

A. General approach.

Free acetate is present in small but fairly constant amounts in animal tissues, and is readily utilized by certain tissues. These facts together with the circumstance that like the ketone bodies, acetate can arise from the degradation of fatty acids (by the action of deacylase on acetyl CoA) raise the question as to whether acetate plays a similar role as the ketone bodies in providing fuel for respiration (Krebs, 1961a).

The metabolism of acetate has been extensively studied in certain organisms, such as in yeast, in some strains of bacteria, and in ruminant mammals. In the latter, acetate is one of the main end-products of bacterial fermentation of carbohydrates in the rumen, is absorbed as such into the blood stream, and constitutes a significant source of energy (for review see Annison and Lewis, 1959). In the non-ruminant, however, while it has long been recognized that acetate is rapidly oxidized (Lusk, 1921), there is a paucity of quantitative data on acetate metabolism. This is due to the fact that the concentrations of acetate in these animals are relatively low (Lundquist, 1962; Annison, 1954b) and the methods used for its determination have been neither sufficiently sensitive nor specific (e.g. Friedemann, 1938; Black, 1949; Rose, 1955).

The aim of the work described here was firstly to attempt to develope a method for the determination of acetate in the amounts likely to be present in non-ruminant tissues, and subsequently to study its metabolic behaviour in these animals in the hope that this might increase the understanding of its physiological significance (cf. Krebs, 1961a).

The potential metabolic significance of acetate is obvious from the fact that aerobic oxidation of the acetyl group via the tricarboxylic acid cycle makes available two-thirds of the total energy of combustion of both fatty acids and of carbohydrates (Krebs and Kornberg, 1957). Thus if made available to tissues and if utilized by them, acetate would be an effective respiratory fuel. Furthermore, acetate is readily soluble, diffuses freely into most cells, and is relatively non-toxic, advantages over the long-chain fatty acids which it shares with the ketone bodies (Krebs, 1966).

B. Ready utilization of acetate by animal tissues.

Since Lusk (1921) observed the ready utilization of large oral doses of acetate in the dog, a wealth of data has accumulated on acetate oxidation in non-ruminants, largely obtained from experiments with radioactive isotopes (for brief review, see Annison, 1964).

After injection of labelled acetate in rats, most of the label (50% to 95%) appeared in the expired CO_2 within a few hours (e.g. Buchanan, Hastings and Nesbett, 1943), acetate thus being oxidized at a rate comparable to succinate (Gould, Sinex, Rosenberg, Solomon and Hastings, 1949)

and more rapidly than glucose or the long-chain fatty acids (Lossow and Chaikoff, 1955; Mayes and Felts, 1967a). That the extrahepatic tissues are capable of oxidizing acetate was shown in the eviscerated rabbit (Wick and Drury, 1952) where its oxidation accounted for half of the total CO_2 output. Elliott, Benoy and Baker (1935), and Elliott, Grieg and Benoy (1987) showed that addition of acetate stimulated respiration in slices of rat liver, kidney and testis. Acetate was confirmed as a fuel of respiration in many tissues, notably in the heart where it could replace glucose (Williamson, 1962, 1964, 1965; Shipp, Matos, Knizley, and Crevasse, 1964) and in kidney where it could replace lactate (Krebs, Speake and Hems, 1965). In brain, acetate was utilized to a small extent especially in the presence of glucose (Beloff-Chain et al. 1962; Quastel, 1965; Gonda and Quastel, 1966) and accounted maximally for 13% of the total oxygen uptake (Lindbohm and Wallgren, 1966). Acetate can also be utilized by the diaphragm (Villee and Hastings, 1949) and by the working skeletal muscle in the dog (Lifson, Umachi, Cavert and Johnson, 1948).

C. Pathways of utilization of acetate.

Acetate as such cannot be directly utilized. It is first activated to acetyl CoA, a compound with a high-energy thiol ester linkage (Lipmann, 1945; Lynen, Reichert and Rueff, 1951). Acetate thus enters the tricarboxylic acid cycle and its metabolism is linked with the degradation ofcarbohydrates, fats and proteins, with the synthesis of

fatty acids, ketone bodies, and cholesterol and with the acetylation of choline, sulphonamides and related compounds (for historical reviews see Lipmann, 1948; Martius and Lynen, 1950). Fig. 1-1 depicts the integration of acetate metabolism with the general metabolic pathways in mammals.

Through acetyl CoA, acetate enters another pool of "active acetate", acetyl carnitine, which is formed by the action of carnitine acetyl-transferase (Friedman and Fraenkel, 1955):

$$\text{Acetyl CoA} + \text{Carnitine} \rightleftharpoons \text{Acetyl Carnitine} + \text{CoA}.$$

The two "active acetate" compounds are in ready equilibrium (Fritz, Schultz and Srere, 1963; Erfle and Sauer, 1967). Bremer (1962a) observed that acetyl carnitine was not directly converted to free acetate in mitochondrial preparations from various tissues. Acetyl carnitine is probably involved in the transport of acetyl groups between subcellular compartments (see Wolf, 1965; Greville, 1966).

The fact that free acetate can be used as a precursor of acetyl groups e.g. for oxidation via the tricarboxylic acid cycle (Kalnitsky, 1949) or for the synthesis of fatty acids (Rittenberg, and Bloch, 1944), cholesterol (Bloch and Rittenberg, 1942) or acetyl choline (Nachmansohn and Machado, 1943), indicates a vigorous activation system for acetate in many tissues. This activation is catalysed by acetyl CoA synthetase (AS) in mammals (Lipmann, Jones and Black, 1952; Jones, Black, Flynn and Lipmann, 1953; Beinert et al. 1953; Hele, 1954). The overall reaction is as follows:

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow[Mg^{++}]{\text{AS}} \text{Acetyl CoA} + \text{AMP} + PP_i$$

The active pyrophosphatases in tissues ensure that this reaction goes in the direction of biosynthesis (see Kornberg, 1962).

Legend to Fig. 1-1. Pathways of Acetate Metabolism in Mammals.

This gives a general outline of the main metabolic pathways involving acetate and the acetyl group.

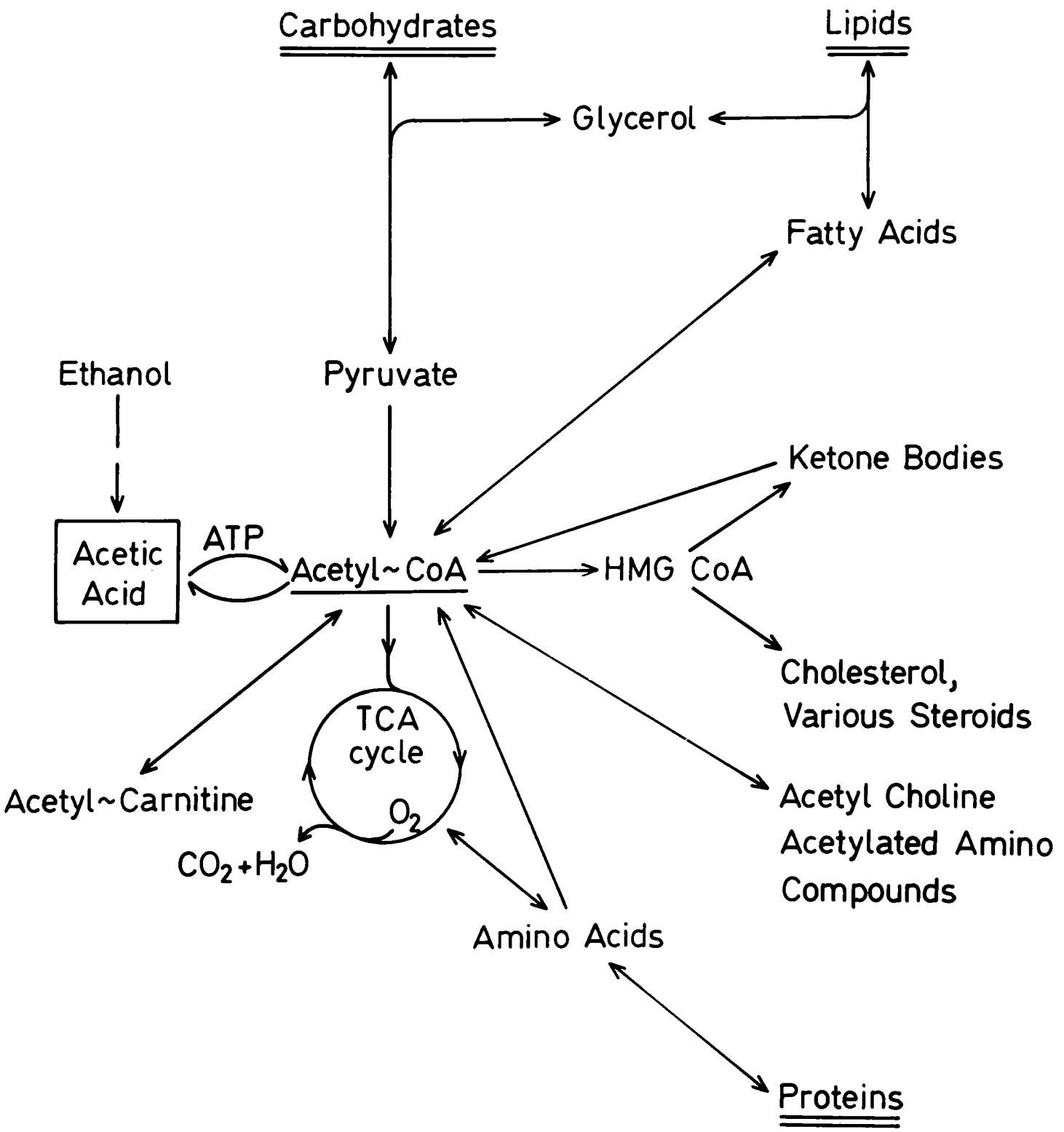
$\longleftrightarrow$ represents one or more steps; reverse processes may occur by different mechanisms.

$\longrightarrow$ represents a process generally considered irreversible.

$\dashrightarrow$ there is also a possibility that ethanol may primarily form acetyl CoA (see the text).

HMG CoA - 3-hydroxy-3-methylglutaryl-CoA.

Fig. 1-1. Pathways of Acetate Metabolism in Mammals.



The properties of the enzyme have been extensively studied in mitochondrial extracts of heart (Campagnari and Webster, 1963; Webster, 1963, 1965a,b, 1966). The enzyme appears to be located mainly within the mitochondria of brain (Schuberth, 1965; Tuček, 1967a,b), but the supernatant fraction also seems to be an important site of action in many tissues (de Duve, Wattiaux, Baudhuin, 1962; Kornacker and Lowenstein, 1964, 1965a,b). It is distinct from the medium-chain and long-chain fatty acid activating enzymes, which do not activate acetate (Mahler, Wakil and Bock, 1953; Kornberg and Pricer, 1953).

D. Origin of free acetate in non-ruminants.

In view of such ready utilization of acetate both for oxidative and synthetic reactions, the question arises as to its origin. While small amounts of acetate may be ingested as in vinegar or in milk products (see Jensen, Quinn,Carpenter and Sampugna, 1967), the acetate content of the diet as such is on the whole negligible. It has generally been assumed, though not proved, that in non-ruminants acetate or the acetyl group arises from endogenous rather than exogenous sources (Fritz, 1961; Sabine and Johnson, 1964), although in 1944, Barcroft, McAnally and Phillipson observed considerably higher levels of acetate in the blood draining the caecum in the pig, and the colon in the pony, than in the arterial blood of these non-ruminants.

There is only scanty information so far on the endogenous production of acetate by isolated tissues, two exceptions being rat erythrocytes

(Hochheuser, Weiss and Wieland, 1964) and mouse tumour tissue (Hepp, Prüsse, Weiss and Wieland, 1966a,b). This is probably largely attributable to the lack of a sensitive method for the small amounts of acetate likely to be involved. Consequently, the enzymic mechanisms of its formation have not been nearly so extensively studied as those of its removal.

1. Acetyl CoA.

The most likely precursor of free acetate is acetyl CoA and the existing evidence seems to suggest that this is the major source:

$$\text{Acetyl CoA} + H_2O \longrightarrow \text{Acetate} + \text{CoA}$$

Korkes, Del Campillo and Ochoa (1952) ascribed the appearance of free acetate from acetyl CoA in heart muscle to enzymic hydrolysis. The deacylase enzyme catalysing simple hydrolysis of acetyl CoA was shown in heart extract by Gergely, Hele and Ramakrishnan (1952). However, in view of the known capacity of heart muscle to utilize acetate (e.g. Williamson, 1962), it is unlikely that deacylase activity in this tissue plays a significant role. The highest specific activity of the enzyme in the rat was observed in the kidney, with intermediate values in liver, brain and testis, and somewhat lower values in muscle and in adipose tissue (Hepp et al. 1966a).

If acetyl CoA is a ready source of free acetate, then precursors of acetyl CoA (e.g. pyruvate or fatty acids) would also be expected to give rise to acetate. This has in fact been observed. Krebs and Johnson (1937) found that pyruvate was converted by anaerobic dismutation

to acetate when added in high concentrations to preparations of liver, kidney, testis, brain and muscle. Aerobic formation of acetate from pyruvate has been observed in tissues, including brain (Long, 1938; Elliott, Scott and Libet, 1942; Coxon, Liebecq and Peters, 1949), erythrocytes (Hochheuser et al. 1964), and tumour tissue, where fatty acids were also converted to acetate (Hepp et al. 1966a,b).

Deacylase (acetyl CoA hydrolase, EC 3.1.2.1.) is distinct from succinyl CoA deacylase (Gergely et al. 1952). But the results of attempts to separate palmityl CoA deacylase (an enzyme of broad specificity) from acetyl CoA deacylase activity in pig brain were rather confusing (Brere, Suebert and Lynen, 1959). In rat liver, deacylase appears to be located 80% in the cytoplasm with a small amount firmly bound in the mitochondria (Székely, 1955).

It is recognized that fatty acids are the major precursors of ketone bodies in the liver (see Krebs, 1966), but it would be of interest to see which of pyruvate (and hence carbohydrate) or fatty acids makes the greater contribution towards acetate formation by the deacylase pathway.

2. Ethanol.

The biological conversion of ethanol to acetate is a well known phenomenon, which accounts for the spoiling of wine. In man, ingestion of ethanol causes a rapid rise in blood acetate level (Lundquist, 1960; Hochheuser et al. 1964). This conversion takes place in the liver since alcohol dehydrogenase is almost exclusively located in this organ in man

(Schmidt, Schmidt and Wildhirt, 1958), as well as in the rat and other mammals (Büttner, 1965; and see Sund and Theorell, 1963), and was directly demonstrated by infusion experiments in humans (Lundquist, Tygstrup, Winkler, Mellemgaard and Munck-Petersen, 1962). The metabolite of ethanol which appeared in perfused rat liver (Forsander, Räihää and Suomalainen, 1960) was subsequently recognized as acetate (Lundquist, 1962).

As in bacteria, ethanol is first converted to acetaldehyde. But this intermediate does not accumulate, due to the extremely low K_m value of aldehyde dehydrogenase (K_m less than 10^{-5}M acetaldehyde, Racker, 1949), and to the relatively greater activity of aldehyde dehydrogenase than of alcohol dehydrogenase, as observed in rat liver and kidney (Büttner, 1965). While in certain bacteria, e.g. Clostridium kluyveri (Burton and Stadtman, 1953) and possibly Pseudomonas aeroginosa (Heydeman and Azoulay, 1963) acetyl CoA is first formed, free acetate appears to be the primary end-product in mammals (see Jakoby, 1963). But, since the pH optimum of the enzyme is around 10, the reaction is usually studied under strongly alkaline conditions (Maxwell and Topper, 1961; Erwin and Deitrich, 1966) where acyl CoA's are known to be highly unstable (Stadtman, 1957).

Lundquist, Fugmann, Kläning and Rasmussen, (1959) studied the anaerobic reaction of acetaldehyde in rat liver suspension in the presence of pyruvate (to prevent ethanol formation). The liver was homogenized in a buffer whose maximum pH is calculated as 7.7, but was

probably closer to 7.0 on addition of the anoxic liver. Rapid formation of free acetic acid accounted for all the acetaldehyde which disappeared. In view of the mild conditions, this experiment makes intermediate formation of acetyl CoA seem unlikely. Furthermore, no CoA requirement could be shown for mammalian aldehyde dehydrogenase (Jakoby, 1963; Erwin and Deitrich, 1966), with one exception. Brady (1958) observed a CoA-dependent reversible reaction catalysed by a pigeon liver extract:

$$\text{Acetaldehyde} + NAD^+ + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + NADH + H^+$$

The CoA-independent reaction described by other authors has been found to be irreversible, and it is not clear whether they represent the action of the same or of two different enzymes.

In summary, it is evident that ethanol is oxidized very rapidly to the acetyl level in the liver, but it is not yet fully settled that free acetate rather than acetyl CoA is the primary end-product.

3. Other sources.

Other potential pathways of acetate formation exist in mammals, which are apparantly CoA-independent. Acetyl choline is hydrolysed to form free acetate as part of the acetyl choline cycle in the nerve cell; the cycle is completed by the reactivation of acetate to acetyl CoA and subsequent acetylation of choline (see Nachmansohn, 1955). It has been calculated that acetyl choline esterase in dog brain if acting maximally and continuously could release 2 - 5 μmoles of acetate/g. tissue/hr. (McIlwain, 1966). However, the enzyme only acts intermittently, on stimulation of the nerve cell, and the recovery of acetyl choline level

is complete within 10 sec. of stimulation (see McIlwain, 1966), indicating efficient recycling. It is possible that the cell may use preformed active acetyl groups for reacetylation (Nachmansohn, 1959). From *in vitro* studies on intracellular localization of enzymes and on relative rates of synthesis from various acetyl donors, Tuček (1967a,b) concludes that citrate may be a more important precursor than acetate. Yet there seems to be no evidence so far for release of free acetate from nerve tissue.

Acetyl choline esterases in serum and other tissues (see Wilson, 1960), glutathione thiolesterase (Kielley and Bradley, 1954), and various acetyl esterases (see Hofstee, 1960) have been described. But most of these enzymes have been studied with synthetic substrates such as p-nitrophenyl acetate (Aldridge, 1953) which makes it difficult to assess their physiological role if any.

Many of the amino sugars are extensively acetylated but very little is known of their catabolism, (see Balazs and Jeanloz, 1965; Davidson, 1966). A slow degradation of N-acetylglucosamine 6-phosphate was described in pig kidney extracts (Leloir and Cardini, 1956; Comb and Roseman, 1957, 1958), and has been found in P815 murine mast cell tumours (C.A. Pasternak, unpublished observations), yielding free ammonia and acetate, (EC 5.3.1.11.). The concentration of the free amino sugars, however, is low, as they are largely protein-bound (see Balazs and Jeanloz, 1965). And relative to carbohydrates and fats, acetylated amino sugars have a slow metabolic turnover (Macbeth, Bekesi, Sugden and

Bice, 1965; cf. Mayes and Felts, 1967a), thus it does not seem likely that they make a major contribution to free acetate formation.

There is evidence also, for acetylation of certain proteins and amino acids (for review see Olsen, 1966) but the degree and mechanism of deacetylation of these is virtually unknown.

E. Site of acetate formation in the body.

By analogy with the ketone bodies, which are also extensively utilized by the extrahepatic tissues (see Krebs, 1961a) the liver suggests itself as a likely site of formation of acetate in the body. The presence of free acetate in the bile of the dog (Fonnesu, 1953) might indicate its production in the liver. The liver has been shown capable of converting pyruvate, ethanol and acetaldehyde to free acetate as described above. Attempts to show endogenous production of acetate in the perfused rat liver (Hochheuser et al. 1964) or in rat or mouse liver slices (Hepp et al. 1966a,b) have failed. But this might be due to the particular experimental conditions used. In rat and mouse liver, the specific activity of deacylase though measured at a lower temperature, was 43% higher than that of acetyl CoA synthetase (Hepp et al. 1966a,b) suggesting that formation of free acetate might be of importance in this organ.

F. Summary.

1. The general approach adopted in this project was to study the

metabolic behaviour of acetate in non-ruminants in the hope of making some contribution to the understanding of the physiological role of acetate in these animals. A review of the literature on the available methods makes it clear that the first requirement is the development of a sufficiently sensitive method for the determination of acetate at the low levels found in non-ruminant tissues.

2. It is well established that acetate is readily oxidized by selective tissues, and some of the data leading to this conclusion are reviewed. The pathways of metabolism of acetate and of the acetyl group are briefly outlined.

3. The possible precursors of acetate and the sites of its formation in the body are discussed, but the information on both these points is far from complete.

CHAPTER 2. DEVELOPMENT OF A METHOD FOR THE MEASUREMENT OF ACETIC ACID

CHAPTER 2

DEVELOPMENT OF A METHOD FOR THE MEASUREMENT OF ACETIC ACID

I Introduction.

The accurate assay of acetic acid in biological materials has until recently been hampered by many factors. The chief obstacles have been its low concentration in tissues, and the non—specificity of many of the assays, permitting interference by substances present in tissues, especially other volatile acids. So, in most cases the actual assay is preceded by a step separating acetate from these substances. Such steps usually involve some loss of acetate.

Methods for measurement of acetate make use of such properties as its volatility (e.g. steam distillation, microdiffusion and gas-chromatographic techniques), its acidic properties (many of the preceding techniques are followed by a titration step), and the enzymic reactions it undergoes (enzymic assays).

Many of the earlier workers e.g. Wieland and Jennen (1941) used the method of Friedemann (1938) based on steam distillation. This method was used to measure total volatile fatty acids while individual acids could be identified by their combined distillation curves and partition coefficient between ether and water. It could not be used for an accurate determination of acetate. However, it is still often

used as a preparative step for more sensitive and specific methods (Elsden, 1946; Annison, 1954b).

Elsden (1946) developed a column chromatographic method which he found satisfactory for volatile fatty acid analysis in sheep rumen. After steam distillation, the acids were separated on a silica-gel chromatogram treated with bromcresol green indicator. They were detected as yellow bands, each being estimated on elution by titration. However, acetic acid could only be eluted with a strong solvent mixture which leached the indicator, and had to be determined by difference.

Black (1949) described a micro-method for measurement of acetate and other volatile acids, by microdiffusion in 50 ml. flasks at over 100° for 14 - 16 hr. By this method 0.2 - 2.0 μequiv. of acetate were recovered from biological material with an average error of 0.03 μequiv. But the method lacked specificity in that other volatile fatty acids, β-hydroxybutyrate, and lactate, interfered.

Conway and Downey (1950) used microdiffusion in the compact units of Conway to measure blood acetate. They added anhydrous sodium sulphate to increase the volatility of acetic acid - the high salt concentration lowered the vapour tension of acetic acid while formation of the decahydrate of sodium sulphate removed some of the water. Diffusion could thus take place at room temperature and was complete in about 8 hr. Serlin and Cotzias (1955) adapting this method added sufficient sodium sulphate to dry the acetate sample completely and accelerate diffusion even further. The method has also been modified by Lundquist,

Fugmann and Rasmussen (1961), and along with the method of Kirk (1950) is the basis of the microdiffusion-distillation technique of Bartley (1953). Microdiffusion and-distillation methods will be discussed in greater detail in the experimental section.

Rose (1955) suggested adaption of his acetokinase (AK) assay for measurement of acetate in biological material. Acetokinase is a bacterial enzyme catalysing the formation of acetyl phosphate thus:

$$\text{Acetate} + \text{ATP} \underset{Mg^{++} \text{ or } Mn^{++}}{\overset{AK}{\rightleftharpoons}} \text{Acetyl phosphate} + \text{ADP}$$

The enzyme has low affinity for acetate (K_m = 0.3 M for the E. coli enzyme, Rose, Grunberg-Manage, Korey and Ochoa, 1954). The reverse reaction is favoured with an apparent equilibrium constant:

$$K = \frac{[\text{Acetyl phosphate}][\text{ADP}]}{[\text{Acetate}][\text{ATP}]} = 0.006$$

However, the reaction was performed in the presence of excess hydroxylamine which trapped the acetyl phosphate as the hydroxamate which could then be measured colorimetrically by its purple complex with ferric ion (Lipmann and Tuttle, 1945). But the assay is non-specific, since the enzyme reacts, though at a reduced rate, with propionate which then also reacts with hydroxylamine.

Acetokinase reaction is usually the first step in more recent enzymic assays, e.g. that of Bergmeyer and Moellering (1966), which will be discussed in the experimental section. Lundquist et al. (1961) followed a microdiffusion step by enzymic formation of acetyl

sulphanilamide, but this reaction cannot be brought to completion, and many standards must be run with each assay, Lundquist (1963).

Acetic acid was among the compounds separated and detected in the first published report on gas-liquid chromatography by James and Martin (1952). Gas chromatographic methods for the quantitative determination of acetate have subsequently been developed with increasing sensitivity, see e.g. Annison (1954b); Erwin, Marco and Emery (1961); Baumgardt (1964); Medzihradsky and Lamprecht (1965). These methods will also be discussed in the experimental section.

II Experimental.

A. Microdiffusion.

1. Principle.

The microdiffusion technique adopted was largely that of Serlin and Cotzias (1955), with some of the modifications suggested by Lundquist et al. (1961), and other alterations.

