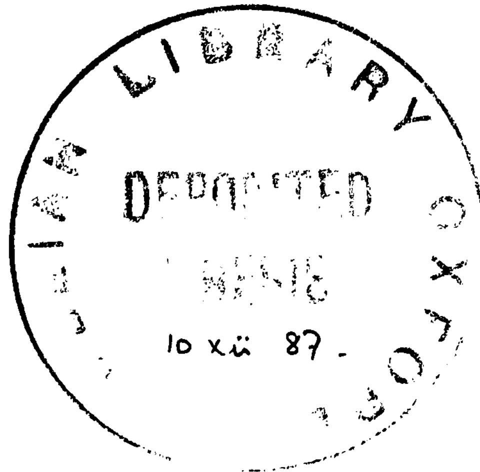
MEASUREMENTS OF PLASMA ACETATE CONCENTRATIONS IN HUMANS, WITH REFERENCE TO DIABETES, DIETARY COMPOSITION AND BOWEL FUNCTION.

Thesis Submitted in Part-fulfillment of Requirements

For the Degree of Doctor of Philosophy

of the

University of Oxford.



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Abayomi Olusola Akanji

Brasenose College.

ABSTRACT

This thesis examined aspects of production and utilization of acetate in humans via measurements of plasma concentrations in different circumstances with particular attention to changes in diabetes.

Circulating plasma acetate was measured by a modified acetate kinase-based enzymatic spectrophotometric method with adequate sensitivity and specificity for levels encountered in human plasma.

Fasting plasma acetate was increased in diabetics and correlated with glucose and indices of glucose disposal. Levels increased further when they were fed different high-fibre diets. The rise in acetate levels after lactulose ingestion correlated with changing breath hydrogen excretion in subjects with suspected malabsorption. Plasma acetate levels increased during fat infusion, and conversely, fell with suppression of fatty acid levels during euglycaemic clamping. Insulin appeared to promote acetate production from glucose by enhancing glycolysis and acetyl CoA availability, although its activity in reducing lipolysis had an opposite effect. The hepatic formation of acetate from ethanol did not appear influenced by prior chlorpropamide intake.

Glucose tolerance was unaffected by a 150mmol/hr acetate load, but acetate tolerance was impaired when glucose was simultaneously available. Adipose tissue lipolysis was suppressed during acetate infusions as evident

from reduced levels of glycerol and non-esterified fatty acids. Blood 'ketone body' levels were increased, suggesting direct conversion from acetate. Possibly as a result, fat oxidation assessed from gaseous exchange, was reduced with infused acetate.

Acetate utilization was impaired in diabetic patients from higher fasting plasma levels and slower metabolic clearance. The defect in diabetes was probably due to both over-production and under-utilization, and could be related to the enhanced lipolysis, hyperglycaemia and a reportedly reduced hepatic activity of acetyl CoA synthetase.

It was concluded that acetate is derived from both colonic fermentation and endogenous catabolism of glucose and fatty acids and appears rapidly metabolisable in humans. Some areas of further interest in human acetate metabolism were highlighted.

DEDICATION

This thesis is dedicated to the memory of my late father Michael Baderinwa Akanji.

ACKNOWLEDGEMENTS

My most grateful thanks to :

(i) Dr Derek Hockaday for his highly effective supervision, support and guidance throughout all the stages involved in the preparation of this thesis, and especially for arranging for me to come from Ibadan, Nigeria and have the benefit of research training under him at Oxford;

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate.
AK	acetate kinase.
ALDH	aldehyde dehydrogenase.
AMP	adenosine 5'-monophosphate.
ANOVA	analysis of variance.
ATP	adenosine 5'-triphosphate.
Co A	coenzyme A.
CP	chlorpropamide.
CPAF	chlorpropamide alcohol flush.
EDTA	ethylene diamine tetra-acetic acid.
GH	growth hormone
GLC	gas liquid chromatography.
HBAlc	glycosylated haemoglobin.
HDL-C	high density lipoprotein cholesterol.
IDDM	insulin dependent diabetes mellitus.
ivGTT	intravenous glucose tolerance test.
KBs	ketone bodies.
LCAT	lecithin : cholesterol acyl transferase.
LDH	lactate dehydrogenase.
MCR	metabolic clearance rate.
NAD	nicotinamide adenine dinucleotide, oxidised.
NADH	nicotinamide adenine dinucleotide, reduced.
NADP	nicotinamide adenine dinucleotide phosphate, oxidi
NADPH	nicotinamide adenine dinucleotide phosphate, reduc
NEFA	non-esterified fatty acids.

NIDDM	non-insulin dependent diabetes mellitus.
OGTT	oral glucose tolerance test.
PEP	phosphoenolpyruvate.
PK	pyruvate kinase.
SEM	standard error of mean.
SD	standard deviation.
TCA	tricarboxylic acid cycle.
VFA	volatile fatty acids.

ENZYME NOMENCLATURE

Acetate kinase (ATP : acetate phosphotransferase) EC 2.7.2.1.

Acetyl CoA deacylase (hydrolase) EC 3.1.2.1.

Acetyl CoA carboxylase (acetyl CoA:carbon dioxide ligase, ADP-forming) EC 6.4.1.2.

Acetyl CoA synthetase (acetate thiokinase, acetate:CoA ligase, AMP-forming) EC 6.2.1.1.

Acyl phosphate hexose phosphotransferase EC 2.7.1.61.

Adenylate kinase (myokinase, ATP:AMP phosphotransferase) EC 2.7.4.3.

Alanine aminotransferase (L-alanine:2-oxoglutarate amino-transferase) EC 2.6.1.2.

Alcohol dehydrogenase (alcohol:NAD oxidoreductase) EC 1.1.1.1.

Aldehyde dehydrogenase (aldehyde:NAD oxidoreductase) EC 1.2.1.3.

Arylamine acetyltransferase (acetyl CoA:arylamine N-acetyl-transferase) EC 2.3.1.5.

Aspartate aminotransferase (L-aspartate:2-oxoglutarate amino-transferase) EC 2.6.1.1.

ATP-citrate lyase (citrate cleavage enzyme, ATP:citrate oxalo-acetate lyase) EC 4.1.3.8.

Citrate synthase (citrate oxaloacetate lyase) EC 4.1.3.7.

CoA transferase (acyl CoA:acetate CoA transferase) EC 2.8.3.8.

Fatty acid synthetase (acyl CoA synthetase, acid:CoA ligase, AMP-forming) EC 6.2.1.3.

Glucose oxidase (b-D-glucose:oxygen 1-oxidoreductase) EC 1.1.3.4.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NAD 1-oxidoreductase) EC 1.1.1.49.

Hexokinase (ATP:D-hexose-6-phosphotransferase) EC 2.7.1.1.

Lactate dehydrogenase (L-lactate:NAD oxidoreductase) EC 1.1.1.27.

Malate dehydrogenase (L-malate:NAD oxidoreductase) EC 1.1.1.37.

6-Phosphofructokinase (ATP:D-fructose-6-phosphate-1-phosphotransferase) EC 2.7.1.11.

Phosphotransacetylase (acetyl CoA:orthophosphate acetyltransferase) EC 2.3.1.8.

Pyruvate dehydrogenase (pyruvate:ferricytochrome b oxidoreductase) EC 1.2.2.2.

Pyruvate kinase (ATP:pyruvate 2-0-phosphotransferase) EC 2.7.1.40.

CHAPTER I

INTRODUCTION

INTRODUCTION

Acetate has been studied extensively in relation to dairy products and the brewing industry. It is the major source of energy for ruminant animals, and also a major component of spoiled wine. Acetic acid gives vinegar its characteristic flavour.

The earlier work on acetate metabolism was in microbiology and related to the metabolism of yeasts and bacteria. With time, it was discovered that the microbial flora in the rumen of ruminants caused extensive fermentation of the vegetable and plant carbohydrate components of the diets of these animals to produce short-chain or volatile fatty acids (acetate, propionate and butyrate) which, on absorption and subsequent oxidation, constitute over half the caloric needs of the hosts (Annison and Armstrong, 1970). Although non-ruminant herbivorous animals had also been shown to support extensive hindgut bacterial communities, the importance of volatile fatty acids (VFAs) in providing for the energy needs of these animals was recognised only recently (Henning and Hird, 1970).

In omnivorous animals like Man however, acetate has always been assumed to be a relatively insignificant metabolite. Because the early methods of measurement were rather insensitive or non-specific, and because there are only low levels of acetate in the blood and tissues of this group of animals, early attempts at metabolic

characterisation proved difficult. It was assumed that acetate was only an intermediate in the trans-mitochondrial movement of acetyl-CoA (Newsholme and Start, 1973). Recently, however, Ballard (1970) and Knowles, Jarrett, Filsell and Ballard (1974) measured substantial quantities of acetate in omnivorous blood and found it metabolically active with a rapid turnover. As they believed the contribution from the diet probably to be small, most of the circulating acetate was assumed to arise endogenously.

The situation in Man still remains uncertain as most of the earlier studies were on rats and livestock animals. It is thus of interest to attempt to study further the sources of circulating acetate in Man as well as its metabolic implications.

SOURCES OF ACETATE IN MAN

Possibly important sources of plasma acetate in Man are: (1) DIET: with recent attempts at increasing dietary fibre intake in "Western" Man, it seems certain that a lot more vegetable and plant polysaccharide will pass undigested through the small intestine to be fermented in the colon. Hitherto, the beneficial effects of dietary fibre on lipid and carbohydrate metabolism had been thought due mainly to their local and mechanical effects on gut motility and nutrient absorption. It is speculative whether the absorbed products of fermentation - acetate and the other VFAs - contribute to this observed benefit.

(2) ENDOGENOUS PRODUCTION: there is ample evidence for endogenous acetate production in the rat. Krebs and Johnson (1937) showed that anaerobic dismutation of pyruvate to acetate occurred in liver slices and many other tissues. This reaction also occurs in mature erythrocytes which lack pyruvate dehydrogenase (Hochheuser, Weiss and Wieland, 1964). Acetate is produced by heart (Davis 1968, Knowles, Jarrett, Filsell and Ballard 1974), brain (Coxon, Liebecq and Peters 1949), liver tumours (Hepp, Prusse, Weiss and Wieland 1966) and perfused liver (Seufert, Graf, Janson, Kuhn and Soling 1974) from precursors of acetyl-CoA which include NEFAs.

Acetate production occurs through hydrolytic cleavage of acetyl-CoA by acetyl-CoA hydrolase (Hepp, Prusse, Weiss and Wieland 1966, Seufert, Graf, Janson, Kuhn and Soling 1974, Knowles, Jarrett, Filsell and Ballard 1974).

Other likely endogenous sources are the tissue acetylated compounds e.g. acetylcholine (McIlwain 1966), acetylcarnitine and acetylated amino-compounds (Keane 1967), acetylaspartate and acetylglutamate (Ballard 1972). However, these compounds are present in only small amounts in blood and contribute only minimally to circulating plasma acetate concentrations (Ballard 1972).

(3) ETHANOL: acetate is produced from ethanol by mammalian liver. The source of ethanol may be endogenous, from

fermentation (Krebs and Perkins 1970, Perkins 1970, Blomstrand 1971), or otherwise, infused or ingested (Lundquist 1960, Lundquist, Fugmann, Klaning and Rasmussen 1959, Crouse, Gerson, De Carli and Lieber 1968).

(4) PHARMACOLOGICAL USES OF ACETATE IN MAN : Recently, acetate has found pharmacological use in the correction of metabolic acidoses of diverse aetiology. Mion, Hegstrom, Boen and Scribner (1964) showed that patients could rapidly metabolise acetate to bicarbonate and since that observation, acetate has become the source of buffer in haemodialysates, and proved more stable and convenient to use than bicarbonate (Mansell and Wing 1983).

Prior to its use in dialysis, Mudge, Manning and Gilman (1949) had suggested using sodium acetate as a source of fixed base, and it has indeed been used to correct acidosis secondary to severe diarrhoeal diseases in adults (Cash, Toha, Nalin, Huq and Phillips 1969) and infants (Ekblad, Kero and Takala 1985).

Each of these various sources of acetate will be discussed in more detail in the later chapters in relation to specific studies.

CHAPTER 2

PRESENT STUDY

A. GENERAL AIMS.

This thesis attempts via the plasma concentration of acetate under a variety of circumstances to investigate some aspects of the endogenous and exogenous production of acetate, and to document any contribution of circulating acetate to intermediary metabolism in non-diabetic and diabetic human subjects. Plasma acetate levels were measured by a modified enzymatic spectrophotometric method, which was validated for use here. Values so obtained were used to draw inferences on the relationship of plasma acetate to other blood intermediary metabolites. The drawback of such an approach, emphasised throughout the thesis, is that plasma levels do not directly reflect either substrate production or utilization rate alone, but are a resultant of the two processes. Nonetheless they produce qualitative ideas on metabolic interactions.

All the studies were of human subjects, in whom there is relatively little published information other than in relation to uraemic haemodialysis. The reports in this thesis probably represent the first attempts at relating acetate metabolism to those of glucose and fat in a qualitative and a quantitative sense. Some areas in which routine acetate measurement may have a clinical utility were also explored.

B. STRUCTURE OF THE THESIS

The thesis is divided into 8 chapters. A general introduction to the subject forms the body of Chapter 1, while in the present chapter (2), the broad aims and structure of the thesis are presented. The reference range of normal acetate values in human, non-ruminant and ruminant mammals is given in Chapter 3 as well as a historical perspective of the various techniques available for the estimation of plasma, blood and tissue acetate levels. Chapter 4 presents the general aspects of recruitment of the volunteers, conduct of individual experiments and the procedure and timing of the collection of specimens. The factors that influenced the choice of an enzymatic method for acetate assay, its procedure and validation studies are also considered in this chapter. The methods for measuring the other substrates and hormones analysed in the thesis conclude Chapter 4. Chapters 5, 6 and 7 represent the main experimental section of the thesis. They respectively present findings on the endogenous metabolism of acetate, its exogenous sources and its utilization in humans. Each of the chapters is divided into 2 sections : (i) a detailed review of current literature on the subject; (ii) an experimental section with its specific aims, and the conduct, results and conclusions of each study.

The general conclusions reached from the various studies and an overview of human acetate metabolism, with an insight into areas of further interest constitute the concluding chapter (8).

CHAPTER 3

ACETATE AS A PLASMA METABOLITE

A. REFERENCE RANGE OF ACETATE VALUES.

Plasma acetate is currently measured by enzymatic or GLC methods. Each technique has numerous variations, and this fact probably accounts for the wide variability in the 'normal' values obtained in fasting and post-prandial humans, rat and ruminant animals (Tables 3.A.1 and 3.A.2).

The values obtained generally tend to be higher with enzymatic methods than with GLC. Another important variable is the nature of the specimen analysed viz plasma, serum or whole blood; and indeed the state of the subject, fasting or fed. Although Tollinger, Vreman and Weiman 1979) did not detect any differences on GLC analysis of plasma and whole blood stored at 4°C, it is conceivable that the concentration of acetate might differ between plasma and erythrocytes.

Differences may also be related to the sex and age of the subjects studied. The results reported are however contradictory. While Trivin, Lenoir, Bretaudiere and Sachs (1982), using a similar acetate-kinase based enzymatic acetate assay method, found significant differences in plasma acetate values between males and females, Laker and Mansell (1978) could not detect any such difference with a GLC method. Also, while Skutches, Holroyde, Myers, Paul and Reichard (1979) obtained reduced acetate values with increasing age using an acetate kinase assay method, Smith, Humphreys and Hockaday (1986) found no such difference with the method used here.

TABLE 3.A.1 : RANGE OF FASTING ACETATE VALUES IN OMNIVORES (MAN AND RAT).

		RANGE OF ACETATE VALUES (mmol/l)	REFERENCES.
..ENZYMATIC ASSAYS			
. untreated plasma	Man	0.00 - 0.31	Bartelt et al 1985, Trivin et al 1982*, Smith et al 1986.
i. deproteinised plasma	Man	0.13 - 1.17	Skutches et al 1979
	rat	0.14 - 0.25	Buckley 1974
ii. deproteinised plasma and distillation	Man	0.026 - 1.00	Lundquist 1962, Port et al 1975
	rat	0.14 - 0.15	Hermann et al 1985
v. deproteinised whole blood	rat	0.17 - 0.40	Knowles et al 1974
. deproteinised whole blood and distillation	Man	0.026 - 0.40	Seufert et al 1984 Korri et al 1985
. GLC METHODS			
. deproteinised whole blood and distillation	Man	0.029 - 0.038	Tollinger et al 1979
i deproteinised plasma and distillation	Man	0.023 - 0.60.	Nielsen et al 1978, Whitfield 1969, Desch et al 1977, Kveim et al 1977, Laker and Mansell 1978, Vreman et al 1978, Tollinger et al 1979, Vreman et al 1980, Dankert et al 1981, Richards et al 1982, Pomare et al 1985.
ii.alcohol and ether extraction of plasma	rat	0.21 - 0.27	Remesy and Demigne 1974.

* pooled blood donors, probably non-fasting.

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iii. alcohol and ether extraction of plasma	rat	0.21 - 0.27	Remesy and Demigne 1974.

* pooled blood donors, probably non-fasting.

TABLE 3.A.2 : RANGE OF WHOLE BLOOD ACETATE VALUES IN RUMINANTS

	RANGE OF ACETATE VALUES (mmol/l)		REFERENCES
	Fed	Fasted	
A. ENZYMATIC ASSAYS			
Cow	0.64-0.79	0.08-0.21	Hochheuser et al 1964
B. GLC METHODS			
Sheep	1.00-1.32	0.43	Annison 1954, Annison et al 1967.
Cow	0.49-0.60	0.25	Simkins et al 1965, Kronfield 1968.
Goat	1.60	-	Annison and Linzell 1964.

B. ASSAY METHODS FOR ACETATE.

Numerous methods for the assay of plasma, blood and tissue acetate levels have been described over the years. The methods hitherto used encountered problems because of the relatively low concentrations of the metabolite in the samples studied, coupled with the inherent non-specificity of those techniques which allowed interference by other tissue components, especially the other VFAs (butyrate and propionate).

The properties of acetate used in its determination are (Buckley 1974) :

1. volatility, useful in steam distillation, micro-diffusion, gas chromatography;
2. acidity, useful in titrimetric analysis;
3. enzymatic reactions, since acetate is an important metabolite in many species.

The older methods , many of which are no longer used are:

a. Steam distillation, used to measure total VFAs. The individual acids were then identified by their combined distillation curves and partition coefficients between ether and water (Wieland and Jennen 1941, Friedeman 1938, Elsden 1946, Annison 1954). These methods are unable to measure acetate accurately, although they still find occasional use as a preparative step for more sensitive and specific methods;

b. Column chromatography on silica gel of steam distillates

of VFAs (Elsden 1946);

c. spectrophotometric assay : lanthanum nitrate and acetate produce lanthanum acetate which reacts with molecular iodine to produce a blue coloured adsorption complex (Hutchens and Kass 1949). This method can only measure acetate levels greater than 3mmol/l, higher than the usually encountered blood or plasma levels.

d. microdiffusion in glass flasks at high temperatures for prolonged periods of time; or in Conway compact units with added anhydrous sodium salts to increase volatility of acetic acid at room temperature over relatively short time periods (Conway and Downey 1950). This method lacks specificity due to interference from other VFAs, lactate and 3-hydroxybutyrate.

e. microdiffusion and distillation methods have been described by Bartley (1953), but the high temperature and low pH requirements of these methods produced interference from the other VFAs, lactate, pyruvate, bicarbonate and hydrochloric acid, necessitating preliminary procedures (Keane 1967).

The methods in current use are:

i. GLC with various modifications (James and Martin 1952, Annison 1954, Metcalfe 1960, Medzihradsky and Lamprecht 1965). The major problems with this procedure have been (Keane 1967):

a. the requirement for large initial sample volumes,

b. "ghosting" or the appearance of peaks corresponding to the acids with injection of water,

c. poor separatory power of the columns used for the various VFAs,

d. poor reproducibility and linearity of response,

e. poor sensitivity,

f. need for regular maintenance of the chromatograph and its accessories,

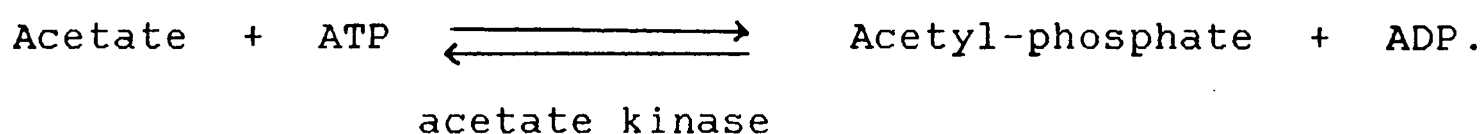
g. need for 'markers' such as isovalerate to distinguish acetate peaks,

h. elaborate sample preparation procedures such as lyophilization and steam distillation.

ii. Enzymatic assay techniques (Buckley 1974). Three key enzymes activate acetate, producing intermediates which are then acted upon by coupling systems (Fig 3.B.1).

a. <u>Acetate kinase</u>.

Acetate kinase derived from Escherichia coli can be used with many coupling systems. The first reaction (Rose, Grumberg-Manago, Korey and Ochoa 1954) is:

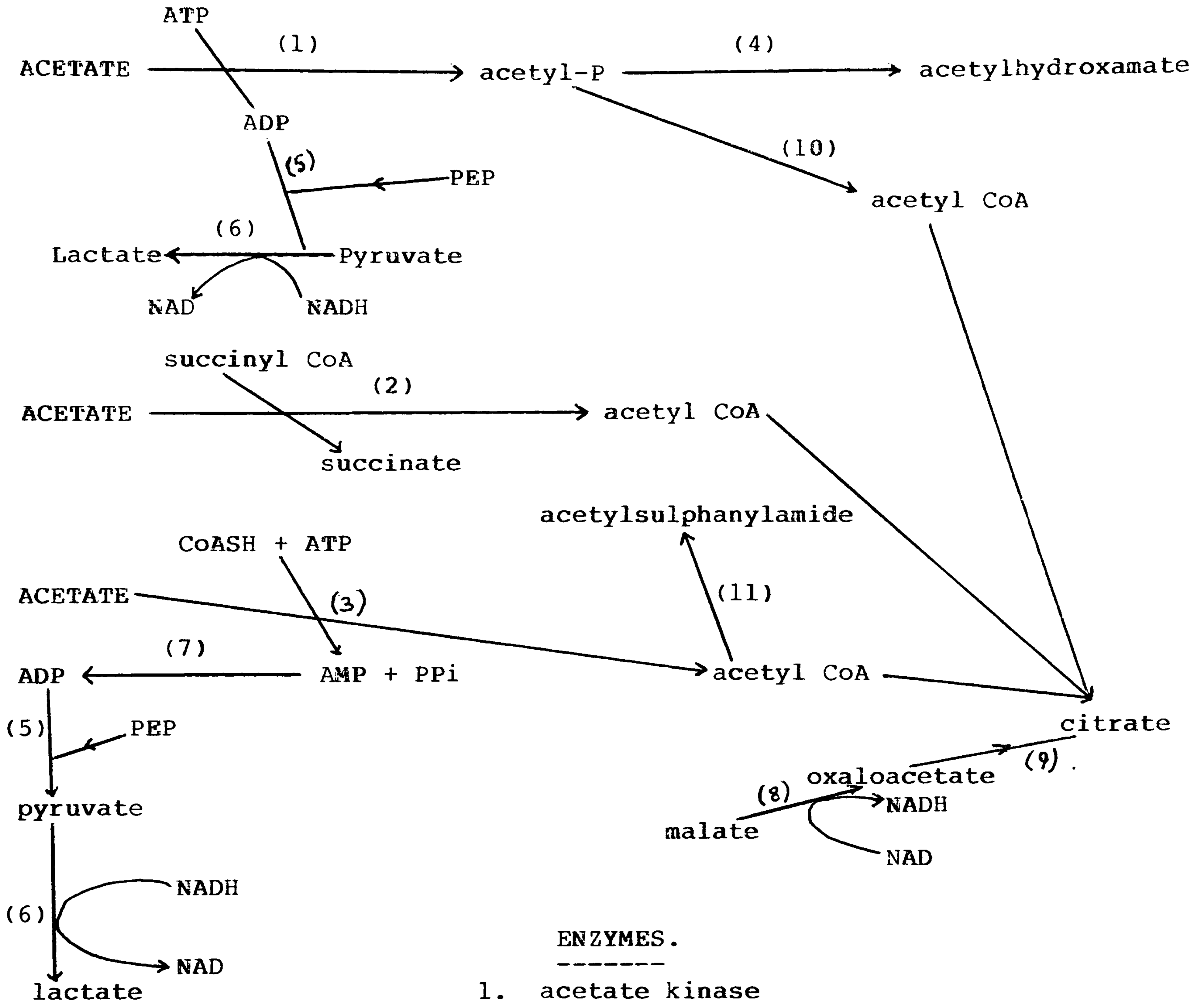


Subsequent reaction steps then include :

1. acetylation of hydroxylamine by acetyl phosphate which gives hydroxamate, measurable colorimetrically from its purple ferric complex (Lipmann and Tuttle 1945). This method is insensitive for acetate concentrations in the physiological range (Holz and Bergmeyer 1970);

FIG 3.B.1 : ENZYMATIC ASSAY METHODS FOR ACETATE

Three key enzymes (1,2,3) can activate acetate to give a product capable of being acted upon by coupling systems:



ENZYMES.

1. acetate kinase
2. CoA transferase
3. acetyl CoA synthetase
4. non-enzymic
5. pyruvate kinase
6. lactate dehydrogenase
7. adenylate kinase
8. malate dehydrogenase
9. citrate synthase
10. phosphotransacetylase
11. arylamine acetyltransferase

2. coupled acetyl phosphate formation to citrate through phosphotransacetylase and citrate synthase. This reaction is continued by measuring NAD reduction caused by the displacement of the malate dehydrogenase equilibrium, since oxaloacetate is drawn off by citrate synthase (Bergmeyer and Moellering 1966, Seufert, Graf, Janson, Kuhn and Soling 1974, Korri, Nuutinen and Salaspuro 1985, Bartlett and Kattermann 1985).

3. ADP liberated in the reaction reacts with phosphoenolpyruvate in the presence of pyruvate kinase to produce pyruvate. The subsequent conversion of pyruvate to lactate by lactate dehydrogenase is measured by NADH oxidation (Ballard, Filsell and Jarrett 1972, Knowles, Jarrett, Filsell and Ballard 1974, Trivin, Lenoir, Bretaudiere and Sachs 1982, Smith, Humphreys and Hockaday 1986).

4. use of acyl-phosphate hexose phosphotransferase, which catalyses the transfer of phosphate from acetyl-phosphate to hexoses (Buckley 1974). This enzyme has a low Km for acyl-phosphate and glucose, and in generating free acetate from acetyl phosphate, is used in a cycling system for acetate with glucose-6- phosphate dehydrogenase. The enzyme is not commercially available.

The main disadvantage of the acetate kinase system is the use of numerous enzymes in the coupling system with acetate kinase, with the possibility of increasing contamination with the other enzymes such as LDH and alanine

aminotransferase. As samples for acetate determination usually contain ample amounts of substrate for these enzymes, there is a large blank absorbance change with a substantial drift in absorbance obscuring the end-point (Buckley 1974).

Also, acetate kinase has a high Km (0.3mol/l) for acetate (Rose, Grunberg-Manago, Korey and Ochoa 1954), and the resulting need for high enzyme concentrations makes the procedure expensive. The enzyme may also react, albeit at a reduced rate with propionate (Lipmann and Tuttle 1945) likely to be present in measurable amount in portal blood samples.

b. CoA transferase.

Coupling of CoA-transferase from Propionibacterium shermanii to the malate dehydrogenase reaction has been used to measure acetate concentrations in bacterial metabolic products (Schulman and Wood 1971).

c. Acetyl CoA synthetase.

This enzyme is useful in the following assays:

i. pigeon liver preparations containing acetyl CoA synthetase and arylamine acetyl transferase catalyse the reaction involving the production of acetylsulphanylamide from acetyl CoA (Lundquist 1961, Lundquist 1973, Murthy and Steiner 1973). The rate of production and amount of the amide produced is then related to the level of free acetate present in the tissue sample. This method is, however, relatively insensitive, and the impurity of the enzyme

preparations makes elaborate sample purification necessary.

ii. acetyl CoA synthetase purified from beef heart mitochondria or yeast has a Km for acetate of 0.008mol/l and appears better than acetate kinase for use in enzymatic acetate assay methods. The initial results obtained with its use were unsatisfactory however, due to poor enzyme stability, and poor reproducibility of results on tissue specimens as against pure acetate solutions (Buckley 1974).

CHAPTER 4

MATERIALS AND METHODS

A. SUBJECTS.

All the studies were done in the Sheikh Rashid Diabetes Unit, The Radcliffe Infirmary, Oxford or otherwise on the medical wards of the Radcliffe Infirmary, John Radcliffe Hospital and the Renal Unit, Churchill Hospital, Oxford. Where necessary, the approval of the Oxford Area Ethical Committee was obtained, and all the subjects studied voluntarily gave informed consent.

The control subjects were healthy University students and laboratory and hospital workers; the diabetic patients were recruited from the hospitals' diabetic clinics and wards. Uraemic patients were recruited from and studied at the Renal Unit of the Churchill Hospital, Oxford; patients with chronic diarrhoea were out-patients of the gastroenterology unit at the John Radcliffe Hospital, Oxford.

None of the subjects had any known endocrine or metabolic disorder other than diabetes and none was on oral contraceptives or hormones known to affect glucose metabolism, other than the usual antidiabetic medications such as insulin, sulphonylureas and metformin, a biguanide. Also, none of the subjects regularly smoked cigarettes. For each subject, anthropometric indices of height (m) and weight (kg) were measured and so either the body surface area (m^2) or body mass index (kg/m^2) could be calculated. All the studies were performed on overnight fasted (>10hr) subjects in a room whose temperature was controlled at 23°C.

Unless otherwise stated, venous blood samples were taken from a cannula inserted into an antecubital vein and kept patent with isotonic saline. The catheter was always inserted at least 30min before collecting the first sample from the semi-recumbent subject. Two fasting blood specimens were taken, 10min apart and the subject's fasting value calculated as the mean of these two determinations. Samples were divided into two aliquots - one into a cold heparinised plastic tube, and the other (5ml) into a previously weighed plastic universal tube containing 5ml 10% (v/v) ice-cold perchloric acid and then shaken vigorously. Both tubes were kept on ice before centrifugation in a cold centrifuge. Plasma was stored frozen at -20°C until analysis for glucose, acetate, insulin, growth hormone and NEFAs. The deproteinised blood (in perchloric acid) was kept at 4°C until analysis of the neutral extracts for the "intermediary metabolites" (lactate, pyruvate, acetoacetate, 3-hydroxybutyrate and glycerol) within 5 days of sample collection.

Baseline investigations of full blood count, urea, electrolytes, creatinine, calcium, phosphate, cholesterol, total thyroxine and cortisol were done on each subject by the hospitals' routine biochemistry and haematology services.

B. MATERIALS.

The following chemicals, all reagent grade, were used:

a. from Sigma (St Louis, Mo, U S A):

Trizma base, Trizma hydrochloride, acetoacetic acid, L(+)-lactic acid, acetyl phosphate;

b. Analar grade reagents from BDH Chemicals, Poole, England:

Tris, KOH, EDTA, sodium metabisulphite, magnesium chloride hexahydrate, hydrochloric acid, anhydrous sodium acetate (Aristar grade), perchloric acid, alanine, glycine, glycerol, bovine serum albumin;

c. from Boehringer Mannheim GmbH, W Germany :

lactate dehydrogenase, 3-hydroxybutyrate dehydrogenase, NAD, NADH, pyruvate kinase, glycerokinase, phosphoenolpyruvate, acetate kinase, glucose oxidase, adenosine-5-triphosphate.

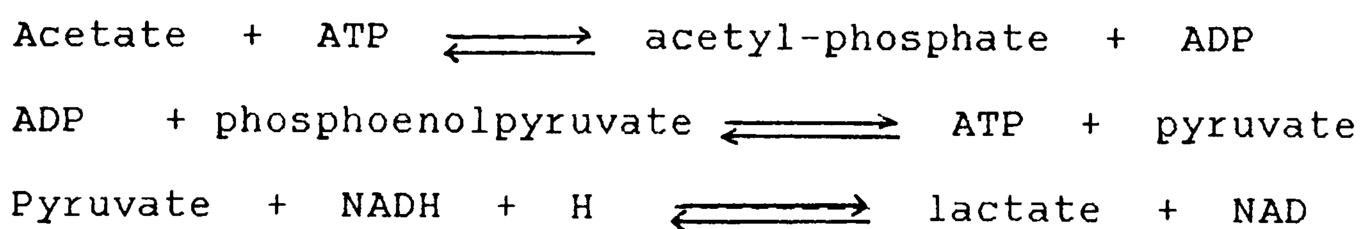
Sodium propionate (Analar) was a gift from Dr D Williamson, MRC Metabolic Research Laboratory, Radcliffe Infirmary, Oxford; acetyl-carnitine was also a gift from Dr A Kerbey, the Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford.

C. METHODS.

(a) Acetate.

(i). Choice of Method.

In view of the earlier described problems with GLC, which also requires, at least initially, significant capital outlay, an enzymatic spectrophotometric method was chosen for acetate estimation in these studies, based on modifications of the methods of Buckley and Williamson (1977) and Trivin, Lenoir, Bretaudiere and Sachs (1982). The enzymes, glucose-6-phosphate dehydrogenase and acyl phosphate hexose phosphotransferase, used in the method of Buckley (1974) were not used in the studies so as to prevent cycling and thereby avoid obtaining inconsistently high results. Instead, the method relied on the acetate kinase reaction (Fig 3.B.1,(1),(5),(6)) according to the following equations:



$$\text{Acetate} + \text{ATP} \rightleftharpoons \text{acetyl-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \rightleftharpoons \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H} \rightleftharpoons \text{lactate} + \text{NAD}$$

It was found necessary to introduce a sample preparative stage (not found in the method of Trivin, Lenoir, Bretaudiere and Sachs (1982)) to prevent the over-estimation of acetate due to contamination of commercial preparations of acetate kinase with hexokinase (Smith, Humphreys and Hockaday 1986). Sample

deproteinization and other purification procedures were found unnecessary and this made the method simple and rapid to perform, estimating 5-8 plasma samples in about 4hr.

Reagents.

The reagents used were 0.2mol/l Tris HCl pH 7.8, sodium acetate 0.1mol/l, magnesium chloride 0.1mol/l, ATP, phosphoenolpyruvate, NADH, pyruvate kinase (2000units/ml), lactate dehydrogenase (3000units/ml), acetate kinase (1000 units/ml) and glucose oxidase (100units/ml). Disposable plastic tubes and cuvettes were used throughout.

A working reagent was prepared from: 20ml Tris HCl, 1ml magnesium chloride, 100mg ATP, 25mg phosphoenolpyruvate, 8mg NADH, 40μl pyruvate kinase and 150μl lactate dehydrogenase. This reagent, with pH corrected to 7.4 with 1mol/l HCl, was then incubated for 30min at room temperature to ensure completion of any side reactions due to contamination of the enzyme preparations.

Standard acetate solutions were prepared from anhydrous sodium acetate kept dessicated at 4°C. A 100mmol/l stock acetate standard was prepared in distilled water and frozen in suitable aliquots. These aliquots were then used to prepare an intermediate 10mmol/l standard from which working standards of 2, 1, 0.5 and 0.25mmol/l could be prepared. Distilled water was used as a zero standard and carried through the sample preparation procedure.