As in the method of Serlin and Cotzias, water was used as a trapping solution in the centre chamber and sodium sulphate as a drying agent in the outer chamber of Conway units, and the procedure was initiated at 4°. However as Lundquist et al. have observed, better results were obtained with larger amounts of sodium sulphate (10 g.) and with longer diffusion time at room temperature. It was also found necessary to lower the pH of the sample to below 2.5. Other factors were varied as described below.

Microdiffusion was followed by automatic titration of the centre chamber contents.

2. Materials.

CO_2-free water, used for trapping acetic acid in the centre chamber and for making up standard NaOH solution for titration, was prepared by boiling distilled water for 30 min., and stored with a soda-lime stopper for not longer than 2 days.

Standard NaOH, 0.005, 0.0025 N etc. was prepared fresh daily from a stock solution. The latter, made up from a BDH ampoule, was 1 $\pm$ 0.001 N, and was stored at 4° with a soda-lime stopper.

Standard Acetate, was prepared by dilution of molar stock solution (the exact molarity of which was determined by electrometric titration with 1 N-NaOH). The acetate-containing samples for diffusion were acidified with either $HClO_4$ or H_2SO_4.

Na_2SO_4 anhydrous, analar grade, supplied by BDH, was stored at 100° until 24 hr. before use, then at 4° (covered) until required - it was completely anhydrous.

Conway Units no. 1, and lids, (supplied by Gallenkamp) were used.

Conway Oscillation Table (Gallenkamp MD 150) with an extra spring arm attached to the geared unit, maintained a speed of 40 oscillations/min. under the weight of 12 loaded units.

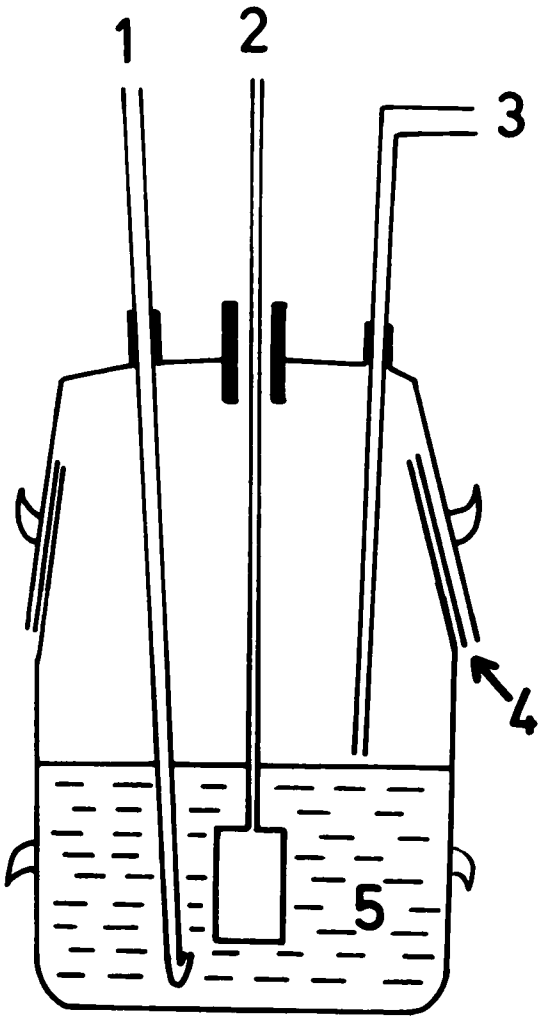
Radiometer Titrator (type TTT 1b) and Titrigraph (type SBR 2b) were used. The titration vessel was modified as described below.

3. Method of titration.

The titration vessel of the Radiometer Titrator was slightly modified to ensure CO_2-free conditions (see Fig. 2-1). A stirrer with steel shaft and glass paddle was fitted to the stirring motor. The vessel, of 5 ml. capacity, was attached to its lid by a ground glass joint with a close-fitting Teflon sleeve, and secured with rubber bands. Through openings in the lid, the stirrer, the capillary tip of the titrator admitting NaOH, and a capillary tube attached to a nitrogen cylinder entered the vessel. The opening for the stirrer was quite loose-fitting and lined with rubber tubing, permitting free vibration of the shaft and allowing escape of nitrogen blown in during titration. The tip of the nitrogen tube was just above the surface of the solution being titrated, and the flow of nitrogen adjusted so that it just made a slight depression on the surface, without bubbling. This ensured a CO_2-free atmosphere above the titration fluid without causing escape of the rather volatile acetic acid. Titration was performed immediately after adding the solution to the vessel. pH 6.73 (99% conversion of acetic acid to acetate ion) was taken as the end point. 0.5 μequiv. of standard acetic acid could thus be titrated with an accuracy of ± 10%. The accuracy was greater with larger amounts and less with smaller amounts of acetate.

Titration of CO_2-free water always gave a zero or negligible reading.

Fig. 2-1. Modified Titration Vessel.



The titration vessel of a Radiometer Titrator was modified to ensure CO_2-free conditions during titration, as described in the text.

1. NaOH from titrator enters here.
2. Mechanical stirrer.
3. N_2 admitted here (capillary tip is just above the surface of the solution).
4. Ground-glass joint fitted with Teflon sleeve.
5. Solution for titration.

4. Use of Conway unit.

The Na_2SO_4, units, and all solutions were cooled at 4^o for at least an hour before use. 1 ml. of CO_2-free water was placed in the centre chamber of the units and 10 - 12 g. of Na_2SO_4 in the outer chamber. A sample containing an exactly known amount of acetic acid (0.5 - 5.0 µmoles) in 0.5 - 2.0 ml. of solution of pH usually below 2.5, was placed on to the Na_2SO_4 and the units were immediately sealed with vaselined lids. Blanks were set up similarly but omitting acetate. The units were now left at 4^o for 15 - 20 min. to ensure rapid crystallization of the decahydrate, then left on the oscillation table at room temperature (or at 36^o) for various lengths of time. After diffusion, the contents and washings of the centre chamber were transferred with a Pasteur pipette to a titration vessel, giving a final volume of 3 - 5 ml. Titration was performed as described above.

A rigorous technique was followed for the cleaning of the units. They were brought to the boil in Pyroneg detergent, rinsed, soaked in hot aqua regia for a few hours, and thoroughly rinsed with distilled water. They were now clean in the 'acid-alkali' sense of Conway (1962).

5. Recovery of known amounts of acetic acid.

After overnight diffusion at room temperature the recovery of 0.5 - 2.0 µmoles of acetate was completely erratic (see Table 2-1). With 0.5 µmole, recovery varied between 78 and 157%, while with 1 µmole there was also a wide scatter, but the mean recovery was considerably lower. Recovery of 2 µmoles was somewhat better. The discrepancies in

Table 2-1. Recovery of various amounts of acetic acid by microdiffusion.

(The method was based on that of Serlin and Cotzias, 1955). 10 g. Na_2SO_4 (anhydrous) was placed in the outer, and 1 ml. CO_2-free water in the centre chamber of Conway No. 1 units. Known amounts of acetic acid in 2 ml. of solution, pH 0.4 - 1.2, were added to the outer chamber. Microdiffusion was initiated at 4° (15 - 20 min.) then continued overnight at 25°. Centre chamber contents were titrated against NaOH, as described in the text.

Acetic acid added (μmoles)	% Recovery (Mean ± S.E.M.)	No. of determinations
0.5	121 ± 21.7	(5)
1	51 ± 14.5	(6)
2	99 ± 6.7	(4)

these early results are considered partly due to poor technique.

In an effort to improve acetate recovery, the following factors were varied:

a) Temperature and duration of diffusion. The diffusion temperature (after 20 min. at 4°) was raised from 25° to 36° (see Table 2-2). It was observed that maximum diffusion had occurred within the first 3 hours, and that during the subsequent 3 - 6 hr. there was no further recovery of acetate. But recovery of 1 - 5 µmoles was even lower than at 25° and generally well below 60%.

b) pH. Both Serlin and Cotzias (1955), and Lundquist et al. (1961) buffered their samples for diffusion at pH 3. It was found that if the sample pH were over 3 (e.g. 3.1), there was considerable lag in acetate diffusion at 36° (see Table 2-3). None diffused in the first 4.5 hr. and only 15% in 18 hr. On lowering the pH to 2.7 or below, there was much more rapid diffusion. At pH 2.7, 99% of the acetic acid is in the free acid form. Conway and Downey (1950) used samples of pH below 1, but no further improvement was observed if the sample pH were below 2. A pH of 2 - 2.5 seemed satisfactory.

c) Sealing of the Conway units. Vaseline was replaced by liquid-solid paraffin mixture (Conway, 1962) and no improvement was observed. It was thought that gas might be escaping on transferring the units from the lower to the higher temperature, due to expansion, and that this might be prevented by applying weights to the lids. So steel weights of about 150 g. were secured to the lids and units by

Table 2-2. Recovery of acetic acid by microdiffusion at raised temperature (36°) for various lengths of time.

Units were prepared as described in Table 2-1. The sample pH was between 0.5 and 2.7. Microdiffusion was initiated at 4° (15 - 20 min.) and continued at 36°. For further details see the text. % Recoveries are expressed as mean ± S.E.M., with the no. of determinations in parentheses. [In this and in the following tables, whenever there were only 2 or 3 determinations, the mean is given, with the range in square brackets.] (-) denotes no experiment.

Acetic acid added (μmoles)	% Recovery after microdiffusion at 36° — Diffusion time (hr.)					
	2	3-3½	4	5-5½	6-6½	9
1	42 ± 11.6 (5)	59 ± 7.2 (7)	12.5 [2,23] (2)	54 ± 8.5 (5)	37 ± 6.0 (6)	(-)
2	38 [37,39] (2)	31 [15,47] (2)	37 ± 10.2 (4)	37 [31,43] (2)	11.5 [11,12] (2)	39 [30, 48] (2)
5	(-)	19 [18,20] (3)	20.5 [19,22] (2)	23 (1)	(-)	(-)

Table 2-3. Effect of pH on acetic acid recovery by microdiffusion.

The general procedure was as described in Table 2-2. Acetate-containing samples were of various pH values as indicated. Unless stated otherwise, diffusion was carried out for 3 - 4 hr.

Acetic acid added (μmoles)	pH of sample	% Recovery (Mean ± S.E.M.)	No. of determinations
2	3.5*	15 ± 1.9	(7)
	1.5	26 ± 8.0	(4)
1	3.1**	0	(3)
	2.5	64 ± 5.9	(6)

* 18 hr. diffusion.

** from ½ to 3 hr. diffusion.

rubber bands. However, during shaking at the warmer temperature the weights caused the lids to slip about with resulting leaks.

d) Sodium sulphate.

(i) Quantity. For efficient drying of 2 ml. samples at least 10 g. must be present (if less than 6 g. were used no acetic acid diffused). But it was quite difficult to fit this amount into the units without contacting the vaseline of the lids, which of course interfered with diffusion.

(ii) Quality. Analar grade only was used, as the ordinary grade contains alkali, which owing to the large amount of sulphate, might neutralise the acetate and thus prevent diffusion. A 10 g. sample of the former, in solution, required 0.975 m-equiv. acid to reach pH 4.0. Some of the salt was recrystallized from an acidic aqueous solution, but this made no difference to the results.

Seeding the anhydrous salt with 5% decahydrate (according to Lundquist) was actually found to diminish the recovery of 2.5 μmoles of acetate (from 60% to about 20%).

e) Initiation at 4°. To ensure complete crystallization, the initial period at 4° was prolonged for 1 hr. (as recommended by Lundquist) instead of the usual 15 - 20 min. This was followed by diffusion at 36° for 3 - 6 hr., and the recovery of 5 μmoles of acetate was quantitative and much less variable (see top line of Table 2-4).

f) Size of sample. The sample size was varied between

Table 2-4. Effect of sample size on acetic acid recovery by microdiffusion.

The general procedure was as described in Table 2-3, except that the initial period at 4° was prolonged (1 hr.). This was followed by the usual 3 - 6 hr. diffusion at 36°.

Sample size (ml.)	Acetic acid added (μmoles)	% Recovery (Mean ± S.E.M.)	No. of determinations
2.0	5	101 ± 3.7	(3)
1.0	5	71 ± 0.9	(4)
	2.5	60 ± 3.6	(8)
0.5	2.5	81 ± 1.0	(51)

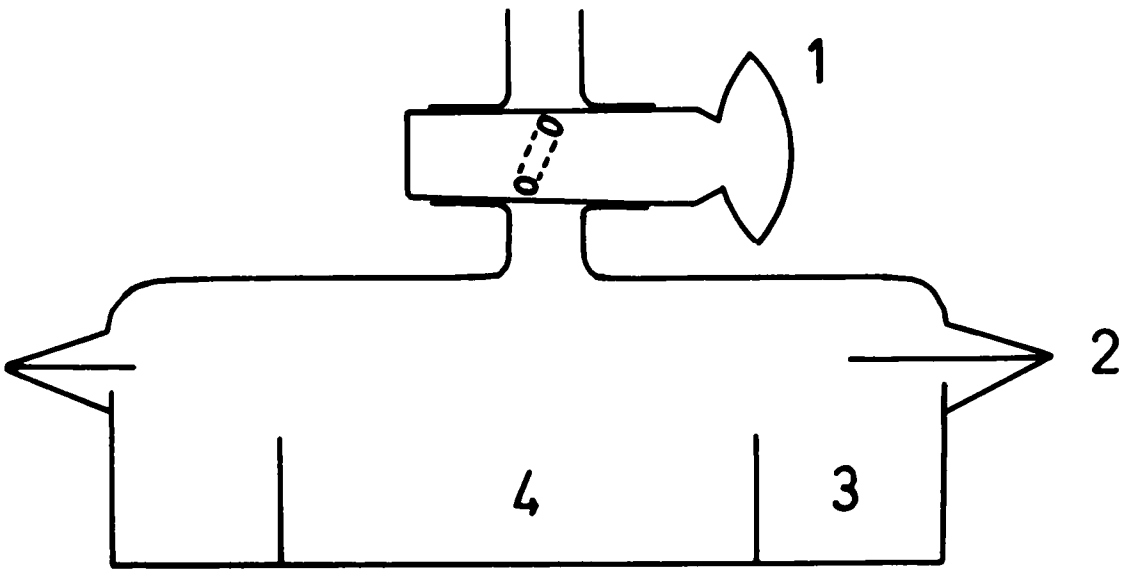
0.5 and 2.0 ml. It was observed that with the smaller sizes the sodium sulphate had a drier, more powdery appearance after diffusion. Quantitative results appear in Table 2-4. There appears to be no simple correlation between recovery of acetate and sample size. With the larger amounts of acetate (5 μmoles) recovery was quantitative from 2 ml. and dropped to 70% from 1 ml., whereas with 2.5 μmoles the recovery actually increased by about 20% on decreasing the sample size from 1 to 0.5 ml.

g) Shape of microdiffusion unit. Enlarged units were made, to take a 3 ml. sample (see Fig. 2-2). A stopcock was fitted in the lid, so that the unit could be evacuated (thus increasing the volatility of acetic acid, cf. Bartley, 1953). 2 ml. of water was required to cover the centre chamber floor, but this had largely disappeared during 2 hr. diffusion. 79 - 85% recovery of 3.6 μmole was obtained with these units i.e. less than with the original units (diffusion being complete in 2 - 3 hr.).

6. Summary.

Using the microdiffusion technique the recovery of up to 2 μmoles of acetic acid from simple aqueous solutions was incomplete and variable. The results for diffusion of 2 ml. samples at room temperature had a very wide scatter. If the diffusion were carried out at raised temperature (36°), after 1 hr. at 4°, the results were less variable but never quantitative for less than 5 μmoles of acetate (see Table 2-4).

Fig. 2-2. Enlarged Microdiffusion Unit.



The microdiffusion unit was adapted from the standard Conway Unit, but was designed to take larger samples and to be evacuated.

1. Stopcock (for evacuation of the unit).
2. Phlange (ensures maintenance of the vacuum).
3. Outer chamber (filled with Na_2SO_4, takes a 3 ml. sample).
4. Centre chamber (holds 2 ml. of fluid).

The recovery of 2.5 μmoles was only 81%, but since this was fairly constant a correction could be made. However, this amount of acetate is well in excess of that expected to be present in mammalian blood and tissue samples (where levels could be as low as 0.1 μmole/ml.). Thorough experimentation with the 7 factors described above did not increase the sensitivity sufficiently.

Also, as Conway points out (1962), propionic, butyric and formic acids, if present, would cause considerable interference. There are likely to be at least small amounts of these acids in mammalian systems. Acetoacetate might also interfere as mentioned by Lundquist et al. (1961), by releasing CO_2.

While microdiffusion can be used to measure higher levels of acetate in the absence of these interfering substances, as in incubation or perfusion media (e.g. Alexander, Britton and Nixon, 1967), only by the use of many standards and corrections for acetate losses could it be used for slightly lower levels, and the method did not appear suitable for the determination of the very low levels of acetate in blood and tissues.

B. Microdistillation.

1. Principle.

In 1953, Bartley designed a microdiffusion-distillation method for acetate determinations, based on the principles of the methods of Conway and Downey (1950) and of Kirk (1950). The acetate sample of pH below 1

was placed on Na_2SO_4 in a distillation bulb maintained at 50° while the attached receiver tube was kept at 0°, and the entire unit was then evacuated. The combined effect of raised temperature and reduced pressure markedly accelerated acetate diffusion, which was found to be complete in 20 min. The acid contents of the receiver were estimated by titration.

Bartley obtained 100% recovery of 2.0 μmoles of acetate added to tissue extract. However, with less than 1 μmole of acetate the recoveries varied between 96 and 104% (which he partially attributed to the diffuse end-point of the indicator used). By altering the titration and other conditions it might be possible to improve the accuracy with smaller amounts of acetate.

The higher temperature and lower pH used in this method, as compared with the microdiffusion methods of the previous section, would also be expected to cause greater interference from other volatile acids in biological samples. Thus Bartley found that pyruvate and HCl (formed on acidification of incubation media) interfered, but could effectively be removed by the addition of silver perchlorate and 2,4-dinitrophenyl-hydrazine, respectively. Lactate has also been found to cause considerable interference, as did CO_2 from high-bicarbonate saline. So, means of removing these interfering substances were looked for.

2. Materials.

CO_2-free water, standard NaOH and acetate solutions, and Na_2SO_4 were as in section A. The acetate-containing samples were acidified (pH about 0.5)

with 6 N H_2SO_4 before distillation.

2,4-dinitrophenylhydrazine. A saturated solution in 2 N-H_2SO_4 was just under 0.1 M. It was filtered hot.

1.0 M-$AgClO_4$ solution.

$Ce(SO_4)_2$ saturated solution in 6 N-H_2SO_4.

Microdiffusion-distillation units.

(i) 'Original' units - exactly as described by Bartley (1953).

(ii) Enlarged units - as (i) but the distillation bulb, only, was enlarged. Its diameter was increased by a factor of 1.5,i.e. its volume enlarged by a factor of 3.375. The enlarged bulb held 11 - 13 g. Na_2SO_4 without danger of spilling into the receiver.

Beckman Spinco grease (Cat. No. 003-301063 from Beckman Instruments Ltd., Glenrothes, Fife, Scotland) was found most satisfactory for sealing the joints of the units, the vacuum being almost invariably maintained throughout diffusion.

3. Procedure.

The microdiffusion-distillation technique in original units was as described by Bartley (1953), using 0.5 ml. samples. Diffusion was allowed to continue for 30 min. With the enlarged units, 2 ml. samples were used and were efficiently dried off. Again, distillation was complete within 30 min.

The contents of the receiver were automatically titrated against NaOH as in section A.(p. 18).

Blank solutions, similar to the analytical solutions, but omitting

acetate were put through the same procedure.

The units were cleaned as follows: surplus grease was wiped off and the remainder was removed with White Spirit (BDH). They were soaked in hot aqua regia for 15 - 20 min., and finally rinsed thoroughly in distilled water.

4. Effect of enlarging the units on recovery of known amounts of acetate.

The original units as described by Bartley are only capable of taking up to 0.5 ml. samples for distillation. Incomplete recoveries of acetate from aqueous solution was obtained with these, so the enlarged units (p. 25) were used. Results appear in Table 2-5. With the latter units, better recoveries were obtained with 2 ml. than with 3 ml. samples. Recovery of 2.0 - 2.5 μmoles was satisfactory, but the results were usually too large at 1 μmole (100 - 109%).

5. Interfering substances.

a) Pyruvate. Pyruvate would be expected to be roughly equimolar with acetate in tissues, but it no longer interfered in test systems after treatment with dinitrophenylhydrazine (cf. Bartley, 1953). Interference of pyruvate and its effective removal are shown in Table 2-6.

b) Hydrochloric acid. HCl would be formed on acidifying any medium containing chloride ion e.g. bicarbonate saline of Krebs and Henseleit (1932) (0.126 M-Cl^-), plasma (0.103 M-Cl^-), and tissue homogenates. The HCl would volatilize during subsequent drying of the

Table 2-5. Microdistillation: Recovery of acetic acid from aqueous solutions.

The method used was that of Bartley (1953). The acetate-containing solution was acidified with H_2SO_4 (final pH below 1.0). 0.5 ml. samples were tried out in the original, small units, and 2 or 3 ml. samples in the enlarged units. 30 min. distillation was followed by titration of the receiver flask contents. For further details see the text.

Units used	Sample size (ml.)	Acetic acid added (μmoles)	% Recovery (Mean ± S.E.M.)	No. of determinations
Small (original)	0.5	3.9	100 ± 1.9	(4)
		3.6	103 [100,106]	(2)
		2.5	80.5 ± 6.5	(13)
Enlarged	2.0	2.5	101 [101]	(3)
		2.0	99 [95-106]	(3)
		1.3	103 [100,106]	(2)
		1.0	109	(1)
	3.0	2.6	65 [48,82]	(2)
		1.3	76	(1)

Table 2-6. Interference of pyruvate in microdistillation procedure, and its removal with 2,4-dinitrophenylhydrazine.

Microdistillation procedure, in small units, was as described in Table 2-5.

a) 0.5 ml. of acidified samples containing pyruvate were used.

b) To some solutions containing 10 μmoles pyruvate (with or without acetate) 4 ml. sat. dinitrophenylhydrazine (a large excess) was added to precipitate pyruvate phenylhydrazone (see Bartley, 1953), and 0.5 ml. samples of the final mixture were distilled as above.

Acetic acid added (μmoles)	Pyruvic acid added (μmoles)	Dinitrophenyl-hydrazine added	Volatile acid found in receiver		No. of determinations
			Amount (μequiv.)	% of initial pyruvate	
None	3.6	None	2.55	71	(2)
None	2.5	Large excess	0.068	3.7	(2)
2.56	2.5	Large excess	2.45	-	(2)

sample, thus interfering with acetate determinations. To all saline samples containing 0.201 m-equiv. Cl^-, 1.0 ml of 1 M-$AgClO_4$ solution was routinely added (cf. Bartley, 1953). If the $AgClO_4$ were omitted an extra 0.6 - 1.4 μequiv. of volatile acid distilled from 2 ml. samples of saline, presumably HCl.

c) Bicarbonate. Despite CO_2-free conditions during titration (see p. 18) the values for blanks were high - 0.28 μequiv. acid from 2 ml. samples, i.e. about 10 times the values obtained by Bartley, although the sample size was only 4 times as large. This was presumably due to CO_2.

Much higher values were obtained with bicarbonate saline (see Table 2-7). An attempt to remove CO_2 at pH 6 was unsuccessful. So the saline was acidified (as for distillation) then shaken at 30° on a mechanical shaker (amplitude 2 in., frequency 112 complete strokes/min.) for various lengths of time. According to Friedemann (1938) one minute aeration with vigorous shaking should be sufficient to remove all the CO_2 without losing acetic acid. The amounts of volatile acid appearing from saline thus treated are shown in Table 2-7. There seemed to be an increasing production of volatile acid with time. Also, acetate recovery from saline after 3 min. shaking, was impaired: 90.5 ± 5.3% recovery of 2.9 μmole (mean ± S.E.M. for 5 determinations), (cf. Table 2-5).

d) Lactate. The concentration of lactate in rat blood is considerably greater than that of acetate (1.0 μmole lactate/ml. from the data of Ronzoni, 1950). Lactate was found to distil in this system,

Table 2-7. Microdistillation: Appearance of volatile acids from water and saline alone.

Microdistillation procedure, in enlarged units, was as described in Table 2-5. Distilled water was acidified and 2 ml. samples were put through the procedure. Bicarbonate saline of Krebs and Henseleit (1932) was acidified, $AgClO_4$ added (to remove HCl), then dinitrophenylhydrazine and $Ce(SO_4)_2$ were added, as when pyruvate and lactate were present. The solution was shaken mechanically at 30° for various lengths of time as indicated, and 2 ml. samples were distilled.