Preparation of Standards and Samples.

To 100µl of plasma in duplicate was added 10µl of glucose oxidase. Tubes were then stoppered, mixed and incubated at 37°C for 30min. The acetate standards were treated similarly. Samples and standards were then removed from the incubator and placed on ice for 10min. At this time, glucose levels were checked to be <0.5mmol/l with a Beckman glucose analyser; higher values for glucose meant further incubation of the samples. Hydrogen peroxide generated during the reaction was removed by addition of 10µl of 65mmol/l sodium metabisulphite.

Procedure.

Duplicate cuvettes were prepared for reference and standard analyses and for all the test samples. To the reference cuvette (reagent blank) was added 1.6ml distilled water and 0.5ml of the working reagent. To the standard and test cuvettes, 1.0ml of distilled water and 1ml of working reagent were added. 100µl of standard or test sample was then added, and the total contents of the cuvette well mixed.

The absorbance at 340nm (LKB Spectrophotometer, Biochrom, Cambridge, U K) was then measured at 5min intervals until steady (after a period of at least 15min to ensure the completion of any non-specific side reactions and be certain of the removal of any pyruvate present in the original sample). 5µl of acetate kinase was then added to

all the cuvettes, except the reference, and the cuvettes again well-mixed. The absorbance at 340nm was then monitored every 5min for 30min. The rate of the reaction was calculated as the ΔA340/min by determining the slope of the regression line of sample absorbance against time. The acetate concentration of each sample was calculated by a computer programme, from a curve relating standard reaction rates to concentration.

The addition of increasing concentrations of glucose to plasma samples prior to the sample preparation stage did not result in any change in measured acetate concentration for glucose values up to 30mmol/l with low (0.1mmol/l) or high (2mmol/l) acetate concentration.

Analytical Recovery of Acetate from Plasma.

1ml of 0.25mmol/l, 0.5mmol/l and 1mmol/l sodium acetate respectively was mixed with 2ml of each of 5 different plasma samples obtained from fasting non-diabetic volunteers. Aliquots of the original plasma, the acetate standards and the plasma/standard mixtures were then analysed, each in triplicate. Percentage recovery was defined as excess amount determined/ excess amount added x 100.

The mean(SD) analytical recoveries of 0.25, 0.5 and 1mmol/l acetate were respectively : 105(3)%, 110(2)% and 110(2)%. Intra-assay coefficient of variation (n=10) was 5.6% and inter-assay coefficient of variation from pooled

plasma samples (n=20) was 7.8%. These values are similar to the mean recovery of 108% and between batch coefficient of variation of 8% reported by Smith, Humphreys and Hockaday (1986), and indicate that this method slightly over-estimates plasma acetate values.

(ii) Validation of Method.

An exchange of 5 plasma specimens was done between our unit and the Cummings' group at the Dunn School of Nutrition, Cambridge. The latter group used freeze transfer, vacuum distillation and GLC to determine plasma acetate levels, and had reported plasma acetate levels which were generally lower than the values we had been obtaining from our enzymatic method (Pomare, Branch and Cummings 1985). This exchange of plasma samples between Oxford and Cambridge was aimed at comparing both the absolute values and rank values obtained by both methods.

Assayed by either method, the ranking order of acetate values was not similar, except in a few of the subjects (rs 0.27, p NS) (Table 4.C.1). Also the absolute values were significantly different with the results from the enzymatic method being about 5-10 times greater than the GLC results.

To investigate the possibility that the enzymatic assay method was grossly over-estimating plasma acetate, we tested the effects of different variables within the assay such as sample preparation and storage, deproteinization, presence of other plasma constituents such as NEFA, lactate, pyruvate

TABLE 4.C.1 : RANKED VALUES FOR PLASMA ACETATE AS MEASURED BY ENZYMATIC (OXFORD) OR GLC (CAMBRIDGE) METHODS

Subjects	Plasma acetate (mmol/l)	
	Oxford	Cambridge
1. normal subject	0.22	0.05
2. NIDDM patient*	0.22	0.06
3. low carbohydrate diet	0.24	0.02
4. low carbohydrate diet	0.28	0.05
5. normal subject	0.28	0.06
6. low carbohydrate diet	0.34	0.02
7. IDDM patient*	0.36	0.05
8. post lactulose intake	0.36	0.12
9. post lactulose intake	0.45	0.04
10. IDDM patient*	0.49	0.14

* The concurrent fasting plasma glucose values for subjects 2, 7 and 10 were 5.4, 9.4 and 9.7mmol/l respectively.

and the "ketone bodies" (acetoacetate and 3-hydroxybutyrate), simple amino-acids, acetyl - groups, organic acetate and another volatile fatty acid, propionate. These constituents had variously been proposed to interfere with either enzymatic or GLC techniques for measuring acetate (Bartley 1953, Lundquist, Fugmann and Rasmussen 1961, Guynn and Veech 1974, Nielsen, Owen Ash and Thor 1978, Tollinger, Vreman and Weiner 1979, Seufert, Mewes and Soeling 1984, Hermann, Herz and Frohlich 1985).

a. Sample Preparation.

Tollinger, Vreman and Weiner (1979) reported that deproteinised whole blood acetate, measured using vacuum distillation and GLC, increased if the specimen was left at room temperature for 2hr before deproteinization, although serum and plasma values of acetate did not change.

To further test this finding, 10ml blood was collected from each of 2 volunteers. Each specimen was divided into 3 aliquots. Aliquot 1 was centrifuged immediately at $4^{\circ}C$; aliquot 2 was kept at room temperature ($20^{\circ}C$) for 10min and then centrifuged on a bench centrifuge; aliquot 3 was left at $20^{\circ}C$ for 4hr before centrifuging at room temperature. All the samples were analysed for acetate on the day of collection.

The results obtained (Table 4.C.2) indicate no significant differences in plasma acetate values in the 3 aliquots.

TABLE 4.C.2: EFFECT OF TIME BEFORE CENTRIFUGATION ON PLASMA ACETATE VALUES IN TWO SUBJECTS

(mean ± SD of 5 determinations)

	Subject 1 (mmol/l)	Subject 2 (mmol/l)
Aliquot 1 (centrifuged immediately at 4 C)	0.18 ± 0.02	0.09 ± 0.01
Aliquot 2 (kept at room temperature for 10min before centrifuging at room temperature)	0.17 ± 0.02	0.09 ± 0.01
Aliquot 3 (kept at room temperature for 4hr, then centrifuged at room temperature)	0.19 ± 0.02	0.11 ± 0.02

b. Sample Storage.

The reported effects of sample storage on acetate values obtained by GLC have been inconsistent. Kveim and Bredersen (1979) showed that storage of heparinised blood samples for 30hr (before centrifugation and plasma separation) at 4°C had no influence on plasma acetate values, but that prolonged storage at room temperature before separation caused a 40% increase in measured plasma acetate; re-analysis of plasma kept at -20°C for 5months however revealed no further change in acetate values. In similar experiments, Nielsen, Owen Ash and Thor (1978) demonstrated that acetate in serum, plasma and aqueous solutions was stable for 10days at 4°C, and at least 3months at -20°C; they also reported that samples could be frozen and thawed as many as 5 times or more without altering stability. There were no differences between serum and plasma acetate, and although erythrocyte acetate was 40-80% of plasma acetate, neither moderate haemolysis nor lipaemia seemed to interfere with the assay.

To attempt to resolve these using our method, 10ml blood was withdrawn from a fasting non-diabetic volunteer into a cold heparinised tube and centrifuged immediately at 4°C. Plasma was divided into 5 aliquots: aliquot 1 was analysed on the day of collection, aliquot 2 was stored at 4°C for 24hr, while aliquots 3, 4 and 5 were stored frozen for 24hr, 7days and 6 months respectively before analysis.

The results (Table 4.C.3) showed that storage of the plasma specimens under the different conditions for varying lengths of time up to 6 months did not significantly influence plasma acetate values.

c. Effect of Sample Deproteinization

Table 3.A.1 shows that initial deproteinization of plasma or whole blood before analysis for acetate by enzymatic or GLC methods has been used in numerous studies. Trivin, Lenoir, Bretaudiere and Sachs (1982) did not consider such a step necessary, and other workers (Teresi and Luck 1952, Bull and Breese 1967, Remesy and Demigne 1974, Spector 1975, Jordan and Phillips 1978) have shown that a low molecular weight fatty acid like acetic acid is only minimally bound to plasma proteins, unlike longer chain VFAs, even as short as butyrate. While deproteinization increases the time for the procedure, the addition of other chemicals tends to reduce the consistency of results obtained. Indeed, potassium perchlorate formed as a result of sample deproteinization with perchloric acid interferes with an earlier reported cycling enzymatic assay for acetate (Buckley 1974).

To investigate the possibility of protein-binding contributing to the variability in results (especially as the method used by the Cambridge group involved a prior deproteinization step), that step was added to the enzymatic assay for this portion of the study.

TABLE 4.C.3: EFFECT OF TYPE AND DURATION OF STORAGE ON PLASMA ACETATE VALUES.

(mean ± SD of 5 determinations)

	Plasma	Acetate (mmol/l)
i.	analysed immediately	0.21 ± 0.02
ii.	stored 24hr at 4 C	0.21 ± 0.02
iii.	stored 24hr at -20 C	0.21 ± 0.03
iv.	stored 7 days at -20 C	0.21 ± 0.02
v.	stored 6 months at -20 C	0.22 ± 0.03

5ml blood was collected from each of 5 subjects, centrifuged immediately at 4° C and the plasma then stored frozen for 1week at -20°C before analysis. Deproteinization of each sample, on the day of analysis, was done by the addition of 1ml 5% (v/v) perchloric acid to 1ml plasma, centrifuging and then neutralizing the supernatant to pH 7.0, with 10% (w/v) KOH. Neutralized, deproteinized and clear plasma extracts as well as untreated plasma were then analysed for acetate.

The results obtained (Table 4.C.4a) indicate that prior deproteinization of plasma samples did not significantly influence the results obtained.

We also measured acetate levels in deproteinized plasma, whole blood and erythrocytes as well as untreated plasma in 6 subjects to assess if these various sample types differed in acetate content. The results obtained (Table 4.C.4b) showed that deproteinized and untreated plasma again gave similar values while whole blood acetate was slightly less (82% of plasma values, $p < 0.01$). Erythrocyte acetate levels were the lowest, being 65% of plasma values and about 80% of whole blood values. This observation has been previously reported (Nielsen, Owen Ash and Thor 1978).

d. Effects of Presence of Acetyl-groups and Organic Acetate.

Tollinger, Vreman and Weiner (1979) remarked that the increase in plasma acetate levels on freezing and thawing of plasma samples may have been due to hydrolysis of acetylated

TABLE 4.C.4a: EFFECT OF PRIOR SAMPLE DEPROTEINISATION ON PLASMA ACETATE LEVELS

	SUBJECTS (acetate mmol/l)						
	1	2	3	4	5	mean(SD)	X(SD)%
i. untreated plasma	0.18	0.41	0.17	0.17	0.69	0.32(0.23)	-
ii. deproteinised plasma analysed immediately	0.19	0.41	0.17	0.24	0.66	0.33(0.21)	109(18)
iii. deproteinised plasma stored frozen at -20 C for 48hr	0.16	0.47	0.24	0.25	0.63	0.35(0.19)	117(27)

X mean of individual values of (ii) and (iii) expressed as percentages of corresponding values of (i).

ABLE 4.C.4b : PLASMA ACETATE VALUES IN PLASMA, WHOLE BLOOD AND RED CELLS OF 6 SUBJECTS.

	Acetate (mmol/l)							
	Subjects (means of 3 determinations)							
	1	2	3	4	5	6	Mean(SD)	X(SD)%
. untreated plasma	0.19	0.25	0.12	0.20	0.31	0.09	0.19(0.08)	-
i.deproteinized plasma	0.19	0.22	0.14	0.19	0.29	0.11	0.19(0.06)	103(14
.ii.deproteinized whole blood	0.17	0.18	0.10	0.17	0.26	0.07	* 0.16(0.07)	82(6)
iv.deproteinized red cells	0.11	0.15	0.08	0.15	0.19	0.06	*+ 0.13(0.05)	65(6)

* $p < 0.01$ compared to plasma (untreated or deproteinized)

\+ $p < 0.01$ compared to whole blood.

X mean of values of (ii), (iii) and (iv) expressed as percentages of corresponding values of (i)

compounds e.g. acetyl CoA, acetylcarnitine and acetyl-aspartate. Of these, only acetylcarnitine is detectable to any extent in plasma (Guynn and Veech 1974, Mitchell 1981, Wolff, Carroll, Thuy, Prodanos, Haas and Nyhan 1986); acetyl CoA is unstable, and acetyl-aspartate is an important intracellular metabolite only in the brain. The effect of adding various amounts of acetylcarnitine to plasma samples was therefore tested.

Our assay technique involves a prior generation of acetyl-phosphate, which, theoretically could react in the assay system as acetate. It was therefore considered that such a reaction might be a cause of over-estimation of acetate values. We thus tested the effect on plasma acetate measurements, of adding various concentrations of acetyl-phosphate to plasma and standard acetate samples.

As "organic acetate" is covalently bound, as in acetate esters, theoretically it should not react in the enzymatic assay for free acetate. This assumption was tested with ethyl acetate.

The above 3 compounds were tested at different sample concentrations; in each case, amounts of 0.25, 0.5 and 1 mmol/l, vastly in excess of what could conceivably be present in human tissues, were analysed alone and also when mixed with plasma (v/v - 1:1), plasma and 1mmol/l sodium acetate v/v 1:1:1) and with only 1mmol/l sodium acetate (v/v 1:1). The following results were obtained:

i. acetylcarnitine was not detectable in the assay system,

the various concentrations used always registered as < 0.01mmol/l acetate; mixtures with plasma and standard acetate similarly did not contribute any additional recovery.

ii. acetyl phosphate at the different concentrations always registered < 0.02mmol/l acetate when used alone or in mixtures with plasma and standard acetate.

iii. similar observations to (i) were made with ethyl acetate.

These results suggest that acetylcarnitine, acetyl phosphate and ethyl acetate do not react as free acetate in the enzymatic assay system, and furthermore that blood and tissue acetylated compounds and organically-bound acetate are unlikely to contribute to the acetate values obtained with this assay.

e. Effect of Simple Amino-acids.

Simple amino-acids such as glycine and alanine present in plasma have been reported to interfere with acetate determination by GLC, especially when there was no preliminary sample purification (Buckley 1974, Lundquist 1961).

To investigate this possibility, the likely effects of alanine and glycine on the enzymatic assay were tested as follows: To 1ml of plasma was added 1ml of each of 1mmol/l alanine and 1mmol/l glycine. Also, 1ml of a 1:1 (v/v) mixture of alanine and glycine (1mmol/l each) was added to

1ml plasma. Aliquots of the original plasma, amino-acids, and the various plasma/amino-acid mixtures were then analysed with the results shown in Table 4.C.5.

These results indicate that alanine and glycine interfere only minimally with the enzymatic assay for acetate.

f. Other "Intermediary Metabolites".

The effects of acetoacetate (0.25mmol/l), pyruvate (0.25mmol/l), glycerol (0.25mmol/l), 3-hydroxybutyrate (0.5mmol/l), lactate (1mmol/l) and NEFAs (linoleic acid 1mmol/l), all in concentrations similar to, or greater than levels in non-diabetic and diabetic blood, were tested by adding 1ml of each solution to 1ml of plasma or standard acetate solution (1mmol/l). The various "pure" metabolites and the plasma/metabolite, standard acetate/metabolite and plasma/standard acetate/ metabolite mixtures were then analysed for acetate.

The results obtained indicate that whichever metabolite was added, the measured acetate was consistently < 0.01mmol/l, and also that none of the various metabolites contributed any additional recovery to plasma or standard acetate used.

g. Propionate.

Acetate kinase has a relatively high Km (0.30 mol/l) for acetate (Rose 1954, Buckley 1974) and has also been

TABLE 4.C.5: EFFECTS OF SIMPLE AMINO-ACIDS ON ACETATE VALUES.

(means of 3 determinations)

	acetate (mmol/l)
i. plasma	0.20
ii. plasma + 0.1mM glycine	0.20
iii. plasma + 0.1mM alanine	0.19
iv. plasma + 0.1mM glycine + 0.1mM alanine	0.19
v. 0.1mM alanine	0.015
vi. 0.1mM glycine	0.011
vii. 0.1mM alanine + 0.1mM glycine	0.011

reported to react with propionate (Lipmann and Tuttle 1945). Although propionate is usually undetectable in peripheral blood (Pomare, Branch and Cummings 1985), unlike the portal venous blood of ruminant and omnivorous mammals (Ballard, Hanson and Kronfeld 1969, Illman, Trimble, Snoswell and Topping 1982), this short-chain fatty acid was tested in the enzymatic assay to exclude it as a possible cause of overestimation of plasma acetate values.

When analyses were done on 0.25 mmol/l propionate alone and mixed with plasma and standard acetate (1mmol/l), there was no additional recovery due to the added propionate, and 0.25mmol/l propionate reacted as < 0.020 mmol/l acetate.

h. Distilled Water.

Bench distilled water and the other reagents used in the determination of acetate viz. components of the working reagent, did not contain detectable amounts of free acetate.

i. Changes in Absorbance of 'Blank'.

The blank sample containing only distilled water changes in absorbance over time, especially during the first 15min after acetate kinase is added. A typical example is as shown in Table 4.C.6. This change is ascribed to impurities in the enzymes causing measurable cross-reactions and is normally minimised by prior incubation of the reaction mixture at room temperature.

To check if these changes in "blank" absorbance

TABLE 4.C.6 : CHANGE IN ABSORBANCE OF STANDARDS, CONTROLS AND PLASMA SAMPLES DURING THE ENZYMATIC ASSAY.

	Time (min)								
	0		5	10	15	20	25	30	Values
Standard 1	.480	.480	.451	.425	.407	.390	.380	.370	0
Standard 2	.480	.480	.444	.410	.385	.362	.346	.332	0.25
Standard 3	.494	.493	.438	.393	.356	.328	.305	.290	0.50
Standard 4	.480	.478	.400	.340	.290	.250	.218	.194	1.00
Control 1	.482	.482	.445	.416	.390	.366	.351	.336	0.21
Control 2	.488	.487	.448	.417	.391	.367	.351	.338	0.23
Sample 1	.493	.493	.458	.428	.405	.382	.368	.355	0.17
Sample 1	.485	.486	.445	.417	.392	.372	.357	.345	0.17
Sample 2	.554	.555	.495	.464	.431	.404	.384	.366	0.42
Sample 2	.553	.552	.480	.445	.415	.388	.368	.352	0.46
Sample 3	.490	.490	.428	.401	.382	.368	.361	.357	0.07
Sample 3	.495	.497	.440	.416	.397	.382	.374	.366	0.07
Distilled water only	.011	.012	.011	.011	.012	.011	.012	.012	-

Values given as mmol/l acetate.

influence the measured acetate values significantly, the concentrations were re-calculated (with the computer programme) by:

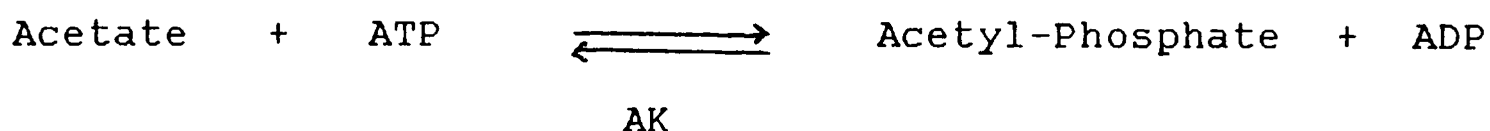
a. keeping the blank sample absorbance 'steady' (Table 4.C.7) and maintaining the other readings;

b. changing all the other readings according to the changes in blank absorbance (Table 4.C.8).

The results obtained (Table 4.C.9) indicate that changes in "blank" absorbance over the duration of the assay did not significantly affect the measured acetate results.

j. Other Reaction Characteristics.

The observation that sample absorbance changes continue till at least the 20th minute of spectrophotometric reading, especially in samples containing relatively high acetate concentrations (>0.5mmol/l), raised the possibility of continuous cycling of the reaction:



$$\text{Acetate} + \text{ATP} \underset{\text{AK}}{\rightleftharpoons} \text{Acetyl-Phosphate} + \text{ADP}$$

possibly through regeneration of acetate from acetyl-phosphate, although it has already been shown in (d) that acetyl-phosphate did not react to any extent in the assay system.

To test this observation further, spectrophotometric readings were continued up to 60min, with the readings shown in Table 4.C.10 and Fig 4.C.1. The results obtained show that the readings became steady between 30 and 40min. As

TABLE 4.C.7 : CHANGE IN ABSORBANCE OF STANDARDS, CONTROLS AND PLASMA SAMPLES DURING THE ENZYMATIC ASSAY.
(blank sample absorbance kept steady)

		Time (min)							
		0		5	10	15	20	25	30	Values
Standard	1	.480	.480	.480	.480	.480	.480	.480	.480	0
Standard	1	.480	.480	.473	.465	.458	.452	.446	.442	0.25
Standard	3	.494	.493	.467	.448	.429	.418	.405	.400	0.50
Standard	4	.480	.478	.429	.395	.363	.340	.318	.304	1.00
Control	1	.482	.482	.474	.471	.463	.456	.451	.446	0.21
Control	2	.488	.487	.477	.472	.464	.457	.451	.448	0.23
Sample	1	.493	.493	.487	.483	.478	.472	.468	.465	0.17
Sample	1	.485	.486	.474	.472	.465	.462	.457	.455	0.17
Sample	2	.554	.555	.524	.519	.504	.494	.484	.476	0.42
Sample	2	.553	.552	.509	.500	.488	.478	.468	.462	0.46
Sample	3	.490	.490	.457	.456	.453	.458	.461	.467	0.08
Sample	3	.495	.497	.469	.471	.470	.472	.474	.476	0.07

Values given as mmol/l acetate.

TABLE 4.C.8 : CHANGE IN ABSORBANCE OF STANDARDS, CONTROLS AND PLASMA SAMPLES DURING THE ENZYMATIC ASSAY.
(changing all other readings according to changes in the blank absorbance)

	Time (min)								
		0	5	10	15	20	25	30	Values
Standard 1	.480	.480	.451	.425	.407	.390	.380	.370	0
Standard 2	.480	.480	.473	.436	.403	.379	.356	.342	0.25
Standard 3	.494	.493	.467	.419	.374	.345	.315	.300	0.50
Standard 4	.480	.478	.429	.366	.308	.267	.228	.204	1.00
Control 1	.482	.482	.474	.442	.408	.383	.361	.346	0.22
Control 2	.488	.487	.477	.443	.409	.384	.361	.348	0.24
Sample 1	.493	.493	.487	.454	.423	.399	.361	.346	0.18
Sample 1	.485	.486	.474	.443	.410	.389	.367	.355	0.18
Sample 2	.554	.555	.524	.490	.449	.421	.394	.376	0.43
Sample 2	.553	.552	.509	.471	.433	.405	.378	.362	0.47
Sample 3	.490	.490	.457	.427	.400	.385	.371	.367	0.09
Sample 3	.495	.497	.467	.442	.415	.399	.384	.376	0.07

Values given as mmol/l acetate.

[TAB]LE 4.C.9 : THE EFFECT ON ACETATE VALUES OF ADJUSTING SAMPLE ABSORBANCE FOR CHANGING BLANK ABSORBANCE.

	Acetate concentration (mmol/l)		
	A	B	C
[Co]ntrol 1	0.21	0.21	0.22
[Co]ntrol 2	0.23	0.23	0.24
[Sa]mple 1	0.17	0.17	0.18
[Sa]mple 1	0.17	0.17	0.18
[Sa]mple 2	0.42	0.42	0.43
[Sa]mple 2	0.46	0.46	0.47
[Sa]mple 3	0.07	0.08	0.09
[Sa]mple 3	0.07	0.07	0.07

A : no adjustments made for changing blank absorbance;

B : assuming that blank readings are steady throughout the procedure and adjusting sample readings on that basis;

C : assuming that blank absorbance changes during the procedure and adjusting sample absorbance for that change.

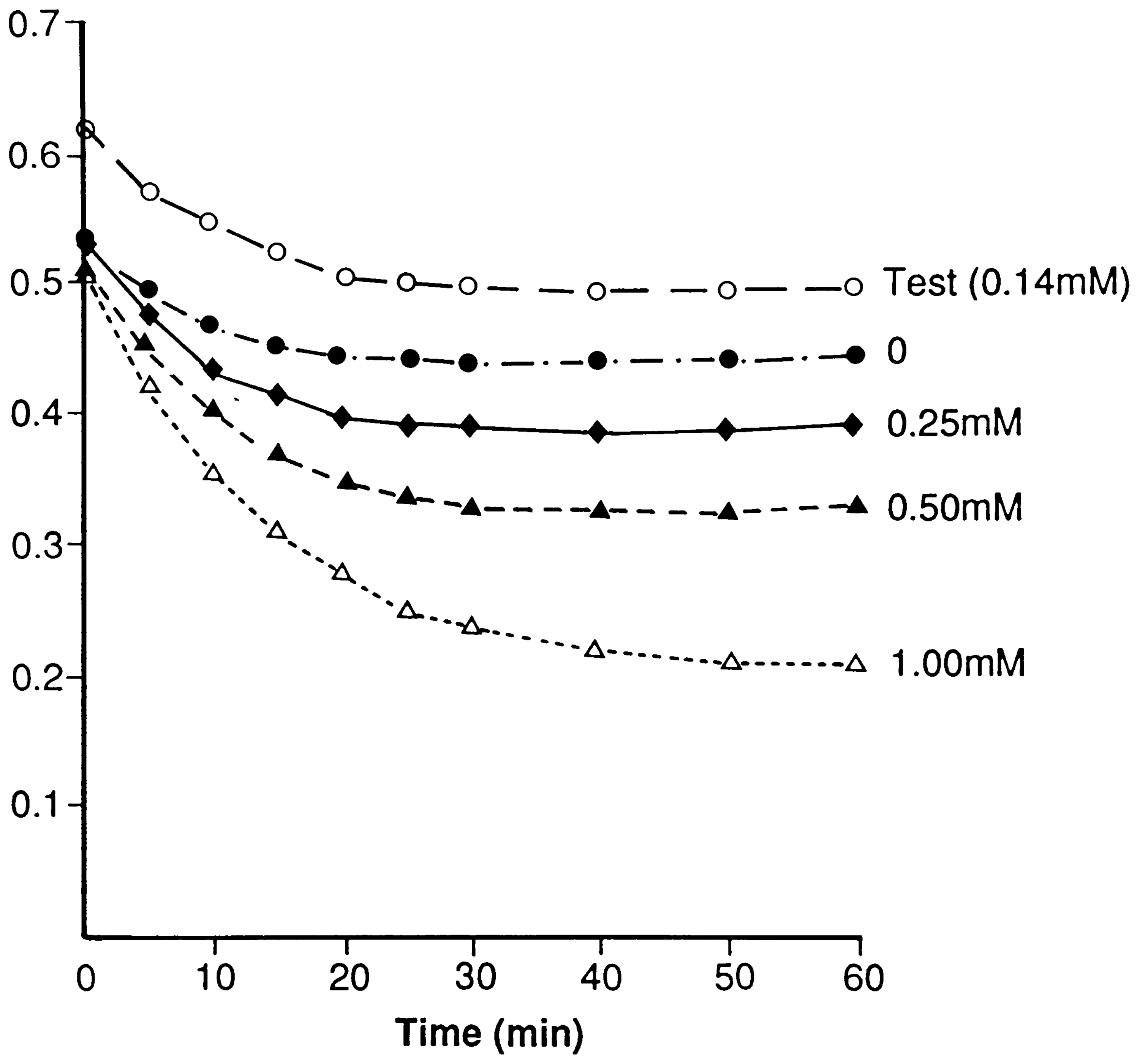
BLE 4.C.10 : ABSORBANCE READINGS ON STANDARDS, CONTROLS AND SAMPLES EXTENDED TO 60 MIN.

		Time (min)										
		0	5	10	15	20	25	30	40	50	60	Values
d 1	.524	.528	.498	.473	.460	.448	.441	.436	.436	.436	.445	0
d 2	.536	.538	.485	.445	.423	.405	.396	.390	.387	.387	.395	0.25
d 3	.539	.538	.464	.410	.378	.355	.342	.334	.330	.328	.335	0.50
d 4	.539	.540	.438	.364	.315	.282	.255	.244	.223	.214	.214	1.00
l 1	.538	.538	.485	.445	.420	.405	.393	.390	.382	.382	.386	0.27
l 2	.540	.540	.486	.445	.424	.410	.396	.392	.390	.389	.395	0.26
e 1	.630	.630	.576	.550	.530	.518	.508	.504	.498	.496	.496	0.14
e 1	.636	.635	.585	.556	.536	.526	.514	.509	.502	.502	.502	0.14
e 2	.610	.610	.555	.530	.514	.497	.492	.485	.484	.486	.488	0.13
e 2	.610	.612	.562	.534	.517	.501	.498	.488	.486	.486	.491	0.12
e 3	.591	.590	.545	.520	.504	.488	.480	.476	.474	.471	.476	0.08
e 3	.592	.593	.548	.522	.505	.491	.484	.478	.474	.475	.480	0.09

Plasma acetate values given in mmol/l.
Std : standard
Ctl : control
Spe : plasma sample.

FIG 4.C.1 :

Change in absorbance with time over 60 min



earlier observed, stabilisation of the readings was earlier in samples with lower acetate values (0-0.25mmol/l). The fact that 30min was the most commonly observed stabilisation time for samples within the usual range of acetate values encountered in the study, made this the chosen time interval for all the experiments reported .

The above validation experiments would suggest that this method of assay for acetate is probably not grossly over-estimating plasma values of the substrate, and furthermore is unlikely to be affected by physiological concentrations of other metabolites in blood. The discrepancy between results obtained by this enzymatic method and GLC methods therefore remains unresolved.

k. Urinary Acetate.

Urine samples were collected pre- and post-acetate infusion, measured and stored in plastic tubes at -20°C without any additive, but analysed for acetate within 1week of sample collection. The assay procedure was as for plasma, other than for a 5-10 times dilution of the urine in 0.1mol/l phosphate buffer (pH 7.4) when, post-acetate infusion, the acetate content of the undiluted specimen was consistently higher than 2mmol/l.

With urine samples, acetate measurement by the enzymatic method also gave consistent results. In 10 subjects (5 diabetic), fasting urinary acetate was 0.06 ±

0.01mmol/1, and rose to 4.5 $\pm$ 1.3mmol/1 after each was infused with 1litre 150mmol/1 acetate over 60min. Values were essentially similar for both the diabetic and non-diabetic subjects studied.

b. OTHER MEASUREMENTS.

Glucose was measured by a polarographic technique using a glucose analyser (Beckman Instruments, High Wycombe, U K). The "intermediary metabolites" were measured by enzymatic methods: lactate and pyruvate (Hohorst, Kreutz and Bucher 1959), acetoacetate and 3-hydroxybutyrate (Williamson, Mellanby and Krebs 1962), glycerol (Kreutz 1962) and NEFA (Shimizu, Inoue, Tani and Yamada 1979).

Insulin was determined by double antibody radioimmunoassay (Morgan and Lazarow 1963, Welborn and Fraser 1960), free insulin (in the diabetics on insulin treatment) by the method of Kuzuya, Horwitz, Steiner and Rubenstein (1977) and growth hormone by the method of Boden and Soeldner (1967). Thyroxine and cortisol estimations were done at the clinical biochemistry laboratory by routine radioimmunoassay.

Glycosylated haemoglobin (HBA1c) estimation was by an isoelectric focussing method with which the range of values for non-diabetic subjects is 4-8% (Jeppson, Franzen and Gaal 1980).

Blood gases (pO_2, pCO_2 , base excess and saturation) were measured on a Radiometer Copenhagen ABL 2 gas meter

calibrated daily with standard solutions.

D. STATISTICAL METHODS.

Results are expressed as means ± SD, except where indicated. Comparison within and between groups was by paired and unpaired Student's t tests and ANOVA with a repeated measures design. Because subject numbers tended to be relatively small and the nature of sample distribution was unknown, correlations were sought by the Spearman's rank method. The level of statistical significance was $p < 0.05$.

The statistical calculations were done using an OXSTAT statistical package on a Zenith Data Systems microcomputer as well as the Statistical Package for the Social Sciences (SPSS) on the main Oxford University computer ICL 2988 with programming help from Mrs Vicky THursfield and Dr Li Zhang.

CHAPTER 5

STUDIES ON ENDOGENOUS ACETATE METABOLISM

A. LITERATURE REVIEW.

It is likely that pathways of metabolism differ between ruminant and non-ruminant mammals, and even among the various species of non-ruminants. The various suggested metabolic pathways are therefore considered further under the following sub-headings:

i. Ruminant Mammals.

Acetate metabolism in ruminants has been extensively reviewed by Annison and Armstrong (1970), Ballard (1972) and Knowles, Jarrett, Filsell and Ballard (1974). The dominant role of glucose in lipogenesis in non-herbivores is largely taken over by acetate in ruminants. This is due to the low hepatic activities of glucokinase and hexokinase, the enzymes responsible for glucose uptake and utilization (Brockman and Laarveld 1985). Also in the absence of adequate levels of ATP-citrate lyase, glucose can supply only limited amounts of acetyl CoA for fatty acid synthesis.

The initial reaction in acetate metabolism is conversion to acetyl CoA in the cytoplasm via acetyl CoA synthetase, an enzyme widely distributed in animal tissues. This enzyme is 2-3 times more active in ruminant adipose tissue than in the corresponding tissue from the rat, reflecting the relatively high rate of acetate conversion to fatty acids in ruminant adipose tissue. Synthesis of long-chain fatty acids requires NADPH which is supplied by glucose metabolism through the pentose phosphate shunt,

since NADP malate enzyme is largely absent from ruminant adipose tissue and liver. High activities of the key pentose phosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been reported in adipose tissue and liver from cow and sheep (Hanson and Ballard 1967).