Sample	Shaking time (min.)	Amount of volatile acid found in the receiver (μequiv.) (Mean ± S.E.M.)	No. of determinations
Distilled water	—	0.29 ± 0.066	(12)
Bicarbonate saline	1	0.083 [0, 0.166]	(2)
	2	0.22 [0.20, 0.23]	(2)
	3	0.62 ± 0.056	(27)
	4	0.16 [0.07, 0.25]	(2)
	5	0.62 [0.51 - 0.76]	(3)

though to a lesser extent than either acetate or pyruvate. When 3.6 μmole was added, about 6.5% distilled over (see top line of Table 2-8). More recently Bergmeyer and Moellering (1966) found that 3 - 4% of lactate distilled when up to 100 μmoles had been added. They carried out the distillation at a higher pH (3.0) which reduced the volatility of lactate (and of acetate), and the distillation time was prolonged for 60 - 90 min.

Ceric sulphate oxidation was considered for the removal of lactate (Gordon and Quastel, 1939; Conway, 1962), the end-products being acetaldehyde and CO_2. 2 ml. of a saturated $Ce(SO_4)_2$ solution was added to a solution containing 1 μmole lactate (cf. Brady, 1961). The mixture was placed in a small flask, immersed in a water bath at 50° and left for various lengths of time, with intermittent swirling and shaking. Excess Ce^{4+} was always present as indicated by the retention of a bright yellow colour (cf. Krebs and Johnson, 1937). Samples were then put through the usual procedure for acetate assay.

When pyruvate was present it was precipitated as in a) above, prior to the addition of $Ce(SO_4)_2$, in order to prevent its oxidation by $Ce(SO_4)_2$ which would produce acetic acid (Krebs and Johnson, 1937).

Before diffusion, the flasks were shaken at 30° [see c) above] to remove the bicarbonate produced from lactate. The results (Tables 2-8, 2-9) were highly variable, but do show that $Ce(SO_4)_2$ oxidation only serves to increase volatile acid production from lactate. This may even be acetate formed by air or $Ce(SO_4)_2$ oxidation of the acetaldehyde

Table 2-8. Microdistillation: Recovery of volatile acid from lactate.

Lactate was added to bicarbonate saline. Then $AgClO_4$ and dinitrophenylhydrazine were added, followed by excess of $Ce(SO_4)_2$-H_2SO_4 solution. Oxidation was allowed to proceed at 50° for various lengths of time, followed by 3 min. vigorous shaking to remove the CO_2 of the saline and that produced by lactate oxidation. 2 ml. samples were distilled in enlarged units as described in Table 2-5.

Lactate added (μmoles)	Oxidation time (min.)	Amount of volatile acid in receiver: Mean and range (μequiv.)	Mean expressed as % of added lactate	No. of determinations
3.60*	None	0.24 [0.15,0.32]	6.5	(2)
1.46	5	0.13 [0 - 0.28]	9	(9)
	10	0.16 [0.10-0.21]	11	(3)
	15	0.12 [0.05-0.24]	8	(3)
0.29	20	0.07 [0 - 0.37]	24	(8)

*present in 0.5 ml. sample; no $Ce(SO_4)_2$ was added; distillation was carried out in small units.

Table 2-9. Microdistillation: Effect of lactate and its oxidation on acetate recovery.

The procedure was as in Table 2-8, except that acetate was also added.

Acetic acid added (μmoles)	Lactic acid added (μmoles)	Duration of $Ce(SO_4)_2$ oxidation (min.)	% Recovery of acetic acid (Mean ± S.E.M.)	No. of determinations
2	0.4	15	107 [106, 108]	(2)
1.5	0.3	15	50 ± 13.1	(4)
1.5	1.5	5	101 [99 - 103]	(3)

produced (cf. Long, 1946; Elsden and Gibson, 1954; Conway, 1962) especially as the reaction was carried out at elevated temperature and with a large excess of $Ce(SO_4)_2$.

e) Formate, propionate. Preliminary experiments showed that these acids are also volatile in this procedure.

6. Summary.

The objective of this investigation had been twofold: (i) to increase the sensitivity of the method, (ii) to remove lactate and bicarbonate interference. To accomplish the former, use was made of an enlarged distillation bulb, taking 4 times as large a sample. Some increase in sensitivity was observed - acetate at 0.65 µmole/ml. in the final sample could now be measured.

Lactate and bicarbonate interference remained obstacles. The presence of bicarbonate produced highly variable results, and acetate recovery in its presence decreased by 10%. Attempts to remove lactate interference by $Ce(SO_4)_2$ oxidation failed, in fact a substance behaving like acetate was formed, so that acetate could not be measured in its presence. Microdiffusion-distillation as a method for the measurement of acetate in mammalian tissue samples where there would be relatively high concentrations of lactate and of bicarbonate was thus considered unpractical.

C. Enzymic Assays.

1. Principle.

The adaption of acetokinase assay (Rose, 1955) for acetate determination has been mentioned in the introduction (p. 15). In brief, it consists of coupling acetokinase reaction with hydroxamate formation and estimation. Before it could be of use for the determination of acetate in mammalian blood and tissues two factors would have to be modified:

(i) an enzyme preparation of very high specific activity must be obtained in order to compensate for the high K_m effect, otherwise the rate of reaction would be exceedingly slow in presence of low concentrations of acetate. This factor was investigated.

(ii) the hydroxamate step should be replaced by a more specific one in view of the interference of propionate.

2. Attempt to obtain a suitable acetokinase preparation.

A crude E. coli extract was prepared by Dr. J.W. Wimpenny and Mrs. H. Cole. Its acetokinase activity, assayed according to Rose (1955), was 41 U./ml. at 37°, with a specific activity of 0.36 U./mg. protein.

Acetate assays were performed by the hydroxamate method (Rose, 1955) on standard acetate solutions free from propionate. The assay system was slightly modified (as suggested by Dr. J. Lascelles) to contain 5 times more ATP, thus ensuring that the enzyme was saturated with this co-substrate (the crude extract contained myokinase).

Various amounts of acetate were used, 0.25 - 1.25, and 2.5 - 10.0 µmoles/assay. While individual assays gave linear results, the response varied widely between assays (between 12.5% and 100% of the acetate reacted) and linearity was not observed over the whole range of acetate, viz. 0.25 - 10.0 µmoles. At the lower limit, values were inclined to be high, which could be partially attributed to the relatively high blank values obtained.

3. Summary and discussion.

The crude E. coli extract was not sufficiently active to ensure complete reaction of small amounts of acetate in a reasonable time (1 hr. incubation). Greater purification would have been necessary. Recently, a commercial preparation has become available with an activity of 80 U./mg. (C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany).

Many more specific alternatives for the hydroxamate step have been suggested, and some of these are described. Acetyl phosphate in the presence of CoA and phosphotransacetylase (PTA) can be readily converted to acetyl CoA. Oxaloacetate reacts with acetyl CoA catalysed by citrate synthase (CS) to form citrate, which can be determined either chemically or optically.

$$\text{Acetyl phosphate} + \text{CoA} \underset{\longleftarrow}{\xrightarrow{\text{PTA}}} \text{Acetyl CoA} + P_i$$

$$\text{Acetyl CoA} + \text{Oxaloacetate} + H_2O \underset{\longleftarrow}{\xrightarrow{\text{CS}}} \text{Citrate} + \text{CoA}$$

This outline was suggested by Beinert et al. (1953) for the assay of

acetate activating system of heart, and by Ochoa (1955) for citrate synthase assay. Chemically, citrate can be measured by oxidative conversion to pentabromoacetone and colorimetric estimation of the latter (Natelson, Pincus and Lugovy, 1948; Stern, 1957).

In the optical assay, malate, NAD and malic dehydrogenase are added as a source of oxaloacetate, and citrate formation is followed by a shift in the malic dehydrogenase equilibrium measured by NAD^+ reduction (at 340 or 366 mμ). The optical density change is related to the acetyl CoA concentration by a factor accounting for this equilibrium shift. There is slightly less NAD^+ reduced than citrate formed and Pearson (1965) has pointed out the necessary corrections.

This latter method has been used by Bergmeyer and Moellering (1966) for their sensitive determination of acetate in biological materials. For quantitative conversion of acetate to acetyl CoA their assay mixture contains only 9 - 450 nmoles of acetate and about 15 U. acetokinase, and under these conditions the reaction is complete within 1 hr. They thus claim measurement of down to 10 nmoles of acetate and have obtained 99.4% recovery of 0.1 - 0.3 μmole added to muscle homogenate.

Due to contamination of acetokinase with small traces of lactic dehydrogenase, the lactate and pyruvate occurring in tissues interfere in this method. Pyruvate can be removed by enzymic conversion to lactate and the latter by a microdistillation method (modified from that of Bartley, 1953). These authors find that only 3 - 4% of the lactate distils (see p. 28) and this amount is just below the limit for

interference in the subsequent optical assay.

Mammalian acetyl CoA synthetase has a relatively low K_m for acetate ($K_m = 8 \times 10^{-4}$ M, Webster, 1965a), and might lend itself to sensitive assays. However it is not yet readily available in a sufficiently purified form.

D. Gas Chromatographic Methods.

1. Principle.

James and Martin (1952) utilized a gas chromatographic principle outlined by Martin and Synge (1941), to separate and detect volatile fatty acids. They used 10% stearic acid in silicone as liquid phase, nitrogen as carrier gas, with a column temperature of 137°. The free fatty acids emerged in order of increasing molecular weight, $C_1 - C_{10}$, and were detected by automatic titration.

The method was developed later using similar columns by Annison (1954a,b); Crowther, Fulton and Joyner (1954); van de Kamer, Gerritsma and Wansink (1955); Hawke (1956). Separation of the more volatile methyl esters was achieved by James and Martin (1956), and by Vorbeck, Mattick, Lee and Pederson (1960) using a polyester liquid phase. Polyester columns predominated for the analysis of acetic and other volatile fatty acids but on account of their polarity, adsorbed the acids too strongly and thus caused 'tailing' of the peaks. This was remedied by adding phosphoric acid to the liquid phase (see Metcalfe, 1960, 1963). Phosphoric acid is now a standard ingredient of most polyester columns. Terephthalic acid has been used

in the same way e.g. by Byars and Jordan (1964); Lester (1964); Grey and Stevens (1966).

Most of the earlier systems, due to the nature of the columns and/or detectors, required that the fatty acids be in a volatile organic solvent such as ether or acetone, (see Averill, 1963; Grey and Stevens, 1966) or in methanol, (Canwin, 1965). This involved elaborate extraction procedures for samples from aqueous biological materials (Hankinson, Harper, Mikolajcik, 1958; Erwin et al. 1961) and careful storage of the prepared samples e.g. as suggested by Nelson, Emmanuelson and Brooks (1965). For these extractions large initial samples were required, e.g. 20 ml. of sheep blood (Annison, 1954a), or a few litres of culture medium (Crowther et al. 1954).

A great advancement came with the development of gas-liquid chromatographic systems which could take aqueous samples of acetate (e.g. Erwin et al. 1961; Averill, 1963; Withers, 1964). Such methods were extended to the determination of acetate in mammalian tissues, e.g. by Baumgardt (1964), Lester (1964) and by Medzihradsky and Lamprecht (1965).

The column described by Baumgardt (1964) contained neopentyl glycol succinate (NPGS) treated with phosphoric acid as a liquid phase, and coupled with flame ionization detection it was utilised to measure short chain fatty acids in rumen fluid and blood. This system was investigated with a view to acetate measurement in non-ruminants, after some preliminary work on another column.

2. Use of diethyl glycol adipate column. (Preliminary experiments).

a) Method and response. A diethyl glycol adipate (DEGA) column according to the recipe of Lindsay Smith, Norman and Radda (1964) was investigated. A Perkin-Elmer F11 Gas Chromatograph with Flame Ionization Detector was used (see Section 3a). This column was found suitable for ethereal solutions of short chain fatty acids which appeared as well defined peaks shortly after injection. Optimal conditions were established (see Table 2-10).

The response to individual acids was estimated from the drawn peak height (more accurate than the actual height especially with smaller peaks), and the response constant, k, was calculated according to Baumgardt (1964):

$$k = \frac{\text{Peak Height x Attenuation}}{\text{Concentration x Volume Injected}} = \text{cm./nmole.}$$

[peak height is expressed in cm., concentration in nmoles/µl., volume injected in µl.] The response to acetic acid over a range of 0.25 - 1.0 nmole injected was linear as shown in Table 2-10. The response to propionate was also linear.

The column, however, was not suitable for aqueous solution of these acids - in fact, any traces of water in the ethereal solution caused tailing of the ether peak so that it masked the subsequent acetate peak. So sodium sulphate was added to the ethereal solution as a drying agent, and various methods of ether extraction were attempted.

Table 2-10. Response of diethyl glycol adipate column to ethereal solutions of acetic acid.

The general gas chromatographic procedure was as described in the text. Optimal conditions established for the diethyl glycol adipate column were: Column temp. 100°; gas pressures, N_2, 23; H_2, 17; air, 42 p.s.i.; attenuation: 5X. 1 µl. injections of standard acetic acid solutions in ether were made. The response constant, k, is calculated according to Baumgardt (1964), from the drawn peak heights (see text).

Acetic acid conc. (µmole/ml.)	Actual height of peak (cm.)	Drawn height of peak (cm.)	Response constant, k (cm./nmole)	No. of injections
0.25	2.5	3.1	61.2	(2)
0.5	4.7	5.6	56.3	(3)
1.0	10.1	11.6	58.0	(3)
2.0	20.5	23.0	57.5	(3)

b) Ether extraction experiments. The aim was to extract known amounts of fatty acids from acidified aqueous solutions into ether. (Propionate was provisionally used as a marker in these experiments).

(i) Simple extraction - was found to be extremely inefficient. Never more than 60% of the acetate appeared in the ether phase after repeated extraction. Friedemann (1938) extracted only 13.5%, and Black (1949) achieved extraction of 71% into a relatively large volume of ether.

(ii) Continuous extraction - (using peroxide-free ether, and a micro-apparatus to hold just 2 ml. of extract) also gave poor results (see Table 2-11). Furthermore, acetate was produced by breakdown of unknown substances within the ether on prolonged boiling.

(iii) Lyophilisation - and subsequent uptake of sodium acetate into acidified ether were carried out according to Erwin et al. (1961). The ether was acidified with conc. H_2SO_4, or by bubbling HCl from a generator through it. But both acids had disastrous effects on the column, and numerous large peaks appeared (Erwin et al. were using another type of column).

Thus the diethyl glycol adipate column, while it gave good resolution of fatty acids in ether, was considered impracticable for analysis of aqueous solutions, as extraction of the short chain acids into ether was found to be incomplete and unreliable.

3. Use of neopentyl glycol succinate column.

[Note. This method is basically that of Baumgardt (1964). The column recipe, gases, solvent and method of detection (flame ionization) were

Table 2-11. Recovery of acetic and propionic acids from aqueous solutions by continuous extraction into ether, followed by gas-liquid chromatography.

Acidified aqueous solutions, equimolar in acetic and propionic acids were subjected to continuous extraction in a micro apparatus, for various lengths of time. The ethereal extracts were analysed by gas-liquid chromatography with a diethyl glycol adipate column (as described in Table 2-10).

Amount of each acid added (μmole)	Extraction time (hr.)	% Recovery		No. of experiments
		Acetic acid	Propionic acid	
1	2	29	25	(1)
	2½ - 3	140	99	(2)
3	1/4	38	45	(3)
	2	87	93	(4)
	2½ - 3	78	73	(1)
5	½	28	42	(1)
	1½	43	59	(1)
	2	63	66	(1)
10	3	62	88	(1)

the same, except that

(i) the column support material was Chromosorb W instead of firebrick.

(ii) hydrogen and air were supplied from cylinders instead of a generator.

(iii) a filter-drier was not found necessary for the nitrogen.

The operating conditions used were those found most suitable for the particular column.]

a) Materials.

F11 Gas Chromatograph with hydrogen flame ionization detector, manufactured and supplied by Perkin-Elmer, Ltd., Beaconsfield, Bucks., England, was used.

Servoscribe Potentiometric Recorder, Type RE511, Fabr. No. R740, C.P. Goerz Electro, was supplied by the Industrial Division of S, Smith and Sons (England) Ltd., Kelvin House, Wembley, Middlesex. This recorder features a variable range (it was operated at 2 mV), variable chart speeds (it was used at 30 mm./min.), and chart width 20 cm.

Gases (all supplied by British Oxygen): Hydrogen, nitrogen and air, in cylinders. (The laboratory supply of compressed air caused background noise and clogging of the detector jet due to contamination by traces of oil.) Reduction valves were coupled to the normal cylinder heads, allowing very sensitive control of gas pressures.

Column (supplied, packed, by Perkin-Elmer). The recipe is based on that of Baumgardt: neopentyl glycol succinate and H_3PO_4 on Chromosorb W (60 - 80 m), in the ratio 20:2:78, packed into a stainless steel column, 1/8" o.d., 2 m. long. The maximum temperature of the column is 240°.

Before use it was aged for 2 days at about 170°, with nitrogen flow at 10 p.s.i.

Injection syringes. Hamilton 1 µl. graduated microsyringes, type 7001-N, also available from Perkin-Elmer, were employed.

b) Operating conditions. Optimal conditions were established and the gas pressures adjusted from time to time. The conditions mostly used were:

(i) Column temperature - 125° was found optimal. With higher temperatures there was more tailing of the peak eluted by water, while at lower temperatures the retention times were longer and the peaks consequently broader. The temperature was checked with a thermocouple and shown to be steady, within 0.25° during a chromatographic run of 3 injections.

(ii) Injection block temperature - at the position of the tip of the syringe during injection, was 231 - 232° (measured with a copper Constantan Thermo-couple).

(iii) Gas pressures - Nitrogen at 17 p.s.i., had a flow rate of 40 ml./min. at the detector head (measured with a bubble flowmeter). Hydrogen, 17 p.s.i., had a flow rate of 31 ml./min. Air, 30 p.s.i., or later at 22 p.s.i., had too fast a flow rate to measure.

(iv) Sample size - 0.2 - 0.7 µl. (With larger injections tailing of the water peak interfered with the acetate peak).

(v) Solvent - water.

c) Behaviour of the column.

(i) With respect to water and short chain volatile fatty acids. Water is not detected by flame ionization detection, but it causes the elution of volatiles from the column giving characteristic 'water peaks' just after injection.

Formic acid is not resolved by this column. Acetic acid is the first compound to be eluted, with a retention time of just under 3 min. Propionic, and the other mono-basic acids appear soon afterwards, and are well resolved. They emerge in order of increasing chain length, the more volatile iso- forms preceding the n- isomers (see Table 2-12). The peaks of propionic and isobutyric acids slightly overlap.

(ii) Ghosting, or the appearance of peaks corresponding to the fatty acids on subsequent injection of water alone was checked for daily. Unlike Baumgardt's observation this did not occur with the present column. Sometimes a very small acetate peak appeared which disappeared on thoroughly cleaning the injection block and was not therefore a property of the column.

(iii) Separatory power of the column, is described as the Height Equivalent to one Theoretical Plate (H), by Martin and Synge (1941). It is assayed by calculating the number of theoretical plates (n) in a column of length (l). This was done for acetic and isovaleric acids with this column, by the simplified calculations of Bayer (1961), from data of 11 injections of standard solutions of the acids.

Table 2-12. Response of neopentyl glycol succinate column to short-chain fatty acids.

The general procedure for the use of the neopentyl glycol succinate column was as described in the text. Results are calculated from the data of injections of standard aqueous solutions of the fatty acids.

Acid	Response constant, k ($cm.^2$/nmole)	Approx. retention time (min,)
Acetic	87.2	2.9
Propionic	182.8	4.3
Isobutyric	258.2	5.0
Butyric	244.2	6.6
Isovaleric	279.4	8.4
Valeric	269.9	11.2
Hexanoic	162.4	15.2

$$H = \frac{n}{l}, \qquad n = 16 \times \frac{[\text{Retention Time}]^2}{[\text{Base Width of Peak}]^2}.$$

for <u>acetic acid</u>: $n = 952$, $H = 2.10$ mm.

for <u>isovaleric acid</u>: $n = 963$, $H = 2.08$ mm.

The column is thus satisfactory for both acids.

d) <u>Calculations</u>. The peak areas were calculated by triangulation (taken as drawn height x base width), and the retention time was measured from the moment of injection. Peak areas were considered more accurate than heights since:

(i) during the analyses, slight fluctuations in carrier gas flow rate caused very slight broadening or narrowing of the peaks with inverse effects on the heights, while the areas remained virtually constant.

(ii) especially with smaller peaks at the most sensitive range of the amplifier used, a measurement of area by triangulation was more accurate, as the background noise caused perturbation of the peaks.

Another possible method of measuring peaks would be to cut out and weigh them, but again with smaller peaks there would be difficulties, and the process would be just as time-consuming as triangulation. Since the baseline is not prefectly steady, a disc-integrator could not be used to measure peak areas.

Injections of standard solutions of the acids were made after every 3 or 4 analytical samples. It was found that the response to standards varied slightly each time the gases were relit, but over a single run was quite constant. Thus the response constants, k, of the acids were

calculated from the mean data of each run, according to Baumgardt, inserting peak areas instead of peak heights:

$$k = \frac{\text{Mean Peak Area x Attenuation}}{\text{Volume Injected x Concentration}} = \text{cm.}^2/\text{nmole.}$$

[peak area in $cm.^2$, concentration in nmole/μl, volume injected in μl.] Using k, the absolute concentration (c) of acids in analytical samples could be calculated from their peak areas (cf. Baumgardt):

$$c = \frac{\text{Peak Area x Attenuation}}{\text{Volume Injected}} \times \frac{1}{k} = \text{nmole}/\mu\text{l.}$$

But it was more usual to add a known amount of a marker acid at the initial step of biological sample preparation and to measure the peak area ratios of acetic acid/marker acid in the final solution. This is compared with the area ratios obtained with standard solutions equimolar in both acids. Then, assuming parallel recovery of both acids, the amount of acetate in the initial biological sample is determined:

$$\text{Acetate } (\mu\text{mole}) = \frac{\text{Acetate/marker Peak Area in Analytical Sample}}{\text{Acetate/marker Peak Area in Equimolar Standard}} \times \frac{1}{\text{Marker Added } (\mu\text{mole})}.$$

e) Reproducibility and linearity of response. Two consecutive injections were made of each sample. Their peak areas were nearly always in close agreement unless the peaks were very small. If there were more than 10% difference between the peak area ratios further

injections were made, and the discrepant results discarded. The slight variations were due to differences in injection size and to errors in measurements especially of the very small peaks. The long-term reproducibility over a whole run was also very good e.g. for 11 standard injections interspersed throughout 54 injections over 3 days, the S.E.M. of the ratios was ± 3.3% (see Table 2-13).

Linearity of response to acetate and isovalerate is shown in Fig. 2-3, over a 5-fold range. By the use of standards, the method was applicable over a much greater range. Linearity was also observed with the other volatile fatty acids.

f) Sensitivity. The chromatographic technique could determine final acetate concentrations of 0.5 µmole/ml., in a 0.5 µl. injection, i.e. 0.25 nmole. The final solution represented a 6-fold concentration of the biological medium e.g. blood, so the complete method could measure acetate at 0.08 µmole/ml., the accuracy for the whole procedure being ± 29% at worst, for a single determination.

g) Maintenance of the chromatograph and accessories. The recommendations of Baumgardt were carried out with respect to rinsing the syringe, flushing out the column (though smaller volumes of water were used) and changing the septum.

After every few runs the injection block was cleaned by squirting analar acetone through it, with the column disconnected.

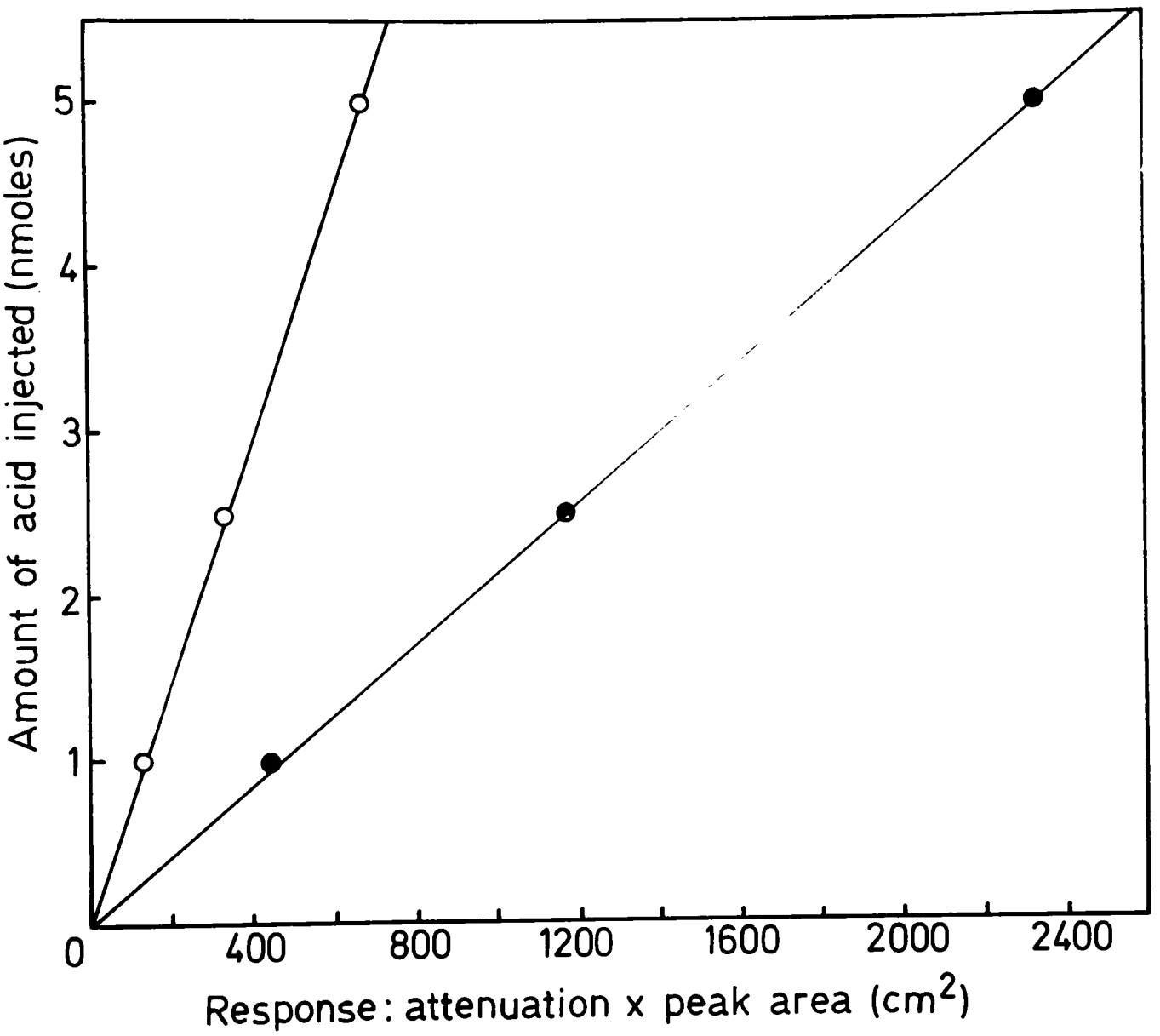
The detector cylinder was gently cleaned from time to time with acetone-soaked cotton wool., as white deposits of potassium phosphates and other matter gradually accumulated on it, marring the response.

Table 2-13. **Use of neopentyl glycol succinate column: Reproducibility of response to acetic and isovaleric acids.**

Injections of 0.3 µl. of solutions of acetate and isovalerate both at 2 µmoles/ml., were made over a period of 3 days. Attenuation: 5X. For further details see the text. Results are expressed as means ± S.E.M. for 11 injections.

Acetate peak area ($cm.^2$)	Isovalerate peak area ($cm.^2$)	Acetate/Isovalerate peak area ratio
13.47 ± 0.471 (± 3.5%)	48.13 ± 1.862 (± 3.9%)	0.280 ± 0.0091 (± 3.3%)

Fig. 2-3. Linearity of Response of Neopentyl Glycol Succinate Column to Acetic and Isovaleric Acids.



Injections were made of 0.2 - 0.5 µl. of equimolar standard solutions of acetic and isovaleric acids (together). Attenuation 10x - 50x. For further details see text.

o—o response to acetic acid.

●—● response to isovaleric acid.

The flow restrictors, gas leads, and detector jet were periodically cleaned by drawing acetone, then air through them. The flow restrictors occasionally got blocked and were replaced.

h) Use of isovalerate as a marker. Isovalerate was not detected in any of the biological media examined (e.g. rat blood, liver), even though this acid can be metabolised in mammals to produce ketone bodies (Meister, 1965) and it has been postulated from isotope experiments that the amino acid leucine is metabolised via isovaleryl CoA in rat liver preparations (Coon, 1950; Coon, and Bachhawat, Robinson, 1955).

In the gas chromatographic system used, isovalerate appeared quite soon after acetate (retention time approx. 9 min.) and was well separated from the other volatile fatty acids e.g. butyrate and hexanoate, which were sometimes used as substrates in experiments. The column is just as efficient in separating isovalerate as acetate (see p. 40).

Its usefulness as a marker throughout the preparation of biological material for gas chromatography was demonstrated in many experiments on the recovery of added acetate and isovalerate from rat blood, liver homogenates, perfusion media (see later Table 2-14). Acetate recovery in terms of isovalerate recovery (calculated as on p. 41) was mostly between 92 and 105%, whereas the absolute acetate recovery was about 30% lower, showing that isovalerate losses do parallel those of acetate during sample preparation, thereby justifying the use of isovalerate as a marker.

The % recovery of isovalerate was calculated with each analysis.

If it were less than 50%, the results were considered unreliable and were discarded.

4. Sample preparation for gas-liquid chromatography.

a) Unsuitability of lyophilization. Baumgardt (1964) using a similar column for the determination of acetate has adopted the preparative method described by Erwin et al. (1961). This involves protein precipitation of blood by sulphuric acid-sodium tungstate reagent, addition of NaOH followed by lyophilization of the sodium salts, and finally acidification with the minimum volume of H_2SO_4. (This method had proved unsuccessful with a diethyl glycol adipate column as previously described.) The method was found unsuitable for the present column since an excess of sulphuric acid as would be required to safely acidify the volatile fatty acids in unknown solutions, was detrimental to the column, producing large peaks which interfered with those of acetate and of isovalerate. Any traces of tungstate likewise damaged the column. Another protein precipitant, perchlorate, was tried instead. KOH was added to the protein-free supernatant and this was left standing at 0° for 30 min. to allow complete precipitation of perchlorate. Yet again large peaks appeared, especially during subsequent injections of water. It seemed that traces of perchlorate were having a delayed action on the column.

It is also possible that some of the interference in the gas chromatography just described, was caused by other organic compounds of

blood remaining after protein precipitation e.g. glucose, pyruvate, lactate. Also, the final solutions were often coloured (yellowish) possibly due to the presence of carotenes and other pigments. It was obvious that greater purification of biological samples was required.

b) Steam distillation.

(i) Principle. Steam distillation of protein-free supernatants had been used for gas chromatographic analysis of volatile fatty acids of sheep blood by Annison (1954b). Annison found that steam distillation at a pH of just under 3 gave complete recovery of the lower volatile fatty acids (including formate) with negligible interference from HCl, lactate, pyruvate and β-hydroxybutyrate. For the present experiments since formate recovery is not required, steam distillation at pH 3 - 3.5 should be satisfactory (pK acetic acid = 4.73).

(ii) Method. [This method is based on that of Dr. E.F. Annison, personal communication, except that alkaline trapping agent is added to the collecting flask prior to distillation, and the final volume is 10 times as large.] Protein-free supernatant is adjusted to pH 3 - 3.5 (bromo-phenol blue) with 2N-KOH, the final volume being under 10 ml. The solution and rinsings are quantitatively transferred to a Markham still. The first 100 ml. of distillate is collected in a flask already containing 0.1 ml. of 2N-KOH. The lead from the condenser of the still is actually immersed in the alkaline trapping solution, thus preventing escape of any free volatile acids during distillation. The aqueous solution of the potassium salts (pH about 11) can be reduced to 1 - 2 ml. with

minimal loss of volatile acids, by careful boiling (first in an Erlenmeyer flask, then in a small beaker). Finally the solution is transferred using a Pasteur pipette, to a small tube (1 cm. x 4 cm.) inserted in a Seroblock at 50°, and brought to dryness under a stream of nitrogen. The dried salts are carefully dissolved at 0°, in 0.4 ml. of water using a glass rod to remove them from the sides of the tube. Then 0.1 ml. of 30% orthophospheric acid is added slowly, to form the free acids (final pH below 1). The tube is sealed with a Suba-seal cap, and stored at -20° until required for gas chromatographic analysis. The frozen sample can be stored for about 2 months without loss of volatile fatty acids.

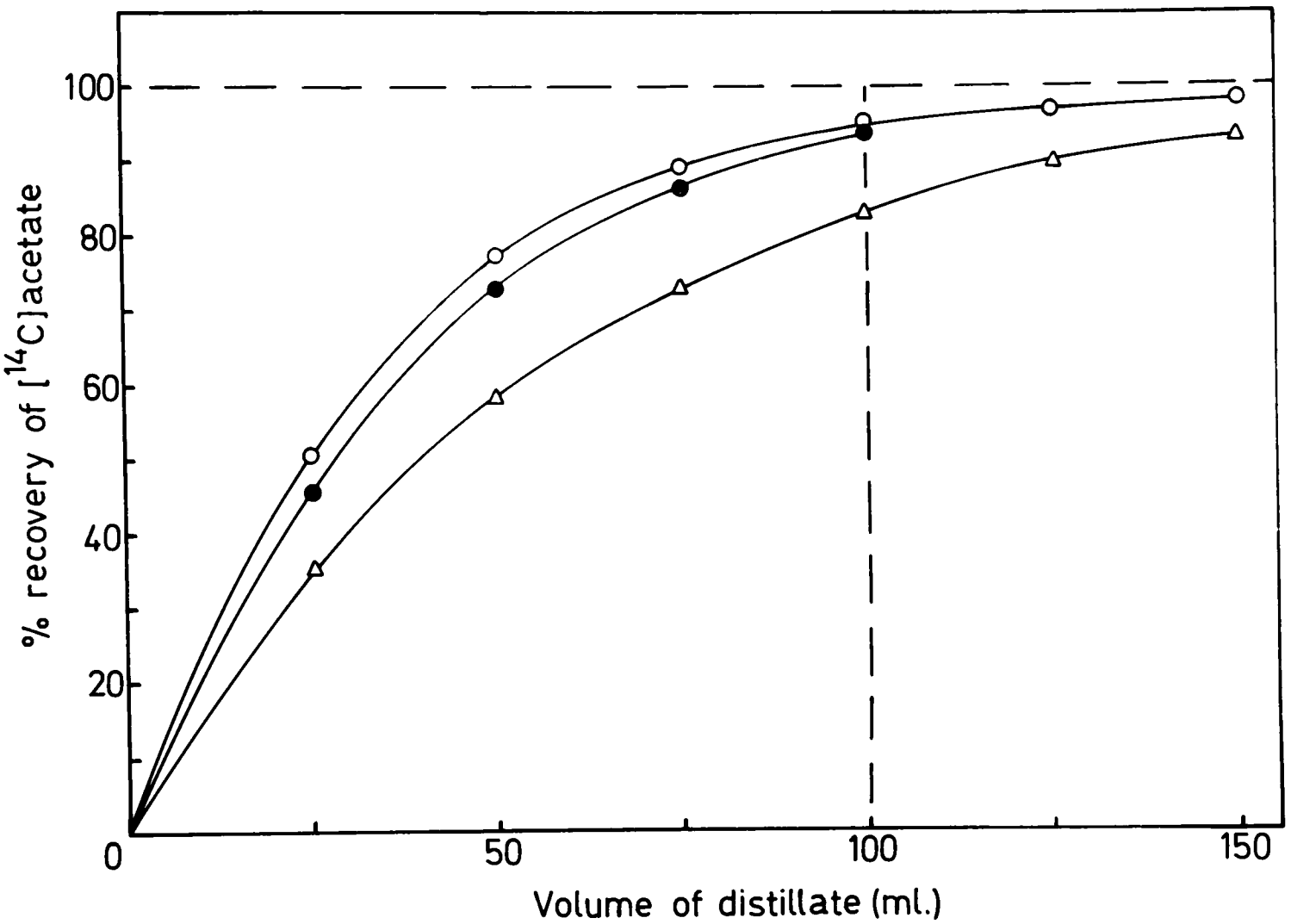
These final solutions have a small amount of colloidal white precipitate, presumably potassium phosphate. This gradually accumulates in the detector jet which has to be cleaned out every 2 - 3 weeks during continuous use, but it does not otherwise interfere with the analyses.

(iii) Recovery. The progress of acetate recovery by steam distillation was followed using [2-^{14}C]acetate (see Fig. 2-4) and measuring isotope recovery in successive 25 ml. samples of distillate.

In 3 experiments, the first 100 ml. of distillate contained 92.3 - 95.0% of 3 or 10 μmoles of added [2-^{14}C]acetate. A further 50 ml. of distillate only contained another 3.8% of acetate and would add to the chances of losses during subsequent evaporation, so it was decided to collect 100 ml. only each time.

The volume taken for distillation was critical. With more than 10 ml., distillation was slower and recovery poorer, e.g. with 25 ml. there

Fig. 2-4. Recovery of Acetic Acid by Steam Distillation.



Solutions of pH 3 - 3.5, containing 3 or 10 μmoles of [2-^{14}C]acetic acid in 10 or 25 ml. volumes were steam distilled as described in the text. Successive 25 ml. aliquots of distillate were collected, without KOH, 0.4 ml. samples of which were added to 10 ml. of scintillation fluid (Butyl PBD, see Materials and Methods) and were counted on a Beckman Liquid Scintillation System (model 1650).

○—○ recovery of 3 μmoles from 10 ml.

●—● recovery of 10 μmoles from 10 ml.

△—△ recovery of 3 μmoles from 25 ml.

was a mean recovery of only 81.6% of 3 μmoles of [2-^{14}C]acetate in the first 100 ml. of distillate (see Fig. 2-4).

The more volatile acids, propionate, isovalerate etc. distil more rapidly (see McClendon, 1944), i.e. at this stage, acetate recovery is the rate-limiting process,

(iv) Blanks. All experiments were accompanied by blanks put right through the procedure, 1 or 2 μmole of isovalerate was added initially as in the analyticals (no isovalerate peak appeared when this acid was omitted). There was always a small acetate peak representing about 0.1 μmole, and its origin was investigated as follows: 100 ml. of distilled water was brought to pH 11 with KOH, evaporated to dryness and analysed by gas chromatography. A similar acetate peak appeared. Thus the 'blank' acetate apparantly arose during the evaporation of the distillate to dryness, even though caution was taken not to contaminate the atmosphere with acetic acid.

c) Preparation of biological samples.

(i) Blood. [Note. The preparation of blood is also according to Dr. E.F. Annison, personal communication, except that for convenience, proteins are removed by centrifugation rather than by filtration.] To 3 ml. of freshly collected rat blood at 0° is added exactly 0.1 or 0.2 ml. of 0.01 M isovalerate (i.e. 1 or 2 μmoles) as a marker. The blood is now hemolysed with 15 ml. of distilled water to ensure measuring acetate of whole blood, both cells and plasma. (Annison and Lindsay, 1961 have observed uniform distribution of acetate between the cells and plasma of sheep blood.)

2 ml. of freshly prepared 25% w/v metaphosphoric acid is added as a protein precipitant and the solution is mixed and allowed to stand for 20 min. It is centrifuged at 0°, at 3000 r.p.m. for 15 - 20 min., and the supernatant poured off. The protein precipitate is resuspended in about 2 ml. water, centrifuged again, and the washings combined with the supernatant. The latter is brought to the first faint phenolphthalein pink with 2N-KOH and reduced to 2 - 3 ml. in a rotary evaporator just below 60°. Then the pH is adjusted to 3 - 3.5 (bromophenol blue) with 30% H_3PO_4. The sample is now ready for steam distillation (p. 45) and subsequent gas chromatographic analysis.

(ii) Perfusion medium etc. A 1 ml. sample of perfusion medium is taken, and since the protein and erythrocyte content is equivalent to that of blood it is treated as one-third of the volume of blood [see (i)]. As the final volume of supernatant and washings is under 10 ml. the rotary evaporation step is unnecessary. (Solutions of albumin were treated similarly.)

(iii) Tissues. Perchloric acid is the long-established protein precipitant for frozen powder of freeze-clamped tissue. Acetate recovery was satisfactory using perchlorate (see Table 2-14). Isovalerate is added as soon as the tissue powder-perchlorate mixture has thawed. After 20 min. at 0° the proteins are removed by centrifugation at 3000 r.p.m. for 15 - 20 min. If other metabolites e.g. lactate, pyruvate are to be measured the supernatant is neutralised and an aliquot removed for these analyses. The remainder is brought to pH 3 - 3.5 for steam distillation

Table 2-14. Recovery of acetic acid from biological material, by steam distillation and gas-liquid chromatography (neopentyl glycol succinate column).

Sample preparation and gas chromatographic procedure were as described in the text.

Biological Material	Acetic acid added (μmoles)	% Recovery Mean	Range	No. of determinations
Perfusion Medium	1	92.6	92.3-95.1	(3)
	2	102.3	92.0-116.3	(4)
Liver Homogenate	1	105.5	81.0-134.3	(4)
	10	99.6	97.9, 101.2	(2)

as on p. 45. Rotary evaporation is not required, the supernatant volume from up to 3 g. tissue being under 10 ml.

5. Summary.

Coupling of the steam distillation technique of Annison with the gas-liquid chromatographic system of Baumgardt (1964) has been found a satisfactory method for the determination of acetate in biological samples. With isovalerate as a marker, the mean recoveries of 1 - 10 μmoles of acetate from tissue homogenates and perfusion media lie between 93% and 105% (Table 2-14). The accuracy of the overall procedure is ± 29% at worst, and the limit of sensitivity is 0.08 μmole.

The method is specific for acetate. The acetate peak is well resolved from those of the other short chain fatty acids, and even if small amounts of other volatile acids (β-hydroxybutyrate, acetoacetate, pyruvate and lactate) come over at steam distillation, these acids were shown not to interfere in the chromatographic system.

Unlike another gas chromatographic system investigated (diethyl glycol adipate column), the present one has the advantage of taking aqueous samples, thus cutting out the need for cumbersome extraction procedures.

In the early experiments with this system the scatter of results was very large e.g. for 4 duplicates in each case the range of recovery of 0.25 μmole of acetate from blood was 75 - 187% and of 1 μmole was 107 - 156%. Also at this stage the results were rather high, the mean recovery in 19 experiments being 128.8%. However, some months later the

results had improved, the scatter was much less and the recovery more nearly 100% (Table 2-14). The reason for this trend was thought to be improvement in technique, and settling of the column and chromatograph with age.

Other volatile fatty acids (e.g. propionate, butyrate) present in some of the biological materials examined could be assayed simultaneously with acetate.

CHAPTER 3. ACETATE IN RAT BLOOD AND ITS ORIGIN

CHAPTER 3

ACETATE IN RAT BLOOD AND ITS ORIGIN

I Introduction

An approach to the study of the metabolism of a substance in an organism is the determination of the level of the substance in the blood and any changes in that level induced by applying altered physiological or pathological conditions. More detailed information may be obtained by examining its level in blood taken from different parts of the animal and by studying arterio-venous differences across various organs.

This approach was adopted for initial studies on the metabolism of acetate in the rat. The aim of the work described in this chapter was to establish the blood acetate level in the normal animal, to find out something of the factors affecting or controlling this level and to elucidate if possible, the origin of blood acetate.

Table 3-1 is a collection of some published data on blood acetate levels in different species. Some of the discrepancies within the data for a given species (e.g. man) may be partially attributed to differences in the methods of determination. A number of the earlier methods, especially, were neither sufficiently sensitive nor specific. Thus the values of Fonnesu (1951) (from dichromate oxidation and photometry) are excessively high. Excluding the latter results, which are 10-fold higher than the rest, the levels in human blood lie between 0.025 and

Table 3-1. Some published blood acetate levels in different species.

[Note. In this table GLC = gas-liquid chromatography.]

Animal	Observation No.	Acetate level (μmoles/ml. blood†) Post Absorbtive	Fed	Method of Determination	Reference
Man	1	0.80	1.25	Dichromate oxidation	Fonnesu (1951)
	2	0.025	0.038	Microdiffusion + Enzymic assay	Lundquist (1962)
	3	0.03–0.20*		Vacuum Distillation + GLC	Lester (1964)
	4	0.034–0.134*		as in No. 2	Hochheuser et al. (1964)
	5	0.064*		Enzymic assay	Bergmeyer&Moellering(1966)
Sheep	6	0.43	1.32	Steam Distillation + GLC	Annison (1954,b)
	7	0.84*		Lyophilization + GLC	Baumgardt (1964)
Cow	8	0.25	0.60	as in No. 7	Simkins et al. (1965)
	9	0.08–0.21	0.64–0.79	as in No. 2	Hochheuser et al. (1964)
Rat	10	0.195*		as in No. 2	Hochheuser et al. (1964)

† Serum in Nos. 2 and 5; plasma in Nos. 3 and 4. *Dietary state unspecified.

0.20 µmole/ml. - generally below 0.1 µmole/ml. These values were obtained by various enzymic methods (Lundquist, 1962; Hochheuser et al. 1964; Bergmeyer and Moellering, 1966) or by gas-liquid chromatography (Lester, 1964). In the fasted sheep the level was 0.4 - 0.8 µmole/ml., rising after feeding to 1.3 (Annison, 1954b; Baumgardt, 1964), was similar in cows (Simkins, Sultie and Baumgardt, 1965; Hochheuser et al. 1964), and was about 0.2 µmole/ml. in the rat.

Two trends emerge from these data. Firstly, the values are in general higher in ruminants (sheep, cow) than in non-ruminants (rat, man). Secondly, in ruminants, feeding causes a rise in blood acetate but there is not sufficient evidence to establish whether this is the case in non-ruminants. These observations bear out the well-known theory of acetate formation by ruminal flora and its subsequent absorption into the bloodstream (Phillipson and McAnally, 1942; Barcroft et al. 1944; Elsden, 1945), but leave unanswered the question of the origin of blood acetate in non-ruminants.

II Experimental.

A. Methods.

1. Treatment of animals.

a) Feeding and fasting. The fed rats were allowed free access to normal 'rat-cube' diet. They were found in this laboratory to eat overnight between 5 pm. and 6 am. (C. Start, unpublished observation).

Blood samples were normally collected between 10.30 am. and 2 pm.

The fasted rats were deprived of food for either 24 or 48 hr. as stated, and allowed free access to water.

b) High-roughage (bran) diets.

Bran diet (i) [for 4 days before the experiment]. The rats were fed bran ground up and mashed with a little water, and drinking water ad lib. They ate virtually nothing for the first 48 hr., ate avidly for a further 48 hr. and were then used for the experiments.

Bran diet (ii) [for 6 days before the experiment]. The rats were fed 95% bran, and 5% ordinary rat cake ground together with a little water, for 3 days, followed by 3 days on bran and water only [diet (i)]. The rats adapted themselves immediately to this diet and ate well throughout.

c) Neomycin treatment. 0.5% neomycin sulphate (for dispensary purposes, from E.R. Squibb and Sons, Liverpool) was added to the drinking water of rats on a normal diet, for 2 days before the experiments.

This dosage was used by Capps, Shetlar and Bradford (1966) to restrict the activities of rat intestinal flora to a minimum. Similar doses were found to cause no abnormalities in intestinal morphology or xylose absorption (Broitman, Flint and Zamcheck, 1967) being well below the amounts known to cause renal toxicity (Emmerson, Pryse-Davies, 1964). Neomycin is known to cause malabsorption in man (Jacobson, Chodos and Faloon, 1960; Rothfeld and Osborne, 1963). But after 48 hr. of treatment the rats appeared healthy, and free of intestinal lesions, though the caeca were distended with gases, and contained a larger amount of more

fluid contents.

2. Collection of blood.

a) Cannulation of the aorta. Rats can be approximately 50% exsanguinated by cannulation of the aorta. 8 - 12 ml. of blood was collected per rat depending on the size of the animal.

The animal was anaesthetised with diethyl ether. 100 µl. of heparin solution was injected into the left saphenous vein. With the animal on its back, a mid-line abdominal incision was made and the aorta was exposed by moving the gut to the animal's left and gently teasing the surrounding connective and adipose tissue aside. The aorta was clamped with a small bulldog clip at the level of the renal arteries, held distally with a forceps and a cut made in its wall. The cannula, an 8 in. length of Portex Nylon Tubing No. 3, cut obliquely to give a sharp point, was inserted into the aorta and the bulldog clip released. The blood was collected in a tube dusted with heparin powder and standing in crushed ice. It was shaken to ensure mixing with the heparin and thus coagulation was always prevented.

b) Cannulation of the portal vein. The portal vein was cannulated to collect blood from the viscera.

The animal was anaesthetised with Nembutal and 100 µl. of heparin was injected into the saphenous vein, as in liver perfusion experiments. A mid-line abdominal incision was made and the portal vein exposed by placing the gut to one side. The vein was tied off immediately below the

liver to prevent bleed-back. Two loose ligatures were passed around the portal vein half-way between the tie-off and the point at which it receives its tributaries from the various visceral organs. The portal vein was then cannulated with a hypodermic needle, 16 gauge, into which was inserted about 8 in. of Portex Flex Nylon Tubing No. 2. Both ligatures were tied, securing the cannula in place. Care was taken not to push the tip of the cannula beyond the branching point of the vein. Blood was collected as from the aorta (above), and despite the slower flow rate, up to 8 ml. could be collected.

3. Preparation of caecum contents.

The caecum was removed from a living (anaesthetised) or freshly killed rat, usually after exsanguination via the aorta or portal vein. The semi-solid contents were quickly extracted and deproteinised with the same weight of $MgSO_4.7H_2O$ (Annison, 1954b). The suspension was left standing for 30 min. at 0°, then spun for 15 - 20 min. at 2000 r.p.m. 5 - 10 ml. of the supernatant was brought to pH 3 - 3.5 (bromophenol blue) with KOH, added while whirling, to prevent localised concentration of alkali with precipitation of $MgSO_4$. This solution was steam distilled and analysed for volatile fatty acids in the usual way.

B. Results.

1. Aortic blood acetate under various conditions.

The results of analyses on aortic blood appear in Table 3-2. The

Table 3-2. Rat blood acetate under various dietary and pathological conditions.