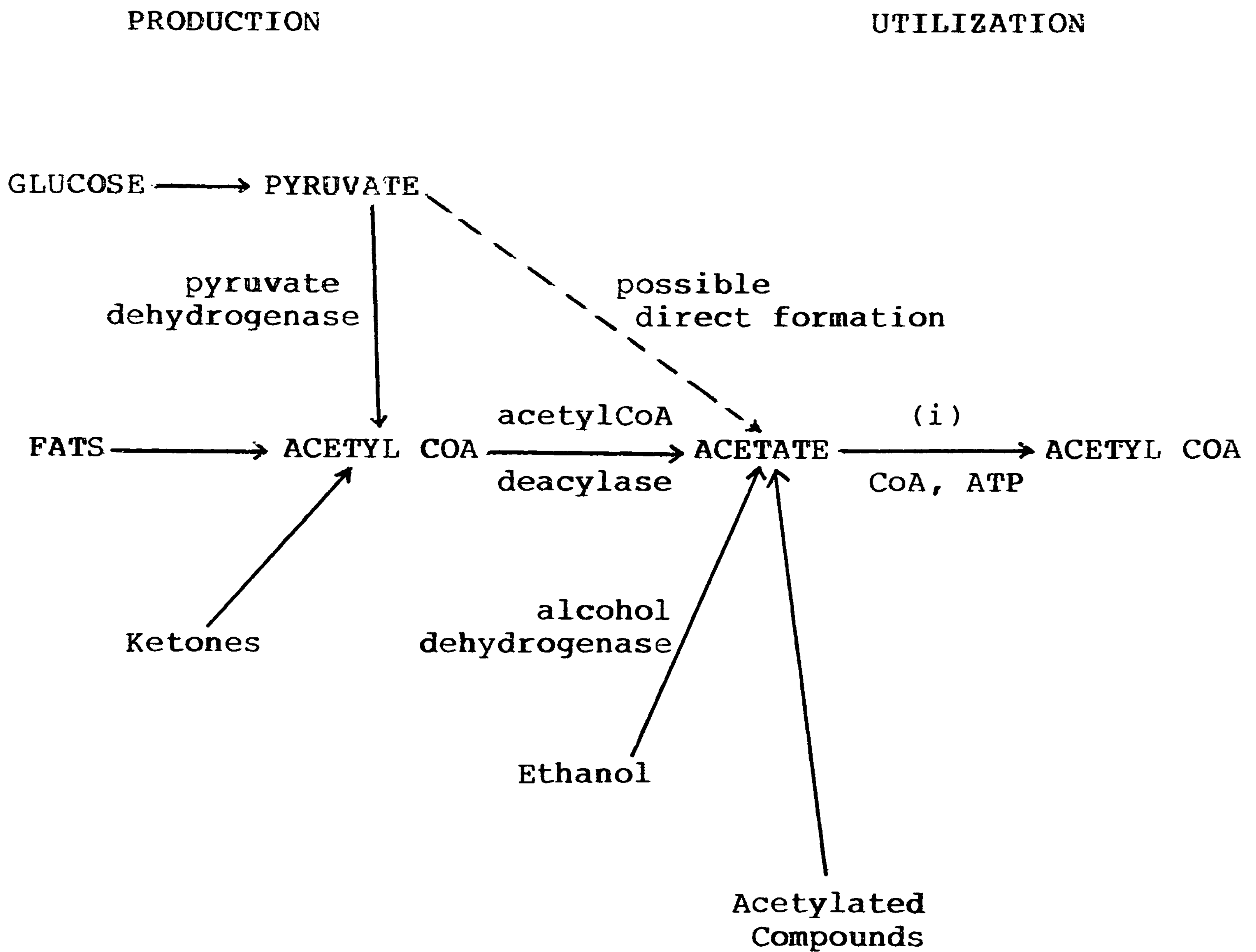
A portion of the acetyl CoA could be converted back to free acetate by the enzyme acetyl CoA hydrolase (Ballard 1967). This is the likely pathway of conversion of fatty acids to acetate, and there is supportive evidence in the observation that substantial incorporation of radioactivity into acetate occurs during infusion of radio-labelled palmitate and other NEFAs (West and Annison 1964).

The pathways of production and utilization of acetate in mammals are demonstrated in Fig 5.A.1.

Metabolism.

VFAs (including acetate) are efectively used as energy sources for maintenance and productive purposes. They account for about 80% of total energy supply to ruminants. Calculated theoretical efficiency of acetate utilization for synthesis (fattening and growth) is about 78-80% (Annison and Armstrong 1970) provided that propionate is available to be metabolised as glucose through the pentose phosphate shunt. Metabolism probably differs between different animals, and even within the same animal, between different organs.

FIG 5.A.1 : ENDOGENOUS ACETATE METABOLISM IN MAMMALS.
(after Ballard, 1972)



(i) acetyl CoA synthetase

Generally, propionate appears mainly gluconeogenic, being responsible for half the glucose formed in ruminants. Studies on mature ruminant and non-ruminant mammals (Ballard, Hanson and Kronfeld 1969) indicate that propionate is mainly converted within the gut wall to lactate which, in turn, is metabolised via pyruvate and oxaloacetate to phosphoenolpyruvate and finally, glucose. The remaining propionate is converted to oxaloacetate through succinate, fumarate and malate. Acetate and butyrate are not ordinarily glucogenic, but probably spare glucose utilization for oxidation and lipogenesis.

Uptake of acetate contributes 25% of milk fatty acid carbon in the mammary gland of the lactating ewe (King, Gooden and Annison 1985) and in goats, but more than in cows. Radioactive studies on the production rates of acetate and glucose in sheep (Annison and White 1962) showed that, in fed and starved animals, endogenous acetate production was 25-50% of total acetate utilization, an amount significantly reduced by raised concentrations of blood glucose and acetate. This suggests that the oxidation of fatty acids contributes substantially to the endogenous production of acetate in sheep. In bovine mammary tissue (Forsberg, Baldwin and Smith 1984), acetate utilization followed first-order kinetics with affinities in the physiological concentration range of acetate. The availability of glucose had a large "permissive" effect on acetate for conversion to fatty acids. Stearate generally

increased acetate metabolism, which also progressed in the absence of 3-hydroxybutyrate. There was also an appreciable conversion of acetate to glyceride and glycerol. These various observations suggest the possibility of a bidirectional glycolytic flux limiting glucose conversion to fatty acids, and also that the "ketone body" 3-hydroxybutyrate may not be required as an initiator of fatty acid synthesis when acetate is available at least in cow mammary tissue.

Acetate metabolism is different between intramuscular adipose tissue (IM) and subcutaneous adipose tissue (SC) (Smith and Crouse 1984). Thus, while acetate contributed 70-80% of acetyl units to IN VITRO lipogenesis in SC, the contribution in IM was only 10-25%; an opposite relationship was obtained with glucose - SC 1-10%, IM 50-75%. These observations suggest different regulatory processes in different types of adipose tissue, and furthermore that glucose and acetate function as competitive substrates depending on the metabolic milieu.

Bartos, Skarda and Dolezel (1968), Laarveld, Chaplin and Brockman (1985) and Brockman and Laarveld (1985) have, on different occasions, studied the effect of insulin on glucose, acetate and ketone body metabolism in ruminant mammals. In the goat, respective infusions of insulin, glucose, and glucose with insulin, increased the rate of acetate utilization, the highest rate being found after glucose with insulin; acetate utilization appeared

generally dependent on glucose metabolism. On the other hand, it was shown with the glucose clamp technique on bovine mammary gland that insulin did not alter acetate metabolism by direct effects on the mammary gland. However, again during euglycaemic clamping in sheep, the net hepatic production of acetate and 3-hydroxybutyrate was reduced to a greater extent than would be accounted for by a reduction in provision of NEFAs to the liver, suggesting a possible direct effect of insulin on acetate metabolism.

Tolerance Studies.

Acetate tolerance studies show that this metabolite is rapidly removed from the blood of ruminants. The rate of disposal (after injection of 5mmol/kg body weight acetate) appears to depend on the age of the animal, as it is faster in the newborn compared to older lambs (Jarrett and Filsell 1960). This difference is comparable to that seen in glucose tolerance and insulin sensitivity and while it may be merely a consequence of the small size of the young (doses were per unit body weight, not surface area), it may be that young lambs behave similarly to non-herbivorous animals. In mature sheep and possibly other ruminants, prior administration of glucose leads to a more rapid rate of disposal of acetate from the blood than occurs in control experiments without exogenous glucose (Jarrett and Filsell 1961). This reinforces the belief that glucose subserves a permissive action on acetate catabolism in ruminant mammals.

Studies in Diabetic Ruminants.

Jarrett and Porter (1957) showed that on induction of a diabetic state, as with alloxan treatment or pancreatectomy in sheep, acetate tolerance was impaired, more so than those of propionate and butyrate. Acetate uptake was low in the hind limb of severely diabetic sheep and became rapidly restored by insulin treatment (Ballard 1972). Similar observations have been described in non-ruminant mammals (Ciaranfi and Fonnesu 1954).

These abnormalities in acetate metabolism induced by diabetes have been ascribed to a reduced rate of lipogenesis in diabetic adipose tissue and provide evidence for a role of insulin (which is absolutely or relatively deficient in diabetes mellitus) in acetate metabolism. The specific mechanism for this effect has not been defined.

ii. Non-ruminant Mammals.

Non-ruminant mammals like the rat also have a high capacity to metabolise acetate and the other VFAs. Acetyl CoA synthetase occurs at high activities in heart, liver and kidney cortex of rats where it is mostly cytosolic in the liver and mitochondrial in the heart (Knowles, Jarrett, Filsell and Ballard 1974). Acetyl CoA hydrolase is also widely distributed in rat tissues with the highest activity in the liver where it is mainly mitochondrial. Its activity is increased in fasting. Acetyl CoA synthetase (acetate

thiokinase) probably subserves a regulatory role in lipogenesis in rat liver (Murthy and Steiner 1972).

As in ruminants, acetate is produced by non-ruminant tissue from hydrolysis of acetyl CoA. Knowles, Jarrett, Filsell and Ballard (1974) suggested that release of acetate from tissues occurs when TCA cycle activity is restricted, so that the circulating acetate redistributes oxidisable substrate through the body, analogous to the function of "ketone bodies" formed by the liver. Isolated rat livers exhibit continuous net release of free acetate enhanced by intra-portal infusions of hexanoate and oleate (Seufert, Graf, Janson, Kuhn and Soling 1974); in mouse ascites tumour cell suspensions and hepatocarcinomas, acetate was found to be an end-product of aerobic metabolism of pyruvate and palmitate (Hepp, Prusse, Weiss and Wieland 1966).

Non-ruminants also derive at least part of the circulating acetate and other VFAs from their diet (Chapter 6.A). The VFAs so absorbed were shown to have metabolic implications. Anderson and Bridges (1984) in examining the effects of different VFAs on rates of glucose production and glycolysis in isolated rat hepatocytes, showed that acetate and butyrate increased, while propionate reduced gluconeogenesis from lactate, and furthermore that, while acetate and butyrate reduced glycolysis, propionate increased glucose breakdown by glycolysis. It was therefore inferred that propionate which also inhibits hepatic acetate metabolism, acts to increase glucose

utilization and reduce glucose production contrary to observations in ruminant mammals, in which propionate is predominantly glucogenic (Annison and Armstrong 1970). The metabolic use of the VFAs may therefore differ between ruminant and non-ruminant mammals.

Metabolism.

Circulating acetate influences synthesis and breakdown of fats and carbohydrates through different metabolic processes. Randle, England and Denton (1970) in studies on perfused rat hearts, showed that during steady state perfusion (with 5mmol/l acetate), acetate accounted for 80% of oxygen utilization and completely suppressed glucose oxidation, with significant increase in TCA cycle turnover rate. Acetate oxidation also increased muscle concentrations of acetyl CoA, glutamate, alanine and glucose-6-phosphate, with reduced output of lactate. Other studies on isolated female rat hepatocytes showed that acetate inhibited glycolysis and stimulated fatty acid synthesis (Nomura, Iguchi, Sakamoto and Harris 1983). No relationship was found between acetate and hepatic ketogenesis in studies on livers of fed male rats perfused with whole rat blood containing various acetate concentrations (Snoswell, Trimble, Fishlock, Storer and Topping 1982), although oleate infusion reduced hepatic lactate uptake with increasing acetate in the perfusate.

Radioactive acetate is recoverable in NEFAs and other

circulating lipids. Furthermore, Snoswell, Trimble, Fishlock, Storer and Topping (1982) showed with perfused fed male rat livers that, with a perfusate acetate concentration less than 0.25mmol/l, there was a net acetate production by the liver, and when the concentration was higher than 0.25mmol/l, net uptake by the liver took place. Such observations had earlier been made by Hepp, Prusse, Weiss and Wieland (1966) and Buckley and Williamson (1977), and were thought due to variations in amount and activity of the enzymes acetyl CoA synthetase and acetyl CoA hydrolase. The liver may therefore have an important role in regulating circulating blood acetate. Acetate taken up by the liver appears to compete directly with lactate for lipogenesis and oxidative metabolism. It may also act like "ketone bodies" (especially 3-hydroxybutyrate) in stimulating gluconeogenesis from lactate as shown in rat hepatocyte perfusion experiments (Menahan, Ross and Wieland 1968, Kummel 1984).

There are only a few studies on whole-body acetate metabolism in non-ruminant mammals. Karlson, Fellenius and Kiesling (1975) showed that the rate of acetate uptake by the perfused hind-quarter of the rat increased with increasing initial amounts of acetate in the perfusion medium, suggesting that the plasma level of free acetate was one factor in skeletal muscle regulation of acetate uptake. In the rat muscle model used, infused acetate accounted for 25-45% of oxidative metabolism. With simultaneous glucose

(5.5mmol/l) and acetate (2.0mmol/l) perfusion of the rat hind-quarter, acetate contribution to oxygen consumption was about double that of glucose, although total oxygen consumption and tissue ATP levels were only slightly elevated. Acetate did not appear to affect glucose uptake. However insulin, while not affecting acetate uptake, significantly increased acetate oxidation (Karlson, Fellenius and Kiesling 1976). Simultaneous acetate (2.0mmol/l) and palmitate (1.0mmol/l) perfusion (Karlson, Fellenius and Kiesling 1977) again in the rat muscle model, showed that acetate contributed up to 3-4 times more than palmitate to total oxygen consumption, and also significantly inhibited palmitate oxidation. These observations on the rat suggest that available acetate could spare glucose and fatty acid oxidation.

In dogs anaesthesized with sodium pentobarbitone, blood acetate was not influenced by loading with glucose and insulin or fat emulsion with heparin, nor indeed with adrenaline or noradrenaline stimulation, despite the various changes in NEFA levels under the different conditions (Kveim, Nesbakken and Bredersen 1979). In other acetate infusion experiments on anaesthesized (again with pentobarbitone) dogs, the plasma levels of plasma glucose, pyruvate and alanine decreased while levels of lactate, citrate, the "ketone bodies" and NEFAs increased although there was no consistent change in insulin concentrations (Ward, Wathen, Harding and Thompson 1985). In the

chloralose anaesthesized dog, Liang and Lowestein (1978) showed that Tris-acetate infusions (in isotonic saline, pH 7.4, and Tris-pyruvate infusions as control) increased tissue levels of AMP and arterial blood and skeletal muscle citrate and malate, and also significantly increased cardiac output and coronary blood flow; observations not made with pyruvate. These effects on the circulation were ascribed to increased cleavage of ATP to AMP accompanying activation of acetate to acetyl CoA. All these observations in the dog (a carnivore), while occasionally contradicting findings in the rat (an omnivore), would further suggest that major interactions exist in the metabolism of acetate, glucose and fat.

In the long term, these effects may be explained on the basis of alterations in enzyme activities. Stanley and Newsholme (1985) have demonstrated that long-term ingestion of guar gum by mice significantly increased the hepatic activities of glucose-6-phosphate dehydrogenase, malate dehydrogenase and 6-phosphofructokinase in mouse liver, enzymes involved in hepatic lipogenesis, a process possibly influenced by acetate derived from the enhanced colonic fermentation. Also, consumption of fibre by rats has been shown to enhance the insulin responsiveness of adipocytes through an action on the basal and stimulated pyruvate dehydrogenase activity (Ogunwole, Knight, Adkins, Thomaskutty and Pointer 1987). One postulated mechanism of insulin action is via the generation of a putative second

messenger that stimulates the activity of the alpha subunit of mitochondrial pyruvate dehydrogenase by a dephosphorylation reaction (Jarrett and Seals 1979). It is of interest to speculate that the fermentative end-products of dietary fibre contribute to these effects on insulin action.

Tolerance Studies.

Acetate is rapidly metabolised by non-ruminant mammals, even more so than mature ruminants (Ballard 1972). Smyth (1947) and Ciaranfi and Fonnesu (1954) respectively measuring intravenous acetate tolerance in cats and dogs, showed that the disposal of an administered load is rapid, and by first-order kinetics. The urinary excretion of acetate accounted for only a small part of the acetate disappearing from the blood stream. Disappearance rate of acetate was however dependent on the initial blood concentration of the substrate. The liver accounted for about half of acetate utilization (Smyth 1947, Demigne, Yacoub, Remesy and Fafournox 1986) while it was not metabolised to any extent by the kidney. Other observations in nephrectomised and eviscerated cats suggested that skeletal muscle and brain account for about 50% of acetate metabolism.

Studies in diabetic non-ruminant mammals.

The plasma acetate level is raised in diabetic dogs

(Ciaranfi and Fonnesu 1954) and rats (Elwood, Marco and van Bruggen 1960, Buckley and Williamson 1977). Acetate tolerance tends to be reduced in diabetic dogs (Ciaranfi and Fonnesu 1954) and acetate infusion produced a higher and more prolonged increase in levels of "ketone bodies" in diabetic as compared to normal dogs (Seufert, Graf, Janson, Kuhn and Soling 1974).

These observations in diabetes are probably related to changes in enzyme and hormonal activities. Acetyl CoA synthetase activity is reduced in livers of alloxan-diabetic rats, an effect reversed by insulin treatment (Murthy and Steiner 1972). As TCA cycle activity and lipogenesis are inhibited, and adipose tissue lipolysis increased by the insulin deficiency (absolute or relative) in diabetes, acetyl CoA levels will rise with consequently increased formation of the "ketone bodies" or alternatively of acetate, if acetyl CoA hydrolase activity is unchanged. The magnitude of the conversion to acetate is yet unquantified, neither has the role of acetate been established in uncontrolled diabetes, where there is unrestrained lipolysis and ketogenesis.

B. EXPERIMENTAL SECTION

SPECIFIC AIMS

The studies described in this chapter were aimed at investigating the relationship of the fasting acetate level with other blood metabolites and hormones. Specific changes in the levels of acetate secondary to alterations in glucose and NEFA values were also examined to acquire an insight into the interactions between these different substrates.

C. THE FASTING PLASMA ACETATE LEVEL AND ITS RELATIONSHIP WITH OTHER BLOOD INTERMEDIARY METABOLITES IN NON-DIABETIC AND DIABETIC SUBJECTS.

AIMS.

Acetate, by its liability to one-step conversion to acetyl CoA, potentially occupies a prime position in intermediary metabolism, especially of glucose and fatty acids. We therefore proposed to test for any relationships that may exist between the circulating concentrations of glucose, the glycolytic products (lactate and pyruvate), NEFAs, glycerol and products of fatty acid oxidation (KBs), while correlations with fasting levels of the two hormones, insulin and growth hormone, most likely to influence the metabolism of these various substrates, were also sought.

SUBJECTS

Overnight fasted healthy, non-diabetic laboratory workers and University students and diabetic patients (insulin-treated or not) were studied. The diabetic patients had varying glycaemic control, as they presented consecutively at the routine diabetic clinics, or for periodic assessment as part of a 10yr prospective study of long-term complications at the Sheikh Rashid Diabetes Unit (Hockaday, Hockaday, Mann and Turner 1978) or otherwise newly diagnosed or poorly controlled in-patients. All the subjects were on a regular Caucasian diet, although increased dietary fibre was advised in the diabetics, with

varying degrees of compliance. The diabetic subjects had not taken their daily insulin or other antidiabetic medications before the fasting blood samples were drawn.

Comparisons between groups were by Student's t tests, and Spearman rank correlations (rs) were used to explore the relationships between levels of the various blood metabolites.

RESULTS.

i. All Subjects.

191 subjects aged 46.7 ± 17.0yr were studied. 77 were non-diabetic and 114 diabetic. In the whole group, fasting acetate correlated with glucose (rs 0.28, $p < 0.0001$), blood lactate (rs 0.17, $p < 0.05$) and negatively with growth hormone (rs -0.27, $p < 0.05$), but not with age, pyruvate, acetoacetate, 3-hydroxybutyrate, glycerol, NEFAs or the ratios lactate/pyruvate and 3-hydroxybutyrate/acetoacetate. These latter ratios respectively reflect the cytoplasmic and mitochondrial redox state of the hepatocyte.

The correlations with glucose, lactate and growth hormone were at least partly due to the difference between the diabetic and non-diabetic subjects, hence the sub-groups of non-diabetics and diabetics were further considered in relation to age, sex and type of antidiabetic treatment.

ii. Non-diabetic Subjects.

Of 77 non-diabetic subjects aged 31.8 ± 10.8yr, 56 were male (age 30.1 ± 10.2yr) and younger than the 21 females

(age 35.9 ± 11.0yr). The fasting values (mmol/l) for the various metabolites were : glucose 5.0 ± 0.4, acetate 0.17 ± 0.07, NEFAs 0.47 ± 0.39, lactate 0.77 ± 0.33, pyruvate 0.072 ± 0.035, acetoacetate 0.022 ± 0.029, 3-hydroxybutyrate 0.042 ± 0.039 and glycerol 0.074 ± 0.030. Fasting insulin and growth hormone (mU/l) were respectively 9.3 ± 6.4 and 5.1 ± 6.7. All these values were similar in males and females.

Considering all the subjects as a whole, or relative to sex, there was no significant correlation between acetate and age, the various metabolites, insulin and the indices of the cellular redox state. Growth hormone was however negatively correlated (rs -0.39, $p < 0.05$).

iii. Diabetic Subjects.

Of 114 diabetic subjects aged 56.8 ± 12.3yr, 89 (53M) were NIDDM (age 59.0 ± 10.4yr, none was insulin-treated) and, as expected, older ($p < 0.01$) than the 25 (15M) IDDM patients (age 43.7 ± 14.8yr). The mean fasting levels (mmol/l) of the different metabolites were : glucose 9.3 ± 4.9, acetate 0.22 ± 0.12, NEFAs 0.69 ± 0.30, lactate 0.98 ± 0.43, pyruvate 0.087 ± 0.032, acetoacetate 0.053 ± 0.061, 3-hydroxybutyrate 0.185 ± 0.259 and glycerol 0.103 ± 0.048. Fasting levels of insulin (NIDDM) and growth hormone were repectively (mU/l) : 11.8 ± 7.8 and 4.5 ± 3.7.

The values for these different metabolites did not differ significantly between NIDDM and IDDM, nor between the sexes, excepting glycerol in NIDDM, where males had generally lower values than females (0.091 ± 0.043 v 0.122 ±

0.047, $p < 0.01$). In the whole group, acetate correlated only with glucose (rs 0.36, $p < 0.001$), a relationship also seen in the NIDDM (rs 0.31, $p < 0.001$) and IDDM (rs 0.51, $p < 0.05$) separately. There was no correlation with age, duration of diabetes, the other metabolites and hormones and the indices of the cellular redox state. These relationships were uninfluenced by sex.

iv. Non-diabetic and Diabetic Subjects.

The non-diabetic subjects were younger ($p < 0.0001$) and had lower plasma acetate levels ($p < 0.001$) than the diabetics. As expected, the fasting values for glucose, NEFAs, lactate, pyruvate, glycerol and the `KBs' were greater in the diabetics (all $p < 0.001$). The lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios were similar in the two groups.

DISCUSSION.

This study confirms previous observations in ruminants (Ballard 1972), rats (Buckley and Williamson 1977) and humans (Smith, Humphreys and Hockaday 1986) that plasma acetate is increased in diabetes, often in relation to the degree of glycaemia (Seufert, Mewes and Soeling 1984). This increase is presumably due to an increased availability of acetyl CoA from enhanced adipose tissue lipolysis (Knowles, Jarrett, Filsell and Ballard 1974) and probably from an increased activity of acetyl CoA hydrolase in diabetic livers (Seufert, Graf, Janson, Kuhn and Soling 1974). As

acetyl CoA synthetase activity is reduced in diabetes (Murthy and Steiner 1972), it is additionally likely that the consequently reduced acetate utilization rate would contribute to the raised acetate levels in the disease. There is also the likelihood of some contribution from the high dietary fibre intake encouraged for diabetics, although admittedly patients generally complied poorly to that advice in this study.

The other changes differentiating the diabetic from the non-diabetic states are raised levels of the glycolytic products, lactate and pyruvate, as well as of substrates derived from increased lipolysis i.e. NEFAs, glycerol and the KBs, coupled with a relative or absolute lack of insulin. Of all these, significant correlations with acetate were seen only with glucose and more weakly, lactate. This raises interesting possibilities.

Acetyl CoA is derived mainly from glycolysis and fatty acid oxidation, the relative contribution of each depending on the duration of fasting. The finding of a simple relationship with glucose and its glycolytic product, lactate, might thus be in keeping with the belief that endogenous acetate is formed from glucose. The other source, NEFAs, presents a paradox. Acetate levels rise with lipid infusions (Thesis, Chapter 5.E), and radioactivity in fatty acids is incorporated into acetate (Seufert, Mewes and Soeling 1984); however, acetate also inhibits endogenous NEFA mobilisation from adipose tissue (Crouse, Gerson,

DeCarli and Lieber 1968). This might explain the lack of a simple relationship. Likewise, acetate is probably also not significantly formed from the hydrolysis of acetoacetate or acetoacetyl CoA, and probably more importantly, the lack of a correlation between acetate and acetoacetate levels confirms that acetoacetate was not measured as acetate (Thesis, Chapter 4.C).

No sex difference in acetate values was detected in this study, contrary to some earlier observations using a similar acetate kinase based enzymatic acetate assay method (Trivin, Lenoir, Bretaudiere and Sachs 1982). Also there was no correlation with age, unlike the report by Skutches, Holroyde, Myers, Paul and Reichard (1979), again with an acetate kinase method. Those two studies however had many fewer subjects which may account for the difference. Acetate levels and correlations did not differ between NIDDM and IDDM, suggesting that its metabolism is influenced less by the type of diabetes than by the degree of glycaemia. This is supported by the lack of a correlation with fasting insulin levels, which indeed tend to be elevated above normal in NIDDM.

The finding of a consistent negative correlation with growth hormone in non-diabetics agrees with an earlier observation (Schmitz, Hansen, Hansen, Orskov and Alberti 1982), albeit in uraemic patients during haemodialysis or acetate infusion experiments, that acetate suppresses basal and induced growth hormone secretion. This may have important therapeutic implications, especially in diabetes,

where growth hormone serves a major glucose counter-regulatory function.

CONCLUSION

In conclusion, fasting plasma acetate levels appear to be mainly influenced by variations in glycaemia.

D. HUMAN PLASMA ACETATE LEVELS IN RESPONSE TO ORAL AND INTRAVENOUS GLUCOSE

AIMS

Plasma acetate is probably formed endogenously from glucose and fatty acid oxidation. We therefore aimed to investigate the production of acetate after orally and intravenously administered glucose in non-diabetic and diabetic subjects, and to relate this to changes in the other blood metabolites, insulin and growth hormone as well as some indices of glucose disposal.

Although the liver and most peripheral tissues can produce and release acetate (Ballard 1972, Knowles, Jarrett, Filsell and Ballard 1974), it is likely that the homeostatic adjustment of systemic acetate levels (Buckley and Williamson 1977, Hepp, Prusse, Weiss and Wieland 1966) is predominantly a hepatic function (Snoswell, Trimble, Fishlock, Storer and Topping 1982). It was therefore also proposed to test whether the initially different metabolism of glucose given orally or i.v. might affect the production of acetate and the change in its plasma level relative to that of other blood metabolites and hormones.

SUBJECTS AND METHODS

a. Intravenous Glucose

74 diabetic subjects (age 59.1 ± 10.4yr, BMI 26.8 ± 4.3kg/m^2) volunteered for this study. 42 were male (age 58.4

$\pm$ 11.0yr, BMI 26.8 $\pm$ 3.6kg/m^2) and 32 female (age 60.1 + 9.8yr, BMI 26.7 $\pm$ 5.0kg/m^2). 14 (age 53.8 $\pm$ 10.3yr, BMI 26.2 $\pm$ 5.4kg/m^2) were insulin-treated, 47 (age 59.5 $\pm$ 9.8yr, BMI 27.1 + 3.9kg/m^2) were sulphonylurea-treated and 13 (age 63.7 $\pm$ 11.2yr, BMI 26.2 $\pm$ 4.5kg/m^2) controlled on dietary measures alone. All attended the Sheikh Rashid Diabetes Unit at 10yr from diagnosis for an ivGTT as part of a prospective study of long-term diabetic complications (Hockaday, Hockaday, Mann and Turner 1978).

9 healthy male non-diabetic University students (age 25.8 $\pm$ 4.3yr, BMI 22.2 $\pm$ 2.4kg/m^2) also volunteered for the ivGTT.

Both groups were studied after an overnight fast with a standard ivGTT (20g glucose i.v./m^2 body surface area over 2min into an antecubital vein contralateral to that used for blood sampling). Blood samples were taken fasting, and at 2,3,4,5,10,15,20,25,30,40,50,60 and 90min from the start of the glucose injection. The glucose disappearance rate (K_G rate constant) was calculated by the method of Conard, Franckson, Bastenie, Kestens and Kovacs (1953).

b. Oral Glucose.

8 male non-diabetic subjects (age 34.8 $\pm$ 12.6yr, BMI 23.5 $\pm$ 2.4kg/m^2) volunteered for this study and took oral glucose on 2 occasions. On each occasion, after an overnight fast, each drank 75g glucose dissolved in at least 300ml water (with lemon flavour) over 2min followed by 50ml plain water. Blood samples were taken fasting and half-hourly

thereafter for 2hr.

The area under the glucose/time curve was calculated by the trapezoidal rule. The results obtained on both occasions on individual subjects were similar (on ANOVA) and hence were pooled.

Differences in acetate levels at different time points during (a) and (b) were examined by paired Student's t test and relationships between the change in acetate level and that of the different metabolites and insulin were assessed by Spearman rank correlation coefficients. The effect of gender and type of diabetic treatment on the post-glucose acetate levels was assessed by ANOVA.

RESULTS

a. Intravenous Glucose

In all the subjects, the plasma acetate concentration rose rapidly after intravenous glucose.

i. Diabetic Subjects.

In the diabetics, the rise was from a fasting value (mmol/l) of 0.17 ± 0.07 to 0.27 ± 0.18 ($p < 0.01$) at 5min and a peak level of 0.31 ± 0.61 ($p < 0.01$) at 10min before falling to 0.24 ± 0.38 by 90min. This last value was still greater than the fasting level ($p < 0.01$). Although males tended to have greater rises than females, and the sulphonylurea-treated the highest increases as compared to the insulin- and diet-treated patients (Fig 5.D.1, 5.D.2), these differences were not statistically significant; only

the time effect was significant ($p < 0.0001$).

The respective changes in the blood levels of the different intermediary metabolites after intravenous glucose are shown on Figs 5.D.3 and 5.D.4. The increase in plasma acetate concentration did not significantly correlate at any time point with the change in levels of glucose, lactate, pyruvate, glycerol, 3-hydroxybutyrate, NEFA, insulin or growth hormone. The K_G rate constant however correlated negatively with the fasting plasma acetate level and with values at 60 and 90min after glucose injection (rs respectively -0.24, -0.36 and -0.39, all $p < 0.05$) as well as with the change in acetate levels at 90min (rs -0.26, $p < 0.05$). A similar pattern of relationships was seen when these diabetic patients were considered in relation to gender or type of diabetic treatment.

ii. Non-diabetic Subjects.

In the 9 non-diabetic subjects studied, plasma acetate levels showed a similar pattern to that of the diabetics (Fig 5.D.5), doubling from a fasting value of 0.15 ± 0.05 to about 0.30 at 5 and 10min. Levels started dropping by 30min and had reached the fasting value at 60min. There was no significant correlation between the change in plasma acetate concentration and that of the other hormones and metabolites at any of the time points nor with the K_G rate constant.

b. Oral Glucose.