Acetate was determined by gas chromatography as described in Chapter 2, on duplicate or triplicate 3 ml. samples of arterial blood (cannulation of the aorta). The blood was collected between 10 am. and 2 pm. For further details see the text.

State of animal	Acetate level (μmoles/ml. blood) Mean ± S.E.M.	No. of animals
Fed	0.43 ± 0.110	(8)
Fasting 24 hr.	0.60 [0.66,0.54]	(2)
48 hr.	0.18 ± 0.035*	(3)
High-roughage (bran) diet	0.27 ± 0.016	(6)

* Compared with normal, fed animal, the probability (P) that the difference was due to chance, was calculated by the t test: $0.025 < P < 0.05$.

values in the fed normal animal varied between 0.15 and 0.91 μmole/ml. (mean, 0.43 μmole/ml.), the determinations being performed over a period of 5 months. The results of duplicate analyses were mostly in very close agreement. The blood from fed rats usually contained traces of other volatile fatty acids, notably propionate (at less than one-fifth of the acetate level), and sometimes traces of butyrate and valerate appeared also. Branched-chain acids never appeared in aortic blood.

Fasting caused no significant changes in the acetate concentration. The slightly higher levels after 24 hr. fasting were not significantly different. After 48 hr. fasting, P, the probability that the difference was due to chance (calculated by the t test) was just below 0.05. No other volatile fatty acids appeared in the blood of fasted rats.

Bran, consisting almost entirely of roughage, could presumably only be digested by the fermentative bacteria of the intestines, especially of the caecum. A bran diet might therefore be expected to raise the volatile fatty acid levels of the caecum contents and possibly of blood. However, the blood acetate was lower if anything in the bran-fed rats and no other volatile fatty acids appeared. There was no difference observed between the results obtained with bran diets (i) and (ii) (see p. 53).

2. A possible intestinal origin of acetate.

a) Principle. The portal blood drains the stomach, intestines (including the caecum) and associated organs (the spleen and pancreas). Analysis of acetate in the portal blood should reveal whether the

intestine was an important source of blood acetate, cf. the studies of Barcroft et al. (1944) on the volatile fatty acid content of blood draining different sections of the alimentary tracts of larger animals (sheep, pig, pony). Comparison of the acetate in blood from the aorta and the portal vein might also shed light on uptake of acetate by the liver.

b) Acetate in portal blood.

[It was not possible to collect blood from both the aorta and portal vein of the same animal, so comparisons were made between the mean results obtained with different animals.]

It was first established that portal blood contains no isovalerate, so this acid could still be used as a marker. In the fed animal the total volatile fatty acids (2.48 μmole/ml., taken as the sum of all these acids analysed by gas chromatography) was about 6 times as high as in the blood from the aorta, and the acetate (1.71 μmole/ml.) was roughly 4 times as high (see Table 3-3). Propionate, butyrate and valerate which appeared in mere traces in aortic blood together accounted for 16 - 45% of the total volatile fatty acids in portal blood. No branched-chain acids or hexanoate appeared, unlike the findings of Annison and Pennington (1954) for blood draining the sheep rumen.

c) Acetate in caecum contents. Acetate at about 24 μmoles/g. represented 50% of the total volatile fatty acids of caecum contents (see Table 3-4). There were relatively larger contributions by propionate and butyrate than in portal blood, and isobutyrate, isovalerate, valerate and hexanoate were present. Each of the new acids appearing in

Table 3-3. Effect of neomycin treatment on rat portal blood acetate and other volatile fatty acids.

Volatile fatty acids, were measured by gas chromatography as for acetate (Chapter 2). "Total VFA" = the sum of volatile fatty acids thus measured. Cannulation of the portal vein, and oral treatment with neomycin (antibiotic) were as described in the text. The table shows the actual results of the volatile fatty acid analyses, with the means for acetate and total VFA. (- denotes no detectable acid.)

State of animal	Rat No.	Individual volatile fatty acid levels (μmoles/ml. blood) Acetate	Propionate	Butyrate	Valerate	Total VFA (μmoles/ml. blood)
Normal	1	1.40	trace	0.90	0.26	2.56
	2	2.02	0.23	0.15	trace	2.40
	Mean	1.71 = 69% of total VFA				2.48
Neomycin-treated	3	0.35	0.05	0.15	-	0.55
	4	0.66	0.16	trace	-	0.82
	5	0.48	0.16	0.18	-	0.82
	6	0.50	0.30	0.48	-	1.28
	Mean	0.50 = 57.5% of total VFA				0.67

Table 3-4. Effect of neomycin treatment on acetate and other volatile fatty acids of rat caecum contents. Volatile fatty acid analyses and neomycin treatment were as in Table 3-3. Caecum contents were prepared for analysis according to Annison (1954b) (see text). 2 of the 6 complete analyses in normal rats appear in the table; the means for acetate and total volatile fatty acids are from the 6 experiments. All 4 analyses with neomycin-treated animals are shown.

[Note. Since the contents contained isovalerate, these analyses were performed without added marker, so the results only represent about 60 - 95% of the true values.]

State of animals	Rat No.	Individual VFA (μmoles/g. of caecum contents) Acetate	Propionate	Isobutyrate*	Butyrate	Isovalerate	Valerate	Hexanoate	Total VFA (μmoles/g.)
Normal	1	28.5	8.3	small amount	7.8	1.0	1.1	1.4	48.1
	2	29.8	9.0	small amount	5.2	0.2	0.6	0.2	45.0
	Mean(6)	23.6 = 52.2% of total VFA							45.2
Neomycin-treated	3	5.12	0.88	-	0.09	trace	-	-	6.09
	4	4.22	2.19	trace	0.55	0.08	-	-	7.04
	5	5.34	4.49	trace	1.74	0.06	0.05	-	11.68
	6	4.35	4.48	trace	1.70	0.91	0.10	-	11.54
	Mean(4)	4.76 = 52.4% of total VFA							9.08

*Isobutyrate peak was not resolved from that of propionate and could not be measured. It was comparable in size with that of isovalerate.

these analyses was identified:

(i) by having the characteristic retention time on the column of the corresponding pure acid,

(ii) by the fact that when pure solutions of the acids were added to the analytical solutions their corresponding peaks coincided to give enlarged single peaks.

The total volatile fatty acid concentration in the caecum contents was about half that of sheep rumen contents (Annison, 1954a,b; Bergman, Reid, Murray, Brockway and Whitelaw, 1965). The overall pattern was very similar to that found by Annison (1954a) for the rumen contents of a sheep fed a casein-rich diet, who suggested that the branched-chain acids arise by bacterial action on dietary amino acids. No further peak appeared in the present analyses between those of iso- and n-valerate where 2-methyl butyrate might be expected to emerge (cf. Annison, 1954a). But the possibility of the presence of 2-methyl butyrate could not be excluded, as a sample of this acid was not available and it is not known whether the column would resolve it from its isomers, iso- and n-valeric acids.

d) Effect of neomycin. The effects of neomycin on the volatile fatty acid composition of portal blood and of caecum contents are summarised in Tables 3-3 and 3-4 respectively.

Neomycin treatment caused the levels of both total volatile fatty acids and of acetate in caecum contents to drop to one-fifth of their normal levels, while hexanoate and the branched-chain acids had almost vanished,

even though these animals were still adequately fed (stomach and intestines were full). Thus neomycin drastically reduced the bacterial activity of the caecum.

In portal blood, neomycin treatment reduced total volatile fatty acids and acetate to about one-third of the normal levels. The acetate was now within the range found in aortic blood, so absorption from the caecum (and the rest of the gut) was minimal, though since propionate and butyrate still appeared in much larger amounts than in aortic blood some volatile fatty acid absorption must have been occurring.

C. Discussion.

1. Blood acetate level in the fed rat.

The acetate levels found in the fed rat blood were lower than those found by Ciaranfi and Fonnesu (1952) using a less sensitive oxidimetric method, and about twice as high as the values obtained by a microdiffusion-enzymic method (Hochheuser et al. 1964).

2. Apparent resistance to dietary alterations.

Several possible explanations suggest themselves for the apparant resistance of blood acetate levels to dietary alterations.

The dietary acetate content as such is not relevant, even allowing for hydrolysis of acetyl groups from proteins and amino sugars.

As pointed out in the methods section, the fed rats normally stopped eating 4 - 8 hr. before the blood was collected, even though they

had free access to food until the time of collection. Maximal rises in blood acetate (of up to 3 times the post-absorptive level) were obtained 3 - 4 hr. after a 2 hr. feed in sheep (Annison, 1954b), and 6 hr. after the start of feeding in cows (Simkins et al. 1965), with a fairly rapid return to the post-absorptive level, within a few hours. The present experiments did not rule out the possibility that a similar though much smaller rise might occur in rats. Lundquist (1962) failed to observe a significant rise in human blood acetate level 2 hr. after a meal rich in fats. The sharp rise after feeding in the ruminant is presumably caused by the lipid content of the diet as well as by bacterial decomposition of carbohydrate.

If the acetate were being produced by bacterial decomposition of feedstuffs, then 48 hr. fasting might not be sufficient to deplete the bacteria of substrate. This explanation is quite possible. It was shown that the bacteria do indeed produce acetate, and it was observed that the caecum where most of the bacterial activity of the gut resides, was full even after 48 hr. fasting.

If acetate is absorbed via the portal system, then the liver might remove acetate before it reaches the general circulation. Indirect evidence already exists that the portal circulation is the major route for acetate absorption based on experiments with medium-chain fatty acids (Fredrickson and Gordon, 1958; Kiyasu, Bloom and Chaikoff, 1952; Hyun, Vahouny and Treadwell, 1967). From the present experiments it seems that the liver (and possibly the heart) remove acetate (this will be further

discussed in section 4).

To summarise, the acetate level in blood from the aorta of the rat remained constant under the various dietary conditions employed, viz. fed (i.e. 4 - 8 hr. after feeding), fasted (for 24 and 48 hr.), and bran-fed. Yet this finding is not incompatible with the idea that blood acetate arises mainly from the intestine. Further experiments showed that bacterial action produces acetate from residual foodstuffs in the caecum, that this process continues even after 48 hr. fasting and that the acetate, absorbed via the portal vein, is largely removed by the liver before appearing in the general circulation.

3. Role of the caecum in acetate absorption.

As early as 1944 Barcroft et al. showed that the blood draining the caecum in sheep and non-ruminants (pig, rabbit) had raised levels of volatile fatty acids, largely acetate, and that the products of fermentation (i.e. volatile fatty acids) in the caecum of the sheep and pig were similar to those in the rumen of sheep.

In the present experiments, the caecum of the rat was shown to contain very high concentrations of acetate (about 60 times more than in arterial blood). Net absorption of acetate into the blood stream occurred so that the blood returning from the caecum (portal blood) was 4-fold richer in acetate. After oral treatment with antibiotic to diminish intestinal flora, the acetate levels were greatly reduced in both the caecum and in portal blood and net absorption of acetate was no longer apparant, demonstrating that part of portal blood acetate is due

to bacterial action in the caecum.

The caecum thus appears to play an analogous role with respect to volatile fatty acids in the rat as the rumen does in ruminants, though on a much smaller scale. The caecum contents in the rats used weighed between 2.3 and 4.5 g. i.e. roughly 1% of the total body weight (350 - 400 g.). From Bergman et al. (1965); Annison and Lewis (1959; p.14), the rumen contents represent 10% or up to 20% of the total body weight in the sheep, and 15 - 30% in cattle (where the body weight is taken as 600 - 900 kg., Spector, 1956a). As already observed, the acetate concentration in the rat caecum is half of that in the sheep rumen (Annison, 1954a,b). Acetate would therefore be expected to make a smaller contribution to total energy output in the rat than in the sheep, as is borne out by the following estimations:

a) In the sheep, Sabine and Johnson (1964) calculated on the basis of intravenous infusion experiments, that the absorbed acetate could account for 40 - 50% of the total carbon burned. By intraruminal infusions of labelled acetate, and allowing for interconversions with other volatile fatty acids, Bergman et al. (1965) concluded that the acetate produced by the rumen was responsible for 42% of the daily energy expenditure, in agreement with the earlier result of Sabine and Johnson.

b) An approximation of the corresponding figure in the rat can be made as follows.

In a rat of 300 g., the flow rate in the portal vein is approximately 12 ml./min. (from Dobson and Jones, 1952; assuming the liver weighs 10 g.).

From Tables 3-2, 3-3, the arterio-portal venous difference for acetate is about 1.2 μmole/ml., representing an approximate splanchnic absorption of 20 m-moles of acetate/day. Since complete oxidation yields 209 kcal./mole, the absorbed acetate could correspond to 4.1 kcal./day.

The rate of metabolism in the rat is 1,040 kcal./sq.m./day. (Carr and Krantz, 1942), or 41.7 kcal./day in a 300 g. animal. Thus, if the absorbed acetate were completely oxidised, it could maximally produce 10% of the basal caloric output in the rat. (Only a very small amount of this acetate would be involved in the biosynthetic reactions of acetyl CoA.)

The following correlation appears between blood acetate values and the digestive systems of various species.

SPECIES	Sheep, Cow	Rat	Man
DIGESTIVE SYSTEM	Large rumen + caecum	Simple stomach + caecum	Simple stomach + relatively small caecum
RELATIVE BLOOD ACETATE LEVEL	High	Intermediate	Low

It remains to be seen whether any of the acetate in human blood is of exogenous origin, e.g. by bacterial action in the intestine.

4. Effect of other organs on rat blood acetate level.

The portal blood contains 4 times as much acetate as that from the aorta indicating uptake of large amounts of acetate by certain organs (as mentioned earlier). Between the portal vein and the aorta the blood passes through the liver, the lungs and the heart. The portal blood, comprises 60 - 70% of the blood supply to the rat liver (Dobson and Jones, 1952; Sapirstein, 1956); this suggests that the liver is largely responsible for removal of the portal blood acetate. Acetate uptake by the lungs is likely to be negligible (but there is not much evidence on this point). The heart which takes up acetate rapidly in vitro (Williamson, 1962) may account for some of the removal, although the coronary blood flow represents only 2.6% of the total cardiac output (Sapirstein, 1956) and the blood which originated in the portal vein has been diluted with that from the rest of the body when it reaches the heart tissue. If it were feasible, analysis of the acetate in blood from the hepatic vein might shed light on the relative uptake by liver and heart.

Since the rat has a small circulatory volume (about 20 ml. in these rats of 300 g.) and relatively large samples (3 ml.) are required per assay, it has not been possible to measure arterio-venous differences across individual organs and thus gauge their contribution (positive or negative) to blood acetate levels. Some arterio-venous difference studies of acetate have been performed in larger animals, and support the idea that acetate from the bloodstream is utilised by extrahepatic tissues

(Fonnesu, 1951; Annison, 1954b; Lundquist, 1962).

D. Summary.

1. Acetate levels were measured in fresh rat blood obtained by cannulation of the aorta under various dietary régimes (fed, fasting for 24 or 48 hr., high-roughage diet). The level remained constant at about 0.4 mM in all conditions. The diet as such was not a source of acetate.

2. However, acetate in the portal blood draining i.a. the caecum was 4 times as high as in aortic blood. The caecum contents contained relatively large amounts of volatile fatty acids (50 μmoles/g.), of which about 50% was acetate (similar to sheep rumen contents, Annison, 1954b; but on a smaller scale).

3. Oral treatment with neomycin(antibiotic) reduced the acetate in the caecum to one-fifth and in the portal blood to one-third of the value in untreated rats.

4. It was concluded that the free acetate in rat blood largely arises by bacterial activity in the intestine and especially in the caecum, and that the liver (and possibly the heart) removes acetate from the bloodstream.

CHAPTER 4. ACETATE IN THE TISSUES OF NORMAL AND ALLOXAN-DIABETIC RATS.

CHAPTER 4

ACETATE IN THE TISSUES OF NORMAL AND ALLOXAN-DIABETIC RATS.

I Introduction.

It is well established that alloxan-diabetes causes major metabolic disturbances in the rat affecting not only the metabolism of carbohydrates but also that of fat (for reviews see Steele, 1966; Randle *et al.* 1966). The metabolism and levels of the acetyl group are therefore disturbed (see e.g. Wieland, Weiss and Eger-Neufeldt, 1964). Furthermore, Hochheuser *et al.* (1964) have reported a significantly raised level of free acetate in the blood of rats with chronic alloxan-diabetes. A study of the effects of alloxan-diabetes on the free acetate levels in blood and in various tissues therefore seemed of interest.

II Experimental.

A. Methods.

1. Treatment of animals.

Large, male rats (250 - 400 g.) were used, as in Chapter 3. In addition, acetate was measured in the livers of female rats (180 - 220 g.) of similar weight to those used in liver perfusion experiments (Chapter 5). Conditions of feeding and fasting, and cannulation of the aorta were as

described in Chapter 3.

a) Alloxan-diabetes. Alloxan.$1H_2O$ (biochemical grade, obtained from British Drug Houses Ltd.) was doubly recrystallized from water by Mr. L.V. Eggleston of this Department. The final formula was probably Alloxan.$4H_2O$. The dosage, based on that of Newsholme and Randle (1964), was 80 mg.of recrystallized alloxan/Kg. body weight. The required amount, dissolved in less than 0.4 ml. 0.9% NaCl was injected intravenously (saphenous) into rats under ether anaesthesia. The animals were left on a normal diet and used 48 hr. later. By this time urinary samples invariably gave positive results for glucose and ketone bodies as tested with Clinistix and Ketostix respectively (both obtained from Ames Co., Division of Miles Laboratories Ltd., Stoke Poges, Slough, Bucks.).

2. Measurement of intermediate levels in tissues.

a) Principle. In order to avoid post-mortem changes in the levels of free acetate, the tissues were freeze-clamped.

Organs were either frozen in situ (liver), or removed and frozen within seconds of making the first incision (kidneys, heart), at -196° with the clamps of Wollenberger, Ristau and Schoffa (1960). The frozen tissue was ground in a percussion mortar (Newsholme, 1962) and extracted with frozen perchlorate (Lowry, Passoneau, Hasselberger and Schulz, 1964) so that it was deproteinized during thawing. Acetate was assayed in the deproteinized solutions as described in Chapter 2. Lactate and pyruvate were usually assayed (see Materials and Methods), and the lactate/pyruvate ratios were calculated as a measure of the redox state of the cytoplasm

(Hohorst, 1960).

b) Anaesthesia. Anaesthesia was induced by drawing air-ether mixture through a translucent box (about 12 in. x 9 in., x 4 in. deep) containing the animal. It is known that barbiturates in doses used for anaesthesia can raise the levels of ketone bodies in blood (Engel and Engel, 1954) and that in larger concentrations they can enhance the rate of ketogenesis in rat liver slices (Mellanby, 1963) and in perfused rat liver (Scholz, Schwarz and Bücher, 1966), so their use was avoided in these experiments.

c) Freezing of the organs.

(i) Liver. The liver was frozen in situ. A strip of skin about 2 in. long was cut from the abdomen of the anaesthetised animal. An incision was made along the median line as far as the xyphisternum. The animal was turned over so that the liver dropped out, and the gut was restrained with the fingers. The liver was clamped between the clamps of Wollenberger et al. (1960), consisting of 2 flat aluminium plates of 12 cm. diameter mounted at the end of tongs 1 m. long, previously cooled to -196° (liquid nitrogen). The time taken from the abdominal incision to freezing the tissue was between 17 and 24 secs., or later 8 - 10 secs. Any unfrozen tissue was chipped off the sides of the clamps with a cooled spatula, and the frozen tissue dropped into a plastic container at -70°.

(ii) Kidney. Since each kidney weighs only about 1 g., acetate was measured in the pooled two kidneys from each animal.

A mid-line abdominal incision was made in the anaesthetised animal.

The gut was placed to the animal's left, the right kidney exposed and a slight nick made in the capsule along the outer curvature with a blade. Applying very gentle pressure with the fingers the kidney was decapsulated and it emerged free from adipose and conneetive tissue. The kidney was cut out at the hilus, placed in opened, cooled Wollenberger clamps and immediately clamped. The gut was now placed to the animal's right, and the left kidney exposed and removed as the right. Meanwhile the frozen right kidney had been deposited in a plastic beaker at - 70°, and the clamps were held open for the left kidney which was now clamped.

Since only 15 - 25 secs. elapsed between removal of the two kidneys, the blood loss from the right renal artery was not considerable, the left kidney was still observed to have good circulation and its colour was bright pink. The time taken from the first incision to the freeze-clamping of the second kidney was 35 - 50 secs. in all (about equal timing for removal of each kidney).

(iii) Heart. Since an average rat heart weighs just under 1 g. and the acetate concentration is lower than in liver, 2 frozen hearts were combined for each assay. Like the kidney, the heart could not be clamped *in situ* but was removed as rapidly as possible and from the measured lactate/pyruvate ratios it was seen that the redox state was still satisfactory.

The animal was anaesthetised as usual and a strip of skin was removed from its thorax. An incision was made into the thorax slightly to the left of the sternum, from the diaphragm towards the neck. The

heart was extracted and placed between the cooled clamps and clamped as described above for the liver. The operation took 7 - 16 secs.

d) Pulverization of frozen tissue. The percussion mortar of Newsholme (1962) was used. The mortar consists of close-fitting stainless steel cylinder and pestle. It stood in 'dry-ice' (-70°) and just before use liquid air was poured into the barrel and the piston slowly dropped in. The frozen tissue was placed in the mortar, the pestle was sharply hammered down, lifted, turned through 180°, and hammered again. This process was repeated about 6 times, and a very fine powder was obtained. The powder was returned to the plastic beaker at -70°. A small sample was removed for dry weight determination, and the rest was extracted.

e) Extraction of frozen tissue powder. Lowry et al. (1964) have stressed the importance of using frozen $HClO_4$ (M.Pt. = -17.8°) in order to achieve acidification (i.e. enzyme inactivation and deproteinization) of the tissue powder immediately on thawing.

The frozen powder, 1 - 3 g., was quickly weighed and added to frozen 6% $HClO_4$ (allowing 2 ml./g. tissue) in a porcelain mortar previously cooled with a few ml. liquid nitrogen. This mixture was rapidly ground with a porcelain pestle until the acid had penetrated the powder and the mixture began to thaw. The mixture was quantitatively transferred to a centrifuge tube at 0°. Marker was added, proteins were removed and the assays were carried out as previously described (p. 48).

B. Results.

1. Speed of extraction.

In these analyses, only 7 sec. (heart) to 50 sec. (max. length for the second kidney) elapsed between the first major incision in the rat and freezing the organ at -196°. And the frozen tissue was extracted into frozen $HClO_4$ at -17.8° within 30 min.

Experiments were performed to test the effect of delayed clamping on acetate levels. One lobe of liver was clamped as usual at zero time. The liver was left in situ, but with its blood supply cut off, and further lobes were clamped after 2 and 5 min. The lactate/pyruvate ratios thus attained after 5 min. anoxia were with one exception 120 - 300. However, partly owing to the small samples taken for the 3 successive clampings, the results were inconclusive as far as acetate was concerned - the apparant level being sometimes increased, sometimes decreased after these short periods of anoxia.

So the following experiment was carried out. A large lobe of liver was clamped at zero time, then the liver was left in situ with the thorax opened up (so that the whole animal became anoxic). The rest of the liver was not freeze-clamped but was extracted after 20 or 30 min. by homogenization (glass-Teflon homogenizer) in 6% $HClO_4$ (between 0° and 25°). The results are shown in Table 4-1. There was a relatively slow increase in acetate due to the delay, of approximately 0.01 μmole/g. fr. wt./min. The level had thus increased by 50 - 150% in 20 - 30 min. anoxia at body temperature. Bergmeyer and Moellering (1966) also

Table 4-1. Tissue acetate level. Effect of delayed extraction.

A large lobe of rat liver was freeze-clamped immediately and extracted as on p. 48. The rest was left *in situ* for 20 or 30 min. with the thorax opened up (so that the animal became anoxic), then extracted by homogenization in 6% $HClO_4$ at 0° (see text). Acetate was assayed by gas chromatography (see Chapter 2).

Extraction Procedure	Freeze-clamping	Homogenization in $HClO_4$ at 0°	
Period of anoxia at body temp.	None	20 min.	30 min.
Acetate level (μmoles/g. fr. wt.)	0.35	0.54	—
	0.17	—	0.41

stressed the necessity of rapid extraction, finding a 3-fold increase when rat liver was left for an hour at room temperature, or up to 25-fold increase when tissue was stored deep-frozen for 4 weeks before extraction.

2. Acetate levels in rat liver under various conditions.

Rat liver acetate levels in various nutritional states and in alloxan-diabetes are shown in Table 4-2. The mean value, 0.43 μmole/g. fr. wt., was identical in fed male (250 - 350 g.) and female (180 - 220 g.) rats. Fasting for 48 hr. did not alter the acetate level, reflecting the unchanged concentration in blood during fasting. Bergmeyer and Moellering (1966) found that liver acetate decreased after 20 hr. fasting. It is not considered likely that the longer fast in the present experiments could explain the discrepancy between the results, since it has already been shown (Chapter 3) that blood acetate is also unchanged after 24 hr. fasting. In bran-fed animals the slight decrease in liver acetate was not significant.

The level in alloxan-diabetes will be discussed in the next section.

There was no correlation of acetate level to the absolute concentrations of lactate and pyruvate nor to their ratio. In fed liver, the concentrations in μmoles/g. fr. wt. ranged between 0.6 and 3.3 for lactate, and between 0.06 and 0.24 for pyruvate, but acetate levels showed no correlation to either set of values. The corresponding lactate/pyruvate ratios generally varied between 6 and 13 (cf. Williamson, Lund and Krebs, 1967), but even when it was as high as 25 or 44, the acetate levels were still 0.42 and 0.38 μmole/g. fr. wt., i.e. well within the

Table 4-2. Acetate levels in rat liver under various conditions.

The livers were freeze-clamped *in situ* in anaesthetised rats, and the frozen tissue was powdered and extracted as described in the text. Acetate was determined as described in Chapter 2. For further details see the text.

State of animal	Sex	Acetate (μmoles/g. fr. wt.) Mean ± S.E.M.	No. of animals
Fed	Male	0.43 ± 0.043	(6)
Fed	Female	0.43 ± 0.031	(7)
48 hr. fasted	Female	0.47 ± 0.057	(5)
Bran-fed	Male	0.30 ± 0.038	(4)
Alloxan-diabetic	Male	1.10 ± 0.152*	(10)

* Significantly different from value in fed male controls. $P < 0.0005$. (P calculated by the t test.)

normal range. In 48 hr. fasted animals the mean lactate/pyruvate ratio increased to 20, while acetate concentration was unchanged.

Similarly, acetate level in heart muscle showed no correlation to that of lactate and pyruvate. The latter metabolites were not assayed in kidney.

3. Tissue acetate levels in acute alloxan-diabetes.

Table 4-3 shows the acetate levels in blood and various organs of normal and acute alloxan-diabetic rats.

a) Blood. Acute alloxan-diabetes was not found to alter the blood acetate significantly. Hochheuser et al. (1964), on the other hand, found that blood acetate was raised by 48% in rats with chronic alloxan-diabetes. But in view of the difference in the condition of the animals this discrepancy is hardly surprising. The animals used in the present experiments, 48 hr. after injection of a more or less lethal dose of alloxan, were in the last stages of acute alloxan-diabetes. Those of Hochheuser et al. were treated with subliminal doses of insulin after the alloxan injection and survived for up to 3 months with a blood sugar level of over 350 mg.%.

b) Organs. In the normal rat, the levels followed the pattern found by other workers, being highest in liver (0.43 μmole/g. fr. wt.), intermediate in kidney (0.25 μmole/g.) and considerably lower in heart muscle (0.09 μmole/g.). These results are in very close agreement with the values obtained by Bergmeyer and Moellering (1966) but are

Table 4-3. Acetate levels in various tissues. Effect of acute alloxan-diabetes.

Acute alloxan-diabetes was induced by a single large injection of alloxan (see text). Analyses were done on the pooled 2 kidneys from each animal, and on pooled hearts from 2 animals. Other details are as in the legend to Table 4-2. All analyses were on male rats. Results are expressed as means ± S.E.M., with the no. of data in parentheses.

Tissue	Acetate level (μmole/g. fr. wt., or μmole/ml. of blood)	
	Normal, fed rats	Alloxan-diabetic rats
Blood	0.43 ± 0.110 (8)	0.50 ± 0.075 (5)
Liver	0.43 ± 0.043 (6)	1.10 ± 0.152* (10)
Kidney	0.25 ± 0.025 (7)	0.36 ± 0.031** (9)
Heart	0.09 ± 0.022 (7)	0.10 ± 0.015 (7)

* Significantly different from fed normal controls. $P < 0.005$ (P calculated by the t test).

** $0.005 < P < 0.01$.

2 - 3 times higher than those of Hochheuser et al. (1964).

In alloxan-diabetes, the acetate level in liver and kidney were significantly raised, by 150% in liver and by 50% in kidney. The level in heart was unchanged.

C. Discussion.

1. Acetate levels in various tissues.

Of all the tissues examined, the liver was found to have the highest concentration of acetate, being roughly equivalent to that in blood from the aorta at about 0.4 mM. This may reflect the fact that the portal vein which contributes about 60 - 70% of the total blood supply to the liver in the rat (Dobson and Jones, 1952) contains 4 times as much acetate as the aorta which supplies the heart and kidney (see Chapter 3). The difference between its concentration in liver and kidney is diminished when the results are expressed in terms of dry weight, being 1.55 and 1.20 μmoles/g. dry wt. respectively. There is a much lower level in heart muscle (4 times less than in liver), the heart being known to utilize acetate very rapidly (Williamson, 1962).

It is of interest to compare the levels of free acetate and of active acetyl compounds in liver and in heart. The following table can be compiled (where the intermediates were measured in each case in the rapidly frozen tissue from fed rats. All values are μmoles/g. fr. wt.):

Organ	Free acetate	Acetyl CoA	Acetyl carnitine
Liver	0.43	0.045	0.061
Heart	0.09	$<$0.010	0.364

The acetate values are from Table 4-3; the acetyl CoA level in liver is from Williamson (1966); the acetyl carnitine values and the estimate of acetyl CoA in heart are those of Pearson and Tubbs (1964). (While the conditions were similar, the results obtained by different authors are not strictly comparable although they do indicate orders of magnitude, e.g. Pearson and Tubbs found that acetyl CoA level in liver was 0.020 µmole/g. fr. wt., less than half the value obtained by Williamson.) Free acetate is approximately 10 times more concentrated than acetyl CoA in both tissues, and is 4 times higher than the total "active acetate" (acetyl CoA + acetyl carnitine) in liver, yet in heart the total "active acetate" outweighs the free acetate by about 4 -fold. This points to an efficient activation system in the heart, and suggests that the liver might be a site of release of free acetate.

2. Tissue acetate levels in alloxan-diabetes.

a) General comment. In acute alloxan-diabetes the acetate levels in liver and kidney were raised by 150% and by 50% respectively, but were unaltered in blood and in heart muscle.

The level of a metabolite must depend on the balance of its formation and removal. So the main known factors involved in the formation and removal of acetate in the rat will be discussed, in relation to the altered levels in diabetes. But certain limitations must be borne in mind. While the activities of enzymes involving free acetate have been measured in individual organs *in vitro* (e.g. Kornacker and Lowenstein, 1964), the acetate level in any single tissue *in vivo*

is influenced to a greater or lesser extent by the rate of metabolism (formation or removal) in other tissues. This was illustrated by Krebs (1966) in relation to ketone body metabolism where an elevated output by the kidney is accompanied by only a slight rise in the plasma concentration, since it can be balanced by an increased rate of oxidation in the peripheral tissues.

b) Factors generating acetate.

(i) Intestinal absorption. In the rat, free acetate is formed by the intestinal bacteria, is absorbed into the portal bloodstream and is partly taken up by the liver (see Chapter 3).

Crane (1961) confirmed *in vitro* an observation made by previous authors *in vivo* (see Wilson, 1962) that intestinal sugar absorption in the alloxan-diabetic rat is actually increased as compared with the normal animal, despite the raised blood sugar level. Thus, in alloxan-diabetes there is likely to be a deficit rather than a surfeit of sugar in the intestine (i.e. a reduction of substrate for the bacteria). The portal blood acetate levels in the alloxan-diabetic rats were not measured in the present experiments. But in view of the observations of Crane and others it is unlikely that the raised acetate levels in liver and kidney were caused by increased production in the intestine.

(ii) Deacylase. Another major factor generating acetate is the deacylase enzyme. As pointed out in the general introduction, its role in acetate formation is unlikely to be significant in such tissues as heart which readily utilize acetate. The kinetic properties of this enzyme have only

been studied in mouse liver so far (Hepp et al. 1966a,b).

Williamson (1966) has measured the level of acetyl CoA in the livers of normal rats and of rats made diabetic by injection of anti-insulin serum. The value rose from 0.160 μmole/g. dry wt. in the normal to 0.220 μmole/g. dry wt. in the diabetic rats, i.e. from approximately 0.045 to 0.061 μmole/g. fr. wt. If the K_m value for deacylase is assumed to be the same in rat as in mouse liver (0.6×10^{-4} M acetyl CoA, Hepp et al. 1966a), then the alteration in acetyl CoA level (0.45 to 0.61×10^{-4} M) is in the range where control of the rate of deacylase reaction is expected. The specific activity of the enzyme in rat liver has been measured (Hepp et al. 1966a). Thus from a reciprocal plot it can be seen that this 35% rise in substrate level would increase the rate by 17%. Increased liver acetyl CoA levels are also characteristic of alloxan-diabetes (Wieland and Weiss, 1963; Tubbs and Garland, 1964). But the control values in these earlier reports were less than half those found by fluorimetric measurement (Williamson, 1966).

It thus seems that the rate of deacylase reaction may be increased in alloxan-diabetes. Yet the idea that deacylase activity affects the concentration of free acetate is made unlikely by the observations that in the fasting state acetyl CoA level in liver rises 2 - 3-fold (Tubbs and Garland, 1964; Williamson, Herczeg, Coles and Danish, 1966) (which should likewise increase the rate of deacylase reaction) but the free acetate level is unaltered (Table 4-2) or slightly decreased (Bergmeyer and Moellering, 1966).

c) Factors disposing of acetate.

(i) Acetyl CoA synthetase. Acetyl CoA synthetase catalyses the first step in the metabolism of free acetate. Its activity measured in a high-speed supernatant of rat liver homogenate was found to decrease to half the normal value in alloxan-diabetic rats (Kornacker and Lowenstein, 1964, 1965b). Such a decrease in activity would tend to raise the level of free acetate in liver cytoplasm. But since the activity of the enzyme was not measured in the mitochondria the effect on the acetate content of whole liver cannot be assessed. [There seems to be no direct evidence for the intracellular distribution of this enzyme in liver. In many other tissues it is mainly located or highly active in mitochondria e.g. in rat brain (Schuberth, 1965) and in beef heart (Hele, 1954; Campagnari and Webster, 1963).]

Again the importance of this enzyme in the control of free acetate level is offset by the fact that a similar decrease in its activity (50%) was observed after 48 hr. fasting (Kornacker and Lowenstein, 1965a) while the acetate level was unchanged (Table 4-2).

(ii) Subsequent metabolism. Whether the total capacity of the body to utilize acetate is altered in alloxan-diabetes is unknown. Disappearance of labelled acetate from the bloodstream of pancreatectomised dogs was reported to be diminished (Ciaranfi and Fonnesu, 1954). In the alloxan-diabetic rat, $^{14}CO_2$ production from carboxyl-labelled acetate was impaired in diaphragm but not in heart muscle *in vitro* (Villee and Hastings, 1949; Pearson, Hsieh, Du Toit and Hastings, 1949). But these observations require confirmation by measurements of absolute quantities of acetate.

(iii) Urinary excretion. Another possible means of disposal of acetate is by excretion in the urine, although in the sheep, urinary loss of acetate is considered negligible (Annison and Lindsay, 1961). Small amounts of acetate have been detected in rat urine (Medzihradsky and Lamprecht, 1966) amounting to about 5 μmoles per day, or about 15 μmoles per day after large oral doses of acetate (assuming a mean daily urinary output of 12 ml. for rats on drinking water only, unpublished observations from this Laboratory, cf. Spector, 1956b).

Increased levels of acetate in the urine were reported in human diabetics (Caselli and Ciaranfi, 1946) and in pancreatectomised dogs (Ciaranfi and Fonnesu, 1954). Urinary acetate was not measured in the present experiments.

d) Conclusions. It appears that in liver the activities of both acetyl CoA synthetase and deacylase may be modified in the direction of increased formation of free acetate in alloxan-diabetes. Yet this evidence is not sufficient to account for the marked increase in acetate levels in liver and kidney (Table 4-3). Since the factors controlling free acetate level in the liver and in other tissues are not yet fully understood, it is not possible to offer a satisfactory explanation for the present findings.

D. Summary.

1. Acetate levels were measured in freshly collected aortic blood and in rapidly frozen tissues from fed and from acute alloxan-diabetic

rats (injection of a single large dose of alloxan). Long delays (20 - 30 min.) in the extraction of tissues caused a slow rise in the free acetate level.

2. The levels in the fed, normal animals were as found by others (Bergmeyer and Moellering, 1966), approximately 0.4 mM in blood and in liver, slightly less in kidney, and lowest in heart (about 0.1 mM). Liver acetate levels were unaltered after 48 hr. fasting, or after bran-feeding.

3. In alloxan-diabetes, the levels were raised by 150% in liver, and by 50% in kidney, but were unaltered in aortic blood, and in heart muscle.

4. Factors generating and disposing of free acetate are discussed, but the mechanism of control of its level is not sufficiently understood to offer a satisfactory explanation for the altered levels in diabetes.

CHAPTER 5. ACETATE AND KETONE BODY PRODUCTION BY RAT LIVER IN VITRO.

The perfusion experiments described in this chapter were performed jointly with Mr. R. Hems whose collaboration is gratefully acknowledged.

CHAPTER 5. ACETATE AND KETONE BODY PRODUCTION BY RAT LIVER IN VITRO.

CHAPTER 5

ACETATE AND KETONE BODY PRODUCTION BY RAT LIVER IN VITRO

I Introduction.

Preliminary experiments with slices, which will be described, confirmed that rat liver can produce acetate from endogenous sources, and at a rate comparable to that of ketogenesis. Since the perfused rat liver had been found to form ketone bodies at a considerably higher rate than in slices (unpublished observations from this laboratory) this system was adopted for further investigations.

Isolated rat livers were perfused *in situ* by the technique of Hems, Ross, Berry and Krebs (1966). Endogenous acetate production was studied and compared with that of ketone bodies in various nutritional states, and the effects of addition of some precursors of the acetyl group (fatty acids, pyruvate, ethanol) were systematically investigated.

II Experimental.

A. Methods.

1. Liver slice experiments.

a) Animals. Small male rats, weighing 100 - 150 g. were used. They were either fed, or fasted for 22 - 24 hr. as stated.

b) Treatment of tissue. Slices of rat liver (80 - 100 mg./ cup) were incubated as described for mouse liver by Krebs, Notton and Hems (1966). Incubations were carried out for 20, 30 or 60 min.

Butyrate, hexanoate or oleate were added in the presence and absence of 1 mM DL-carnitine. The fatty acids were added as solutions of their sodium salts to give final concentrations between 0.5 and 8 mM., and the pH of the medium was unaltered by their addition. At the end of the incubation period, 0.5 ml. of 25% metaphosphoric acid was added, and the content of the cup was homogenized in a glass-Teflon homogenizer. 1.0 μmole isovalerate was added as a marker and the homogenate was treated as described for the acetate assay of tissue homogenates (p. 48).

Where ketone bodies were also estimated, the experiments were performed in duplicate. After homogenization, the media of the duplicate pairs were combined, and centrifuged. An aliquot of supernatant, 4 ml., was set aside for ketone body determinations and the rest was analysed for acetate. It was checked that the presence of metaphosphate ion in concentrations greater than those occurring in deproteinized tissue extracts did not interfere with either β-hydroxybutyrate or acetoacetate determinations.

2. Liver perfusion experiments.

a) Animals. Female rats, weighing 170 - 230 g., were used, either fed, or fasted for 24 or 48 hr.

b) Perfusion technique. The rat liver was perfused in situ

as described by Hems et al. (1966).

c) Dialysis of albumin. The only modification to the perfusion medium of Hems et al. was the prior dialysis of the bovine serum albumin (according to Nishiitsutsuji-Uwo, Ross and Krebs, 1967). Solutions of albumin (10%, w/v) in the saline of Krebs and Henseleit (1932) were thus dialysed, then stored at -20° (for a maximum length of 10 days) until required. Before use, dialysis tubing was boiled in water (with many changes) for about 12 hr. to remove impurities (as recommended by Garland, Newsholme and Randle, 1964). 39 ml. of dialysed albumin solution was used for each perfusion (final volume of medium, 150 ml.). The final albumin concentration was about 2.6% (w/v).

Solutions of albumin (25%, w/v) were similarly dialysed, for binding with fatty acid added as substrate in some perfusions, and a corresponding amount of albumin was omitted from the perfusion fluid initially.

d) Sampling of perfusion fluid. For acetate assays, 1 ml. samples were removed from the collection vessel and treated as described on p. 48. These samples were usually taken initially, and after 30, 60, 90 and 120 min. in control perfusions, or after 40, 100 and 130 min. in perfusions where substrate was added at 38 min. For other analyses (ketone bodies, lactate, pyruvate) 0.5 ml. samples were taken and pipetted into 4 ml. of 2% $HClO_4$, initially and usually every 15 min. throughout the perfusion. The resulting changes in the volume of perfusing fluid were taken into account in calculations of metabolite

content of the medium. The times given in the description of the experiments refer to time elapsed after the start of the perfusion, and not after the addition of substrate.

e) Preparation of substrates for perfusion. The volume of perfusion fluid was initially 150 ml., less the volume of substrate solution (max. 9 ml.) to be added at 38 min.

Addition of 3.75 ml. of 0.2 M ethanol gave a concentration in the medium of 5 mM; 7.5 ml. of 0.2 M freshly prepared sodium pyruvate solution gave a final concentration of 10 mM, and 3 ml. of 0.1 M solution of sodium butyrate produced a concentration of 2 mM. The pH of the medium was unaltered by these additions. 3 ml. of 0.1 M solution of DL-carnitine was usually added with the fatty acids, giving a final concentration of 1 mM of the physiological L-isomer (see e.g. Bremer, 1962a; Fritz and Schultz, 1965).

Long-chain fatty acids (stearate, oleate, linoleate), and octanoate were first solubilized by forming albumin complexes of their sodium salts (see Teresi and Luck, 1952; Goodman, 1958), neutralised solutions of which were added to the perfusions. The amount of fatty acid required for the perfusion was weighed, and 2.7 ml. of water and 0.3 ml. of N-NaOH (or sufficient to neutralise the acid) were added. 1.5 ml. of dialysed 25% bovine serum albumin was mixed with this suspension. It was warmed to 37° and the resulting clear neutral solution was transferred to the perfusion medium.

B. Results.

1. Preliminary experiments with rat liver slices.

[Note. Tables 5-2 and 5-3 each represent a single experiment, and Table 5-1 shows separately the results of 2 experiments (fed and fasted rat). Single analyses were performed in each case. Only two further slice experiments were done as described in the text. This work was of a preliminary nature, showing trends rather than quantitative effects, and the problem was pursued using the perfusion technique.]

a) Time-course of acetate production from endogenous sources and from oleate. Table 5-1 shows the time-course of acetate production both from endogenous sources and from added 2 mM oleate, in fed and fasted rat liver slices. The rate of endogenous production was approximately 3 μmoles/g. fr. wt./hr., which appeared to be maximal in the first 20 - 30 min., then decreased slightly. Oleate caused a definite increase especially in the fasting state, of 2 - 3-fold in this experiment.

Hepp et al. (1966a) observed only a very slight production of acetate (less than 1 μmole/g. fr. wt./hr.) in rat liver slices. But the ratio of tissue to medium (w/v) was 3 times as high as in the present experiments, which may have resulted in incomplete aeration of the tissue and accounted for the smaller production of acetate (see Krebs, Bennett, de Gasquet, Gascoyne and Yoshida, 1963).

b) Acetate production from various fatty acids with and without carnitine. Table 5-2 shows the effects of various fatty acids on

Table 5-1. Time-course of acetate production in fed and fasted rat liver slices, from endogenous sources and from oleate.

Liver slices from a fed rat (a), or 22 hr. fasted rat (b), were incubated in 4 ml. of the saline of Krebs and Henseleit (1932), with $O_2 + CO_2$ (95:5) in the gas phase. Oleate was added at 2 mM concentration. The results are of single experiments. For further details see the text. The initial values (equivalent to blank acetate values) have been subtracted.

State of animal	Substrate added	Acetate produced (μmoles/g. fr. wt.) Time of incubation (min.)		
		20	30	60
(a) Fed	None	1.59	4.73	3.31
	Oleate	—	—	5.04
(b) Fasted	None	1.61	2.08	3.08
	Oleate	3.33	4.79	8.41

Table 5-2. Acetate production in rat liver slices from various fatty acids, with and without carnitine.

The general experimental conditions are described in Table 5-1. Slices from a fed rat were incubated for 1 hr.

Substrate added	Acetate produced (μmoles/g. fr. wt./hr.) No Carnitine	1 mM DL-Carnitine
None	3.13	1.76
Butyrate (2 mM)	3.05	6.70
Hexanoate (1 mM)	4.72	2.67
Oleate (2 mM)	5.45	3.03

acetate production in the presence and absence of carnitine. Butyrate, in the absence of carnitine, had no effect on acetate production at 2 mM. concentration (Table 5-2) or at 5 or 8 mM (further experiments). Hexanoate and palmitate at 1 mM and 2 mM respectively did enhance acetate formation (Table 5-2).

The slight suppression of endogenous acetate production in the presence of carnitine (Table 5-2) was not confirmed in a subsequent experiment. The response of acetate production from fatty acids to the addition of carnitine was equally erratic (Tables 5-2, 5-3) and these results are considered inconclusive. Carnitine has been found to stimulate acetate production, both endogenous and from added fatty acids, in mouse tumour tissue (Hepp et al. 1966a,b). But from their data it is not possible to distinguish between the effects due solely to carnitine and those due to other cofactors (ATP, NAD^+, CoA) added simultaneously.

c) Comparison of acetate and ketone body production. The ketone body production by fed rat liver slices both in the absence of oleate and with various concentrations of this acid was compared with that of acetate (see Table 5-3). The total ketone body production rate (taken as the sum of acetoacetate and β-hydroxybutyrate) was up to 100% higher than that of acetate, and increased fairly linearly with oleate concentration between 0.5 and 2 mM. Addition of carnitine increased ketogenesis from 0.5 mM oleate, but its effect was not studied further.

The rate of endogenous ketogenesis in this experiment, 4.3 μmoles/g.

Table 5-3. Comparison of acetate and ketone body formation from oleate in rat liver slices.

The general experimental conditions are described in Table 5-1. Slices were incubated for 1 hr. The rat was fed. 1 mM DL-carnitine was added in cup No. 3 only. Ketone body results are the sum of β-hydroxybutyrate and acetoacetate.

Cup No.	Conc. of added Oleate (mM)	Acetate produced (μmoles/g. fr. wt./hr.)	Ketone bodies produced (μmoles/g. fr. wt./hr.)
1	None	2.74	4.29
2	0.5	3.76	5.98
3	0.5 (+ carnitine)	2.67	6.84
4	1.0	3.11	6.30
5	2.0	9.89	9.35

fr. wt., was comparable with the optimal rate observed in rat liver homogenate with added cofactors (3.1 μmoles/g./hr., Ontko, 1967).

2. Rat liver perfusion experiments.

a) Requirement for dialysis of albumin. Table 5-4 shows the acetate and long-chain fatty acid contents of crude and dialysed albumin, and of defatted albumin (purified by Dr. M.J. Weidemann, according to the method of Garland et al. 1964).

The Fraction V serum albumin used is prepared by Armour Laboratories by the procedure of Cohn and his associates (1946). The fractionation involves a gradual acidification to pH 4.8, by successive additions of acetate buffers. According to Cohn et al. (1946), the unpurified Fraction V precipitate contains approximately 75 m-moles of free acetic acid and 45 m-moles of Na acetate per 250 g. of protein (i.e. 0.5 m-moles of total acetate/g. of protein; the concentration in the final washed precipitate is not stated). Because of the high molar ratio of total acetate : albumin, of approximately 34:1, and since the pH is subsequently raised (Cohn et al. 1946), it is quite likely that considerable amounts of acetate are bound to the albumin (see Teresi and Luck, 1952; Fredrickson and Gordon, 1958), accounting for the high residual acetate content of crude albumin (34 μmoles/g., Table 5-4).

The bound acetate would provide a high initial value (a total of 133 μmoles in the perfusion medium) and might conceivably interfere with acetate metabolism in the liver (evidence which will be discussed on p. 99,

Table 5-4. Removal of acetate and long-chain fatty acids bound to bovine serum albumin.

Bovine serum albumin was dialysed for 48 hr. against bicarbonate saline (see the text), or purified according to Garland et al. (1964). Long-chain fatty acids were determined by the method of Dole and Meinertz (1960), modified according to Kelley (1965). The figures for acetate equivalents of long-chain fatty acids were obtained by assuming that the mean chain length is 16 carbon atoms.

Treatment of albumin	Acetate content (μmoles/g.)	Long-chain fatty acid content (μmoles/g.)	Acetate equivalents (μmoles/g.)
None	34	2.0	16
Dialysis	0	1.3	10
Purification of Garland et al. (1964)	12	0.2 (approx.)	1.6 (approx.)

shows that a high level of circulating acetate may suppress acetate production, or be taken up by the perfused liver). Crude albumin also contained long-chain fatty acids at 2 μmoles/g. (a total of 62 "acetate equivalents" in the medium). Albumin-bound fatty acids represent readily oxidizable substrate for mammalian tissues (Goodman, 1958; Fredrickson and Gordon, 1958), as was specifically demonstrated with rat liver mitochondria (Björntorp, Ells and Bradford, 1964).