The plasma acetate level rose from a fasting value of

Plasma acetate levels after iv glucose in diabetic subjects

FIG 5.D.1

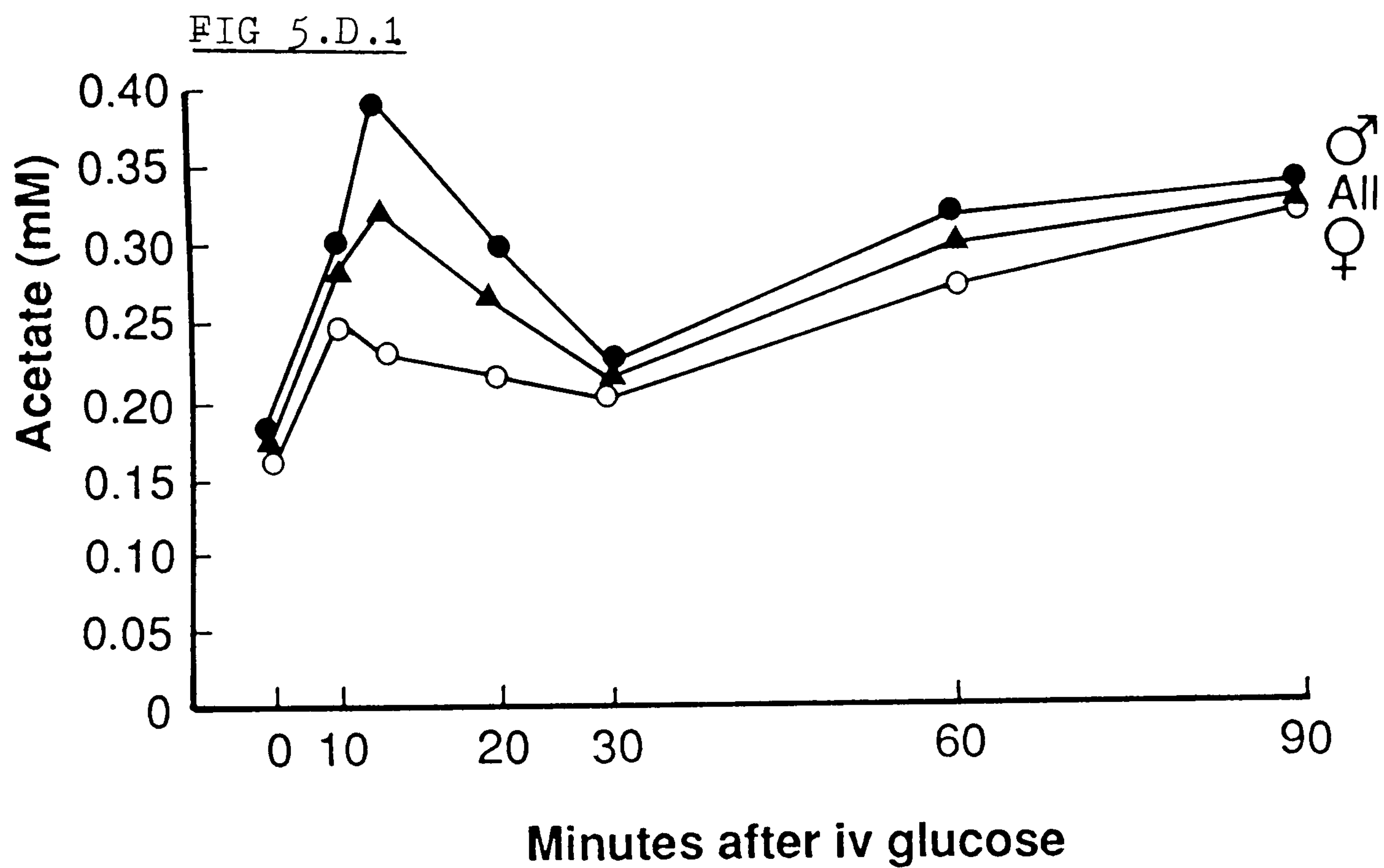


FIG 5.D.2

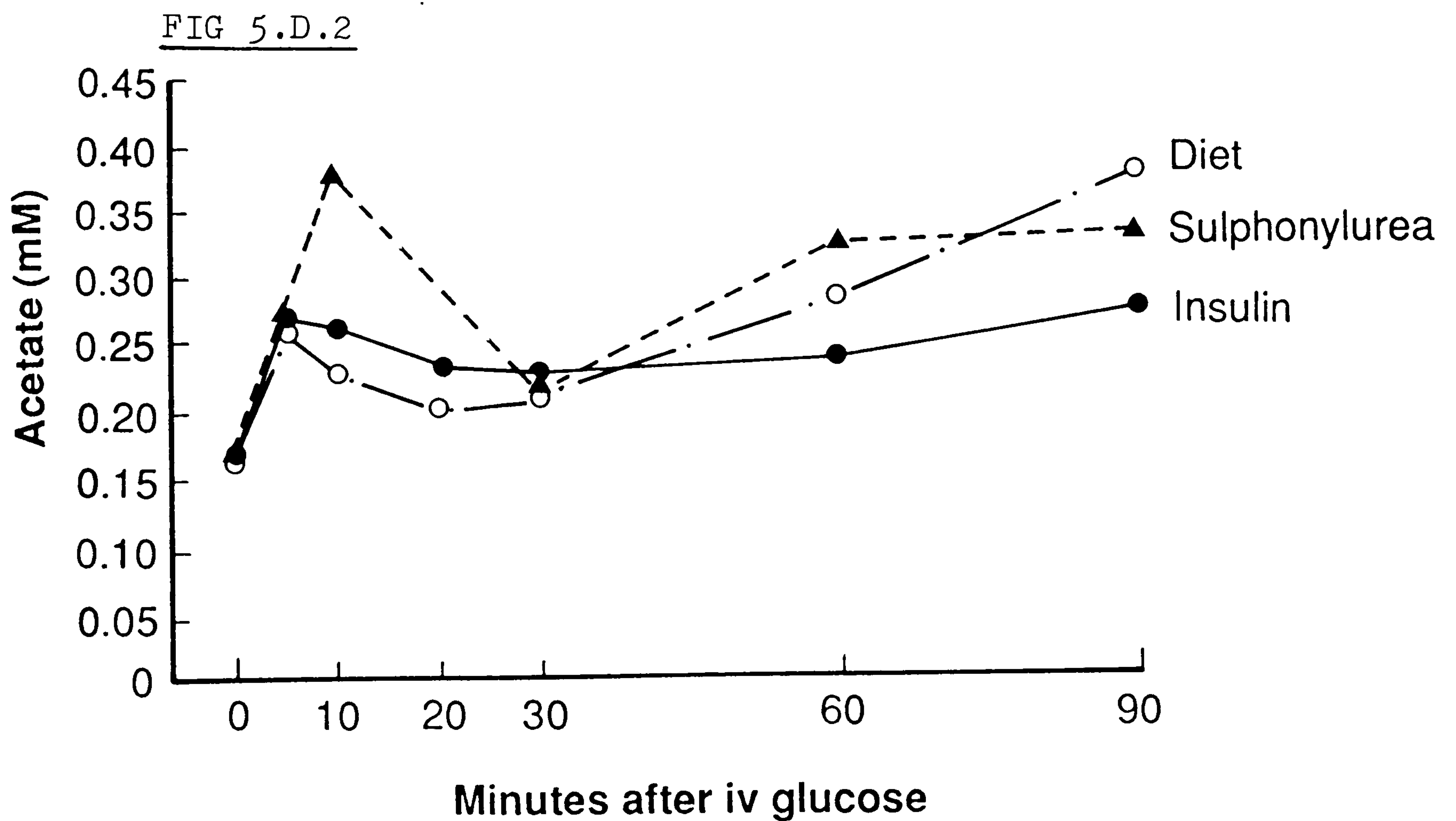


FIG 5.D.3 : **Plasma acetate levels and other metabolites and hormones after iv glucose in diabetic subjects (n=74)**

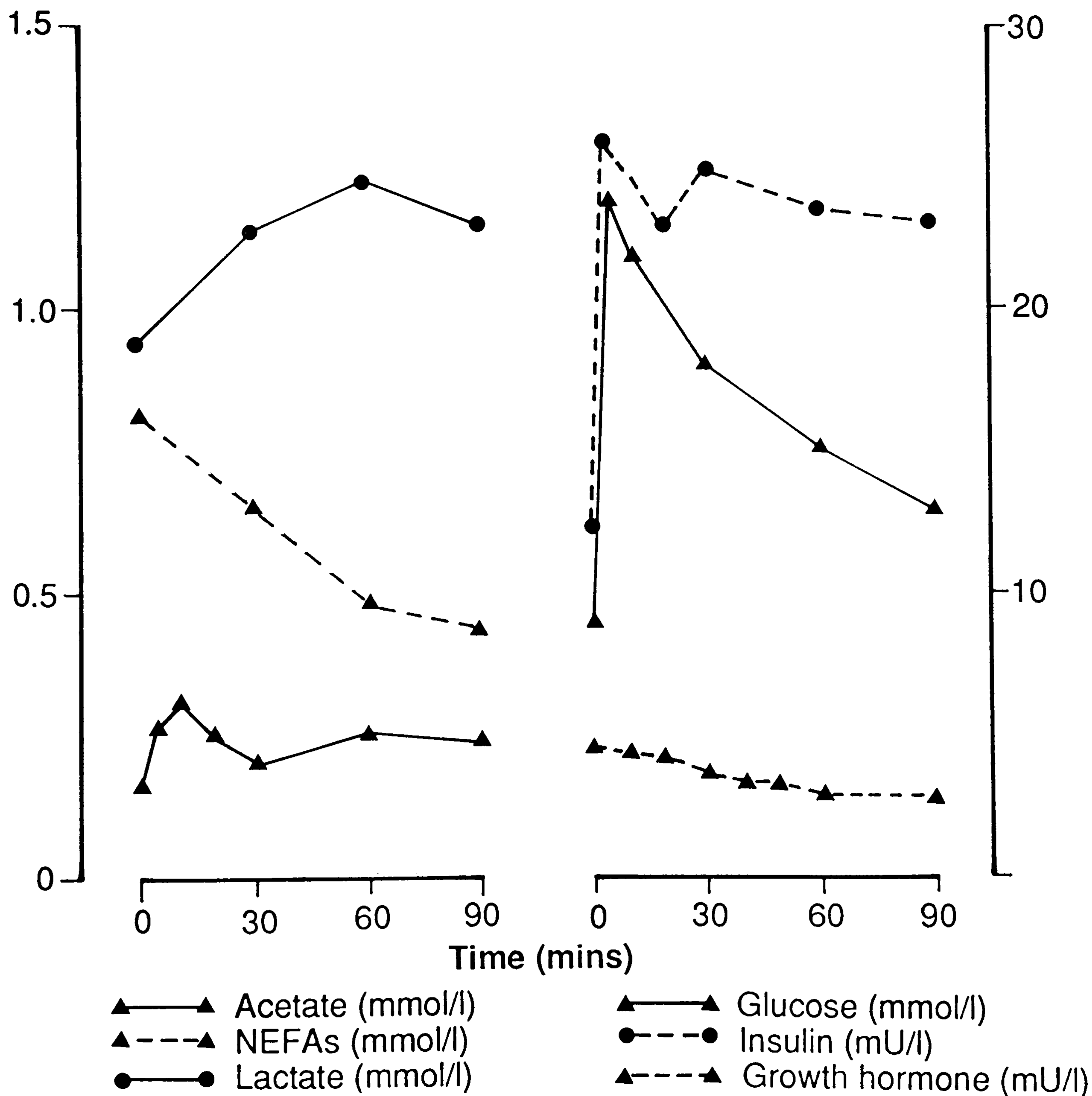


FIG 5.D.4 : **Blood metabolites after iv glucose in diabetic subjects**

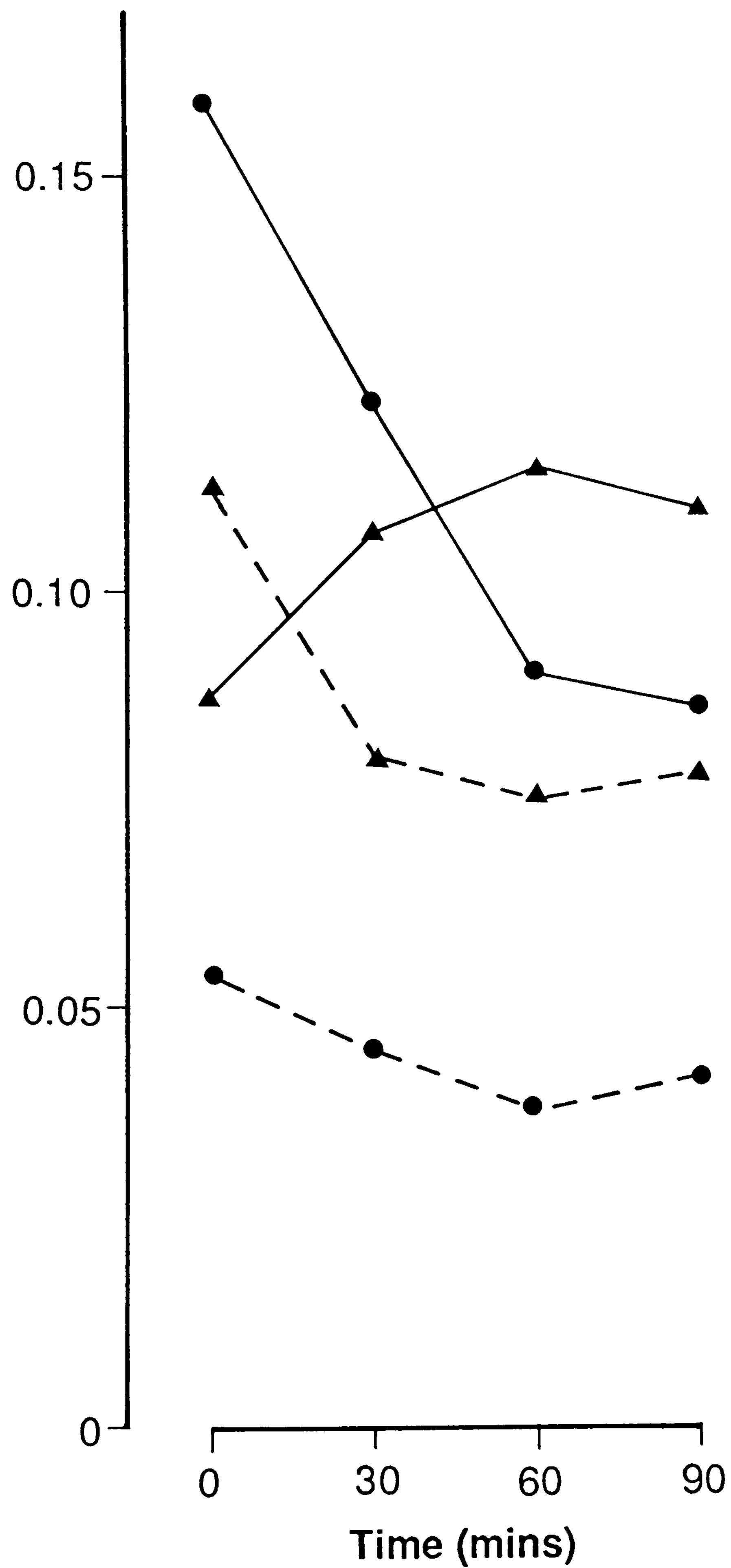


FIG 5.D.5: **Plasma acetate levels after intravenous glucose in non-diabetic subjects**
(n=9, means ± SEM)

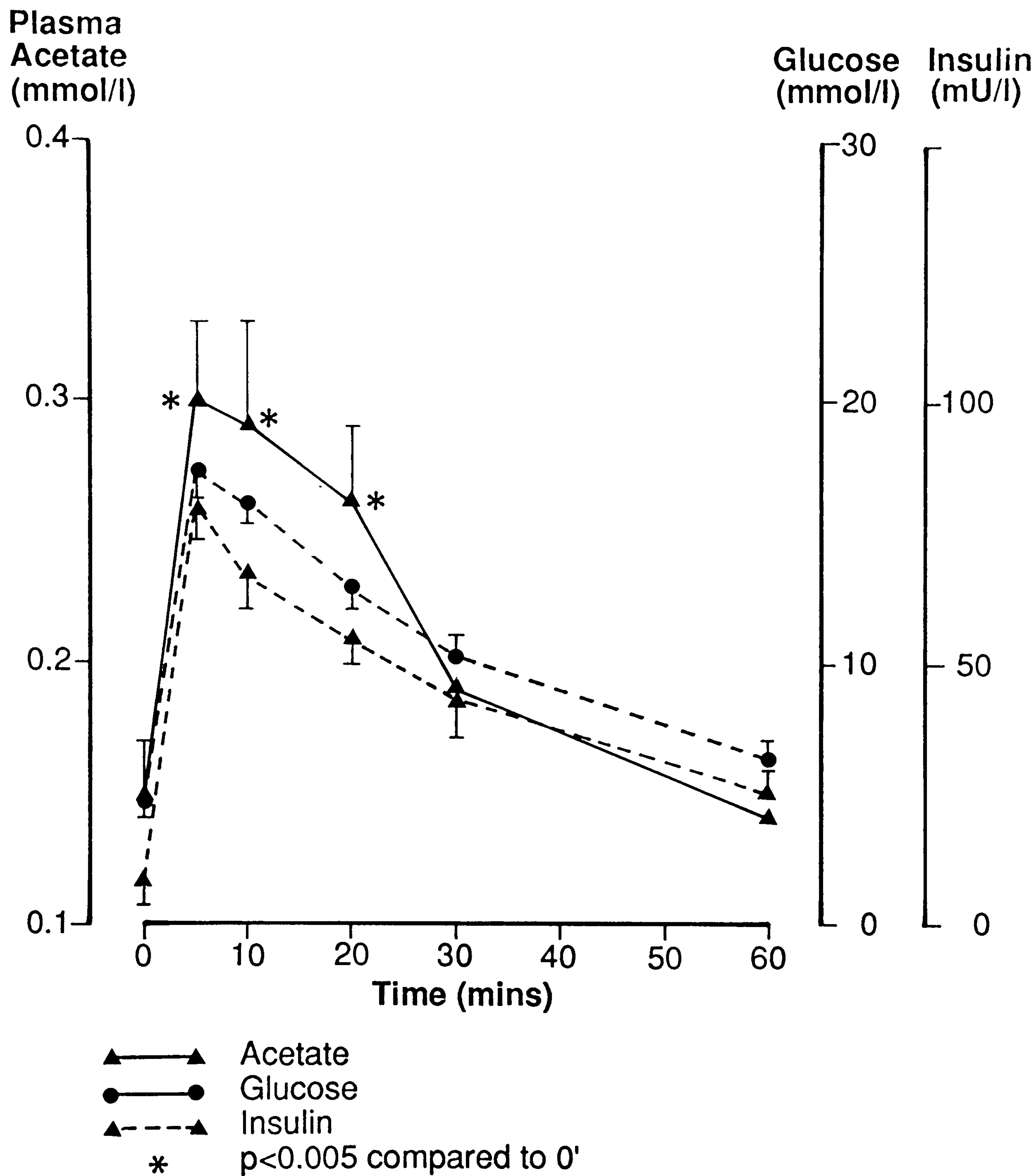
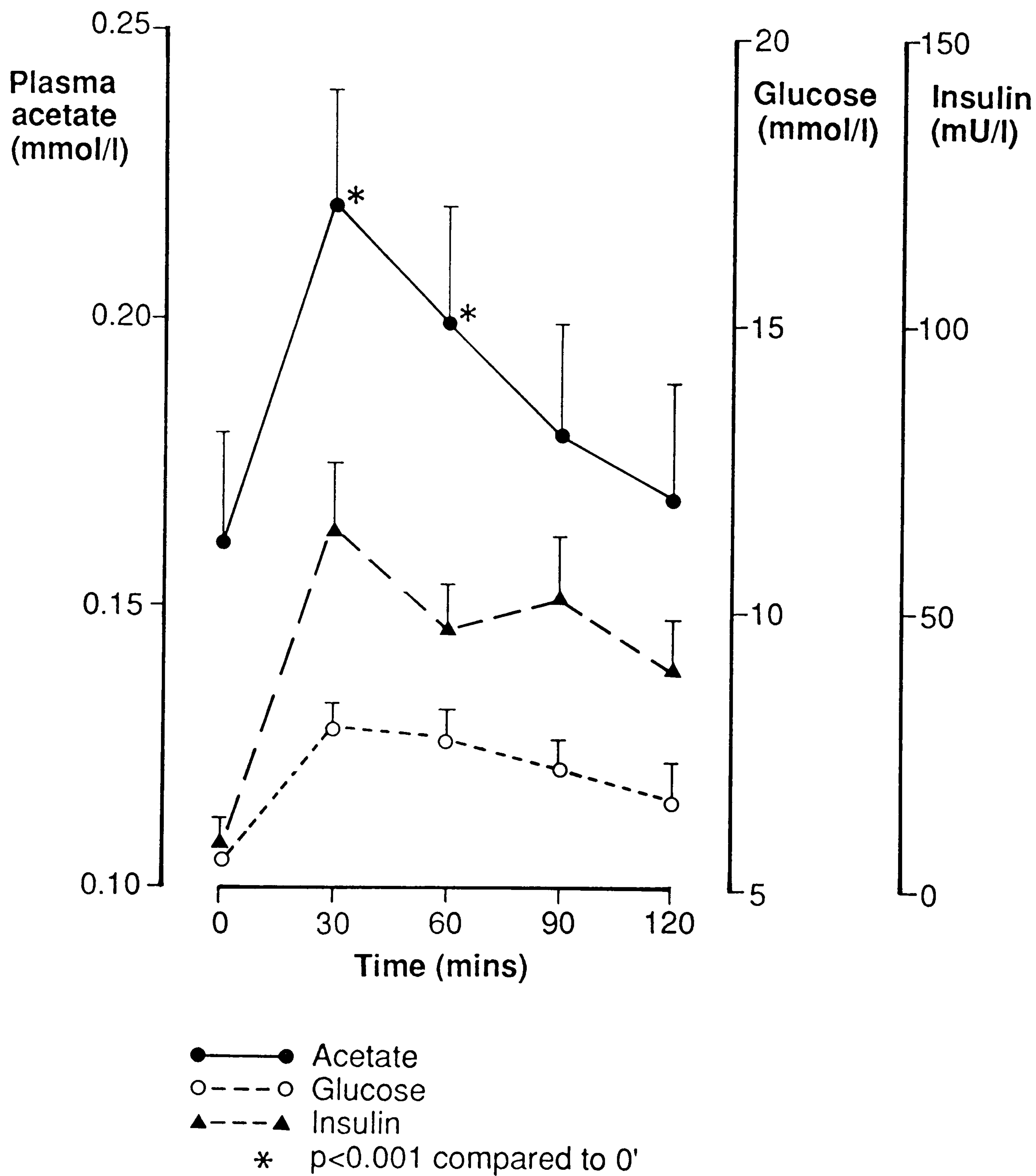


FIG 5.D.6 : **Plasma acetate levels after oral glucose in non-diabetic subjects**
(n=16, means ± SEM)



0.16 ± 0.07 to 0.20 ± 0.09mmol/l ($p < 0.001$) at 30min and 0.20 ± 0.08 ($p < 0.001$) at 60min after non-diabetic subjects ingested 75g glucose. The values however fell to near-normal range at 90min (0.18 ± 0.08) and 120min (0.17 ± 0.07), both p NS (Fig 5.D.6).

The change in acetate concentration correlated significantly with the change in plasma glucose levels at 30min (rs 0.54, $p < 0.05$), 60min (rs 0.53, $p < 0.05$) and 120min (rs 0.49, $p < 0.05$) and also with the change in blood pyruvate levels at 120min (rs 0.60, $p < 0.05$), incremental plasma insulin levels at 90min (rs 0.49, $p < 0.05$) and 120min (rs 0.40, $p = 0.05$) and the total area under the glucose/time curve at 60min (rs 0.56, $p < 0.05$) and 120min (rs 0.50, $p < 0.05$). There was no correlation at any of the time points with the change in blood levels of lactate, KBs, glycerol or NEFA.

DISCUSSION

These results indicate that the plasma acetate level rises when the plasma glucose concentration increases from either oral ingestion or intravenous injection. This rise is quantitatively higher with i.v. glucose, although the plasma glucose level is also higher then, and this despite the greater load (75g) given orally than intravenously (about 40g). The difference in pattern of increase in acetate concentrations probably reflects the prime role of the liver in glucose and acetate metabolism. Increased rate of glycolysis and consequently greater conversion of

pyruvate to acetyl CoA probably occurred from the glucose loads. The acetyl CoA, in turn, can be converted to acetate by acetyl CoA hydrolase (Knowles, Jarrett, Filsell and Ballard 1974).

The change in acetate levels correlated most consistently with the indices of glucose utilization whichever way glucose was administered. Thus, the diabetics with the greater K_G had the smaller increase in plasma acetate and vice versa. Similarly, non-diabetics with the lesser post-oral glucose areas under the glucose/time curves had the smaller rise in plasma acetate level.

There is a controversy regarding the metabolism of glucose whether given orally or i.v. It was initially believed that ingested glucose is extracted predominantly by splanchnic tissues (Perkey and Kipnis 1967, Jackson, Peters, Advani, Perry, Rogers, Brough and Pilkington 1973), with the net effect that only a relatively small proportion is eventually released to the systemic circulation by the liver. It was then hypothesized that a 'gut factor' subserved this function (DeFronzo, Ferrannini, Wahren and Felig 1978). Other studies in dogs (Bergman, Beir and Hourigan 1982, Abumrad, Cherrington, Williams, Lacy and Rabin 1982) and humans (Felig, Wahren and Hendler 1975, Katz, Glickman, Rapoport, Ferrannini and DeFronzo 1983, Katz and McGarry 1984) failed to confirm that hypothesis. Intravenously given glucose is believed, on the other hand, to be oxidized primarily by the peripheral tissues to

gluconeogenic substrates like lactate (DeFronzo, Ferrannini, Hendler, Wahren and Felig 1978, DeFronzo, Jacot, Jequier, Maeder, Wahren and Felig 1981), an observation confirmed by Radzuik (1981, 1982) who reported that the same amount of glycogen was produced in humans with glucose given orally or i.v., and also that with either form of administration, about 10% of glucose was taken up by the liver, the rest being diverted to the peripheral tissues. Later reports from the same group (Radzuik and Inculet 1983) however further confused the issue in suggesting from human forearm glucose uptake studies, that the major proportion of the gluconeogenic substrates was splanchnic in origin, whichever way glucose was given.

The results reported here appear in accord with that belief, in that the formation of acetate from glucose (which may also reflect the extent of glycolysis, analogous to lactate) was not as dependent on how glucose was administered as it was on the rate of glucose utilization.

While the plasma acetate level returned to the basal range by the second hour after oral or intravenous glucose in the non-diabetic subjects, it remained elevated by 90min after i.v. glucose compared to the fasting value in the diabetics. This accords with earlier observations (Chapter 5.C) that the acetate level rises with impairment of glucose tolerance and provides further evidence for a reduced rate of acetate utilization in diabetes (Seufert, Graf, Janson, Kuhn and Soling 1974, Buckley and Williamson 1977, Smith, Humphreys and Hockaday 1986). The biochemical basis for

this has been considered in Chapter 5.A.

The lack of a correlation with glucose during the ivGTT probably only reflects swamping of the metabolic pathways by the relatively high glucose levels achieved in that procedure. With oral glucose where plasma levels are lower than in the ivGTT, significant correlations between acetate and glucose were seen at multiple time points; acetate and pyruvate also correlated at one time point. Levels of the latter may indicate the conversion of glucose to acetyl CoA. The late relationship established with insulin after oral glucose probably underscores the role of that hormone in the interaction between acetate and glucose, though the details are largely unknown.

CONCLUSION

The results show that the plasma acetate concentration rises with glucose levels after oral or intravenous administration of glucose. This rise is highest in the subjects (diabetic and non-diabetic) with the slower rates of glucose disposal and might offer further evidence for the impaired acetate metabolism in diabetes.

E. PLASMA ACETATE LEVELS IN RESPONSE TO INTRAVENOUS FAT OR GLUCOSE/INSULIN INFUSIONS IN DIABETIC AND NON-DIABETIC SUBJECTS.

AIMS

Much of the earlier work on endogenous acetate metabolism has been in ruminants and rats. Since the metabolite is likely to have increased importance, not only because of increased colonic production from increased intake of dietary fibre but also as a potential source of energy, it was proposed to investigate its production under various conditions in humans and in relation to KBs and NEFAs.

Links between plasma glucose, acetate and NEFAs and blood 'KBs' concentrations in non-diabetic and non-insulin dependent diabetic subjects were examined under two experimental conditions:

i. in non-diabetic subjects, increased plasma NEFA levels with glucose and insulin essentially held constant, as in lipid and heparin infusions;

ii. in non-diabetic and diabetic subjects, suppressing lipolysis and increasing glucose flux despite euglycaemia as in the hyperinsulinaemic euglycaemic clamp; indices of tissue insulin sensitivity could also be derived by this method.

SUBJECTS

All the studies were performed after a 10hr overnight fast (with water AD LIBITUM). The diabetic subjects omitted their usual drugs on the morning of the study.

i. INTRALIPID Studies.

7 non-diabetic male subjects volunteered for the INTRALIPID/heparin infusion studies. They were aged 32.0 $\pm$ 12.5yr and BMI was 23.3 $\pm$ 2.9kg/m^2. The study consisted of a 90ml/hr INTRALIPID (Kabitvitrum Ltd, Uxbridge, U K) infusion using an infusion pump (Braun Melsungen AG, W Germany); the 20% INTRALIPID used contained per 500ml : energy 4.2mJ, fractionated soya bean oil 100g, fractionated egg phospholipids 6g and glycerol 11g dissolved in sterile water to an osmolality of 350mOsmol/kg water. 200units heparin was also given i.v. initially followed by maintenance heparin at 0.4units/kg/min. These rates of infusion of INTRALIPID and heparin had previously been shown to increase plasma NEFAs to > 2mmol/l (Ferranini, Barrett, Bevilacqua and DeFronzo 1983). Both were infused over 3hr into an antecubital vein cannulated 30min prior to start of infusion. A contralateral antecubital vein was also cannulated for sample collection and kept patent by intermittent flushing with isotonic 150mmol/l saline. Fasting specimens were collected 20 and 30min after cannula insertion and others at 30min intervals for the 3hr duration of the lipid infusion.

ii. Euglycaemic Clamping.

13(6M) non-diabetic and 9(6M) non-insulin dependent diabetic subjects volunteered for the clamp studies. The non-diabetic subjects were aged 41.8 ± 12.6yr with BMI 27.0 ± 4.3kg/m^2. Of the 13 non-diabetic subjects, 7(3M) were obese (age 43.7 ± 11.4yr, BMI 30.6 + 1.8kg/m^2) and 6(3M) of normal weight (age 39.7 ± 14.2, BMI 22.8 + 1.9kg/m^2). The diabetic subjects were aged 52.1 + 10.8yr with BMI 29.4 ± 6.9kg/m^2. All had fasting plasma glucose levels < 10mmol/l, and had never required insulin (6 were satisfactorily controlled by dietary measures alone). 6 of the diabetics were obese (BMI > 28.0kg/m^2). The clinical and clamp parameters of the individual subjects are shown in Table 5.E.1.

All the subjects had a 40mU insulin/m^2 body surface area/min hyperinsulinaemic euglycaemic (at fasting glucose level) clamp (DeFronzo, Tobin and Andres 1979) with Soluble insulin (Humulin S, Eli Lilly and Co Ltd) and 20% glucose in water (Travenol Lab Ltd, Trentford, U K) infused with an infusion pump (Braun Melsungen AG, W Germany) into an antecubital vein; a contralateral antecubital vein was also cannulated for sample collection. Plasma glucose was measured every 5min, and the glucose infusion rate was adjusted to maintain the fasting plasma glucose by an algorithm based on the 'minimal model' of Bergman (Pacini, Finegood and Bergman 1982). Steady states of plasma glucose and glucose infusion rate were usually achieved within

TABLE 5.E.1 : CLINICAL CHARACTERISTICS AND CLAMP PARAMETERS OF THE SUBJECTS STUDIED.

Sex	Age (yr)	BMI (kg m^{2})	Duration of diabetes (yr)	Treatment*	FPG (mM)	MCG (mM)	FPAc (mM)	NEFAs (mM)	Ins (mU/1)	Δ Ins.
A. Non-diabetic										
1 M	56	29.7	-	-	5.6	5.5	0.19	+	21.6	71.1
2 F	25	25.9	-	-	5.4	5.3	0.17	0.35	5.9	63.7
3 F	24	20.9	-	-	5.2	5.0	0.10	0.21	7.1	58.9
4 F	35	22.1	-	-	5.0	5.2	0.20	+	10.1	69.2
5 F	60	29.0	-	-	5.5	5.3	0.16	0.40	9.2	51.0
6 F	40	28.0	-	-	5.2	5.0	0.22	0.70	7.8	47.8
7 F	41	31.9	-	-	5.5	5.4	0.14	0.46	18.7	62.1
8 F	48	32.0	-	-	5.1	5.2	0.16	0.68	10.4	57.4
9 M	59	20.7	-	-	5.2	5.1	0.11	0.80	3.4	61.7
10 M	30	30.5	-	-	5.0	4.7	0.22	0.53	13.8	62.5
11 M	31	33.4	-	-	5.1	5.1	0.18	0.50	20.3	52.8
12 M	42	24.3	-	-	4.8	4.5	0.20	0.42	2.9	52.4
13 M	53	22.9	-	-	5.2	5.4	0.17	0.14	7.5	70.8
Mean	41.8	27.0	-	-	5.2	5.1	0.17	0.47	10.7	54.9
SD	12.6	4.3	-	-	0.4	0.4	0.04	0.22	6.5	18.1

TABLE 5.E.1. (Contd)

Sex	Age (yr)	BMI (kg m^2)	Duration of diabetes (yr)	Treatment*	FPG (mM)	MCG (mM)	FPAc (mM)	NEFAs (mM)	Ins (mU/1)	Δ Ins.
B. Diabetic										
1 F	60	30.5	2	Diet only	9.2	8.0	0.28	0.38	11.7	85.1
2 M	66	27.6	1	Diet only	7.6	7.7	0.20	0.15	20.7	63.1
3 M	54	23.3	1	Glibenclamide	9.5	9.5	0.26	0.34	7.1	63.9
4 F	40	45.2	4	Diet only	6.2	5.8	0.17	0.73	8.6	75.7
5 F	38	21.5	10	Diet only	8.8	9.0	0.23	0.40	4.6	76.2
6 M	67	26.4	19	Chlorpropamide	8.4	8.1	0.25	0.46	12.0	70.8
7 M	43	32.4	1	Diet only	6.8	6.9	0.12	0.31	27.5	91.1
8 M	51	29.7	1	Diet only	6.2	6.2	0.26	0.52	18.6	40.2
9 M	50	28.2	6	Glibenclamide	6.3	6.2	0.32	0.37	12.1	60.7
Mean	52.1 $	29.4	5.0	-	7.7 $	7.4 $	0.23 $	0.41	13.7	69.7 $
SD	10.8	6.9	6.1	-	1.2	1.2	0.06	0.15	7.2	15.0

* extra to dietary advice and exercise as applicable
\+ value not available
$ $p < 0.05$ compared to non-diabetics
FPG fasting plasma glucose
MCG mean clamp glucose
FPAc fasting plasma acetate
Ins fasting plasma insulin
Δ Ins incremental change to peak in insulin values during the clamp

90min, and the clamp in each case lasted 120min. The fasting plasma glucose was well maintained in the non-diabetic and diabetic subjects as seen from Table 5.E.1, where fasting glucose was no different from mean clamp glucose. The coefficients of variation of plasma glucose values during the clamps were 5.4% and 4.8% respectively for the two groups of subjects.

The metabolic clearance rate of glucose (MCR), an index of tissue insulin sensitivity, was calculated from the ratio Steady State Glucose Infusion Rate μmol/(min.kg) (90-120min) /Fasting Plasma Glucose (μmol/ml) and expressed in ml/(kg.min) (DeFronzo, Tobin and Andres 1979).

Significant differences within and between groups were assessed by the Student's t-test for paired and unpaired observations. Spearman rank correlations were sought between the indices of insulin sensitivity, BMI and change in plasma acetate values.

RESULTS

i. INTRALIPID Infusion.

Plasma NEFAs (mmol/l) increased from 0.26 ± 0.08 fasting to peak 2.55 ± 0.47 and mean (over 3hr) 2.25 ± 0.47 levels, about 10 times above basal. Plasma acetate (mmol/l) also increased from 0.14 ± 0.05 fasting to peak 0.32 ± 0.10 and mean (over 3hr) 0.25 ± 0.05 (both $p < 0.01$), about twice the basal level. Total blood KBs (mmol/l) increased with the lipid infusion from 0.083 ± 0.044 fasting to peak

1.012 ± 0.408 and mean (over 3hr) 0.666 ± 0.307 (both $p < 0.001$), increases of about 10 times above base-line. Thus, while the relative increases in KBs and NEFAs, as measured in peripheral venous blood were closely similar, that in acetate was only about one-fifth the increase in the other 2 substrates. Also while NEFAs reached steady levels by the second hour, the rise in levels of KBs and acetate continued. These changes are illustrated in Fig 5.E.1.

Glucose levels (mmol/l) fell minimally (4.9 ± 0.21 to 4.7 ± 0.23, $p < 0.05$) during the lipid infusion, perhaps due to the slight rise in insulin (mU/l) levels (fasting 10.0 ± 3.6 to peak 13.3 ± 5.7, $p < 0.05$) (Fig 5.E.2).

ii. Euglycaemic Clamping.

As a whole, the 13 non-diabetic subjects were younger ($p < 0.05$) and had lower fasting plasma acetate levels ($p < 0.01$) than the diabetic patients, although BMI and fasting insulin and NEFA levels were similar in the 2 groups. The mean increase in plasma insulin during the clamp was greater ($p < 0.05$) in diabetic than in normal subjects (Table 5.E.1).

When the non-diabetic subjects were considered on the basis of their BMI, the 7 obese did not differ in age, fasting glucose, acetate and NEFAs, nor did their mean rise in clamp insulin levels differ from the 6 non-obese. In comparison with the 9 diabetic subjects, the obese non-diabetics naturally had lower plasma glucose levels ($p < 0.001$) and were similar in age, BMI and fasting insulin

TABLE 5.E.2 : THE BODY MASS INDEX (BMI), METABOLIC CLEARANCE RATE - GLUCOSE (MCR) AND STEADY STATE GLUCOSE INFUSION RATE (SSGIR) DURING EUGLYCAEMIC CLAMPING FOR THE SUBJECTS.

	Sex	BMI (kg/m^2)	MCR (ml/min.kg)	SSGIR (μmol/min.kg)
A. Non-diabetic				
i. Normal weight				
1	F	25.9	9.7	52.5
2	F	20.9	14.3	74.7
3	F	22.1	13.0	65.1
4	M	20.7	9.8	51.0
5	M	24.3	12.6	60.5
6	M	22.9	9.0	48.7
Mean		22.8	11.4	58.8
SD		1.9	2.1	10.0
ii. Obese				
1	M	29.7	3.9	21.7
2	F	29.0	3.5	19.1
3	F	28.0	4.4	22.7
4	F	31.9	2.5	13.9
5	F	32.0	5.3	26.8
6	M	30.5	5.9	29.6
7	M	33.4	2.4	12.1
Mean		30.6*	4.0*	20.8*
SD		1.9	1.3	6.4
B. Diabetic				
1	F	30.5	2.9	26.3
2	M	23.3	3.0	28.4
3	F	45.2	4.5	28.1
4	F	21.5	4.6	40.6
5	M	26.4	4.5	37.9
6	M	32.4	2.2	14.7
7	M	29.7	1.3	7.9
8	M	28.2	1.7	10.4
9	M	28.0	8.3	63.0
Mean		29.7*	3.7*	28.6*
SD		7.5	2.2	17.2

* $p < 0.01$ compared to normal weight non-diabetic subjects.

and NEFA values, but they had a smaller mean increase in clamp plasma insulin ($p < 0.05$).