Dialysis of albumin as described in the methods section seemed the most effective treatment (see Table 5-4). It removed all detectable acetate and reduced the long-chain fatty acid content by 35%. The more lengthy purification procedure of Garland et al. (1964) utilized glacial acetic acid which was not removed even after 7 days dialysis against several changes of saline. When dialysed albumin was used there was initially no acetate detected in the perfusion medium.

A rapid method of removing free fatty acids from serum albumin, with acid-charcoal, was recently reported (Chen, 1967). The treatment was equally effective in the removal of medium-chain (C_8) as of long-chain fatty acids, but the removal of acetate was not described.

b) Time-course of acetate production. Acetate was produced by the perfused rat liver, in the absence of substrate, at a considerable though variable rate (see Table 5-5).

With 48 hr. fasted animals the initial rate was 45.1 ± 2.10 μmoles/g. fr. wt./hr. (mean ± S.E.M., measured in 40 perfusions regardless of whether substrate was added at 38 min.). When no substrate was added,

Table 5-5. Endogenous acetate and ketone body production in the perfused rat liver.

Perfusion of rat liver was according to Hems et al. (1966). Rats were fed or fasted as indicated. For further details see the text. Acetate production rate was measured initially (0 - 30 min.) and over the 30 - 60 min. period. Ketone body production (sum of acetoacetate and β-hydroxybutyrate) was linear for the first 90 - 100 min. Results are means ± S.E.M., with the no. of experiments in parentheses.

State of animal	Acetate formed (μmoles/g. fr. wt./hr.)		Ketone bodies formed (μmoles/g. fr. wt./hr.)
	0 - 30 min.	30 - 60 min.	0 - 90 min.
Fed	41.6 ± 8.14 (9)	26.4 ± 6.88 (9)	7.7 ± 1.40 (9)
24 hr. fasted	32.0 ± 6.29 (7)	29.7 ± 7.10 (7)	28.3 ± 3.5 (7)
48 hr. fasted	45.1 ± 2.10 (40)	45.0 ± 5.26* (6)	41.7 ± 5.8 (4)

* Carnitine was added at 38 min. in 2 of these experiments, but did not alter the rate (see p. 89).

the rate was linear for the first 60 min. The rate given in Table 5-5 for the 30 - 60 min. period is the mean for 4 such experiments and for a further 2 where carnitine alone was added at 38 min. (the addition of carnitine caused no inflection in the time-curve of acetate production). The actual rates for the first 60 min. in these 6 experiments varied between 27.6 and 61.3 μmoles/g. fr. wt./hr. as follows:

(i) No substrate: 41.3, 49.3, 56.0, 61.3
(ii) Carnitine only, after 38 min.: 27.5, 34.6

} Mean (6) = 45.0 μmoles/g./hr.

(It was considered that the mean of either group (i) or (ii) alone gave an unreliable value, due to the small number of observations and to the large coefficient of variation of acetate production.)

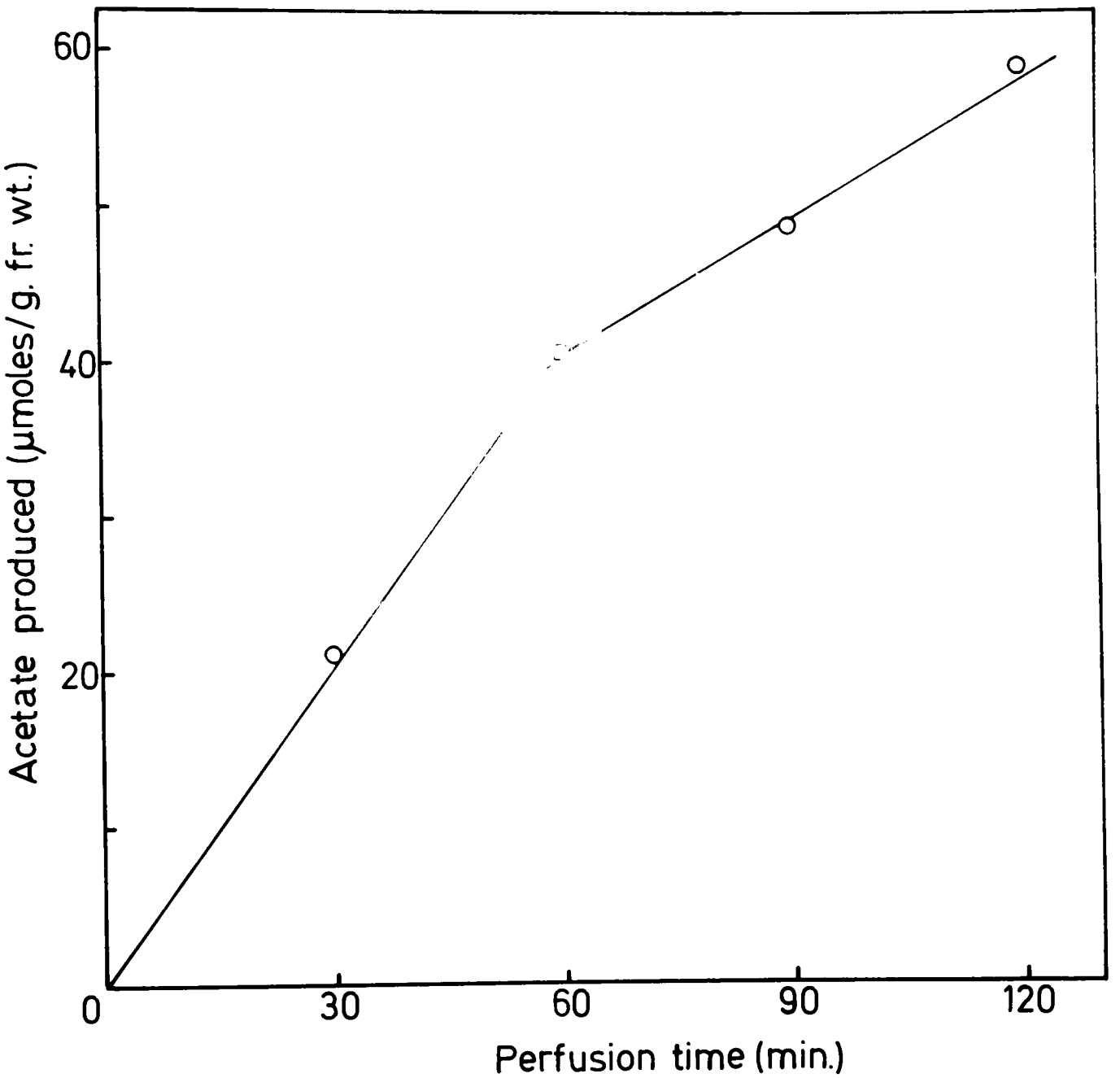
After 60 min. the rate declined and there was usually very little if any acetate produced after 90 or 100 min. Acetate very occasionally started to disappear from the medium in this final period. Fig. 5-1 shows the time-course in a typical control perfusion.

In 24 hr. fasted and in fed animals there was no appreciable difference in the initial rate of production, but the rate fell off more rapidly, decreasing to 30 or 25 μmoles/g. fr. wt./hr. respectively in the 30 - 60 min. interval.

Acetate was the only volatile fatty acid observed in the perfusion medium (except where butyrate was added as a substrate).

c) Hepatic origin of acetate appearing in the medium. That the acetate appearing in the medium was due solely to the metabolic activities of the liver was demonstrated by the following experiments:

Fig. 5-1. Endogenous Acetate Production in the Perfused Rat Liver (48 hr. fasted).



This is a typical curve showing the time-course of acetate production in the absence of substrate. The liver weighed 5.70 g. fr. (1.57 g. dry). Experimental conditions were as described in the text.

150 ml. of medium was circulated through the perfusion apparatus without a rat liver, for 2 hr. The medium was gassed and maintained at 37° as during liver perfusion. No acetate appeared in the medium.

The experiment was repeated with initial additions of 10, 100 and 200 μmoles of acetate, and in all cases the acetate content of the medium remained unchanged. Thus acetic acid was not lost by evaporation in the oxygenator, and the medium was inert with respect to acetate, unlike that of Hochheuser et al. (1964) where the freshly collected rat erythrocytes used in the medium produced acetate.

It was observed in "poor" perfusions, when flow rate was slow, the liver had an unhealthy appearance and the β-hydroxybutyrate/aceto-acetate ratio was low, that acetate production was strikingly reduced, which further implied that acetate appearance in the medium was a result of metabolic activities in the liver.

d) Endogenous ketone body production. Total ketone body production is again taken as the sum of acetoacetate and β-hydroxybutyrate production. A slight disappearance of acetoacetate was observed when 442 or 216 μmoles were incubated with medium under the conditions of perfusion (total loss of 10 - 12 μmoles/hr.) which was not accounted for in these calculations. This loss was probably due to the presence of albumin i.e. an amine-catalysed decarboxylation of acetoacetate (cf. Krebs and Eggleston, 1945) which accounted for a similar observation by Nishiitsutsuji-Uwo et al. (1967).

The rates of ketogenesis from endogenous sources are shown in Table 5-5.

These rates were linear throughout the first 90 - 100 min. then decreased slightly. The mean rates found were 7.7, 24.3 and 41.7 μmoles/g. fr. wt./hr. in fed, 24 hr. fasted and 48 hr. fasted animals respectively. Thus ketogenesis showed a linear increase with duration of fasting in contrast with acetate formation where the initial rate remained virtually unchanged.

The β-hydroxybutyrate/acetoacetate ratio reached a maximum of 1 - 2 in the first 30 min. and gradually decreased to 1.0 or just below it towards the end of the perfusion. There was no detectable difference in the values of this ratio in fed and fasted rats.

Similar ratios have previously been reported for rat liver perfusions, but the rates of ketogenesis were quite different e.g. Forsander, Räihä, Salaspuro and Mäenpää (1965) found no differences between the rates in fed and 48 hr. fasted rats, and Struck, Ashmore and Wieland (1965) observed a comparatively negligible rate with 24 hr. fasted animals. However, in both cases the discrepancies may be explained by the presence of anti-ketogenesis metabolites (glucose or glucose and lactate) in their control media, while the medium used in the present experiments was free of such substances (see Hems et al. 1966).

3. Effects of added substrates on acetate and ketone body production in the perfused rat liver.

a) Fatty acids.

(i) Various fatty acids in the presence of carnitine. In Table 5-6 are shown the effects of addition of 2 mM concentrations of fatty acids

Table 5-6. Acetate and ketone body formation in the perfused rat liver, with added fatty acids and carnitine.

Experimental procedure was as described in the legend to Table 5-5. All rats were fasted for 48 hr. The concentration of added fatty acids was 2 mM, and of carnitine was 1 mM (L-carnitine). Results are max. rates of production after addition of substrate at 38 min., in μmoles/g. fr. wt./hr. (means ± S.E.M., with the no. of experiments in parentheses). In this and in the following tables, where there are only 2 or 3 experiments, results are means with the range in square brackets.

Fatty acid added	Acetate formed	Ketone bodies formed
None (carnitine only)	45.0 ± 5.26* (6)	30.4 ± 3.27 (4)
Butyrate	30.0 [24.7,35.2] (2)	94 ± 14.6 (4)
Octanoate	27.6 [25.0,31.0] (3)	128 ± 6.8 (4)
Stearate	36.3 ± 6.21 (4)	56 ± 9.3 (4)
Oleate	38.0 ± 3.71 (4)	98 ± 4.7 (4)
Linoleate	36.8 ± 3.19 (4)	117 ± 6.3 (4)

* This is the result for 6 control perfusions. In only 2 of these carnitine was added, but did not alter the rate of acetate formation (see p. 89).

(C_4-C_{18}) and 1 mM L-carnitine on acetate and ketone body production by the perfused rat liver.

In contrast with the previous findings with slices, addition of fatty acids did not enhance acetate production in the perfused liver. The rates were sometimes but not consistently reduced (by up to 70%), without altering the mean rates very significantly.

As mentioned earlier (p. 89) carnitine alone did not alter the rate of endogenous acetate production (though in both these experiments the rate was linear until the end). The apparent suppression of endogenous ketogenesis by carnitine was not significant.

Ketone body formation on the other hand was increased approximately 2-fold (56 μmole/g. fr. wt., with stearate), 3-fold (94, 96 μmole/g./hr., with butyrate, oleate) or even 4-fold (117, 128 μmole/g./hr. with linoleate and octanoate). Thus, highest rates were achieved with the intermediate member (octanoate) of the saturated series, and by the polyunsaturated acid (linoleate). Provided that the lower rate with butyrate is not an artifact due to shortage of substrate (which is not likely, see later), the pattern resembles that observed by Björntorp et al. (1964) for inhibition of oxidation by fatty acids in rat liver mitochondria, (intermediate saturated acids and the unsaturated acids caused greatest inhibition).

Löffler, Matschinsky and Wieland (1965) found a marked stimulation of ketogenesis, also, on adding octanoate and oleate in the perfused rat liver. But the rates are not comparable, as these authors used fed rats,

their pre-perfusion was of 3 hours duration and they added the fatty acids by continuous infusion for a further 3 hours.

Addition of fatty acids caused a 2 - 3-fold increase in the β-hydroxybutyrate/acetoacetate ratio, which rose to a maximum of 3 - 4 at 55 - 70 min., and declined slightly towards the end of the perfusions.

Thus the net effect on ketogenesis of added fatty acids was an increased formation of β-hydroxybutyrate with little if any increase in that of acetoacetate, similar to the findings of Löffler et al. (1965) and of Struck et al. (1965) on adding octanoate and oleate to rat liver perfusions.

(ii) Oleate at various concentrations, with and without carnitine. The production of acetate and ketone bodies after the addition of various concentrations of oleate, in the presence and absence of 1 mM carnitine are shown in Table 5-7. Again, there were no appreciable effects on acetate formation. Ketone body production increased only from 67 to 98 μmole/g. fr. wt./hr. as oleate concentration was raised from 0.5 to 2 mM, in the absence of carnitine. Carnitine further stimulated ketogenesis, at the lower concentrations of oleate, but not beyond the maximal rate obtained in its absence (98 μmole/g./hr.). Carnitine did not further increase ketogenesis from 2 mM oleate. These effects on ketogenesis are plotted as a function of oleate concentration in Fig. 5-2.

The maximum β-hydroxybutyrate/acetoacetate ratios were again 3 - 4.

(iii) Time-course of ketone body production from fatty acids. As in the case of endogenous formation, the rate of ketogenesis from added

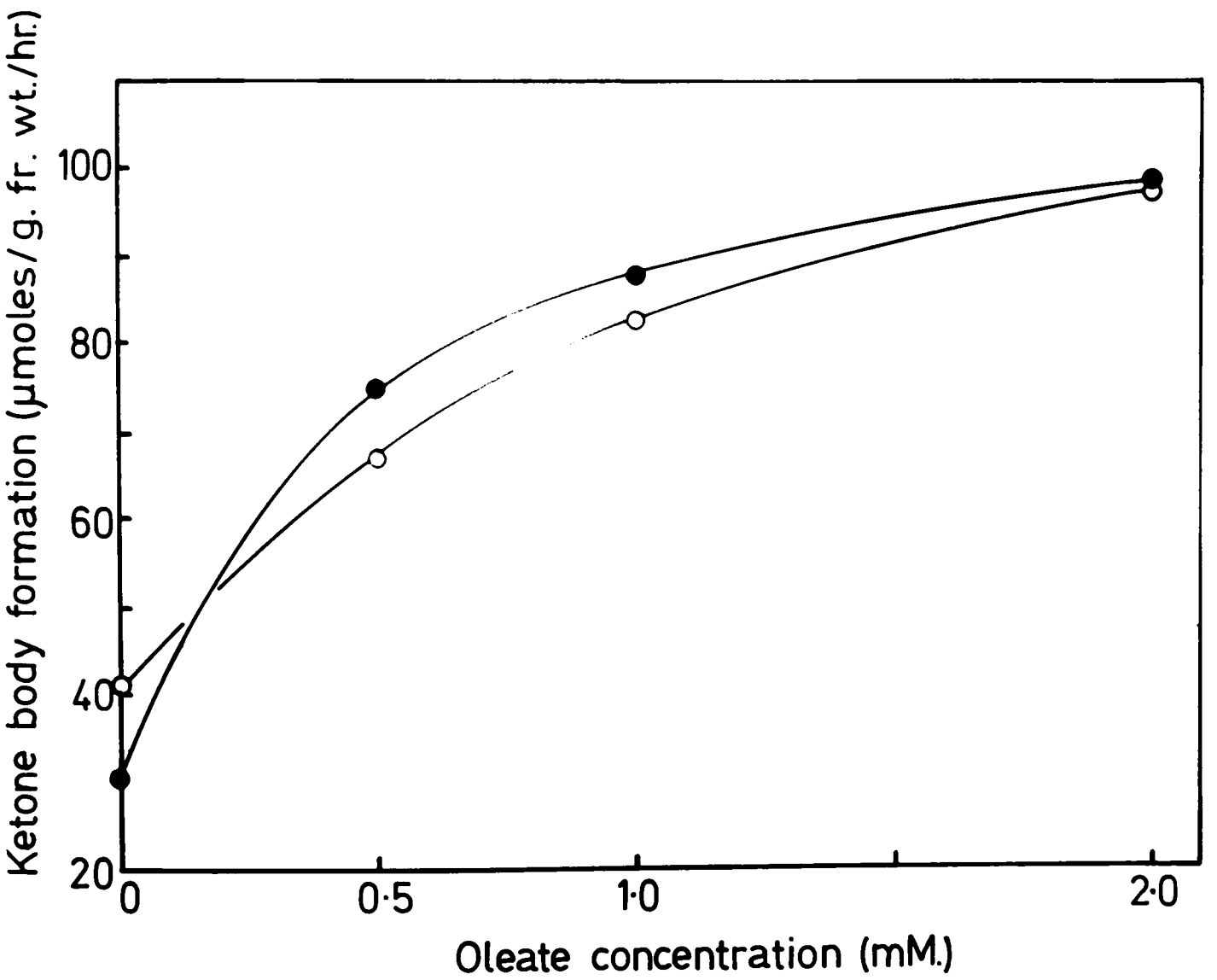
Table 5-7. Acetate and ketone body production in the perfused rat liver. Effects of various concentrations of oleate, and of carnitine.

The perfusion of 48 hr. fasted rats was according to the legend to Table 5-5. Results are expressed as in Table 5-6.

Carnitine added (mM)	Oleate added (mM)	Acetate formed	Ketone bodies formed
None	None	45.0 ± 5.26* (6)	41.7 ± 5.8 (4)
	0.5	34.5 ± 4.82 (4)	67.3 ± 8.2 (4)
	1.0	39.6 ± 10.08 (4)	82.8 ± 5.0 (4)
	2.0	41.6 ± 6.33 (5)	96.0 ± 2.4 (4)
1.0	None	45.0 ± 5.26* (6)	30.4 ± 3.4 (4)
	0.5	42.4 ± 8.59 (4)	75.1 ± 6.2 (4)
	1.0	31.4 [28.6-36.6] (3)	88.1 ± 6.7 (4)
	2.0	38.0 ± 3.71 (4)	98.4 ± 4.7 (4)

* Mean of all control experiments whether carnitine was added or not (see p. 89).

Fig. 5-2. Effect of Oleate Concentration and of Carnitine on Ketogenesis in the Perfused Rat Liver.

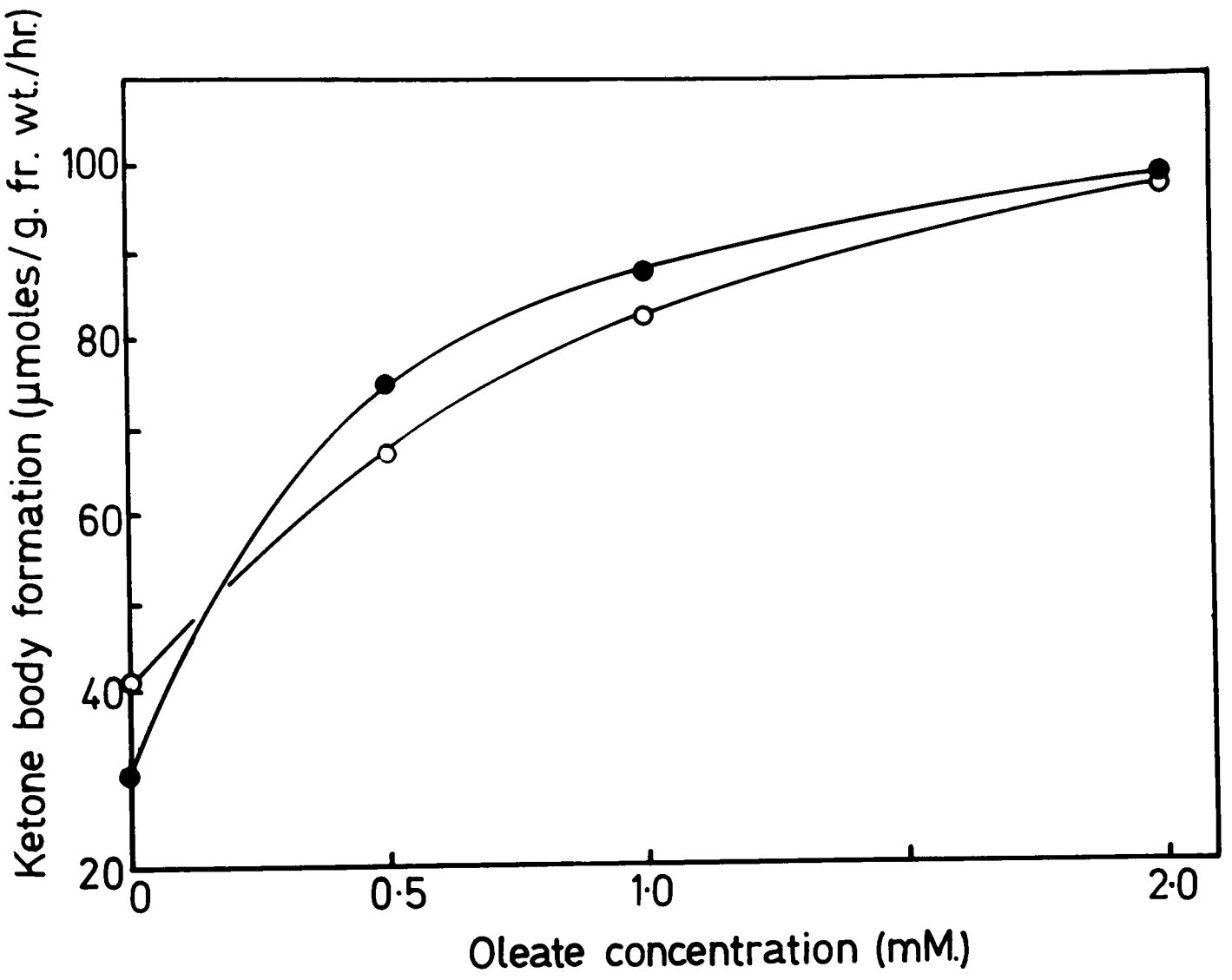


This is a plot of the rate of ketogenesis in the perfused rat liver (48 hr. fasted) against the concentration of oleate added at 38 min. For further details see the text. The data are taken from Table 5-7.

○—○ without carnitine.

●—● with 1 mM L-carnitine.

Fig. 5-2. Effect of Oleate Concentration and of Carnitine on Ketogenesis in the Perfused Rat Liver.



This is a plot of the rate of ketogenesis in the perfused rat liver (48 hr. fasted) against the concentration of oleate added at 38 min. For further details see the text. The data are taken from Table 5-7.

o—o without carnitine.

●—● with 1 mM L-carnitine.

fatty acids was linear until 100 - 115 min. when it decreased slightly. One of the substrates, butyrate, was analysed by gas chromatography as for acetate, and was found to have just disappeared from the medium at 130 min. So the possibility that shortage of substrate might account for the terminal decline in ketone body synthesis was investigated. In an experiment with octanoate, a further 150 μmoles of substrate was added at 100 min. There was an increase in ketone body production during the following 15 - 30 min., after which it stopped again. It was thus apparent that factors other than diminished substrate supply were responsible.

(iv) Balance of metabolites in the presence of fatty acids. The balance of the calculated extra ketone bodies formed in the presence of fatty acids, above the formation in the control, is shown in Table 5-8. On the addition of 2 mM butyrate (representing 300 μmoles of ketone body equivalents), all of which disappeared during the perfusion, a calculated extra 305 μmoles of ketone bodies were formed. With 0.5 mM oleate (representing 338 μmoles of ketone body equivalents) an extra 203 μmoles were formed. Thus there was 100% conversion of added butyrate, and 60% conversion of oleate to ketone bodies. With higher concentrations of oleate there was a lower percentage conversion of added substrate, probably partly due to incomplete utilization of the fatty acid, but this was not measured. Acetate formation did not account for any of the fatty acid utilized (there was no net increase in its formation), and other metabolites were not determined.

Mayes and Felts (1967b) followed the fate of [^{14}C]oleate in the

Table 5-8. Balance between ketone body formation and fatty acid addition in the perfused rat liver.

Livers of 48 hr. fasted rats were perfused as described in the text. Substrates were added at 38 min. The results are the differences between the initial (40 min.) and final (130 min.) amounts of ketone bodies in the perfusion medium (means, 4 experiments in each group).