As the results obtained during the clamp differed somewhat between the diabetic and non-diabetic subjects, they are considered separately.

(a) Non-diabetic Subjects.

Plasma NEFAs (mmol/l) decreased from 0.47 $\pm$ 0.22 to 0.14 $\pm$ 0.11 ($p < 0.0001$) at 30min and subsequently remained at about 0.07 ($p < 0.0001$) for the rest of the study. Plasma acetate (mmol/l) also fell from 0.17 $\pm$ 0.04 fasting to 0.15 $\pm$ 0.07 (p NS) at 30min and then to about 0.12 at 60 and 90min (both $p < 0.001$). Values then rose by 120min to 0.16 $\pm$ 0.07 (p NS compared to fasting value, but $p < 0.001$ compared to 90min value (Fig 5.E.3b).

This trend of change in levels of acetate and NEFAs was uninfluenced by obesity (Fig 5.E.3a).

The steady state glucose infusion rate (µmol/min.kg) in the non-diabetic subjects was 38.3 $\pm$ 21.2. This rate was, as expected higher in the normal weight than in the obese (58.8 $\pm$ 10.0 v 20.8 $\pm$ 6.4, $p < 0.001$). The MCR for the 13 non-diabetic subjects was 7.41 $\pm$ 4.21, and again as expected, the non-obese individuals had higher values than the obese (11.42 $\pm$ 2.14 vs 3.97 $\pm$ 1.30, $p < 0.001$) (Table 5.E.2). These two parameters indicate greater insulin insensitivity in the latter.

(b) Diabetic Subjects.

Again, plasma NEFAs (mmol/l) fell from 0.41 ± 0.15 fasting to 0.20 ± 0.09 at 30min and 0.12 ± 0.09 at 60min, both $p < 0.001$; it then remained steady around this level for the rest of the study. Plasma acetate (mmol/l) also fell from 0.22 ± 0.06 fasting to 0.15 ± 0.03 at 30min ($p < 0.005$) and remained at this level for the rest of the study. There was no increase to fasting levels at 120min as seen with the non-diabetic subjects. Overall, plasma acetate levels remained higher in the diabetics and plasma NEFAs showed a slower rate of decline during the clamp, when compared to the non-diabetic subjects (Fig 5.E.3b).

The steady state glucose infusion rate in the diabetic subjects was 28.6 ± 17.2μmol/min.kg and MCR 3.65 ± 2.20ml/kg.min. These parameters were, as expected, lower than for all the non-diabetic subjects (both $p < 0.05$) or the normal weight non-diabetics alone (both $p < 0.01$) but closely similar to the values for the obese non-diabetic subjects (both p NS). This indicates a similar degree of insulin insensitivity in the obese non-diabetic and mild non-insulin dependent mostly obese diabetic subjects.

c. Non-diabetic and Diabetic Subjects

The change in plasma acetate levels in the non-diabetic (all, and with respect to obesity) and diabetic subjects, during the second hour of euglycaemic clamping (all nondiabetics : 0.04 ± 0.03mmol/l, non-obese non-diabetics : 0.04 ± 0.04, obese non-diabetics 0.03 ± 0.03; diabetics

FIG 5.E.1 :

Change in plasma acetate, NEFAs and blood ketone bodies during intralipid/heparin infusion
(n=7, mean ± SEM)

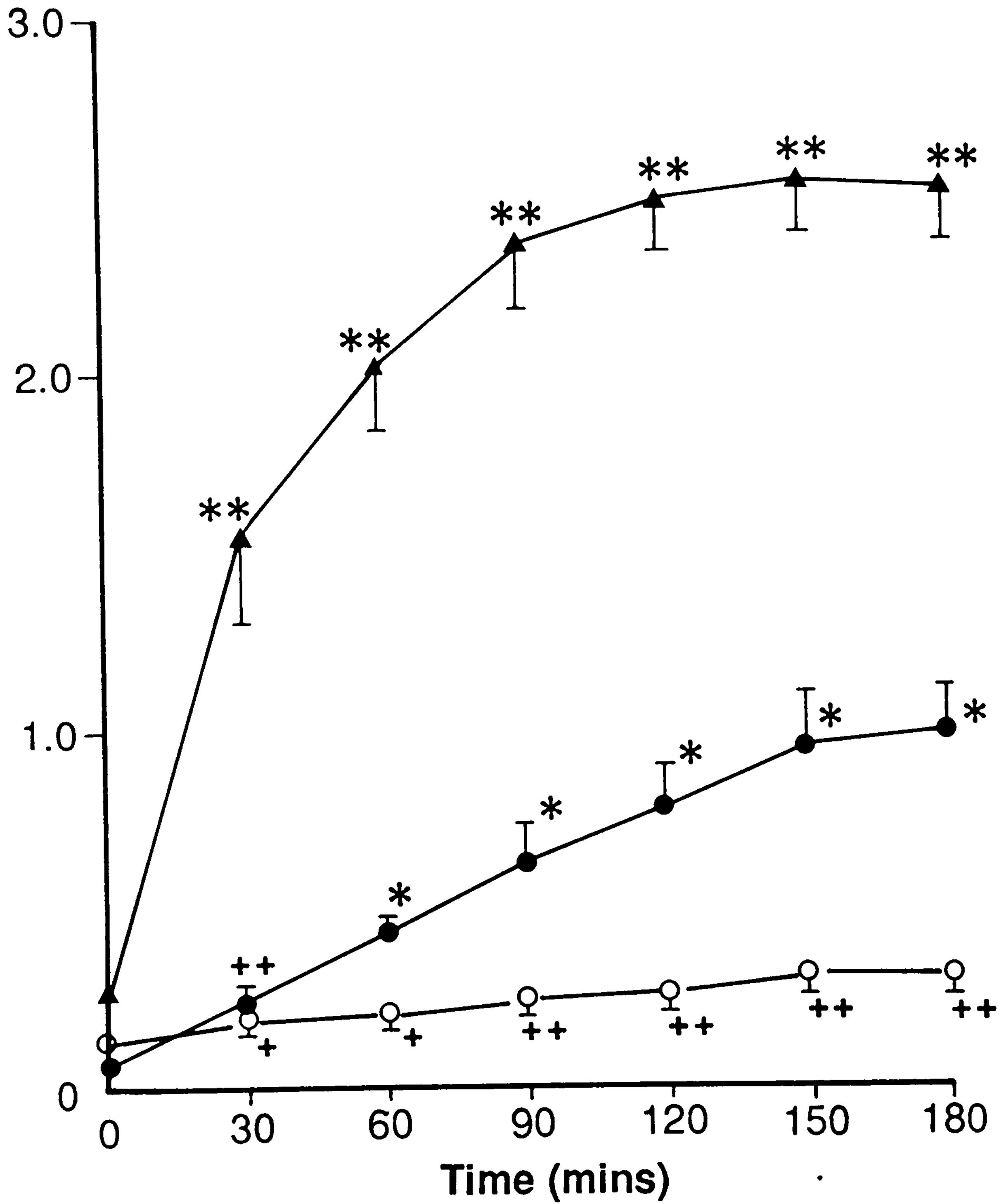


FIG 5.E.2 :

Change in plasma glucose and insulin levels during intralipid infusion
(n=7, mean ± SEM)

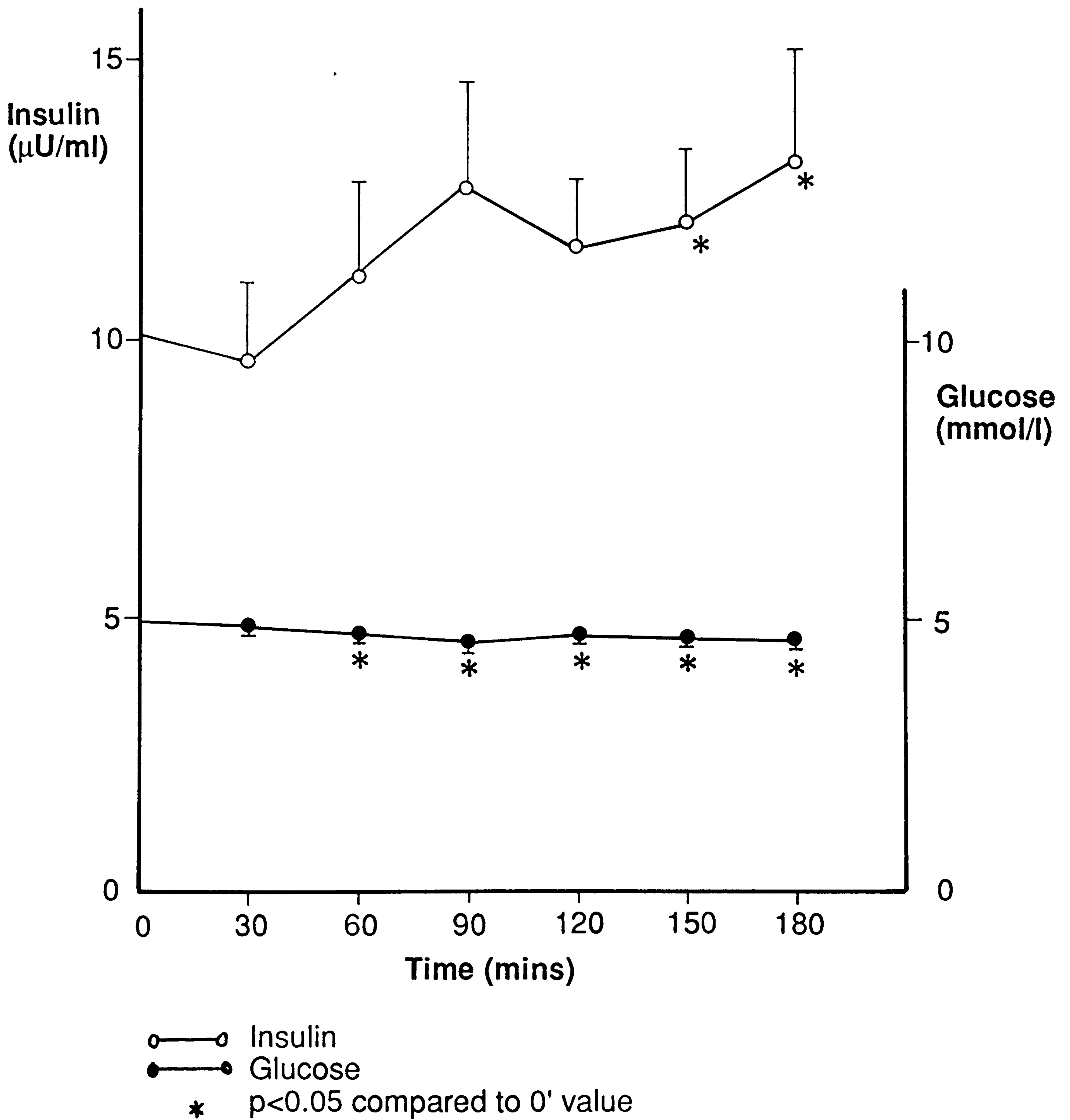
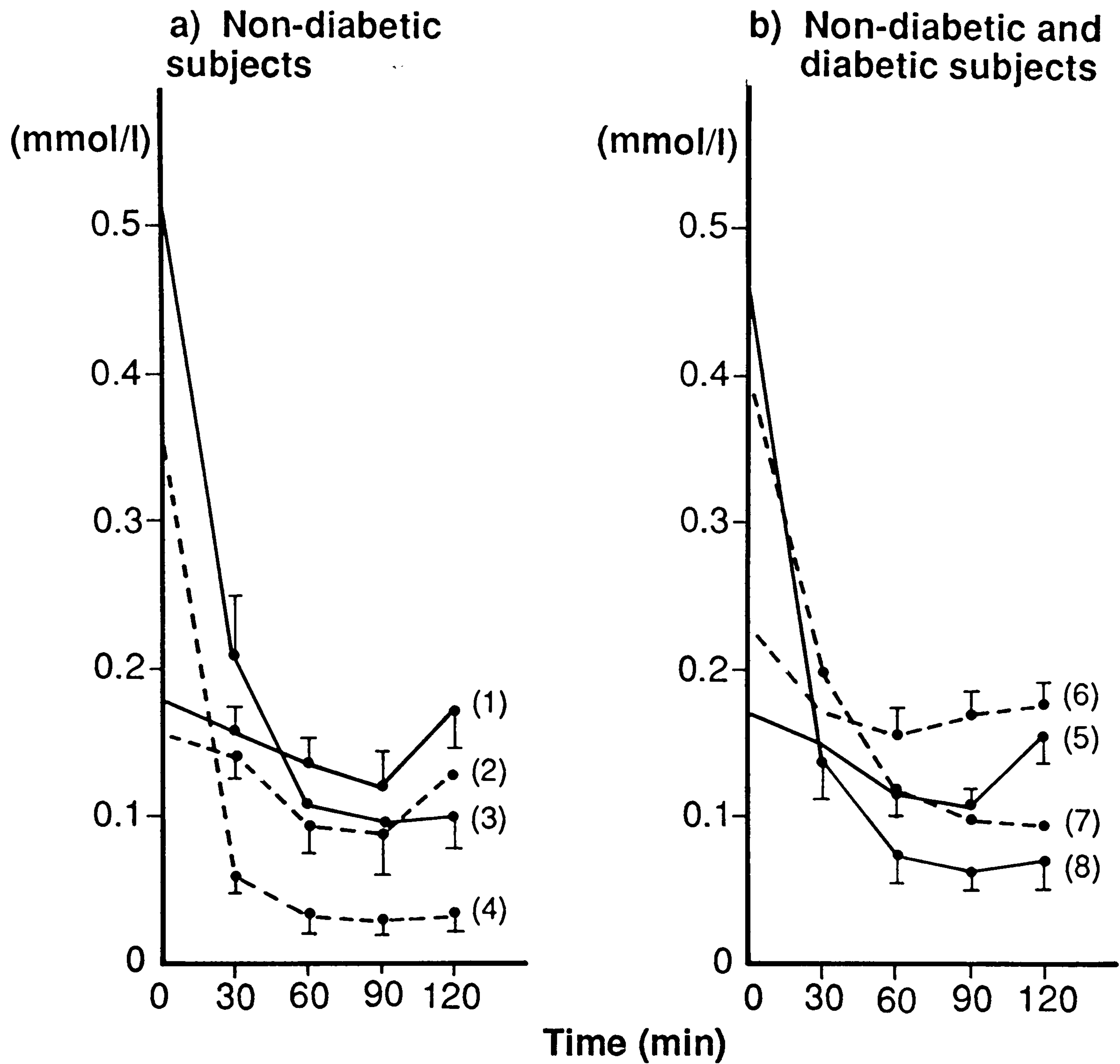


FIG 5.E.3 **Plasma acetate and NEFAs during euglycaemic clamping in non-diabetic and diabetic subjects**



(1) •——• Obese,acetate (n=7)
(2) •- - -• Normal weight, acetate (n=6)
(3) •——• Obese, NEFAs (n=7)
(4) •- - -• Normal weight, NEFAs (n=7)
(5) •——• Non-diabetic subjects, acetate (n=13)
(6) •- - -• Diabetic subjects, acetate (n=9)
(7) •——• Non-diabetic subjects (obese + normal weight), NEFAs (n=13)
(8) •- - -• Diabetics, NEFAs (n=9)

mean ± SEM

0.007 $\pm$ 0.04) did not correlate significantly with either the BMI or the two parameters of tissue insulin sensitivity, steady state glucose infusion rate and the MCR. However the MCR and BMI were significantly negatively correlated in the non-diabetic subjects (rs -0.84, $p < 0.001$) and all the subjects (diabetic and non-diabetic, rs -0.63, $p < 0.005$) but not in the diabetics (rs -0.37, p NS).

DISCUSSION.

INTRALIPID Study.

During lipid and heparin infusions to increase circulating plasma NEFAs ten-fold, the levels of KBs increased to a similar extent but acetate only doubled. This observation is similar to earlier results in humans from radioactive oleate infusion (Seufert, Mewes and Soeling 1984).

Peripheral blood levels of substrates reflect both production and utilization rates, and the relative levels of acetate and KBs might therefore be interpreted as indicating either or both of these. However, the turnover rates (μmol/min.kg) of acetate and KBs, determined in the same subjects by either the elimination kinetics of infused radioactive acetate and acetoacetate or with radioactive oleate infusions were closely similar (acetate 1.26-1.35, KBs 1.06-1.22) (Seufert, Mewes and Soeling 1984). This suggests that the production rate is probably the main determinant of blood levels when both substrates are elevated in blood. Inevitably, with multiple substrates,

there is bound to be competition for utilization (Randle, Garland, Hales and Newsholme 1963).

Both acetate and KBs suppress adipose tissue lipolysis and fatty acid metabolism (Robinson and Williamson 1980, Crouse, Gerson, DeCarli and Lieber 1968). KBs also suppress glycolysis (Ferranini, Barrett, Bevilacqua and DeFronzo 1983), while acetate inhibits KB utilization (Karlsson, Fellenius and Kiessling 1977). It may therefore be inferred that, when these substrates are present at increased levels, acetate is the preferred metabolite and capable of almost complete oxidation (Skutches, Holroyde, Myers, Paul and Reichard 1979). The other substrates are then diverted into lipogenesis, especially when insulin levels are adequate. This observation has physiological implications. In acidotic states accompanied by increased adipose tissue lipolysis e.g. diabetic ketoacidosis, levels of acetate, KBs and NEFAs are high and acetate is present mainly as acetic acid, which, with a lower pK than the keto-acids, should consume increased buffering capacity. It is therefore beneficial in respect of pH regulation in plasma to oxidise acetate preferent ially, moreso that oxidation of acetate generates equimolar amounts of bicarbonate (Lewis, Tolchin and Roberts 1980).

Euglycaemic Clamping.

This study shows that, in both non-diabetic and diabetic subjects, plasma acetate falls with NEFAs during insulin administration. This observation, coupled with the

results obtained during the lipid infusions, would suggest that, at least in the fasting state, endogenous acetate is formed from fatty acids. In non-diabetic subjects acetate levels returned to fasting values during the second hour of the insulin clamp but no such rise was observed in the diabetics. In both groups, glucose flux was increased. Since NEFA levels were still low in the second hour, it is unlikely that thay contributed to the later rise in acetate levels. The likely source is the increasing glucose flux, causing more acetyl CoA to become available from pyruvate in the peripheral tissues and liver. Increase in plasma acetate levels with orally or intravenously administered glucose has already been demonstrated (Thesis, Chapter 5.D). The rise towards normal values in the non-diabetic subjects is further evidence for the role of the liver and peripheral tissues in maintaining acetate levels within a narrow range (Buckley and Williamson 1977, Hepp, Prusse, Weiss and Wieland 1966) and its absence in diabetic patients may indicate failure of this regulatory mechanism.

A much simpler approach may be followed. The 120min acetate values in the diabetics and the non-diabetics are the same. Perhaps this is the level that occurs in either group after substantial exposure to their usual glucose levels and approximately equal insulin concentrations. This is also the usual fasting level of non-diabetics, indicating homeostasis of the acetate level at least as achieved after 2hr of a euglycaemic

hyperinsulinaemic clamp (although it is uncertain if it would have risen even more in the non-diabetics with continued clamping). The abnormality is thus thought to be the fasting acetate level in the diabetic. If this is considered as consequent on their hyperglycaemia, this factor is nullified once much higher insulin levels have acted for a considerable time and NEFA lavels have stabilised at low levels.

This observation in diabetics has not been reported previously. The plasma acetate level is increased in diabetes, and fasting levels correlate directly with plasma glucose (Thesis, Chapter 5.C). The lack of a later rise in acetate levels during the clamp in diabetics however defies easy explanation. It is probably not due to the associated tissue insensitivity to insulin as the results show that similar degrees of insulin resistance, as assessed by either the metabolic clearance rate for glucose or steady state glucose infusion rate, occur in obese non-diabetic subjects, yet the latter exhibit changes similar to those in the thinner normal subjects. It is also probably not due simply to differences in BMI or MCR especially as no relationship could be demonstrated between the change in acetate levels in the second hour of the clamp and both these variables. Furthermore, the difference observed in insulin sensitivity is probably not due simply to varying body mass since the respective slopes for the regression curves relating BMI to MCR differed between the non-diabetic and diabetic subjects. Thus, if the defect in glucose utilization as reflected in

the MCR is principally at the uptake level, it is conceivable that differences still exist between non-diabetic and diabetic subjects at the post-receptor level, diverting glucose into either glycogenesis or glycolysis. A reduced rate of glycolysis in diabetics, might then result in slower formation of acetyl CoA, and hence acetate. Indeed, preliminary results from studies on lactate levels during the clamp showed that the blood lactate level about doubled in 5 non-diabetic subjects (3 obese) while it tended to fall in 3 NIDDM subjects.

CONCLUSION.

In conclusion, (i) plasma acetate levels in humans rise with lipid infusions and conversely fall when NEFA levels are low; these changes, while quantitatively less than those observed for KBs, could be important in conditions of insulin lack and enhanced lipolysis. (ii) diabetic patients do not show a secondary rise in acetate levels towards their usual level (after their initial fall) during euglycaemic clamping, unlike obese and normal-weight non-diabetic subjects; this change is not due to insulin resistance. This could represent post-receptor differences. Alternatively, since the diabetic and non-diabetic levels are the same after 2hr, it could be that the procedure has abolished a difference present in the fasting state.

CHAPTER 6

EXOGENOUS SOURCES OF ACETATE

A. LITERATURE REVIEW.

1. Diet.

i. The Dietary Fibre Theory.

Trowell and Burkitt (1975) working in East Africa, attributed the relatively low prevalence in Africans of such "Western" diseases as irritable bowel syndrome, appendicitis, colon cancer, hiatus hernia, coronary heart disease , diabetes mellitus and cholelithiasis to the high fibre content of the traditional African diet. Since this observation, there have been attempts to increase fibre intake in Caucasian populations. Dietary fibre, in this context, is as defined by Cummings (1981b): 'plant cell wall polysaccharides and lignin which resist digestion by the enzymes of the human gut.'

Increased fibre, at least of "gel-forming" type, taken as part of a mixed meal reduces post-prandial glucose and insulin responses in normal and diabetic subjects (Jenkins, Goff, Leeds, Alberti, Wolever, Gassull and Hockaday 1976, Anderson and Lin Chen 1979, Jenkins and Jenkins 1984, Anderson 1985). These effects are also observed with "pure" (synthetic) fibre preparations such as guar and pectin (Uusitupa, Tuomilehto, Kartunnen and Wolf 1984, Jenkins and Jenkins 1984). Natural and synthetic dietary fibre produces long-term cholesterol-lowering effects in dyslipidaemias. Many of these hypolipidaemic effects were described even before the effect of fibre on carbohydrate metabolism was observed (Fahrenbach, Riccardi, Saunders, Lowrie and Heider

1965, Palmer and Dixon 1966, Jenkins, Leeds, Newton and Cummings 1975, Kritchevsky 1978, Penagini, Velio, Vigorelli, Bozzani, Castagione, Ranzi and Bianchi 1986, Tuomilehto, Voutilainen, Huttunen, Vinni and Homan 1980, Pacy, Dodson, Kubicki, Fletcher and Taylor 1984, Tuomilehto, Kartunnen, Vinni, Kostianen and Uusitupa 1983, Aro, Uusitupa, Voutilainen and Korhonen 1984, Jenkins and Jenkins 1984, Krotkiewski 1984), and were often accompanied by weight reduction especially in obese subjects (Tuomilehto, Voutilainen, Huttunen, Vinni and Homan 1980, Ullrich and Albrink 1985).

Apart from these effects with single meals, the beneficial effect of increased fibre intake continues for as long as the fibre intake is increased (Anderson 1985). Increased fibre taken at a meal improves the glucose tolerance of subsequent meals (Jenkins, Wolever, Taylor, Griffiths, Krzeminska, Lawrie, Bennett, Goff, Sarson and Bloom 1982, Trinick, Laker, Johnston, Keir, Buchanan and Alberti 1986, Jenkins, Wolever, Nineham, Sarson, Bloom, Ahern, Alberti and Hockaday 1980). These observations, and the finding of improved glucose and insulin kinetics with fibre intake (Hall, Bolton and Hetenyi 1980) suggest that dietary fibre, by uncertain mechanisms, influences intermediary metabolism.

An increased fibre intake has energy implications. Unavailable carbohydrate is usually taken as having no energy value in the calculation of daily individual caloric

requirements although Southgate (1973) remarked that the effect of the unavailable carbohydrate on the apparent digestibility of the other energy yielding constituents of the diet is not negligible and may become very important for diets containing large amounts of plant foods. That indeed now seems the case (Heaton 1973).

ii. Mode of Action of Dietary Fibre.

Dietary fibre probably acts by influencing the absorption and subsequent utilization of carbohydrates and fats. The local physiological effects in the gut include (Anderson and Lin Chen 1979) :

- (i) altered food transit time with faster rate of passage through the gut (but see (a) below);
- (ii) water absorption by the fibre and consequent swelling;
- (iii) cation binding;
- (iv) adsorption of organic substances such as bile salts;
- (v) gel formation;
- (vi) colonic microbial digestion of fibre with production of VFAs;
- (vii) altered digestion and absorption of nutrients with alterations in secretion of gut hormones.

To these effects may be added (Stephens and Cummings 1980) changes in colon metabolism of minerals, nitrogen and bile salts, which effects are mediated by some fibre passing through the gut undigested and holding water within its

cellular structure.

Eastwood and Kay (1979) proposed that the physico-chemical properties of the different types of dietary fibre were responsible for their different actions. They suggested that fibre consists of a sponge matrix with specific physico-chemical properties dependent on the structure and composition of its components. These properties may be passive (gel-formation, water-holding capacity, matrix formation) or active (cation exchange, bile-acid adsorption and anti-oxidant activity). Specific examples include:

(a) gel formation, as with pectin and mucilages, causing delayed gastric emptying, increased mouth-to-caecum transit time and delayed small intestinal absorption;

(b) water holding capacity, as with lignins and similar polysaccharides, causing increased faecal transit time and weight, increased intraluminal pressures and influencing reabsorption of the colonic faecal electrolytes;

(c) matrix formation, with most types of fibre, enhancing caecal bacterial metabolism with increased fermentation and production of VFAs - acetate, propionate and butyrate;

(d) bile acid adsorption, with lignin and pectin, causing increased faecal steroid and blood cholesterol turnover;

(e) cation exchange and anti-oxidant activity, with acidic

polysaccharides and lignin;

(f) digestibility, as with the non-starch polysaccharides, producing VFAs which contribute to colonic and portal energy availability and alter the chemical environment of the colon.

These various effects, mainly local, contribute to the beneficial effects of dietary fibre in diabetic glycaemic control. The effectiveness in hyperlipoproteinaemias may be linked to altered intestinal handling of cholesterol due to binding and sequestration of bile acids and altered hepatic cholesterol and lipoprotein metabolism (Anderson and Lin Chen 1979). Guar gum has particularly been shown to increase intestinal unstirred layer thickness and to inhibit intestinal convective solute movement (Penagini, Velio, Vigorelli, Bozzani, Castagione, Ranzi and Bianchi 1986), effects likely significantly to reduce intestinal nutrient absorption.

Dietary fibre therefore has numerous properties which may cause major local effects on the gut, and by so doing, produce the observed metabolic effects. Such effects are often long-term.

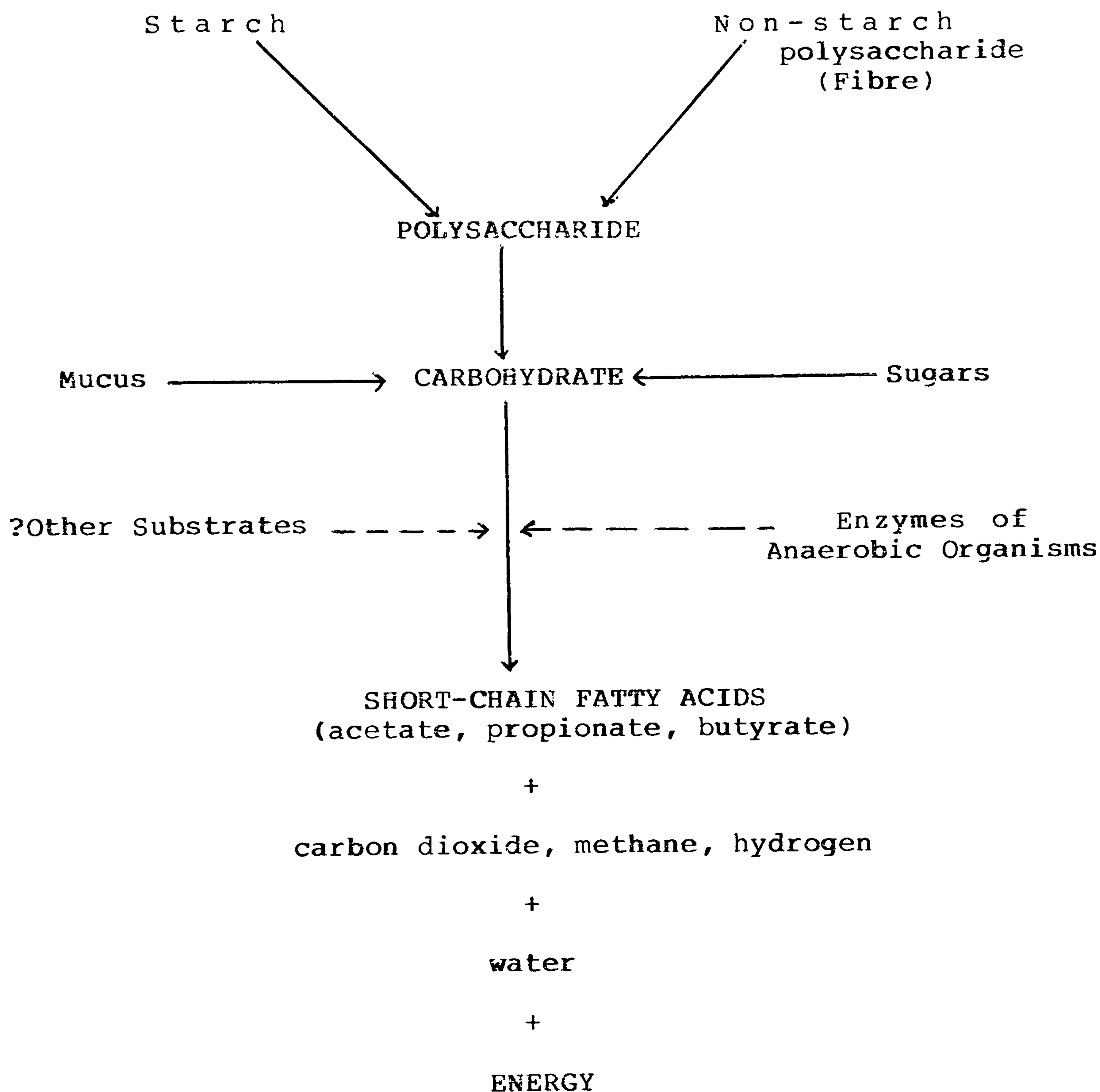
VFAs constitute the major products of bacterial fermentation of dietary fibre in the colon of mammals and when absorbed are available for metabolism. Indeed, VFAs account for virtually all energy requirements in ruminants (Annison and Armstrong 1970)

iii. Fermentation in Man

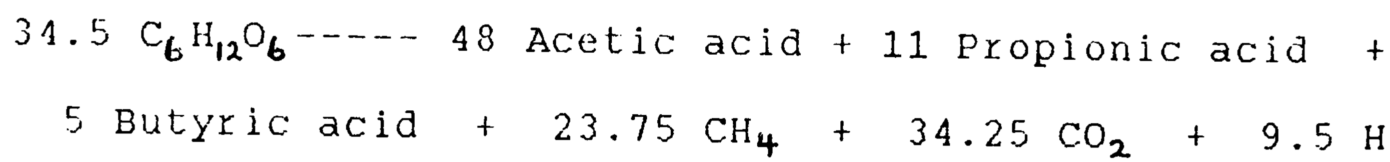
There is substantial evidence that a fermentative process similar to that in non-ruminant herbivorous and omnivorous animal species takes place in the human large intestine (Cummings 1983, 1984, Wolin 1981). Dietary fibre is normally not digested by mammalian small intestinal enzymes but is available for microbial fermentation in the colon. The end- products of this process of fermentation are similar to those of the rumen. VFAs - acetate, propionate, and butyrate, are produced along with the gases, carbon dioxide, methane and hydrogen.

Fermentation is sustained by many of the colonic species of bacteria. The human colon contains a substantial mixed culture of bacteria estimated at 10^{10} - 10^{11}/g colonic and faecal material, 99% of which are anaerobes (Moore, Cato and Holdeman 1978). The major species present are Bacteroides spp. (Salyers, Vercelloti, West and Wilkins 1977a), Bifidobacterium, Peptostreptococci, Lactobacilli, ruminococci, coprococci, eubacteria and fusobacteria (Salyers, Vercelloti, West and Wilkins 1977b). Most of these organisms are saccharolytic. Cummings (1983) estimated that the amount of fermentable carbohydrate available to the large bowel flora in subjects on Western diets is about 20-40g/day. In populations with high dietary fibre intake, the amounts could be substantially greater. A general outline of human colonic fermentation is shown in Figure 6.A.1.

FIG 6.A.1 : GENERAL OUTLINE OF FERMENTATION IN THE HUMAN COLON (after Cummings, 1983).



Based on faecal VFA concentrations determined by Zijlistra, Beukema, Wolthers, Byrne, Groen and Dankert (1977), Wolin (1981) proposed an equation for mammalian colonic fermentation as follows:



$$34.5\ C_6H_{12}O_6 \text{-----} 48 \text{ Acetic acid} + 11 \text{ Propionic acid} + 5 \text{ Butyric acid} + 23.75\ CH_4 + 34.25\ CO_2 + 9.5\ H_2O.$$

However, analyses of molar ratios of VFAs deduced from the equation in relation to concentrations observed in colonic contents indicate that more VFA is produced than is present in faeces, suggesting that VFA is absorbed (Cummings 1983, 1984). This absorption across the colonic wall is accompanied by transport of blood bicarbonate into the colon (McNeil, Cummings and Jones 1978, Hoverstad 1986). Most of the hydrogen and methane produced in the colon are absorbed into the blood and ultimately removed from the lungs by expiration (Levitt 1969, Bond, Engel and Levitt 1971), although a significant proportion is excreted in flatus.

Other non-ruminant mammals also derive part of their circulating VFAs from dietary sources. Illman, Trimble, Snoswell and Topping (1982) in comparing daily variations in concentration of VFAs in the splanchnic blood vessels of rats fed diets high in pectin and bran content, found VFAs to be absorbed in considerable amounts from these dietary sources, more so with pectin (highly degradable) than bran (rich in lignin and generally resistant to bacterial action). Acetate was considered a major hepatic metabolic fuel transported in the splanchnic circulation in proportion

to apparent digestibility of the fibre type. Demigne, Yacoub and Remesy (1986) also showed increased absorption of acetate and propionate in rats on high fibre diets and added that propionate and butyrate were completely cleared from portal blood by the liver.

Measurement of breath excretion of hydrogen and methane is used widely in clinical and physiological studies of fermentation in humans (Cummings 1983, Hanson and Winterfeldt 1985, Read, Al-janabi, Bates, Holgate, Conn, Kinsman, McFarlane and Brown 1985, McNamara, Levine, Levitt and Slavin 1985).

iv. Significance of Fermentation.

This subject has been reviewed by Cummings 1982, 1984 and Wolin 1981. It still remains unclear how important to human metabolism the end-products of fermentation are:

a. volatile fatty acids are important sources of energy in ruminants and could be important in humans when their production is accentuated with increased dietary fibre intake. This subject is considered in further detail later.

b. gas production as a consequence of fermentation has no known metabolic significance. More people produce hydrogen than do methane.

c. with fermentation, faecal nitrogen is diverted into microbial protein synthesis, with the net effect that people on high fibre diets have reduced faecal

ammonia levels (Cummings, Stephens and Branch 1981), an observation also made in patients with liver cirrhosis and taking lactulose (Weber 1979). Fermentation thus leads to significant changes in colonic nitrogen metabolism, an observation of importance, especially as the lowering of colonic ammonia concentration and absorption is considered important in protecting against colon carcinogenesis (Visek 1972).

d. effects on steroid metabolism, possibly by changes in colonic pH, since steroid 7-α-dehydroxylase activity is inhibited by low pH (Midtvedt and Norman 1968) and also by sequestration of bile acids (Thornton and Heaton 1981).

v. Volatile Fatty Acids in Man.

a. Production.

VFAs are produced from carbohydrate fermentation by colonic anaerobic bacteria. The pathways of breakdown are similar to those in the rumen involving initial hexose catabolism via anaerobic glycolysis to pyruvate or alternatively through the pentose phosphate shunt. An important intermediate metabolite in these reactions is pyruvate, but very little is found in the gut as it is rapidly converted to a series of end-products - acetate, propionate, butyrate, carbon dioxide, hydrogen, methane and water. Acetate is usually formed by the oxidative decarboxylation of pyruvate; butyrate derives from reduction

of acetoacetate formed from acetate. Production of propionate involves fixation of carbon dioxide to form succinate which is subsequently decarboxylated, or alternatively, from lactate and acrylate. These various fermentation reactions are shown in Fig 6.A.2 and 6.A.3.

Evidence for the site of VFA production in Man is provided by Mitchell, Lawson, Davies, KerrGrant, Roediger, Illman and Topping (1985) who measured VFAs in digesta taken from the stomata of patients after large bowel surgery. Total VFA was highest in the patients with transverse colostomy and lowest in those with ileostomies; intermediate levels were found at the sigmoid colostomy site. This suggests that most VFA production is in the caecum and ascending colon.

Oral intake of broad-spectrum antibiotics with resulting bowel antisepsis significantly diminishes colonic VFA production and portal and peripheral blood VFA levels in healthy human subjects (Hoverstad, Carlstedt-Duke, Lingaas, Norin, Saxerholt, Steinbakk and Midtvedt 1986) and rats (Keane 1967, Buckley and Williamson 1977, Hermann, Herz and Frohlich 1985).

b. Absorption.

The total VFA concentration in faeces is about 75 mmol/kg (range 30-190mmol/kg), making it the main anion in normal human faeces, and presumably in the colon. Acetate is quantitatively the most important VFA (Hoverstad, Fausa,

FIG 6.A.2 : PATHWAYS OF FERMENTATION OF MAJOR CARBOHYDRATE CONSTITUENTS OF PLANTS TO 3C UNITS.
(after Leng, 1970)

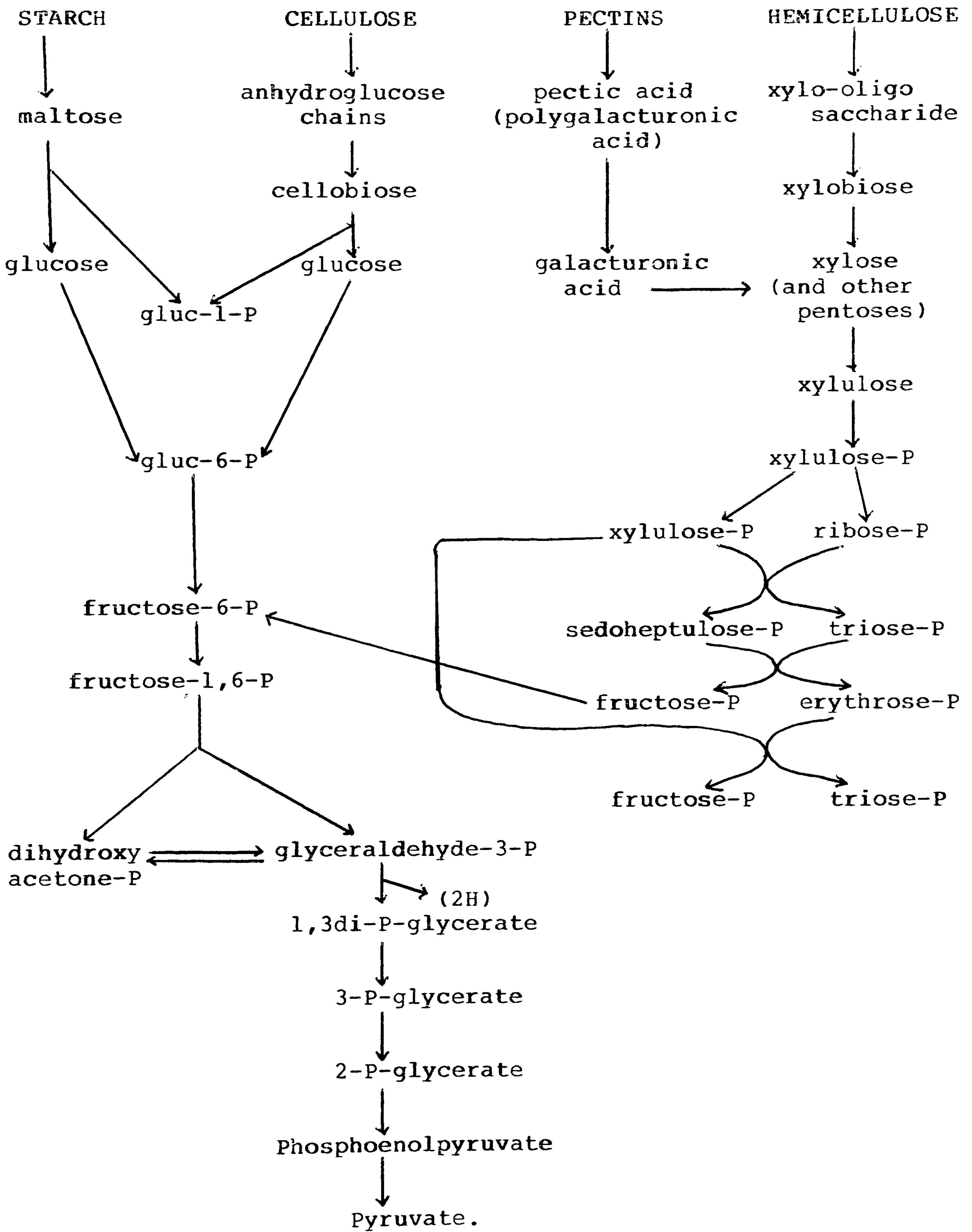
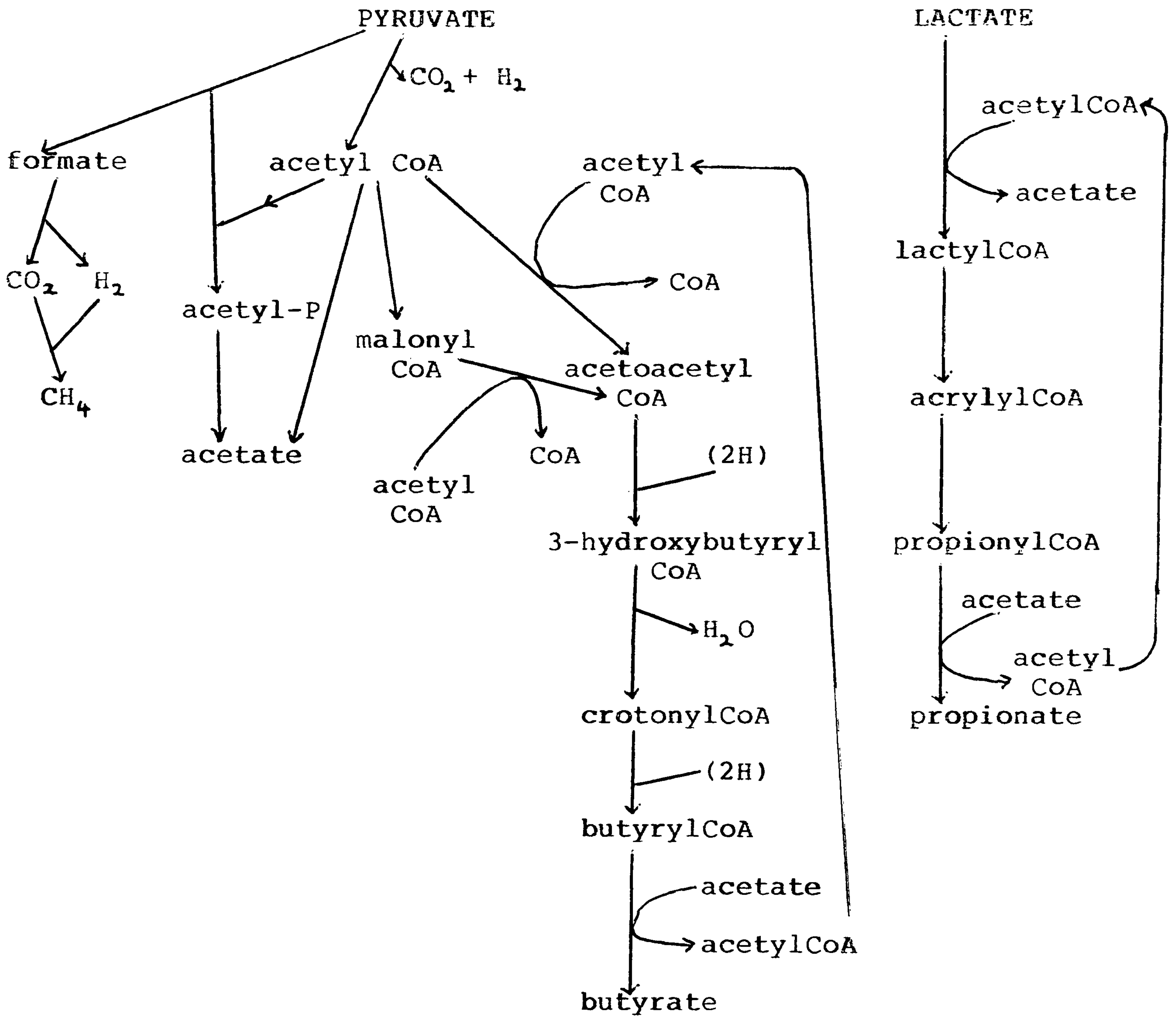


FIG 6.A.3: PATHWAYS OF DEGRADATION OF 3C UNITS DURING FERMENTATION
(after Leng, 1970)



Bjorneklett and Bohmer 1984, Hoverstad 1986). At least 300mmol/day VFA is produced by individuals on regular Western diets (Cummings 1983) and probably a lot more, >600mmol/day, in populations on higher fibre diets (Hoverstad 1986). It is thus apparent that a highly significant part of total VFA produced is absorbed.

Indeed, studies using bolus instillation of mixtures of VFAs in the human colon (Dawson, Holdsworth and Webb 1964), radioactive carbon dioxide breath tests using radio-labelled VFAs (Hoverstad, Bohmer and Fausa 1982), in vitro perfusion of the proximal jejunum (Dawson, Holdsworth and Webb 1964, Schmitt, Soergel and Wood 1976), dialysis bag techniques (McNeil and Cummings 1979) and in vitro colonic perfusion (Roediger and Moore 1981) have confirmed that most VFA is absorbed by the colonic epithelium. Net movement of these acids out of the colonic lumen is more rapid than net sodium transport (McNeil, Cummings and James 1978); transport from the lumen is associated with the appearance of bicarbonate with the stimulation of sodium absorption and is independent of bulk water flow (Cummings 1981a).

The absorbed VFA is to a large extent metabolised by the liver and colonic wall. Dankert, Zijilstra and Wolthers (1981) showed that the concentration of acetic acid is 3-4 times greater and propionic and butyric acids 10 times greater in portal than in peripheral venous blood, suggesting major hepatic extraction. Also, Pomare, Branch and Cummings (1985), in demonstrating increased levels of

arterial and venous plasma acetate in subjects given different fibre or lactulose preparations, did not detect any significant levels of propionate and butyrate in peripheral blood. This accords with the other observations that acetate is quantitatively the most important VFA in peripheral blood; propionate and butyrate are almost completely cleared from portal blood by the liver (Cummings 1981b, 1981a, Demigne, Yacoub and Remesy 1986, Illman, Trimble, Snoswell and Topping 1982). In individuals with hepatic cirrhosis, where large amounts of VFA bypass the liver through portal-systemic anastomoses however, breath and arterial VFA is higher than in normal subjects, and, indeed, propionate and butyrate tend to be present in measurable amounts in the peripheral blood (Chen, Mahadevan and Zieve 1970).

The significance of the differential metabolism of these VFAs remains largely unknown.

c. Implications of VFA Absorption.

As in ruminants where VFAs constitute the most important source of energy, the VFAs absorbed in humans probably contribute to energy metabolism. Cummings (1984) suggested that VFAs could be a considerably important energy source in those countries where daily fibre intake is as high as 100g.

Both the ruminal and colonic epithelium metabolise VFAs, especially butyrate and propionate. Roediger (1980a)

demonstrated with isolated human colonic epithelial cells that butyrate was an important energy source for the colonic mucosa, accounting for the major part of energy needs even in the presence of glucose. Reduced butyrate oxidation in colonic epithelial cells has been implicated as a possible cause of ulcerative colitis (Roediger 1980b).

Butyrate has also been demonstrated to show anti-neoplastic properties, prolonging doubling time and reducing rate of growth of human large bowel cancer cells (Kruh 1982). This is probably via an effect on cellular enzymes resulting in stabilisation of chromatin structure during cell division and suppression of cell proliferation. This antineoplastic function of VFAs is controversial. Although human epidemiological studies indicate an inverse correlation between high fibre consumption and low colon cancer rates, case control studies have consistently failed to confirm any protective effect due to fibre (Jacobs 1986). Fermentable fibre supplements such as pectin, oat bran, guar gum and alfalfa were shown to enhance tumour growth in laboratory animals with chemically induced colon cancer (Jacobs 1986). Indeed, butyrate IN VIVO stimulates cell proliferation in rats and humans, although opposite effects were observed IN VITRO (Jacobs 1986, Sakata and Yajima 1984, Sakata 1986). This is probably because butyrate acts IN VIVO as a luminal trophic factor, and furthermore may modify colonic microbial metabolism by reducing the luminal pH; both these effects promote colon carcinogenesis. On the

other hand, Whitehead, Young and Bhathal (1986) showed that butyrate concentrations were lowest in the sigmoid and descending colon, regions where the incidence of colon cancer was highest, indicating that the VFA may protect against that cancer. It is however possible that different fibre products subserve different functions, since epidemiological studies have demonstrated that cereal fibre shows the most consistent negative correlation with colon cancer. On the other hand, there may be other, yet undetected anti-neoplastic factors in vegetables (Jacobs 1986).

Propionate appears to influence gluconeogenesis (Anderson and Bridges 1984) and has been demonstrated to reduce cholesterol accumulation in the serum and liver of the cholesterol-fed rat. It may therefore mediate some of the hypocholesterolaemic effect of dietary fibre (Chen, Anderson and Jennings 1984).

The role of acetate is even less clear, but being the only VFA found in any significant amount in peripheral blood, it might be expected to influence hepatic and peripheral tissue metabolism. This possibility has been discussed in Chapter 5.

2. Ethanol.

Acetate is produced from ethanol by mammalian liver. Endogenous ethanol derives from rat caecal fermentation and is absorbed into the portal circulation (Krebs and Perkins

1970, Perkins 1970) reaching the liver where it is oxidised to acetaldehyde by alcohol dehydrogenase and then to acetate by aldehyde dehydrogenase. Blomstrand (1971) has also found ethanol in portal blood in humans not taking it orally. Buckley (1974) however indicated that it was unlikely that much acetate escapes the liver to reach the general circulation from the breakdown of endogenous ethanol, as long as the amount of ethanol entering the liver was not enough to affect the hepatic redox state.

Lundquist (1960), Lundquist, Fugmann, Klaning and Rasmussen (1959) and Crouse, Gerson, DeCarli and Lieber (1968) have demonstrated increased human blood acetate level after infusion or ingestion of ethanol. The substrate could, in that circumstance, be an immediately available energy source as has been shown with the exercising human forearm (Lundquist, Sestoft, Damgaard, Clausen and Trap-Jensen 1973).

B. EXPERIMENTAL SECTION

SPECIFIC AIMS

We studied human colonic production of acetate by :

i. assessing fasting and post-prandial acetate levels in diabetic subjects on mixed meals containing different amounts of dietary fibre and,

ii. investigating the time course, duration and extent of fermentation by measuring plasma acetate levels after the ingestion of lactulose, a semi-synthetic, non-digestible disaccharide.

We also examined production of acetate by hepatic metabolism of ethanol and possible effects on this process of chlorpropamide an anti-diabetic sulphonylurea drug thought to influence hepatic aldehyde dehydrogenase activity.

C. CHANGE IN PLASMA ACETATE LEVELS IN DIABETIC SUBJECTS ON MIXED HIGH-FIBRE DIETS

AIMS.

There have been only a few studies on the effects of mixed meals containing natural fibre on fasting VFA levels in animals and probably none in humans. As high dietary fibre is now recommended in the management of conditions such as diabetes, it seemed important to study the effect of increased fibre intake on fasting acetate levels. The effect on acetate levels of increasing refined sugar intake in a mixed high-fibre diet was also studied.

SUBJECTS AND METHODS.

10 diabetic patients (6M) with stable glycaemic control were studied ; 5 were insulin treated and the other 5 controlled with oral agents. They were aged 52.1 $\pm$ 3.7yr with mean duration of diabetes of 13.3 $\pm$ 4.3yr and BMI 24.8 $\pm$ 1.2 kg/m^2 (Table 6.C.1).They were recruited into the study if none of their random plasma glucose values, as recorded in the case-notes, was > 12.0mmol/l, and the last clinic measurement was <10.0mmol/l. Parts of this study which also examined the effect on glycaemic control of regular sucrose intake in the diabetic diet have already been published (Peterson, Lambert, Gerring, Darling, Carter, Jelfs and Mann 1986).

Dietary Methods

The volunteer subjects were assigned in a random cross-over fashion to instruction as to 2 alternate isocaloric

BLE 6.C.1 : CLINICAL CHARACTERISTICS AND TREATMENT OF THE SUBJECTS.

tient	Sex	Age(yr)	BMI	Duration of Diabetes(yr)	Treatment*
[illegible]	F	51	32.2	8	Chlorpropamide
[illegible]	M	63	23.8	5	Chlorpropamide
[illegible]	F	52	23.1	12	Chlorpropamide
	F	66	28.7	5	Glibenclamide
	M	68	27.5	12	Metformin and Glibenclamide
	M	33	28.1	10	Insulin pump
	F	36	23.0	12	Insulin pump
	M	51	24.9	39	Insulin
	M	56	23.9	11	Insulin
	M	45	25.5	7	Insulin
[illegible]		52.1	26.1	12.1	
		11.8	3.0	9.9	

* additional to exercise and diet.

diets, both with increased fibre content (Table 6.C.2):

Diet HF : high fibre diet, 55.7 ± 2.8g fibre/day (as recommended), with 50-55% of the calories as carbohydrate, 30-35% as fat and 20-25% as protein, as recommended by the British Diabetic Association.

Diet HFS : high fibre diet with total calorie and fibre content as in HF, but with 45g of complex carbohydrate in HF replaced by 45g sucrose taken as 15g thrice daily with meals. Carbohydrates such as bread and potatoes were reduced and fibre-rich cereals encouraged to maintain dietary fibre.

Rich sources of soluble fibre, such as dried beans, remained constant throughout. The ratios of simple sugars to complex carbohydrates on diets HF and HFS were respectively 1:5 and 1:2. At the start of the study, patients were individually assessed to determine daily energy requirements and to plan diets HF and HFS. They were also given a sucrose exchange list of 15g portions and a booklet of recipes using high fibre foods in addition to detailed dietary instruction by a dietitian. The average daily total caloric intake was constant on each of these diets and is shown in Table 6.C.2. A typical daily menu plan is shown in Table 6.C.3.

After instruction on each diet, patients had a follow-up interview with the dietitian at 1week. Dietetic advice was available throughout, with contact depending on both the patient's requests and the dietitian's assessment of their understanding of the diets. The patients provided the dietitian with a record of all food and drink consumed

TABLE 6.C.2.: GLYCOSYLATED HAEMOGLOBIN LEVELS AND DAILY FIBRE AND CALORIC INTAKE OF THE SUBJECTS STUDIED.

PATIENT	HBAIC %			TOTAL FIBRE INTAKE/DAY g			TOTAL CALORIC INTAKE/DAY Kcal.
	U	HF	HFS	U	HF	HFS	
1	10.9	10.7	10.4	20.8	38.5	32.9	1230
2	4.7	5.0	5.0	37.6	67.8	63.9	2210
3	10.7	12.0	11.1	30.2	54.6	50.4	1670
4	11.6	12.1	11.4	11.7	31.2	26.1	1240
5	9.0	9.5	9.1	31.5	76.9	71.2	2640
6	11.1	9.2	9.0	34.4	53.3	48.9	1990
7	8.9	10.9	10.5	39.5	68.4	66.4	2510
8	8.7	7.4	8.0	33.0	48.2	43.9	1810
9	14.8	10.4	9.3	32.0	60.1	57.1	2090
10	11.5	10.9	10.3	39.5	57.8	51.5	2200
EAN	10.2	9.8	9.4	31.2	55.7*	51.2*	1959
SD	2.6	2.2	1.9	8.9	13.9	14.3	479

U usual diet
HF high fibre diet
HFS high fibre with sucrose diet
* $p < 0.01$ compared to diet U.

LE 6.C.3 : TYPICAL DAILY MENU PLAN FOR THE SUBJECTS

eal	Diet HF		Diet HFS	
	Food	Wet Weight (g)	Food	Wet Weight (g)
ıkfast	Weetabix	36	Weetabix*	18
	All bran	14	All bran*	20
	Wholemeal bread	70	Wholemeal bread	70
	Margarine	11	Margarine	12
			MARMALADE	22
:k	Digestive biscuits	30	Digestive biscuits	30
·day	Cheddar cheese	30	Cheddar cheese	30
	Tomato	80	Tomato	80
	Lettuce	30	Lettuce	30
	Cucumber	30	Lettuce	30
	Wholemeal bread	105	Wholemeal bread*	70
	Margarine	8	Margarine	6
	Apple	120	Apple	120
			TOFFEES	21
:k	Digestive biscuit	15	Digestive biscuit	15
ıing	Lean roast pork	60	Lean roast pork	60
	Cauliflower	60	Cauliflower	60
	Carrots	50	Carrots	50
	Haricot beans (dry weight)	50	Haricot beans	50
	Boiled potato	150	Boiled potato*	150
	Banana	65	Banana	65
	Custard powder	8	Custard powder	8
	+ art. sweetener		SUGAR	15
:k	Wholemeal bread	70	Wholemeal bread	70
	Margarine	8	Margarine	8
	Tomato	70	Tomato	70
	Digestive biscuit	15	Digestive biscuit	15

Daily allowance of skimmed meal for both diets : 445ml

Foods altered to maintain constant carbohydrate and fibre content

during a 3-day period in the second week, based on household measures or actual weighing as convenient. This was used to verify their understanding and compliance to the recommended diet. A further detailed interview with the dietitian at the end of each period was used to provide an indication of overall acceptability and compliance, which was good in all the subjects studied.

They were first studied on their usual (U) diet, 31.2 $\pm$ 8.9g fibre/day and again after 6 weeks on each of diets HF and HFS with a standard breakfast (450kcal) appropriate to each diet, for 3hr in the morning after a 12hr overnight fast. Fasting samples were taken 30min after cannula insertion, and subsequent samples at 30min intervals after starting to eat the breakfast.

To assess day-to-day and week-to-week variability in fasting plasma acetate levels with the usual Caucasian diet, fasting blood samples were taken from 5 non-diabetic laboratory staff daily for 5 consecutive days. Samples were also taken at weekly intervals for 3 weeks from 7 non-diabetic University students and 6 stable NIDDM patients. No subject drank alcohol during the study period. Plasma was assayed for glucose and acetate.

Areas under the acetate/time curves were calculated by the trapezoidal rule. Results were compared using the Student's t-tests for paired observations. The relationship between acetate and the other metabolites was explored by the Spearman rank method.

RESULTS.

There was no change in body weight during the studies and the glycosylated haemoglobin values on diet U (10.2 ± 2.6%), HF (9.8 ± 2.2%) and HFS (9.4 ± 1.9%) did not differ significantly (Table 6.C.2), but then the test periods were only 6weeks long..

The result obtained for the various plasma metabolites is shown in Table 6.C.4. In the group as a whole, fasting plasma acetate increased on HF ($p < 0.01$) and HFS ($p < 0.005$) compared to U, but the values on the former 2 diets were not significantly different. Fasting glucose decreased from U to HF (p NS) and to HFS ($p < 0.05$); the fasting glucose values on HF and HFS were not significantly different. Fasting NEFAs also decreased on HF (p NS) and on HFS ($p = 0.001$), as compared to U; these values were lower on HFS than on HF ($p < 0.01$). There was no significant change in levels of plasma triglycerides and total cholesterol on the different diets.

Of various metabolites in plasma, glucose has the strongest positive correlation with acetate in the fasting state (Smith, Humphreys and Hockaday 1986, observations in Chapter 5.C) and that relationship was again established here (all diets, rs = 0.48, $p < 0.005$, diet U, rs = 0.62, $p < 0.05$, diets HF and HFS rs = 0.60, $p < 0.01$). The fasting acetate level was therefore related to fasting glucose by deriving an acetate/glucose (A/G) ratio. This ratio (on U) increased on HF ($p < 0.02$) and also on HFS ($p < 0.01$), but

did not differ significantly between HF and HFS (Table 6.C.4). The total area under the acetate/time curve (AUC) over the 180min study period (mmol.min/l) on diet U was unchanged on HF but increased on HFS ($p < 0.05$); values on HF and HFS were not significantly different. The incremental areas on the 3 diets did not change however, indicating that the difference observed in the total areas was due to differing basal values (Table 6.C.4).

The results obtained with the group of 10 diabetic subjects were generally similar to results when sub-grouped into NIDDM and IDDM (Table 6.C.4). The fasting insulin values in NIDDM on the 3 studies were unchanged.

Repeated daily measurements of fasting acetate concentrations in 5 non-diabetic subjects on 5 consecutive days gave a coefficient of variation (CV) of 9.4 ± 2.1%; for weekly fasting acetate values over 3 consecutive weeks in 7 non-diabetic subjects, the CV was 9.2 ± 2.8%, and in 6 diabetic patients, 11.4 ± 3.2%. In these experiments, fasting plasma glucose levels in the subjects, diabetic and non-diabetic, remained essentially unchanged at the different times so that the CV for A/G ratio was closely similar to that for acetate.

DISCUSSION

This study suggests that the chronic dietary fibre intake might have raised portal venous and then hepatic venous levels of acetate (and perhaps other VFAs) derived

TABLE 6.C.4 : FASTING PLASMA VALUES IN THE DIFFERENT SUBJECT GROUPS ON USUAL (U), HIGH FIBRE (HF) AND HIGH FIBRE WITH SUCROSE (HFS) DIETS (means (SD)).

Diet	Acetate (mM)	Glucose (mM)	NEFAs (mM)	A/G	AUC (mM.min)	Δarea (mM.min)	Insulin (mU/l)
ALL (n=10)							
U	0.21 (0.06)	10.8 (4.5)	1.40 (0.70)	0.020 (0.010)	43.5 (16.0)	6.3 (8.6)	
HF	0.28* (0.10)	9.4 (3.2)	1.03 (0.48)	0.032* (0.013)	50.6 (15.4)	4.6 (10.9)	
HFS	0.30* (0.10)	8.9* (4.8)	0.49*+ (0.22)	0.037* (0.010)	60.0* (10.9)	6.5 (14.4)	
IDDM (n=5)							
U	0.22 (0.02)	9.6 (2.9)	1.49 (0.59)	0.025 (0.009)	48.5 (9.7)	9.6 (21.1)	11.1 (6.2)
HF	0.26 (0.07)	9.9 (2.0)	1.14 (0.59)	0.026 (0.009)	52.8 (13.4)	7.1 (9.5)	9.9 (4.8)
HFS	0.29* (0.02)	8.9 (2.6)	0.48* (0.09)	0.035* (0.011)	65.8* (7.5)	14.4 (6.8)	11.3 (9.2)
DDM (n=5)							
U	0.20 (0.11)	12.0 (5.5)	1.30 (0.81)	0.016 (0.004)	38.6 (19.6)	2.9 (9.0)	
HF	0.30* (0.11)	8.9* (4.4)	0.96 (0.44)	0.038* (0.013)	48.4 (17.6)	5.9 (7.9)	
HFS	0.31* (0.13)	8.8 (6.6)	0.50*+ (0.29)	0.039* (0.011)	54.1 (11.0)	1.3 (15.8)	

* $p < 0.05$ compared to usual diet U

\+ $p < 0.05$ compared to high fibre HF diet

AUC: area under acetate/time curve

Δ area : incremental area under acetate/time curve

from colonic fermentation, in turn, reflected in peripheral blood. In earlier studies in humans using pectin (Pomare, Branch and Cummings 1985), acetate levels started rising 6hr after pectin ingestion and peaked by 8-10 hr. This peak should correspond to values obtained after an overnight fast if a `high fibre' dinner was taken. Moreover, if each meal had a high fibre component, the overall cumulative effect on acetate levels might be best detected on the fasting specimen, since fermentation continues for about 20hr. That indeed was the case here.

Fasting acetate values increased by 35 - 40% on high fibre diets with and without added sucrose. This was a substantially greater change than the random variation of normal subjects on the usual Caucasian diet (daily variation 9%), while in diabetic, as well as normal, subjects the weekly variation was 9 - 12%. When the acetate values were related to glucose levels (A/G), the differences were further increased, up to 80%. This could therefore form a basis for the assessment of compliance to high fibre diets.

Fasting plasma NEFA levels decreased during the high fibre diets, moreso with the sucrose diet. It is thus unlikely that the increase in acetate levels was due to conversion from fatty acids. Interestingly, fasting acetate and NEFAs correlated negatively ($r_s = -0.51$, $p < 0.005$), although fasting NEFA levels in the subjects during diets U and HF were rather high. The relationship of fasting acetate and NEFAs however agrees with previous observations, that acetate suppresses adipose tissue lipolysis (Crouse, Gerson,

DeCarli and Lieber 1968). This reduction in NEFA levels by acetate, a product of fermentation of dietary fibre, may contribute to the well-established beneficial effects of fibre on glycaemic control in diabetics, in accord with the glucose-fatty acid cycle (Randle, Garland, Hales and Newsholme 1963).

The 3hr area under the acetate/time curve was increased significantly only on the sucrose supplemented meals, but principally because the absolute fasting acetate values were highest in subjects consuming the sucrose diets, though they differed significantly only from the usual diet. The mechanism for this effect of sucrose is uncertain. A small amount of sucrose taken as part of single meals (Slama, Haardt, Jean-Joseph, Costagliola, Goicolea, Bornet, Elfrably and Tchobroutsky 1984) or regularly as part of a high fibre, low fat diet (Peterson, Lambert, Gerring, Darling, Carter, Jelfs and Mann 1986) does not worsen glucose tolerance in diabetic patients with good glycaemic control as long as total daily calorie intake is maintained. Indeed, normal male rats ingesting sucrose regularly show improved glucose tolerance and tissue insulin sensitivity (Kergoat, Bailbe and Portha 1987). If this observation were also true in human subjects, it is possible that a sucrose induced increased tissue insulin sensitivity might have caused the reduced NEFA levels seen on that diet and by an improved post-receptor insulin action, an increased acetate formation from intracellular glucose metabolism.

The incremental areas over the fasting values were similar with the 3 meals, suggesting that the observed increase in total area was due to the higher baseline levels. This provides a further indication that fermentation of fibre occurs later than 3hr post-meal.

Of interest is the observation that the trend of changes in IDDM and NIDDM were similar, despite the relatively small numbers, suggesting that acetate absorption and/or metabolism in these two groups of diabetics are probably not different as long as glycaemic control is reasonable, as it was in the study.

CONCLUSION

These results show that fasting plasma acetate levels increase in diabetic subjects consuming mixed high fibre diets, and could be useful in assessing compliance to those diets in those patients, especially when expressed in relation to the fasting glucose values. The results also confirm previous observations that the rise in acetate levels and presumably, onset of colonic fermentation, occurs later than 3hr after meals, which might have been observed directly had these studies been continued up to 6-8 hr post-meal. There was a tendency towards lower NEFA and higher acetate levels on the diets that contained sucrose, probably due to increased insulin sensitivity on those diets. There was an inverse relationship between fasting acetate and NEFA values.

D. BREATH HYDROGEN EXCRETION AND PLASMA ACETATE LEVELS AFTER LACTULOSE IN PATIENTS WITH SUSPECTED MALABSORPTION.

AIMS.

Pomare, Branch and Cummings (1985) measured the acetate and breath hydrogen response to increasing oral lactulose doses in 5 normal subjects, and noted a parallel increase in both variables. The same procedure was therefore re-explored in patients with suspected malabsorption who were to have a lactulose breath test as a routine clinical investigation. The aim was to assess the clinical utility of acetate measurements in conditions where breath hydrogen estimation is routine.

SUBJECTS AND METHODS.

9 (6M) clinically stable patients on follow-up at the gastroenterology unit of the John Radcliffe Hospital, Oxford, were studied. Their age range, clinical features and drug treatment are shown in Table 6.D.1. None was on antibiotics or any other medications known to alter gut flora. All were scheduled for routine lactulose breath testing and were overnight fasted. Blood and breath samples were taken 30min after cannula insertion (fasting) and again every 30min after starting to drink (over 2min) 30ml DUPHALAC (Duphar Ltd) containing per 5ml: 0.3g lactose, 3.35g lactulose and 0.55g galactose. Post-alveolar breath samples were collected into sealed 20ml plastic syringes through air - tight rubber tubes. Analysis for breath hydrogen, usually within 24hr of sample collection, was with a Pye Unicam gas

TABLE 6.D.1 :CLINICAL CHARACTERISTICS OF THE SUBJECTS

	AGE (yr)	SEX	CLINICAL FEATURES	SYMPTOM DURATION (yr)	DRUGS
1	34	M	lactose intolerance,	1	-
2	60	M	post-gastrectomy, intermittent diarrhoea, malabsorption	4	-
3	46	M	Crohn's disease,previous right hemicolectomy and ileal resection	17	prednisolone salazopryn
4	41	M	persistent non-infective diarrhoea ? irritable bowel syndrome	2	cortisone acetate
5	81	F	profuse diarrhoea of unknown aetiology	5	lomotil
6	73	M	diabetic diarrhoea ?autonomic neuropathy	3	chlorpropamide metformin
7	41	F	chronic intermittent diarrhoea	2	-
8	70	F	chronic intermittent diarrhoea	2	-
9	66	M	chronic intermittent diarrhoea	5	-
Mean	56.9			4.6	
SD	16.8			4.9	

chromatograph (column temperature 50°C) calibrated with 20ml of 100 vol/million hydrogen-nitrogen mixture with argon gas used as carrier.