Substrate added	None	Butyrate (300 μmoles)	Oleate (75 μmoles)
Substrate in ketone body equivalents (μmoles)	-	300	338
Ketone bodies formed (μmoles)		555	453
Ketone bodies corrected for control (μmoles)	-	305	203
% Substrate converted to ketone bodies	-	102	60

perfused rat liver. They found that in a given nutritional state (fed or 24 hr. fasted), the percentage conversion to ketone bodies was the same whether 50 or 150 mg. of oleate was added, in contrast with the present findings (with 21 to 85 mg. oleate). With fasted animals, labelled oleate was esterified (approx. 20%), oxidized to ketone bodies (approx. 20%) or to CO_2 (15 or 4%); 20% was not utilized and the remaining 18 or 35% was not recovered.

b) Pyruvate. That pyruvate is rapidly utilized by the perfused rat liver is known (Ross, Hems and Krebs, 1967). Added at 10 mM concentration it was without effect on acetate production, but was markedly anti-ketogenic, reducing ketone body formation from 41.7 to 9.6 µmole/g. fr. wt./hr. (see Table 5-9). There was a transient accumulation of lactate on addition of pyruvate, as observed by Ross et al. (1967), and by Hepp et al. (1966a,b) in ascites cell incubation medium. Fig. 5-3 shows the content in the medium of these metabolites, during a typical pyruvate perfusion.

c) Ethanol. Previous experiments with perfused rat liver have employed massive doses of ethanol, 40 - 50 mM (Forsander et al. 1965; Salaspuro and Mäenpää, 1966; Gordon, 1966), concentrations greater than those attained in human plasma after relatively large doses of alcohol (Lundquist, 1960; Hochheuser et al. 1964). In the present experiments 5 mM ethanol was added, a concentration more likely to simulate the chronic effects of ethanol on liver metabolism in alcoholism. The effects of addition of ethanol on acetate and ketone body formation in the

Table 5-9. **Effects of pyruvate and ethanol on acetate and ketone body production in the perfused rat liver.**

Perfusion of rat livers was as described in the text. Results, expressed as in Table 5-6, are compared with those without substrate over the same time period. (-) denotes no determinations.

State of animal	Substrate	Acetate formed	Ketone bodies formed
48 hr. fasted	None	45.0 ± 5.26 (6)	41.7 ± 5.8 (4)
	Pyruvate (10 mM)	41.7 ± 7.26 (6)	9.6 ± 2.21 (6)
	Pyruvate (5 mM) + Ethanol (5 mM)	28.3 [21.0,35.5] (2)	(-)
	Ethanol (5 mM)	45.1 ± 0.84 (4)	23.4 ± 2.9 (4)
Fed	None	26.4 ± 6.88 (9)	7.7 ± 1.40 (9)
	Ethanol (5 mM)	67.2 [55.5,78.8]* (2)	5.15 [5.0,5.3] (2)

* In both these experiments, acetate production ceased at 70 - 100 min., after which acetate disappeared from the medium.

Legend to Fig. 5-3. Time-Course of Acetate and Ketone Body Formation in the Perfused Rat Liver after Addition of Pyruvate.

Pyruvate (1500 μmoles) was added at 38 min. The rat was fasted for 48 hr. The liver weighed 6.53 g. fr. (1.95 g. dry). For further details see the text.

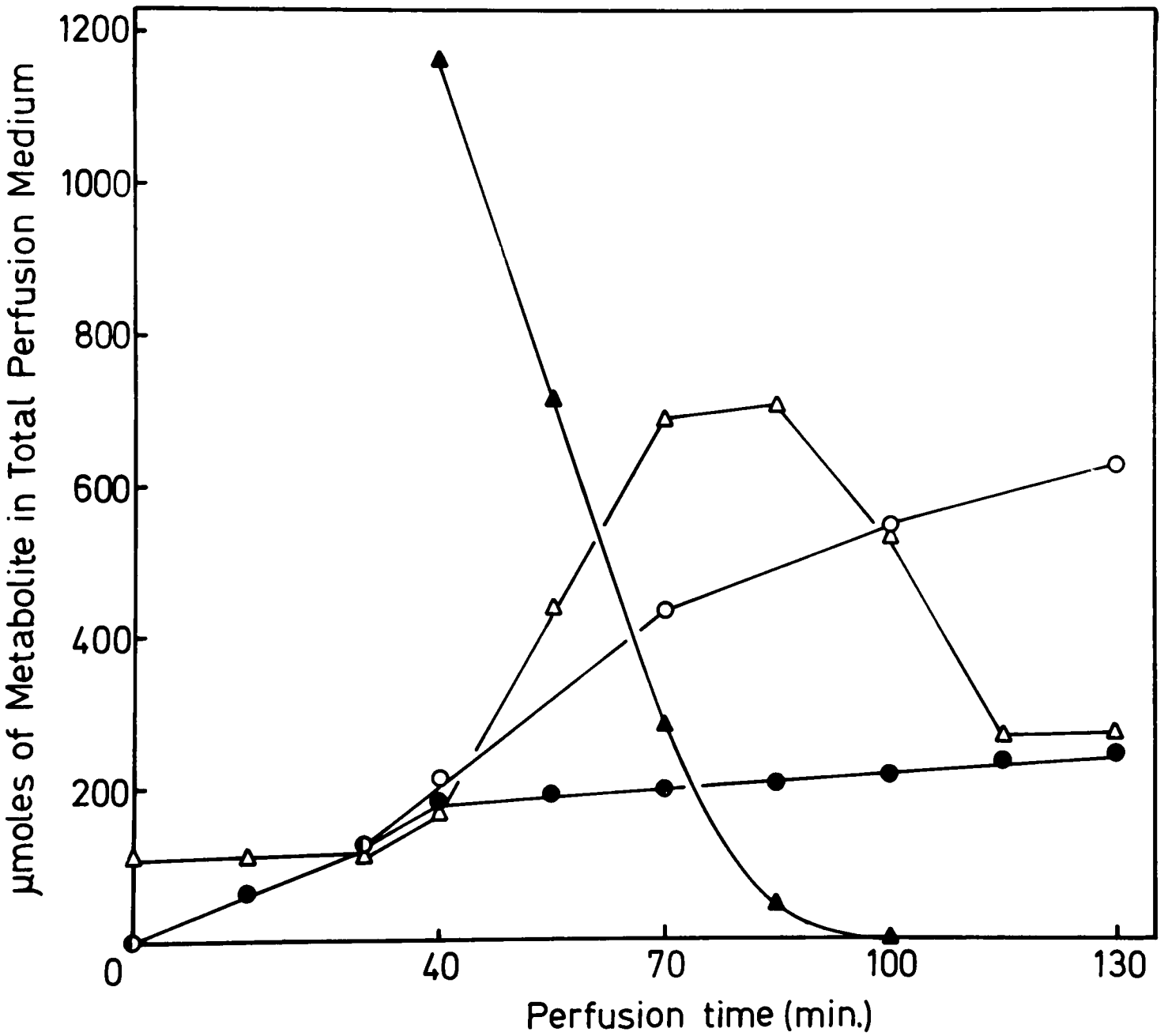
○—○ acetate.

●—● ketone bodies (acetoacetate + β-hydroxybutyrate).

△—△ lactate.

▲—▲ pyruvate.

Fig. 5-3. Time-Course of Acetate and Ketone Body Formation in the Perfused Rat Liver after Addition of Pyruvate.



perfused livers of fed and 48 hr. fasted rats appear in Table 5-9.

(i) Fasted animals. In 48 hr. fasted animals, ethanol was without effect on acetate formation and depressed ketogenesis by about 30%. Ethanol usually caused an initial slight increase (of up to 0.6) in the β-hydroxybutyrate/acetoacetate ratios which was not maintained, showing that it had little effect on the redox state of the mitochondria. Forsander et al. (1965) and Salaspuro et al. (1966) found that addition of 43.5 mM ethanol to a perfusion medium containing 5 - 6 mM glucose, significantly increased ketogenesis in livers of 48 hr. fasted but not of fed rats. In all their experiments with ethanol the β-hydroxybutyrate/acetoacetate ratios continued to rise to values of 7.6 - 9.5 at 180 min., usually by suppression of acetoacetate formation. But it is not possible to make any meaningful comparison between the results obtained with such widely different concentrations of substrate.

Combined addition of 5 mM ethanol and 5 mM pyruvate did not stimulate acetate production (see Table 5-9).

(ii) Fed animals. In fed animals ethanol caused an initial marked increase (2 - 3-fold) in acetate formation which ceased at 70 - 90 min. Thereafter acetate was removed from the medium but did not reach pre-substrate level by 130 min. The disappearance of acetate from the medium can only be attributed to uptake by the liver, as other factors such as removal by the red blood cells or evaporation in the oxygenator have been ruled out (p. 90).

C. Discussion.

[Note. The results on ketone body production in the perfused rat liver will be published and discussed in full by R. Hems et al. Only those aspects which are connected with acetate production will be discussed here.]

1. Acetate production in rat liver slices.

In a few preliminary experiments with rat liver slices, acetate was produced from endogenous sources at an approximate rate of 3 μmoles/g. fr. wt./hr. On the addition of fatty acids, acetate formation increased, up to 3-fold. In general, acetate and ketone body formation in slices moved in the same direction, but were not quantitatively related. Acetate was produced in the absence of carnitine both from endogenous and exogenous sources, while the addition of carnitine had erratic effects on its production. It is recognised that carnitine is an essential factor for the hepatic oxidation of fatty acids in general (Fritz, 1955, 1959; Bremer, 1962b; Ontko, 1967). A specific effect on acetate metabolism has been shown in isolated mitochondria or homogenates of liver (Bremer, 1962a; Bressler and Katz, 1965; Siliprandi, Siliprandi and Ciman, 1965) by formation of acetyl carnitine which is possibly the form in which acetate is transported across the mitochondrial membrane (although this view is contested where transport of acetate from the mitochondria for extramitochondrial syntheses is concerned, Lowenstein, 1963, 1965). Excess carnitine was found to inhibit ketogenesis in vitro by immobilizing acetyl groups as acetyl carnitine (Ontko, 1967). The

liver has an endogenous supply of carnitine (at least 0.25 µmole/g. fr. wt., from Broekhuysen, Rosenblum, Ghislain and Deltour, 1965) but carnitine addition may become limiting in vitro, as found by the authors cited above. In slices, such an effect might vary considerably between different preparations depending on the degree of tissue damage (carnitine is water-soluble and could be lost into the medium). In the perfused liver, a more intact preparation, no effect of added carnitine on acetate production was observed (p. 89, Table 5-7). Thus from the present experiments no conclusions can be drawn as to the effects of carnitine on hepatic acetate metabolism.

2. Acetate production in the perfused rat liver.

a) General pattern. Endogenous acetate production by the perfused rat liver at 45 µmoles/g. fr. wt./hr., was 15-fold higher than in slices. Thus the difference between the two preparations was relatively much greater for acetate than for ketone body production (cf. the data for fed rats in Tables 5-3, 5-5).

The range of acetate formation in six control perfusions with 48 hr. fasted rats was 36% either side of the mean. This variability is partly attributable to experimental error (which can be ± 29% at worst, but is usually much less, see Table 2-14), but is also partly due to biological variations between individual livers. In some perfusions, acetate production failed for no apparent reason, even though ketone body production and ratios were normal (such results were discarded).

The tendency for the rate of acetate production to decrease during

the perfusion could not be explained by a general metabolic deterioration of the liver (see time-course of ketone body production).

The differences between the concentration of acetate in portal blood and in blood from the aorta indicate that the liver does remove acetate from the bloodstream, in vivo (Chapter 3). The final concentration of acetate in the perfusion medium was generally about 3 - 3.5 mM, i.e. twice as high as in portal blood. The rate of acetate production usually started to decline after about 60 min., i.e. when its level in the medium was approximately 2 - 3 mM. However, the values of acetate concentration in the medium have a wide scatter, and it is not possible to decide on the basis of these data whether the decline in production was dependent on the circulating level of acetate or on a metabolic function of the liver. In similar perfusion experiments with fed donor rats (Dr. L.A. Jedeikin, unpublished observations), acetate added at 5 mM or 10 mM concentration was found to be taken up by the liver at a rate of 39 μmoles/g. fr. wt./hr. A study of acetate production or uptake during perfusion with a range of concentrations of added acetate might reveal whether the liver controls the level of circulating acetate, analogous to the remarkable control of circulating amino acid levels observed in perfusion experiments by Schimassek and Gerok (1965).

In contrast with the present findings, Hochheuser et al. (1964) detected no endogenous production of acetate by the perfused rat liver, but observed a rapid uptake even with relatively low levels of added acetate. The rate of uptake increased as the acetate concentration was

raised from 0.13 mM to 1.45 mM, and the maximum rate calculated from their data is approximately 70 μmoles/g. fr. wt./hr. The discrepancy between these results is probably related to differences in the perfusion technique and in the composition of the medium (although their medium contained freshly collected rat erythrocytes which actually produced acetate). The longer period of anoxia in those authors' experiments (apparently 6 - 8 min.) might alter the metabolic status of the liver considerably. In fact, in some initial perfusion experiments where the period of anoxia during the operation was prolonged, acetate production was considerably lower than when this period was later reduced to less than 1 min. of total anoxia, and the results were discarded.

b) Effect of altering the nutritional status. As observed in the previous slice experiments, there was no major difference between acetate production by the perfused livers of fed and fasted rats. With 48 hr. fasted animals the initial rate of production was similar, but was maintained a little longer.

This is another instance where the metabolism of free acetate in the rat appeared to be unresponsive to changes in dietary state (acetate levels intthe blood from both the aorta and portal vein and in the liver were unaltered by such changes, Chapters 3 and 4). It was previously observed that the rate of acetate oxidation in the whole rat was unaltered by feeding a high carbohydrate diet, unlike that of the higher fatty acids (Lossow and Chaikoff, 1955; Mayes and Felts, 1967a). Similarly, Williamson (1962) found that acetate oxidation in the perfused

rat heart was not affected by fasting for 24 hr., in contrast to pyruvate oxidation which was depressed.

c) Lack of stimulation by added substrates in fasted animals. The constancy of the mean acetate production rates in the presence of added substrates in the fasting state, is rather surprising, especially in view of the adaptations of the rate found in the slice experiments. The reason for this difference in behaviour between sliced and perfused liver remains unclear. That it is not due to lack of uptake of substrates by the perfused liver is evident from the facts that fatty acids were converted to ketone bodies (see Tables 5-7, 5-8), pyruvate was converted to lactate (Fig. 5-3) and in the fed animals, ethanol was converted to acetate.

An effect of pyruvate on the observed acetate production might be more likely if this substrate were added with fed donor rats where gluconeogenesis from pyruvate would be considerably less (cf. Krebs et al. 1963; Ross, 1966). Another method of increasing the effective pyruvate concentration would be the addition of glucose to the perfusion, with either fed or fasted donor rats. Neither of these procedures has yet been tried.

d) Relation to ketone body production. The initial rates of acetate and ketone body production by perfused livers of 48 hr. fasted rats were 45 and 42 μmoles/g./hr. respectively, but the similarity ended here, since ketone body production unlike that of acetate was linear for about 100 min., and continued to respond to changes in dietary state and

to additions of substrate as in liver slice experiments. Acetate production remained unaltered whether these substrates were markedly ketogenic (fatty acids) or anti-ketogenic (pyruvate, ethanol).

e) Source of endogenous acetate. From the data of Table 4-2, and taking 6 g. as an approximate mean liver weight, the acetate content of the livers used in these perfusions is calculated as 2.6 μmoles. Since about 500 μmoles of acetate appeared in the medium by the end of the perfusions it is obvious that it was not a washout of preformed acetate, but that acetate was being derived from another source or sources in the liver.

(i) Acetyl CoA. As discussed in previous chapters, the major immediate endogenous precursor of free acetate in mammalian tissues is acetyl CoA, via the action of deacylase. Acetyl CoA is mainly derived in turn from carbohydrate (via pyruvate) and from fatty acids (by β-oxidation). Hepp et al. (1966a,b) showed that both pyruvate and fatty acids increased aerobic acetate production by mouse tumour tissue, and from their findings with arsenite concluded that the long-chain fatty acids were the endogenous source of acetate. However since neither the addition of pyruvate nor of fatty acids stimulated acetate production in the perfused rat liver, it could not be assessed which if either of these substances was was the main endogenous source of acetate.

(ii) CoA-independent pathways. The possibility has been raised (Hochheuser et al. 1964) that pyruvate might be converted to acetate by a CoA-independent pathway in mammalian systems, by analogy to a mechanism

described in yeast (Holzer and Goedde, 1957a). The common intermediate of pyruvit decarboxylating enzymes is hydroxyethyl thiaminepyrophosphate (HETPP) (Holzer, Goedde, Göggel, and Ulrich, 1960; Holzer and Beaucamp, 1961). In yeast cytoplasm, pyruvic decarboxylase converts pyruvate via HETPP to acetaldehyde which can be oxidised to free acetate by aldehyde dehydrogenase (Holzer and Goedde, 1957a). As there is no evidence for the existence of pyruvic decarboxylase in mammals this pathway of formation of free acetate is highly unlikely.

In mammals the aldehydic group of HETPP is first oxidized to the acetyl level then split to acetyl CoA (Korkes et al. 1952) catalysed by the pyruvic dehydrogenase enzyme system (see Reed, 1966). With an analogous enzyme system in yeast mitochondria (Holzer and Goedde, 1957a) and in Aerobacter (Krampitz, Suzuki and Gruell, 1961) no acetaldehyde could be detected.

Oxidation of HETPP by non-specific oxidizing agents (ferricyanide, dichlorophenolindophenol) to form free acetate is possible in vitro (Holzer and Goedde, 1957b; Krampitz et al. 1961; Reed, 1966). But the organization of the pyruvic dehydrogenase system seems to be such that the intermediates are not released, except for acetyl CoA (Reed, 1966). Thus, non-specific oxidation would be improbable where cells are relatively intact as in the present preparations. Furthermore, addition of pyruvate did not increase acetate production in the perfused liver as might have been expected if this non-enzymic reaction were occurring.

Another potential source of acetate is the acetyl moiety of amino

sugars and proteins, but the catabolism of neither of these classes of compounds is fully understood.

The composition and metabolism of amino sugars has been reviewed by Balazs and Jeanloz (1965, 1966). In rat liver the major acetate-containing amino sugars, N-acetylglucosamine and sialic acid are protein-bound. From their data and that of Robinson, Molnar and Winzler (1964), the total amount of these is calculated as approximately 3 μmoles/g. fr. wt. of liver. If complete acetylation is assumed (1 acetyl residue/ glucosamine molecule; 2 residues/sialic acid molecule), amino sugar compounds would contain 4 μmoles of acetyl groups/g of liver i.e. less than 10% of the hourly output of acetate in the perfused organ (Table 5-3). While the half-life of these compounds in liver is quite short (2.5 - 4.4 hr.) it seems that this is due to release of glycoproteins into the circulation rather than to catabolism of the amino sugars (Macbeth et al. 1965). Recent evidence shows that the sugar moiety of these compounds is fairly extensively oxidized in the rat (Robinson, 1967). But deacetylase activity has not yet been characterized in rat liver (Davidson, 1966), although as mentioned in Chapter 1, it has been described in pig kidney (Leloir and Cardini, 1956; Comb and Roseman, 1957, 1958). Until more detailed information is obtained, the acetylated amino sugars could only be regarded as potential sources of relatively insignificant amounts of acetate in the present experiments.

There is increasing evidence for the existence of acetyl groups, usually on the N-terminal acids of certain proteins (for review see

Olsen, 1966). But the potential contribution of acetate from such proteins, if present in the liver, would probably be even less than that from the amino sugars.

Also, non-enzymic hydrolysis of acetyl groups of amino sugars and proteins requires drastic conditions, so that if glycoproteins were released into the perfusion medium they would not interfere with subsequent acetate analyses.

f) Production from ethanol, in fed animals. The addition of ethanol in perfusions of fed rat livers was the only instance where substrate stimulation of acetate production was observed in the present experiments. The rapid increase in acetate appearance in the medium after ethanol addition bears out the *in vivo* findings in humans of Lundquist (1960, 1962) and of Hochheuser *et al.* (1964). The difference in response of fed and fasted livers to ethanol may be explained by the diminished activities of alcohol and acetaldehyde dehydrogenases (the 2 enzymes involved in conversion of ethanol to acetate) in the fasted rat liver (Büttner, 1965). The activities of both were found to be reduced to about 66% of their values in the fed state, after 43 hr. fasting.

D. Summary.

1. Preliminary experiments showed that acetate is produced by rat liver slices at a rate comparable to that of ketone bodies and that production of both is enhanced by added fatty acids.

2. In the perfused rat liver acetate was produced from endogenous

sources at an approximate initial rate of 45 μmoles/g. fr. wt./hr. The rate declined after 60 - 90 min. with 48 hr. fasted donor rats, and after 30 - 60 min. with 24 hr. fasted or fed rats. Ketone body production was similar in fasted rats, but was considerably less in the fed state; it was linear for 90 - 100 min.

3. Tests showed that the perfusion medium did not produce acetate, and that it was not lost by evaporation in the oxygenator, therefore the above figures represent the metabolic activities of the liver.

4. Addition of substrates, whether markedly ketogenic (a range of fatty acids with and without carnitine) or anti-ketogenic (pyruvate, ethanol), had no effect on the rate of acetate production in the perfused liver with one exception. Ethanol (5 mM) enhanced acetate production by the livers of fed donor rats. Pyruvate was not added in fed rat perfusions.

5. The endogenous precursors of acetate in the perfused liver, or the reason for the decline in its production after 60 min. are not known.

CHAPTER 6. ADDITIONAL COMMENTS.

CHAPTER 6

ADDITIONAL COMMENTS

This is not a formal discussion since most of the experimental findings have been discussed at the end of the appropriate chapters. But a few thoughts come to mind on interrelationships between some of the findings, on possible approaches to the further study of one or two of the problems raised by these findings, and on a modification to the method of acetate determination.

A. Metabolic significance of acetate in the rat.

The fact that acetate is a ready fuel of respiration in the rat was pointed out in the introduction. Subsequently it has been shown that free acetate is readily available to rat tissues *in vivo*. But the overall quantitative significance of acetate cannot yet be estimated. A very approximate calculation showed that exogenous acetate (arising mainly by the bacterial activity in the caecum from which it is absorbed into the bloodstream) could maximally account for 10% of the basal metabolic output. But no such estimate for endogenous acetate is yet possible.

Perfusion experiments have shown that the rat liver is capable of producing large amounts of acetate (Chapter 5). Since in these experiments the conditions simulated those obtained *in vivo* as closely

as possible, and the liver was found to maintain its biochemical properties (biosynthesis of glucose and urea, Hems *et al*. 1966; ketogenesis, present experiments) and its physiological functions (e.g. excretion of bromsulphalein, Ross, 1966), acetate production is considered a normal function of rat liver which can also occur *in vivo*. The rate of production of acetate by the perfused liver (approximately 300 µmoles/liver/hr. in rats of 200 g. body wt.) is of the same order of magnitude as the calculated hourly splanchnic absorption (560 µmoles/hr. in 200 g. rats). It is of interest that endogenous production of acetate in the sheep as measured by infusion of isotopic acetate just after feeding, and after complete removal of the rumen contents, appeared to be 25 - 45% of the total entry or production rate (Annison and White, 1962).

However, from blood levels *in vivo* (Chapter 3) it appeared that there was net uptake rather than release of acetate by the rat liver. And in perfusions, acetate release slowed down or ceased long before there was any sign of metabolic deterioration (Chapter 5). Further study on the rather anomalous behaviour of rat liver with respect to acetate might include perfusions with various high levels of added acetate, and acetate assays in both portal and hepatic veins under a variety of conditions including neomycin treatment (to minimise acetate transport to the liver) and alloxan-diabetes (where the liver level of acetate is markedly raised, Chapter 4). It might then be possible to gauge the endogenous production of acetate by the liver and its contribution to the caloric output in the rat under different conditions.

B. Comparison of the rumen and the caecum as sources of acetate.

The calculated rate of acetate absorption (mainly from the caecum) expressed in terms of body weight in the rat is 2.8 m-moles/Kg/hr. Very similar values for absorption from the rumen were obtained in fed sheep by different methods (2.9 - 3.0 m-moles/Kg./hr., Annison and White, 1962; Bergman et al. 1965).

In the sheep, the rumen is the major, and the caecum a relatively minor site of exogenous acetate production (Annison and Lindsay, 1961). Consequently both the entry rates and plasma levels of acetate fluctuate considerably unless the animal is continuously fed, artificially (Bergman et al. 1965). The rat relies mainly on the caecum for its exogenous supply, and since bacterial activity continues in the caecum even days after food is withheld (see Chapter 3) the acetate levels in the plasma and in liver were found remarkably constant after fasts of various durations (4 - 48 hr.).

C. Modification to the method of acetate determination.

Some preliminary experiments seem to indicate that direct gas chromatographic analysis of protein-free supernatants may be possible omitting the steam distillation procedure, despite earlier findings (p.44). It is not yet possible to say whether such analyses will have cumulative ill-effects on the column, but if not it would mean considerable saving of time and labour in the assay of relatively large amounts of acetate (as in samples of perfusion medium) where a concentration step is not necessary.

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