The within-subject differences between time points were compared with the paired Student's t test. Differences between subjects were sought by ANOVA. Spearman rank correlations between plasma acetate and breath hydrogen excretion were estimated for individual subjects and for the whole group.

RESULTS.

There was a rise of varying extent in plasma acetate in all after ingestion of lactulose. In 4, the rise came within the first 30min (Fig 6.D.1). Peak plasma acetate level (mmol/l) was achieved in 8 of the subjects between 150min (0.23 ± 0.12) and 180min (0.23 ± 0.09), both values being significantly different ($p < 0.01$) from fasting values (0.11 ± 0.06) (Fig 6.D.2). One patient (subject 2) peaked at 30min, not surprisingly as she had lost gastric reservoir function from previous gastrectomy and had clinical evidence of intestinal hurry in loose motions containing partially digested food material; she also had the highest percentage post-lactulose acetate rise (Fig 6.D.1). In contrast, subject 3 with previous extensive ileal and colonic resection for Crohn's disease had the lowest fasting level and one of the slowest post-lactulose rises (Fig 6.D.1). In all 9 patients mean post-lactulose plasma acetate levels (0.21 ± 0.09) were significantly higher ($p < 0.025$) than

fasting values (Fig 6.D.3).

Changes in breath hydrogen excretion post-lactulose initially paralleled the observed changes in plasma acetate (Fig 6.D.2). The fasting breath hydrogen (ppm) of 12.7 ± 11.7 rose to 17.4 ± 14.7 (p NS) by 30min and levels peaked at 120min (37.9 ± 17.1) significantly different from fasting values ($p < 0.01$) (Fig 6.D.2). Again, subject 3 with previous extensive gut resection had the lowest fasting (5.0ppm) and mean post-lactulose (13.3ppm) breath hydrogen excretion as well as the slowest rise. Although subject 2 had one of the highest rises in breath hydrogen, the peak level was achieved only at 120min, much slower than the peak observed with plasma acetate (30min).

When all the fasting and post-lactulose acetate and breath hydrogen values of the 9 patients studied were pooled and compared (Fig 6.D.4), the relationship was significant ($rs = 0.39$, $p < 0.01$). Also, when the data was analysed using ANOVA for the regression in individual subjects and in all the subjects pooled together, the relationship was again significant ($p < 0.001$ for the pooled regression line) although the responses of individuals differed ($p < 0.01$) (Fig 6.D.5).

There was no significant difference between fasting and mean post-lactulose levels (mmol/l) of plasma glucose (6.9 ± 1.1 and 7.3 ± 1.5), NEFAs (0.76 ± 0.14 and 0.62 ± 0.10), blood acetoacetate (0.033 ± 0.007 and 0.048 ± 0.017), 3-hydroxybutyrate (0.068 ± 0.030 and 0.096 ± 0.051), glycerol

FIG 6.D.1 : **Plasma acetate profiles in 9 subjects after intake of 20 g lactulose**

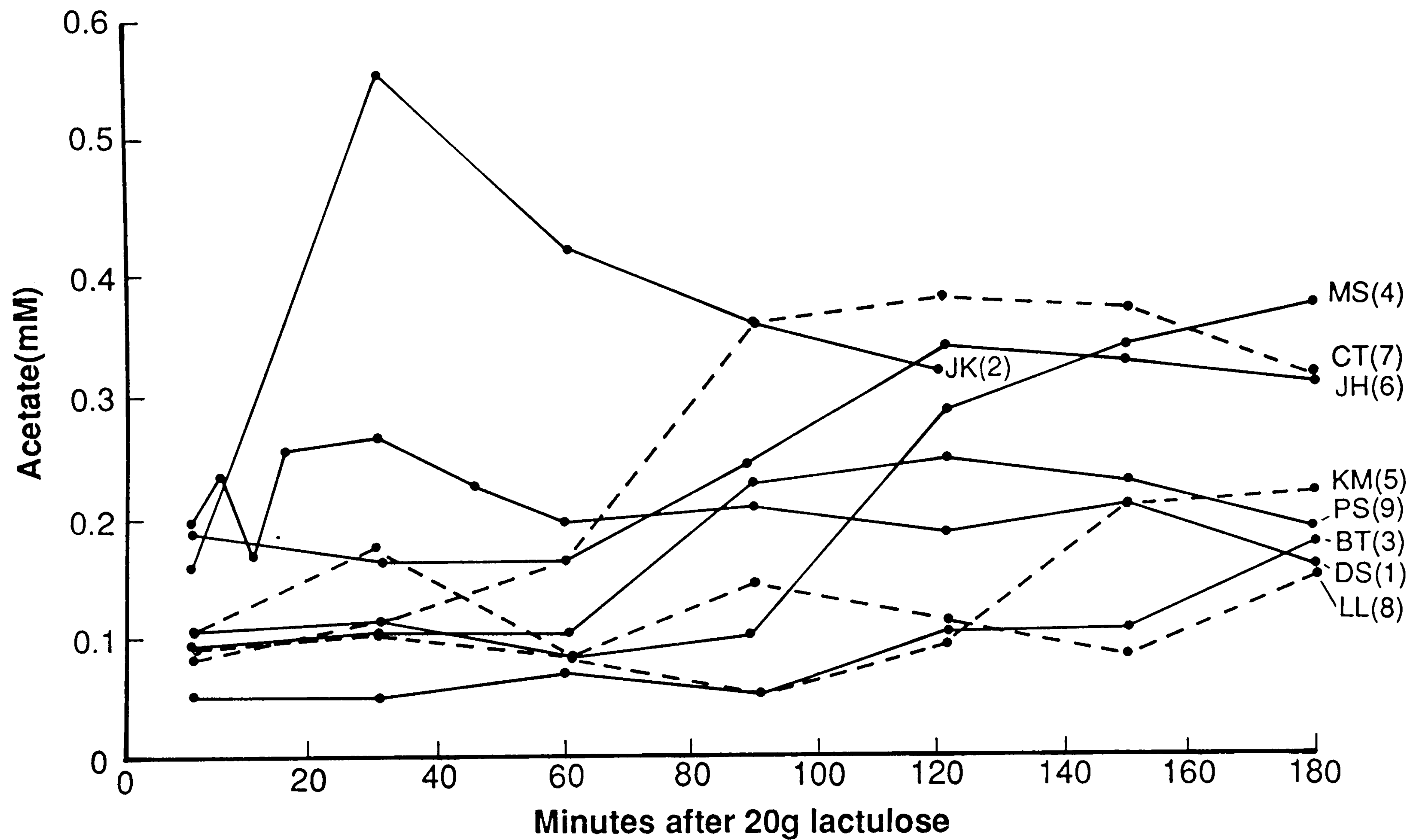
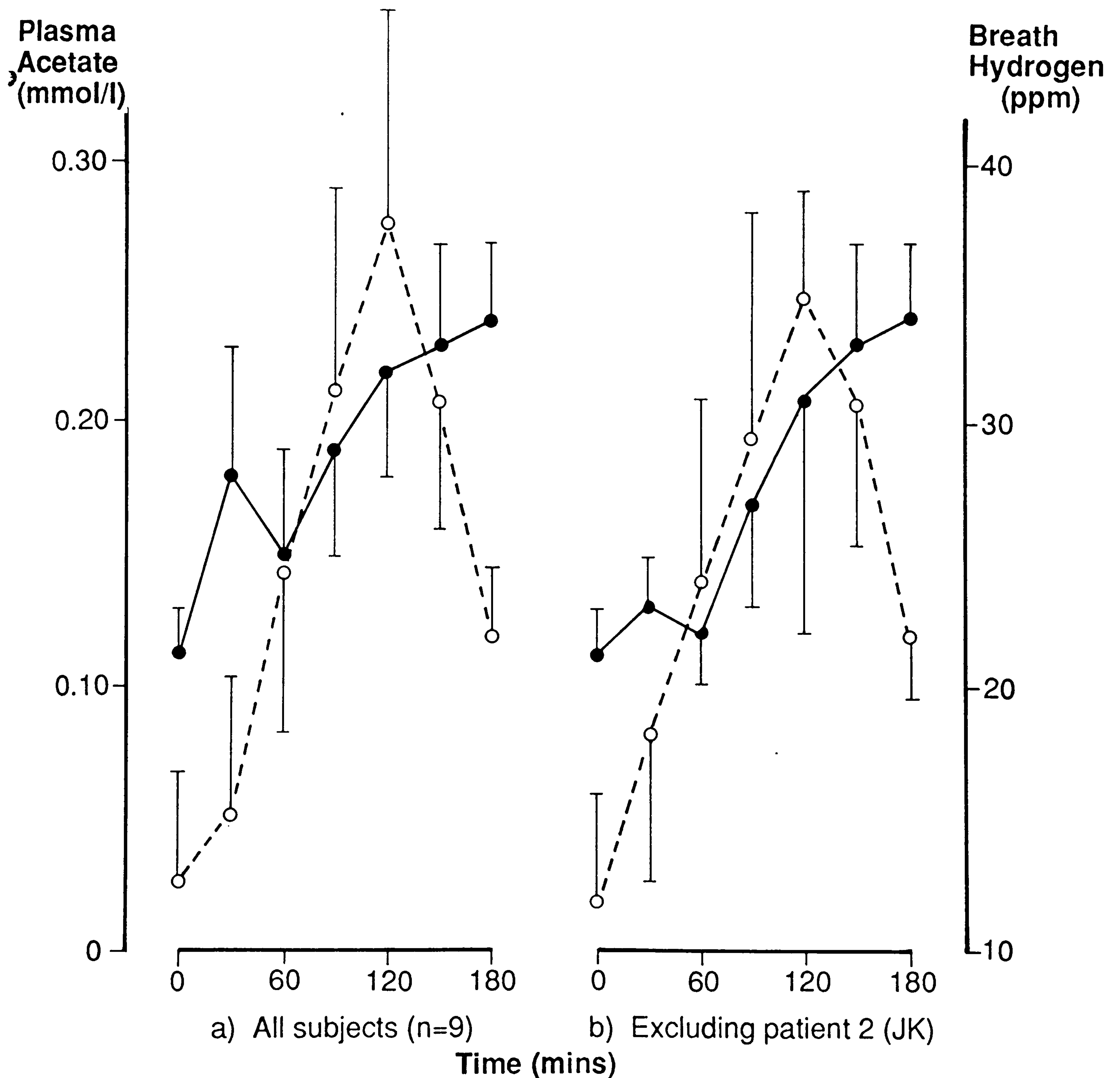


FIG 6.D.2 :

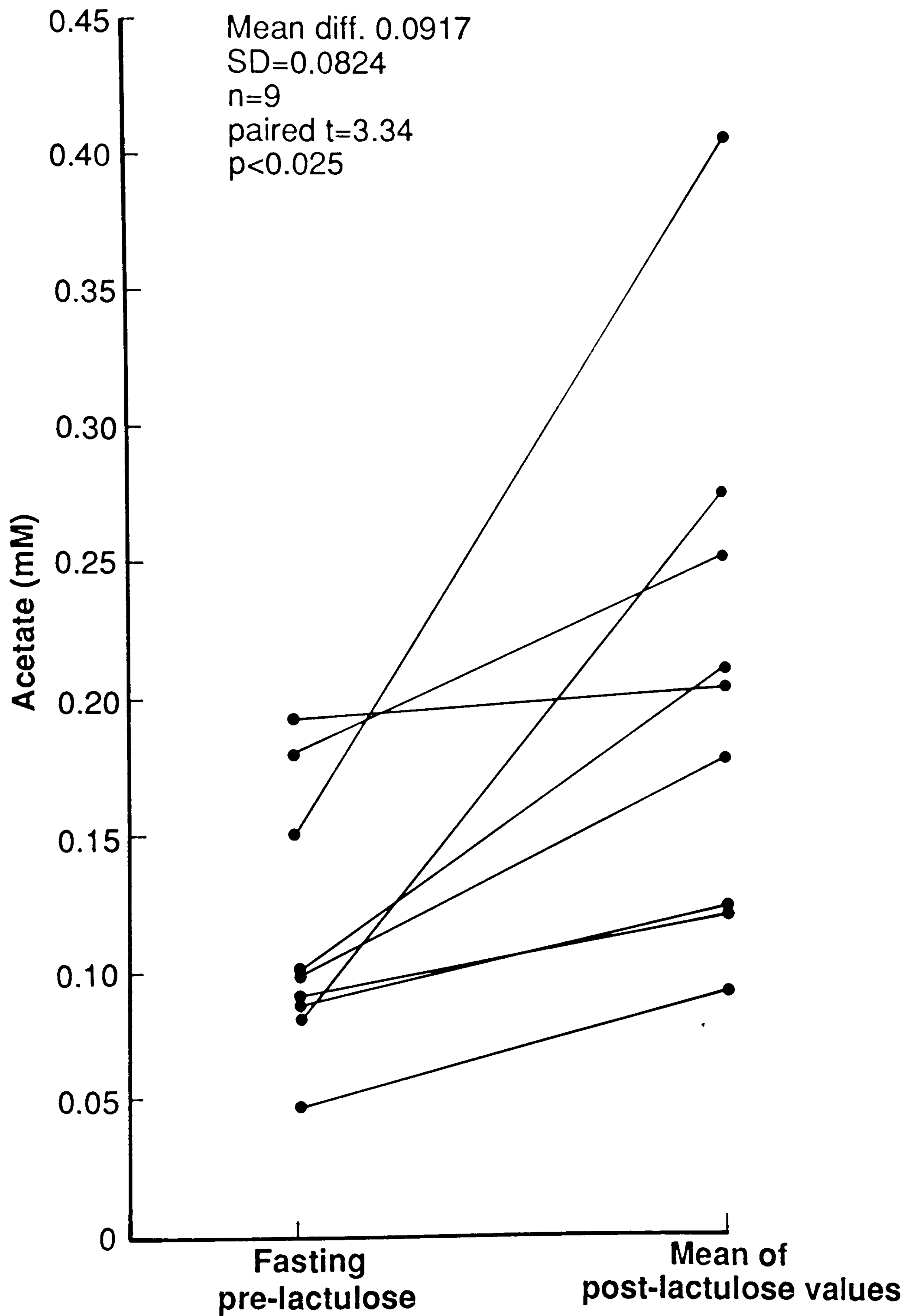
Breath hydrogen excretion and plasma acetate levels after ingestion of 20g lactulose



All time points from 90 min are significantly higher than 0 min value

FIG 6.D.3: **Acetate levels after oral lactulose in the 9 subjects**

Paired t-test pre- v mean post-lactulose acetate



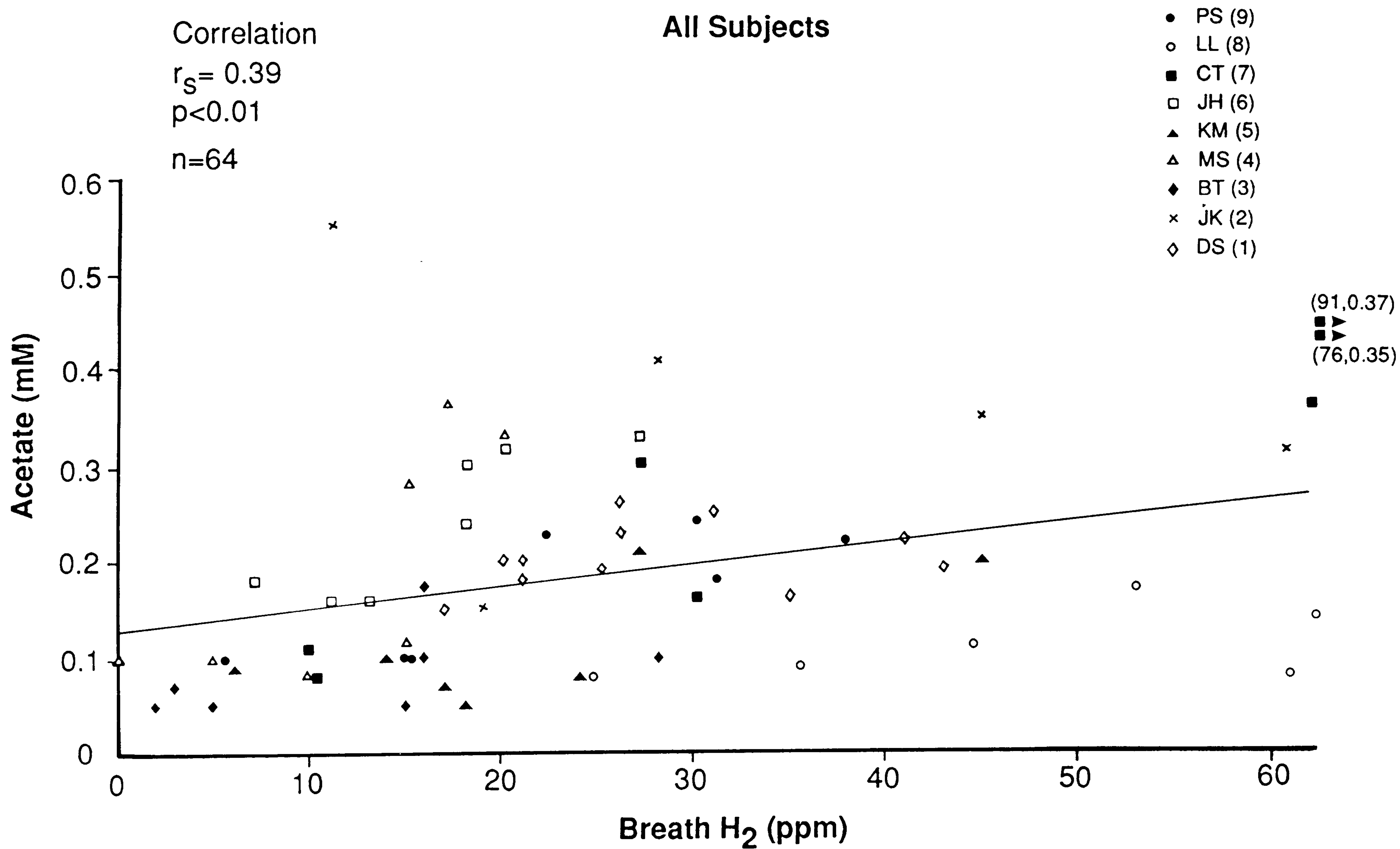
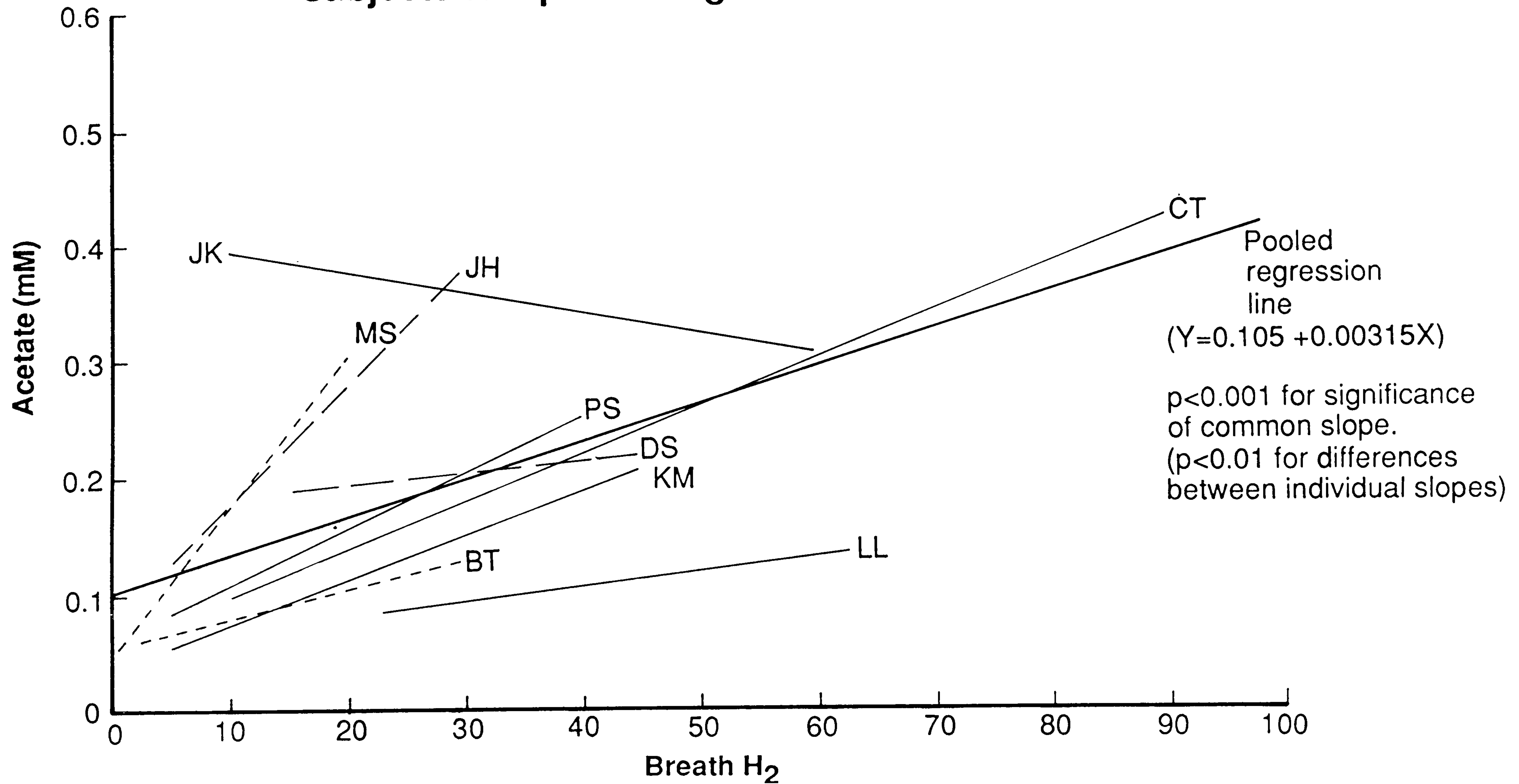


FIG 6.D.4 :
Acetate v H2 Excretion
All Subjects
Correlation
rs= 0.39
p<0.01
n=64
PS (9)
LL (8)
CT (7)
JH (6)
KM (5)
MS (4)
BT (3)
JK (2)
DS (1)
(91,0.37)
(76,0.35)
Acetate (mM)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
Breath H2 (ppm)

FIG 6.D.5 : **Fasting and post lactulose acetate and breath hydrogen - showing individual slopes for 9 subjects and pooled regression line**



(0.092 ± 0.011 and 0.079 ± 0.009), lactate (0.92 ± 0.11 and 0.87 ± 0.10), pyruvate (0.08 ± 0.01 and 0.08 ± 0.01) and insulin mU/l (13.4 ± 3.8 and 13.4 ± 3.4).

DISCUSSION

The lactulose breath test is useful in detecting those cases of malabsorption due to bacterial overgrowth, via an early and prolonged rise in breath hydrogen excretion post-ingestion of lactulose. This test is based on the colonic fermentation of lactulose, a semi-synthetic, indigestible disaccharide, a process which also produces volatile fatty acids. Post-lactulose plasma acetate levels should therefore change in a similar fashion to breath hydrogen. And this was indeed seen in this study.

It was also clear, as in one of the subjects, that the earlier the exposure of lactulose to the colon, e.g. when the reservoir function of the stomach is lost post-gastrectomy, the earlier the rise in acetate. Also, with loss of major portions of the colon, especially the caecum and ascending colon where most fermentation occurs (Mitchell, Lawson, Davies, Kerr Grant, Roediger, Illman and Topping 1985), the production of these fermentation products is reduced.

The substantial rise of both products after 90min confirms that most fermentation occurs in the distal gastrointestinal tract.

In general, the plasma acetate and breath hydrogen values do correlate (Figs 6.D.4, 6.D.5), and the possibility

does exist that one or both can be used for investigation of suspected bacterial overgrowth syndromes. However, the response as measured via the two different metabolic products can differ between subjects (Fig 6.D.5) for, while in 6 of the 9 patients, the acetate and hydrogen values for an individual at the six sampling times correlated significantly ($rs > 0.7$, $p < 0.01$), in 3 there was no such association ($rs < 0.5$, p NS). It may be notable that the 3 patients without a correlation included the post-gastrectomy and hemicolectomy patients as well as the one with lactose intolerance. Thus none of the patients with non-specific diarrhoea (suspected malabsorption) failed to show a significant correlation.

This study has clinical implications. Measurement of plasma acetate is a relatively easily performed biochemical test alternative to breath hydrogen estimation, and could find a place in the investigation of malabsorptive disorders associated with increased bacterial fermentation, but this would require some comparable information on post-lactulose acetate levels in normal controls. From our present point of view, this procedure has potential clinical applicability and efforts are currently underway in that direction.

CONCLUSION

This study shows that patients with suspected malabsorptive syndromes exhibit parallel changes in plasma acetate and breath hydrogen excretion after ingesting

lactulose and reinforces the potential contribution of colonic fermentation to plasma acetate values. The possibility therefore exists for the use of either as a clinical test for quantifying posterior fermentation in humans.

E. THE FORMATION OF ACETATE FROM ETHANOL WITH AND WITHOUT PRIOR CHLORPROPAMIDE INTAKE IN NON-DIABETIC AND DIABETIC SUBJECTS.

AIMS.

It is well known that free acetate is formed by mammalian liver when ethanol is ingested (Lundquist, Tygstrup, Winkler, Mellemgaard and Munck-Petersen 1962, Bode 1978, Korri, Nuutinen and Salaspuro 1985, Nuutinen, Lindros, Hekali and Salaspuro 1985). The process involves an initial oxidation of ethanol to acetaldehyde by the enzyme, alcohol dehydrogenase, and subsequent further oxidation to acetate by aldehyde dehydrogenase. Both enzymes are present in most mammalian tissues, but with the greatest activity in the liver (Bode 1978).

This study attempts to investigate the possible effects of the sulphonylurea drug, chlorpropamide (CP), known to inhibit hepatic aldehyde dehydrogenase (ALDH) activity (Podgainy and Bressler 1968), on acetate levels as measured in human peripheral blood after consumption of moderate doses of ethanol, particularly because of observations previously published in regard to `chlorpropamide alcohol' flushing. This latter group of subjects was studied because of earlier suggestions that the chlorpropamide-alcohol flush (CPAF) had a distinct biochemical basis in increased acetaldehyde levels after taking ethanol (Barnett, Gonzalez-Auvert, Pyke, Saunders, Williams, Dickenson and Rawlins 1981).

SUBJECTS.

6(5M) healthy Caucasian non-diabetic subjects aged 34.7 ± 14.4yr with BMI 24.1 ± 2.9 kg/m^2 volunteered for the study. None had ever experienced any unpleasant facial sensation (flush) on taking ethanol.

5(3M) well controlled non-insulin dependent diabetic patients controlled on dietary measures and CP (age 53.5 ± 7.7yr, BMI 27.5 ± 3.3 kg/m^2, HBAlc 8.4 + 1.3%) and with a definite history of alcohol-induced flushing (hereafter referred to as flushers) were also recruited into the study.

All took 40ml dry sherry (containing 17.5% ethanol), after an overnight fast. Blood specimens were collected fasting and at 10min intervals for 30min. These studies were repeated a week later after each non-diabetic subject took 250mg CP for 5 days, and the diabetic subjects had been without their daily CP for 3 days. All the diabetic subjects flushed on taking ethanol when on CP, but not on taking ethanol alone. The non-diabetic subjects did not flush on either occasion as judged both subjectively and objectively (by cheek colour). None of the subjects took any alcoholic drink during the study period.

Plasma ethanol was measured in the non-diabetic subjects by gas chromatography (Manno and Manno 1978). Similar estimations were not done on the diabetic flushers. Also, plasma chlorpropamide levels were not measured in any of the subjects but compliance of the subjects to the drugs was good, as assessed by telephone calls and direct

questioning.

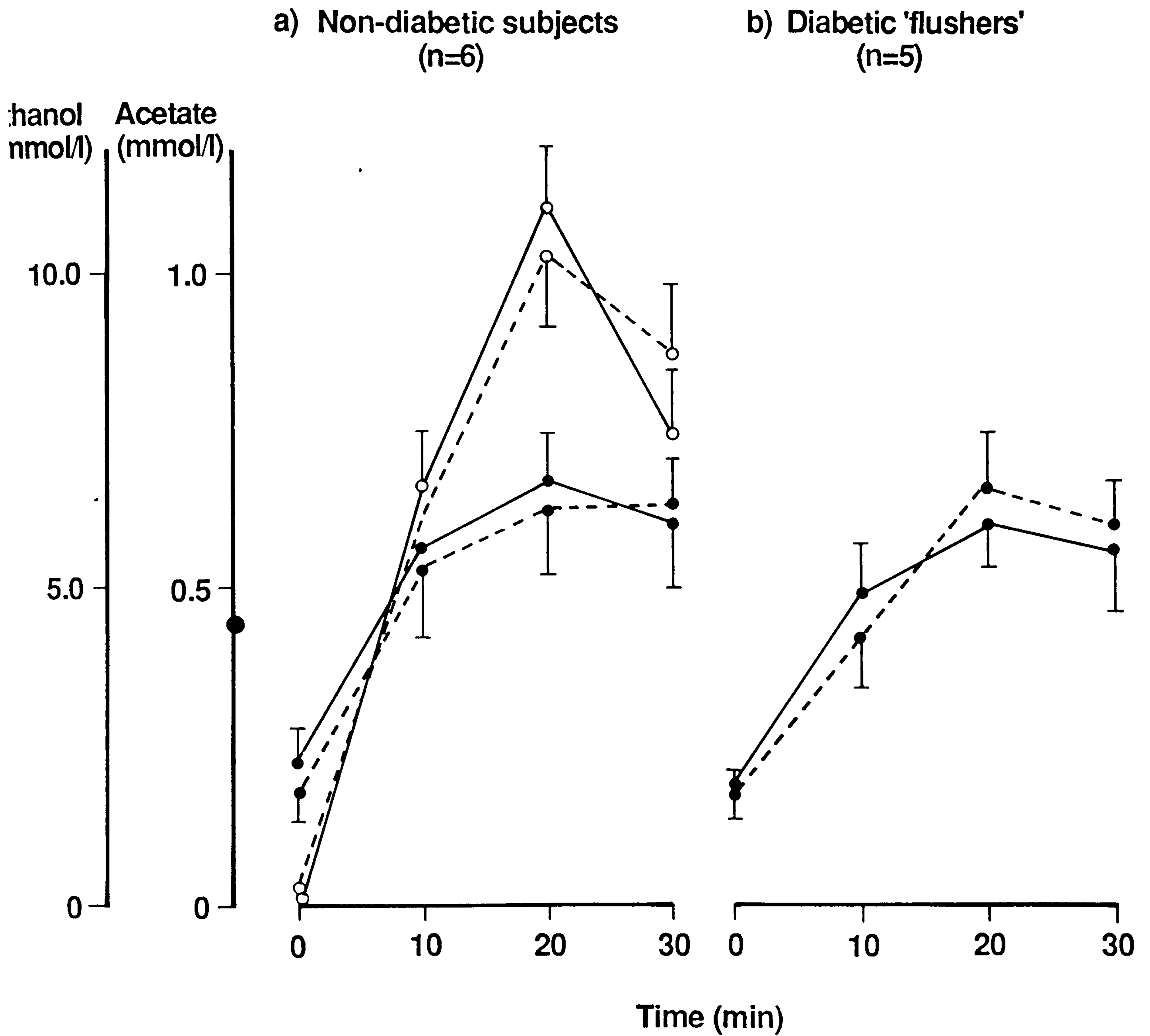
The within-subject comparisons were made by paired Student's t test. The difference in response between the non-diabetic and diabetic subjects was examined by a three-factor repeated measures design ANOVA with one between (diabetes) and two within (treatment and time) variables.

RESULTS.

Before taking CP, the fasting acetate level (mmol/l) in the non-diabetic subjects was 0.22 ± 0.12. The mean increases in acetate and ethanol levels from baseline were respectively 0.38 ± 0.12 and 7.7 ± 3.4 (both $p < 0.001$) over 30min, and mean rises to peak were acetate 0.47 ± 0.14 and ethanol 10.2 ± 6.0 (both $p < 0.001$) after ingesting 7g ethanol in dry sherry. After 5 days of CP, fasting acetate in the same subjects was essentially unchanged at 0.17 ± 0.05. The mean rises in acetate and ethanol levels from fasting over 30min were respectively 0.46 ± 0.14 and 6.6 ± 6.0, and increases to peak respectively 0.56 ± 0.12 and 8.9 ± 7.2 after taking ethanol. These were not significantly different from the changes seen before CP (Fig 6.E.1a). Glucose and insulin levels were essentially unchanged during both studies. There was no significant correlation between acetate and ethanol at any of the time points or during the whole study.

When these studies were repeated in the diabetic 'flushers', similar observations were made, with no difference in acetate levels between the two studies (with

FIG. 6.E.1 :



and without prior CP intake) at any of the time points (fasting 0.18 ± 0.04 v 0.17 ± 0.02 mmol/l, mean increase from fasting over 30min 0.48 ± 0.11 v 0.41 ± 0.09 and to peak, 0.62 ± 0.13 v 0.72 ± 0.18, all p NS) (Fig 6.E.1b). As in the non-diabetic non-flushers, plasma glucose and insulin levels did not change during both studies in the diabetic flushers.

Although these values for mean increase and increase to peak in plasma acetate levels tended to be higher in the diabetic flushers, they were not significantly different from those for the non-diabetic non-flushers.

DISCUSSION

The results confirm previous observations that acetate is produced from ethanol in humans (Lundquist, Tygstrup, Winkler, Mellemgaard and Munck-Petersen 1962, Korri, Nuutinen and Salaspuro 1985, Nuutinen, Lindros, Hekali and Salaspuro 1985). The rise in acetate levels was not influenced by prior CP medication in both the non-diabetic subjects and the diabetic flushers, an observation of some interest.

CPAF remains controversial (Hillson and Hockaday 1984, Johnston, Wiles and Pyke 1984, Waldhausl 1984). While its aetiology is still speculative, the liability to flushing has been related to plasma chlorpropamide concentration (Hillson, Smith, Dhar, Moore and Hockaday 1983), increased post-ethanol acetaldehyde (Jerntorp, Ohlin, Bergstrom and Almer 1980), reduced hepatic ALDH activity (Podgainy and

Bressler 1968), genetic susceptibility (Wolff 1972) and in NIDDM, some protection against microangiopathy (Ohlin, Jerntorp, Bergstrom and Almer 1982). Central to all these possibilities is ALDH whose inhibition would result in elevated acetaldehyde levels, and whose isoenzymes (Murray and Motulsky 1971) could explain the genetic influences. Thus, a raised post-ethanol level of acetaldehyde was proposed as a marker for liability to flushing, since it differentiated `flushers' from `non-flushers' (Barnett, Gonzalez-Auvert, Pyke, Saunders, Williams, Dickenson and Rawlins 1981). However, reduced ALDH activity should also result in decreased formation of acetate when ethanol is taken with CP.

The results obtained in this study are not in keeping with that hypothesis although obviously concerned with plasma concentrations and not total acetate production. Acetate levels were not influenced by CP intake in both the 'non-flushers' and the 'flushers'. If acetaldehyde levels were raised in the flushers, as they might well be, any inhibition of ALDH activity did not affect acetate formation. This could be due to several factors. Raised acetaldehyde levels may have saturated a concentration dependent inhibition of ALDH. The possibility of other pathways of acetaldehyde metabolism also exists. Aldehyde oxidases, capable of converting acetaldehyde to acetate, are present in mammalian liver and capable of aldehyde conversion to acetate (Bode 1978) and it is conceivable that

these enzymes are activated in conditions where ALDH activity is reduced. In any case, ALDH is not at the rate limiting step of ethanol oxidation as alcohol dehydrogenase activity more consistently correlates with ethanol oxidation rates (Lindros, Vihma and Forsander 1972). Otherwise, as proposed by Hillson, Ting, Smith, Yajnik, Crabbe and Hockaday 1987, ALDH may not be consistently inhibited by CP.

CONCLUSION

This study suggests that plasma acetate has no value as a marker for chlorpropamide alcohol flushing.

CHAPTER 7

ACETATE UTILIZATION IN HUMANS

A. LITERATURE REVIEW.

It is likely that healthy humans are as able to use acetate as are the other non-ruminant mammals. The early studies in humans were on uraemic patients undergoing acetate haemodialysis. Acetate oxidation accounted for 40-45% of total caloric expenditure during acetate haemodialysis (Skutches, Sigler, Teehan, Cooper and Reichard 1983) when the level of plasma acetate reached during dialysis was 10-20 times (Mansell and Wing 1983) that in the normal individual on a typical Western diet. Thus, although acetate metabolism may be relatively insignificant as an energy source in healthy humans, that contribution is probably increased in people on high fibre diets who may well be exposed to higher levels of absorbed acetate.

Using infusions of radioactive acetate in healthy men, Skutches, Holroyde, Myers, Paul and Reichard (1979) found that plasma acetate has a rapid turnover rate of 5-8μmol/(min.kg), which correlated negatively with age and positively with the initial plasma concentration. They also showed that about 90% of this turnover was immediately oxidised to carbon dioxide. Richards, Vreman, Zager, Feldman, Blaschke and Weiner (1982) have shown that acetate concentrations fell rapidly on termination of a 4mmol/kg body weight sodium acetate infusion with kinetic modelling consistent with a two-compartment model and first order elimination from the first compartment. The calculated

acetate metabolic clearance rate was 2.31/min. Kveim and Nesbakken (1980) also infused acetate (0.5mol/l mixture of 0.25mol/l sodium acetate and 0.25mol/l acetic acid to pH 4.7) for 60min at 4mmol acetate/kg body weight/hr and observed that the rapid decline of plasma acetate levels post-infusion followed an exponential curve with a plasma half-life of 8min and elimination rate constant of 0.09/min.

These observations suggest that acetate is rapidly metabolised in healthy humans, and this may account for its relatively low circulating level. Indeed, Lundquist (1962) estimated that acetate can be utilized in humans at rates up to 300mmol/hr (1500 kcal/day).

a. Metabolism.

The acetate metabolising enzymes in Man, acetyl CoA synthetase and acetyl CoA hydrolase, are probably similar to those in other non-ruminant mammals, and, as already discussed (Chapter 5), interact with the various pathways for carbohydrate and lipid metabolism. Few specific studies have however been done in healthy humans.

Richards, Vreman, Zager, Feldman, Blaschke and Weiner (1982) showed that there was a significant increase in blood levels of pyruvate, lactate and 3-hydroxybutyrate during acetate infusions in humans, and that this change, with the reduced plasma levels of potassium, calcium and inorganic phosphate, was due to the associated alkalosis, since acetate metabolism generates equimolar amounts of

bicarbonate. Acetate administered intravenously to diabetic subjects reduced the splanchnic glucose output and peripheral glucose concentration and simultaneously increased splanchnic ketone body production and peripheral blood ketone levels, effects also seen with bicarbonate treatment. These observations suggest that the effects were due to alkalosis, rather than the acetate PER SE (Lipsky, Alper, Rubini, Van Eck and Gordon 1954). Surprisingly, in these studies, non-diabetic subjects did not show any of the described changes with acetate or bicarbonate.

Seufert, Mewes and Soeling (1984) reckoned NEFAs to be an important source of plasma acetate in humans. While studying fatty acid metabolism in groups of normal-weight and obese subjects after overnight fasting or more prolonged starvation, they found that up to 5% of turnover of oleate was converted to acetate and about 40% to ketone bodies. There was a significant increase in acetate turnover in long-term starvation, due to the increased fatty acid mobilisation.

There are only few studies on the effect of hormones on acetate metabolism in humans. From its effect on the metabolism of glucose and lipids, insulin should influence acetate use. Acetate was shown to suppress growth hormone secretion in uraemic subjects during haemodialysis and infusion studies by unknown mechanisms, and it is uncertain whether that effect also occurs in non-uraemic subjects (Schmitz, Hansen, Hansen, Orskov and Alberti 1982). Adrenal

corticosteroids may also influence human acetate metabolism since Hennes (1962) demonstrated that adrenal insufficiency was associated with defective synthesis of lipids from acetate and impaired oxidation of acetate to carbon dioxide, effects reversed by adequate steroid replacement therapy. This action of steroids was ascribed to diminished oxidation of reduced nucleotides, a continuous supply of which is necessary for TCA cycle activity (Gallagher 1960).

b. Acetate Metabolism in Human Diabetes.

Acetate levels in diabetic plasma tend to be higher than in non-diabetic subjects (Seufert, Grigat, Poppe and Soling 1978, Smith, Humphreys and Hockaday 1986). In diabetics, fasting plasma acetate correlates positively with blood glucose, lactate and acetoacetate, the strongest association being with glucose (Smith, Humphreys and Hockaday 1986). Despite some contribution to this elevated acetate level from the high dietary fibre intake encouraged in diabetes, the relationship of acetate with glucose and its increase in states of poor glycaemic control and ketogenesis, suggest that most of the excess acetate was from endogenous metabolism.

c. Acetate and Alcoholism.

The plasma acetate level is elevated in chronic alcoholism, and has been suggested as the most sensitive and specific laboratory marker for that condition (Korri,

Nuutinen and Salaspuro 1985).

Studies on Uraemic Subjects.

Since Mion, Hegstrom, Boen and Scribner (1964) observed that acetate is readily converted to bicarbonate in Man, acetate solutions have been increasingly used as haemodialysates (Mansell and Wing 1983). Lewis, Tolchin and Roberts (1980) showed that 90% of acetate administered during haemodialysis is converted to bicarbonate, and that only a small amount of the infused acetate was available for alternative metabolic pathways. These alternative channels are mainly towards lipid synthesis, hence the recent controversy regarding the use of acetate dialysing fluids in elderly patients and diabetics, who already were predisposed to atherosclerotic vascular disease, and in whom further lipid synthesis and deposition could be deleterious (Henrich, Woodward, Meyer, Chappell and Rubin 1983, Mansell and Wing 1983)

Various studies have shown only inconsistent effects of acetate dialysis on blood lipids. Most of the administered acetate load is oxidised to carbon dioxide (Rorke, Davidson, Guo and Morin 1977, Gonzalez, Pearson, Garbus and Holbert 1974, Morin and Davidson 1984/85, Davidson, Rorke, Guo and Morin 1978). While some workers found only a small incorporation of the radioactivity from radiolabelled acetate into lipids of plasma and other tissues (Davidson, Rorke, Guo and Morin 1978, Rorke, Davidson, Guo and Morin

1977, Port, Easterling and Barnes 1979, Kobayashi, Okubo, Marumo and Nakamura 1983), others demonstrated significant acetate incorporation into lipids (Morin and Davidson 1984/85). Total cholesterol and LCAT activity were reduced on acetate as compared to bicarbonate dialysis, although HDL-cholesterol levels remained low during both types of treatment without any effects on levels of apoproteins A and C (Kobayashi, Okubo, Marumo and Nakamura 1983).

a. Metabolism.

The acetate loads during haemodialysis usually approach 5.0mmol/min, the calculated limit of Man's ability to metabolise it. Gonzalez, Pearson, Garbus and Holbert 1974 documented a significant elevation in blood lactate late in acetate dialysis and suggested that acetate follows the anaerobic glycolytic pathway in a retrograde fashion. Glucose-free acetate dialysis was shown to cause a marked decrease in the blood level of glucose, insulin, lactate and pyruvate with an increase in blood "ketone bodies" (Wathen, Keshaviah, Hommeyer, Cadwell and Comty 1978), changes not seen with glucose dialysis. This suggested that the oxidation of acetate and fatty acids increased to meet energy demands, and the increased rate of gluconeogenesis and glycogenolysis acted to prevent critical hypoglycaemia during glucose-free dialysis. Alternatively, the excess acetyl CoA formed from the excess acetate might have been shunted directly into ketogenesis.

Aside from this metabolic effect of acetate during haemodialysis, recent evidence suggests that acetate in peritoneal dialysis fluids may mediate peritoneal inflammatory responses. Human blood monocytes incubated IN VITRO in the presence of sodium acetate produced more interleukin 1 than in the presence of sodium chloride or incubating medium alone, and this at acetate concentrations similar to those encountered on the surface of haemodialysis membranes (Bingel, Lonnemann, Koch, Dinarello and Shaldon 1987) . As interleukin-1 mediates inflammation and promotes fibrosis, its increased production with acetate dialysis with a consequent likelihood to unrestrained inflammation may make acetate undesirable in haemodialysis. This is however not borne out in clinical practice.

b. Tolerance Studies.

As in non-uraemic Man, acetate elimination post-dialysis follows first-order elimination kinetics (Vreman, Assomull, Kaiser, Blaschke and Weiner 1980, Rorke, Davidson, Guo and Morin 1977, Davidson, Rorke, Guo and Morin 1978). The mean half-life of acetate elimination after termination of dialysis was unaffected by uraemia in dogs, although non-uraemic dogs had a faster rate of oxidation of radioactive acetate than the uraemic ones (Davidson, Rorke, Guo and Morin 1978). The situation in Man is probably similar.

c. Studies in Diabetic Uraemic Subjects.

Avram, Lipner, Sadiqali, Iancu and Gan (1976) studied the effect of acetate haemodialysis in diabetic uraemic patients and observed that growth hormone levels fell during dialysis, although growth hormone itself was not dialysable, consistent with the report in nondiabetic uraemic subjects by Schmitz, Hansen, Hansen, Orskov and Alberti (1982). The effect on the other blood "intermediary metabolites" was not reported in those studies.

B. EXPERIMENTAL SECTION

SPECIFIC AIMS

Acetate was infused into human subjects and its kinetic parameters determined under steady state conditions. Estimates of oxidation rates of fat and carbohydrate with and without available acetate were made from the respiratory exchange measurements in order to assess the likely role of acetate in the total energy expenditure.

The metabolic effect of infused acetate was also assessed in non-diabetic and diabetic subjects and in uraemic patients during haemodialysis.

C. ACETATE INFUSIONS IN NON-DIABETIC AND DIABETIC SUBJECTS.

AIMS

The purpose of the present experiments was to determine the metabolic consequences of an acute acetate infusion and the kinetics of acetate utilization in non-diabetic and diabetic subjects. The effect of injected glucose on acetate tolerance was also investigated.

Indirect calorimetry was performed in order to obtain quantitative data on the net rates of carbohydrate and fat oxidation with and without available acetate. An assessment of these rates was made in the subjects after an overnight fast when non-protein energy expenditure was expected to be derived from a combination of fat and carbohydrate oxidation, and again after acetate infusion, when the infused acetate should contribute to total energy needs.

MATERIALS AND METHODS

Protocol

3 studies (1a,b and 2) were performed on each subject : Experiment 1 consisted of two intravenous infusions, an experimental (a) and a control (b), which were performed on each subject. The two infusions were separated by an interval of 1week, and the order of infusions was randomized. The experimental infusion consisted of 2.5mmoles/min isotonic sodium acetate in a volume of 1litre, and was administered over 60min, after which an ivGTT (with 20g glucose/m^2 body surface area) was performed. The control

infusion consisted of 2.8mmoles/min isotonic sodium bicarbonate in 1litre over 60min again followed by the ivGTT. Bicarbonate was chosen as the control infusion because it causes similar changes in blood electrolytes and acid-base balance to acetate. The initial experiments used isotonic saline infusions as control, but because of the different anionic metabolism compared to acetate, it was difficult to implicate acetate rather than alkalosis in the metabolic effects observed. This change in control infusate during the course of the study also accounted for the slight difference in sodium loads administered with the acetate and bicarbonate infusions. Initially, 150mmol/l saline and acetate solutions were used, but the only commercially available sodium bicarbonate solution with a sodium concentration similar to that hitherto used was 167mmol/l; this was thus utilised as the control infusion as it was reckoned that the 17mmol/l difference in sodium concentration should not significantly influence observations. Indeed, as shown later, plasma sodium levels did not change significantly with either the acetate or bicarbonate infusate.

Each infusion was administered by a microdrip infusion device (Braun Melsungen, W Germany). The acetate infusions were done prior to the ivGTT for several reasons : (i) it was reckoned that, in the physiological state, any acetate influence on glucose tolerance would be due to elevated acetate levels from a previous 'high fibre' diet, since fermentation occurs only about 6hr after that meal; (ii)

previous studies in our laboratory (Thompson, 1985, personal communication) showed that acetate simultaneously infused with meals had no effect on post-meal glucose tolerance; (iii) with this protocol, it was possible to assess acetate tolerance with and without glucose.

Experiment 2 consisted of an infusion of 2.5mmoles/min isotonic sodium acetate in 1litre over 60min after an initial rest period of at least 90min. Respiratory exchange measurements were done during the last 60min of the rest period and throughout the acetate infusion period. Respiratory quotient (RQ) and energy expenditure were calculated from the oxygen intake (VO_2) and carbon dioxide production (VCO_2) in the last 15min of each study period, when these values were considered steady.

Isotonic 1.4% (167mmol/l) sodium bicarbonate was obtained from Polyfusor, Boots, Nottingham, U K. Sodium acetate was supplied by the Central Sterile Supply Department of the Oxford Area Health Authority as 10ml ampoules of strong sodium acetate solution (2.5mmole Na/ml), and 60ml of this was diluted in sterile water for injection (Braun Melsungen, W Germany) to 1litre and a final concentration of 150mmol/l acetate, verified at intervals by our enzymatic assay for acetate.

Subjects

9 healthy, male, non-diabetic University students (age 25.5 ± 4.2yr and BMI 22.5 ± 2.7kg/m^2) and 6 male non-insulin dependent diabetic subjects with stable glycaemic control

(age 60.3 ± 7.5yr, BMI 24.9 ± 1.4kg/m^2 and HBAIc 8.1 ± 1.3%) on small daily doses of oral sulphonylureas, volunteered for the studies. All had normal values for routine full blood counts, plasma electrolytes and liver function tests. Also, plasma thyroxine (non-diabetics 93.7 ± 15.7nmol/l, diabetics 98.2 ± 18.5nmol/l) and random plasma cortisol (non-diabetics 401 ± 211nmol/l, diabetics 354 ± 174nmol/l) were within normal limits. All the diabetics and 6 non-diabetics completed the 3 studies.

Sample collection

For Experiment 1, baseline blood samples were obtained prior to each infusion. Further samples were then taken at 20, 40 and 60min of infusion, and subsequently at 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 and 60min after glucose injection. During Experiment 2, baseline blood specimens were again obtained as in (1), and then also at 20min intervals during the infusion and respiratory exchange measurements. Subsequently, further samples were taken at 2, 5, 10, 15, 20, 30, 40, 50 and 60min post-infusion for the determination of the acetate elimination rate. Overnight urine samples were collected prior to Experiment 2, and also at the end of each infusion.

For these studies, arterialised venous blood was obtained from a dorsal hand vein, warmed by a hot air fan and kept patent by regular flushing with isotonic saline. Arterialised venous blood gas (pO_2, pCO_2, base excess and percentage saturation) measurement was done on a Radiometer

Copenhagen ABL 2 gas meter calibrated daily with standard solutions.

Analysis

With the acetate load infused (2.5mmoles/min), steady state plasma acetate levels were achieved by 40min (Fig 7.C.1), and subsequent elimination post-infusion was by first-order kinetics, as previously demonstrated in sheep (Jarrett and Filsell 1960, 1961), dogs (Ciaranfi and Fonnessu 1954, Harper, Neal and Hlavacek 1953) and humans (Kveim and Nesbakken 1980, Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982). The metabolic clearance rate for acetate (MCR, ml/kg.min) was calculated from the ratio : Acetate Infusion Rate (mmol/kg.min)/Steady State Incremental Acetate Concentration (40-60 min) (mmol/ml). The assumptions made were that (i) the endogenous production of acetate was suppressed at this infusion rate; (ii) the metabolic clearance of acetate was independent of the circulating acetate concentration over the range of plasma levels seen (< 2mmol/l), although it approaches saturation at higher concentrations (Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982); (iii) the clearance of infused acetate is similar to that of endogenous acetate.

The elimination rate constant (Kac) was the regression slope of the exponential fitted to the disappearance curve of acetate post-infusion from 0-60min, and plasma half-time (min) was calculated as 0.693/(Kac). The Acetate Turnover Rate (μmol/min.kg) was derived from the product of the

fasting acetate concentration (μmol/ml) and the MCR (ml/min.kg). The elimination rate constant for glucose (K_G) was calculated by the method of Conard, Franckson, Bastenie, Kestens and Kovacs (1953).

Energy expenditure was measured using indirect calorimetry. The volunteers breathed room air through a small Rudolph valve which had a dead space of 16ml. Inspiratory volume was measured with a Parkinson-Cowan dry gas meter. Expired air was passed through a mixing chamber, where it was analysed for percentage oxygen and carbon dioxide using a Servomex 540A paramagnetic oxygen analyser (accuracy $\pm$ 0.02%) and a Gould Mark IV infra-red capnograph (accuracy $\pm$ 0.05%) respectively. The gas analysers were calibrated before, during and immediately after each test with 3 gases of known composition. The values for VO_2 and VCO_2 were calculated from inspiratory volume, mixed expired gas concentrations, known room air gas concentrations, barometric pressure and temperature. RQ was calculated as VCO_2/VO_2 and total energy expenditure (kJ) which includes the contribution from protein oxidation, was calculated from the formula (Elia, Zed, Neale and Livesey 1987) :

$$kJ/O_2 \text{ (litres)} = 15.480 + 5.550 \text{ (RQ)}.$$

Carbohydrate and lipid oxidation rates were calculated from the respiratory gas exchange measurements (Frayn 1983) with the assumption that the daily urinary nitrogen excretion in all the subjects was 8g/day and also that about 90% of infused acetate was immediately oxidised. These assumptions

and others are justified in the `Results' and `Discussion' sections.

Areas under the log insulin/time curve were calculated by the trapezoidal rule. The results were compared by paired and unpaired Student's t tests. A three factor repeated measures design analysis of variance with one between (diabetes) and two within (infusate and time) variables was also performed. In the non-diabetic and diabetic subjects, the relationships between age and BMI and the kinetic parameters of acetate utilization were examined by Spearman rank correlation coefficients.

RESULTS

All the subjects tolerated the infusions well, with no subjective symptoms. Serial pulse and blood pressure measurements remained essentially unchanged.

An important effect of the infusions was haemodilution, indicated by the plasma albumin concentration which fell in all (Table 7.C.1). After correcting for the haemodilution in each case from the change in plasma albumin concentration (about 10%), there were no significant post-infusion changes in the plasma levels of sodium, potassium, chloride, creatinine, urea and total calcium.

With both infusions however, plasma bicarbonate levels increased significantly in the two groups of subjects as expected, while inorganic phosphate decreased (Table 7.C.1). The magnitude and trend of these changes were similar with both infusions, and in the non-diabetics and diabetics.

,E 7.C.1: PLASMA LEVELS OF ALBUMIN, CALCIUM, PHOSPHATE AND BICARBONATE BEFORE AND AFTER ACETATE AND BICARBONATE INFUSIONS IN NON-DIABETIC AND DIABETIC SUBJECTS.
(means ± SD).

	Albumin (g/l)	Calcium+ (mmol/l)	Phosphate (mmol/l)	Bicarbonate (mmol/l)
DIABETIC ECTS (n=6)				
cetate				
Infusion	40.8 ± 3.0	2.20 ± 0.03	1.0 ± 0.1	24.4 ± 3.2
-Infusion	36.1 ± 3.1*	2.15 ± 0.06	0.8 ± 0.1*	25.9 ± 2.9*
icarbonate				
Infusion	40.0 ± 2.6	2.24 ± 0.10	1.0 ± 0.2	24.6 ± 2.4
-Infusion	33.8 ± 1.8*	2.25 ± 0.16	0.8 ± 0.1*	29.0 ± 2.9*
ETIC ECTS(n=6)				
cetate				
Infusion	37.2 ± 8.1	2.13 ± 0.30	0.9 ± 0.2	22.0 ± 1.6
-Infusion	34.6 ± 5.7*	2.15 ± 0.20	0.8 ± 0.2*	27.3 ± 1.6*
icarbonate				
Infusion	39.4 ± 4.5	2.23 ± 0.10	0.9 ± 0.2	22.4 ± 3.2
-Infusion	34.6 ± 5.7*	2.17 ± 0.10	0.8 ± 0.1*	30.6 ± 3.8*

* $p < 0.05$ compared to pre-infusion value.

+ post-infusion total calcium values adjusted for the change in albumin concentration by the method of Berry, Gupta, Turner and Burns (1973).

In all the subjects (diabetic and non-diabetic), arterialised venous blood pH increased from 7.41 ± 0.02 to 7.46 ± 0.01 ($p < 0.01$) on acetate infusions. pO_2 and pCO_2 remained unchanged, and the base excess (mmol/l) increased from 0.40 ± 0.55 to 3.14 ± 0.79 ($p < 0.01$); these changes were consistent with a compensated metabolic alkalosis. The mean oxygen saturation obtained with the arterialised venous blood before and after acetate infusions was about 92%. Blood gases were not analysed during the bicarbonate infusions, but it is likely that any changes would be similar to the observations during acetate infusions.

i. Effect on Other Blood Metabolites

These are shown in Table 7.C.2 and Figs 7.C.2 - 7.C.7. Plasma glucose fell significantly only during acetate infusion in non-diabetic subjects. The blood levels of glycerol and NEFAs decreased during acetate infusions (Table 7.C.2) in the non-diabetic and diabetic subjects. This change was obvious within 20min of infusion and the values had usually returned to fasting levels by the end of the 60min infusion. In contrast, the levels of these two metabolites tended to rise during the bicarbonate infusions. Although the fasting NEFA levels in the diabetic subjects for the bicarbonate studies appeared higher than for the acetate studies, that difference was not statistically significant.

The levels of lactate and pyruvate increased in the non-diabetic subjects during acetate infusion, although they

TABLE 7.C.2:EFFECTS OF ACETATE AND BICARBONATE INFUSIONS ON BLOOD METABOLITES IN NON-DIABETIC AND DIABETIC SUBJECTS.

(means ± SD)

	CONCENTRATION (mmol/l)					
	lactate	pyruvate	total ketone bodies'	glycerol	NEFA	Glucos
A. Non-diabetic subjects (n=6)						
i. Acetate						
Before.	0.62(0.24)	0.063(0.017)	0.039(0.008)	0.086(0.026)	0.31(0.16)	4.8(0
After	0.82(0.26) *	0.072(0.025)	0.051(0.015) *	0.065(0.026) *	0.28(0.09) *	4.5(0
ii. Bicarbonate						
Before.	0.68(0.29)	0.070(0.020)	0.036(0.021)	0.050(0.021)	0.23(0.12)	4.8(0
After.	0.65(0.14)	0.064(0.012)	0.060(0.027) *	0.066(0.030)	0.38(0.19) *	4.8(0
B. Diabetic subjects (n=6)						
i. Acetate						
Before.	1.11(0.38)	0.116(0.042)	0.043(0.016)	0.068(0.020)	0.35(0.07)	7.1(2
After.	1.13(0.48)	0.125(0.037)	0.054(0.014)	0.053(0.018) *	0.27(0.09) *	6.8(1
ii. Bicarbonate						
Before.	1.37(0.54)	0.113(0.039)	0.047(0.023)	0.054(0.022)	0.54(0.23)	6.8(1
After.	0.96(0.44) *	0.103(0.045) *	0.065(0.028) *	0.055(0.028)	0.63(0.20) *	6.5(1

* $p < 0.05$ compared to fasting value.
Before : fasting value
After : mean of values at 20, 40 and 60min.

remained essentially unchanged in the diabetics; conversely, the values fell during bicarbonate infusions. There was thus no significant change in the lactate/pyruvate ratio with either infusate. Total KBs rose in all (diabetic and non-diabetic) whichever the infusion, but the 3-hydroxybutyrate/acetoacetate ratio was unaltered. The trend of changes in all these metabolites (including glycerol and NEFAs) was essentially similar after glucose injection whichever the previous infusion.

There was no consistent change in the plasma level of insulin during these infusions in the diabetic subjects. In the non-diabetics however, there was a fall in insulin levels during both acetate (insulin levels (mU/l) fasting, 8.3 $\pm$ 2.2, mean (over infusion period) of 6.7 $\pm$ 1.9, $p < 0.02$), and bicarbonate (9.2 $\pm$ 1.7 to 6.0 $\pm$ 3.0, $p < 0.05$) infusions (Fig 7.C.8).

Plasma GH levels (mU/l) were essentially unchanged during acetate infusions in the non-diabetic (fasting 3.2 $\pm$ 1.1 to mean over infusion period 3.0 $\pm$ 1.1, p NS) and diabetic (8.3 $\pm$ 8.7 to 4.3 $\pm$ 2.1, p NS) subjects; but while levels again remained unchanged in the diabetic subjects during bicarbonate infusions (7.6 $\pm$ 6.1 to 6.1 $\pm$ 4.5, p NS), there was a fall with this infusion in the non-diabetic subjects (3.0 $\pm$ 1.0 to 2.4 $\pm$ 1.1, $p < 0.01$) (Fig 7.C.9).

ii. Kinetics of Acetate Utilization

In the first 20 min of the acetate infusion, the plasma acetate concentration (mmol/l) rose rapidly from 0.22 $\pm$ 0.01

to 1.12 ± 0.02 ($p < 0.001$) in the non-diabetic subjects and 0.29 ± 0.02 to 1.36 ± 0.02 ($p < 0.001$) in the diabetics. During the remainder of the infusion (Fig 7.C.1), plasma acetate rose more slowly reaching 1.38 ± 0.20 and 1.38 ± 0.22 at 40 and 60min respectively in the non-diabetic subjects, and 1.60 ± 0.29 and 1.65 ± 0.30 in the diabetics at the same times. While these values were significantly higher than the baseline ($p < 0.0001$) and 20min ($p < 0.01$) values, they were not significantly different from each other, suggesting that plasma acetate concentrations were approaching a steady state. Levels subsequently fell rapidly for the first 30min following cessation of the infusion, and then diminished more gradually for the remainder of the study (Fig 7.C.1).

Plasma acetate concentrations did not change significantly during the bicarbonate infusions.

The MCR for acetate (ml/min.kg) in the non-diabetic subjects was 50.4 ± 14.9, higher than seen in the diabetic subjects, 25.0 ± 6.5 ($p < 0.001$). Also, Kac (/min) was higher in the non-diabetic than the diabetic volunteers (0.031 ± 0.003 v 0.026 ± 0.004, $p < 0.01$) and half-time (min) consequently lower (22.6 ± 2.2 v 27.2 ± 3.8, $p < 0.01$). The calculated turnover rate (μmol/min.kg) in the non-diabetic subjects, 8.56 ± 3.65 was also, as expected, higher than observed for the diabetic subjects, 4.92 ± 1.03 ($p < 0.01$) (Table 7.C.3). None of these parameters of acetate utilization correlated significantly with either age or BMI in the diabetic and non-diabetic

TABLE 7.C.3: KINETICS OF ACETATE UTILIZATION IN NON-DIABETIC AND DIABETIC SUBJECTS.

(means ± SD)

	MCR (ml/min.kg)	Basal Turnover Rate (µmol/min.kg)	Kac (min^{-1})	Half-time (min)
Non-diabetic subjects (n=9)	50.4 ± 14.9	8.56 ± 3.65	0.031 ± 0.003	22.6 ± 2.2
Diabetic subjects (n=6)	25.0 ± 6.5*	4.92 ± 1.03*	0.026 ± 0.004*	27.2 ± 3.8*

* $p < 0.01$ compared to non-diabetic subjects

subjects.

iii. Effect of Glucose on Acetate Tolerance

With available glucose, Kac was reduced in all the subjects: diabetic; 0.026 ± 0.004 to 0.021 ± 0.004, $p < 0.001$; non-diabetic subjects; 0.031 ± 0.003 to 0.025 ± 0.004, $p < 0.001$ (Fig 7.C.10). The half-time (min) of acetate was therefore prolonged in both groups of subjects (diabetics to 34.0 ± 7.6, non-diabetics to 27.9 ± 3.9, both $p < 0.001$). These results suggest that the rate of acetate utilization is reduced when glucose is simultaneously available.

iv. Effect of Acetate on Glucose Tolerance

Intravenous glucose tolerance, as assessed by the rate constant, K_G, did not change when non-diabetic and diabetic subjects were infused either with acetate (respectively 2.35 ± 0.71 and 0.78 ± 0.19) or bicarbonate (respectively 2.17 ± 0.84 and 0.71 ± 0.24), all p NS (Fig 7.C.11). There was also no consistent change in post-glucose plasma insulin levels attributable to either infusate. In the non-diabetic subjects, no significant differences were seen in the areas under the log insulin/time curve (mU.min/l) during the first phase (0-10min), second phase (10-60min) or total (0-60min) after glucose injection (Table 7.C.4). The incremental areas from fasting at these various times were also not significantly different. In the diabetic subjects however, although the various total areas differed, being

TABLE 7.C.4: AREAS UNDER LOG PLASMA INSULIN/TIME CURVES DURING INTRAVENOUS GLUCOSE TOLERANCE TESTS AFTER ACETATE AND BICARBONATE INFUSIONS IN NON-DIABETIC AND DIABETIC SUBJECTS.
(means ± SD)

	AREAS (mU.min/l)					
	A	B	C	Δ A	Δ B	Δ C
NON-DIABETIC SUBJECTS (n=6)						
Acetate	42.2(2.1)	167.3(8.0)	209.5(8.1)	27.7(4.7)	80.4(12.4)	142.7(31.3)
HCO_3	44.3(5.1)	185.4(5.2)	229.7(5.4)	29.8(4.5)	112.9(30.4)	105.5(17.0)
DIABETIC SUBJECTS (n=6)						
Acetate	31.3(8.0) *	163.8(32.5) *	195.1(39.9) *	9.5(7.6)	54.8(25.1)	64.3(24.3)
HCO_3	28.3(7.4)	151.3(34.1)	179.6(41.1)	9.9(6.5)	59.3(25.7)	69.2(31.9)

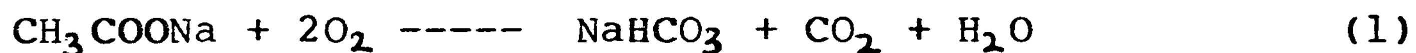
A : first phase insulin (0 - 10 min)
B : second phase insulin (10 - 60 min)
C : total insulin (0 - 60 min)
ΔA : incremental area over fasting for A
ΔB : incremental area over fasting for B
ΔC : incremental area over fasting for C

* p< 0.05

consistently higher with acetate, (Table 7.C.4), the incremental areas were essentially similar indicating that the changes seen with the total areas merely reflected differing fasting levels.

v. Respiratory Exchange Measurements

These were done during the rest period and again during acetate infusion. The values used in these calculations were those obtained during the last 15min of each period, at which time steady state levels of plasma acetate had been established and the rate of utilization was thus similar to the infusion rate. Daily urinary nitrogen excretion was assumed to be 8g (Rafelson, Hayashi and Bezkorovainy 1980, Elia, Zed, Neale and Livesey 1987). It was also assumed that under steady state conditions, about 90% of acetate turnover was immediately oxidised. This appears justified by studies on : (i) bicarbonate generation from infused acetate reflecting acetate utilization according to the equation :



$$CH_3COONa + 2O_2 \text{-----} NaHCO_3 + CO_2 + H_2O \quad (1)$$

during haemodialysis in humans against 30mmol/l acetate dialysate with acetate infusion rates of about 50µmol/min.kg and arterial acetate levels approaching 5mmol/l (Lewis, Tolchin and Roberts 1980, Vreman, Assomull, Kaiser, Blaschke and Weiner 1980, Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982); (ii) the contribution of radioactive acetate to carbon dioxide production during haemodialysis in uraemic humans (Rorke, Davidson, Guo and Morin 1977, Morin and Davidson 1984/85); (iii) non-uraemic humans using primed

continuous infusion of 75-100μCi of 1-^{14}C-acetate, 2.5mCi/mmol specific activity over 6hr (Skutches, Holroyde, Myers, Paul and Reichard 1979); (iv) uraemic and non-uraemic dogs during haemodialysis against 39.5mmol/l acetate dialysate with arterial acetate levels reaching 2.6mmol/l (Davidson, Rorke, Guo and Morin 1978). Similar observations have been made in ruminants e.g sheep, using radioactive acetate infusions (Pethick, Lindsay, Barker and Northrop 1981).

The calculated values for the total energy expenditure (kJ) at rest in the fasting state did not differ significantly from values obtained during acetate infusion in the non-diabetic and diabetic subjects (Table 7.C.5). The basal energy expenditure correlated significantly with BMI in both groups of subjects (non-diabetics: rs 0.94, $p < 0.005$; diabetics: rs 0.89, $p < 0.05$), and with age only in the diabetic subjects (rs 0.89, $p < 0.05$); but it happened in this group that age and BMI correlated significantly (rs 0.99, $p < 0.001$).

The changes in the RQ and oxidation rates of carbohydrate and fat at rest and during acetate infusion are shown in Table 7.C.5. The equations used in the calculations were (Frayn 1983) :

$$c = 4.55 \ VCO_2 - 3.21 \ VO_2 - 2.87 \ n \qquad (2)$$

$$f = 1.67 \ VO_2 - 1.67 \ VCO_2 - 1.92 \ n \qquad (3)$$

where c = carbohydrate oxidation rate (g/min), f = fat oxidation rate (g/min) and n = urinary nitrogen excretion rate (g/min). Since 1mole sodium acetate (82g) consumes

TABLE 7.C.5 :EFFECT OF ACETATE INFUSION ON ENERGY EXPENDITURE AND SUBSTRATE OXIDATION RATES IN NON-DIABETIC AND DIABETIC SUBJECTS. (means ± SD).

	RQ	c (g/min)	f (g/min)	Energy Expenditure (kJ)
NON-DIABETIC SUBJECTS (n=6)				
rest	0.87 ± 0.06	0.16 ± 0.08	0.05 ± 0.04	5.06 ± 1.27
acetate infusion	0.77 ± 0.03*	0.15 ± 0.05	0.02 ± 0.03*	5.47 ± 1.47
DIABETIC SUBJECTS (n=6)				
rest	0.89 ± 0.08	0.17 ± 0.10	0.02 ± 0.02	4.34 ± 0.86
acetate infusion	0.78 ± 0.05*	0.16 ± 0.05	0.01 ± 0.02*	4.54 ± 0.69
ALL SUBJECTS (DIABETIC AND NON-DIABETIC) (n=12)				
rest				4.70 ± 1.10
acetate infusion				5.01 ± 1.19

* $p < 0.05$ compared to rest value.

c carbohydrate oxidation rate

f fat oxidation rate

2moles oxygen (44.8litres) and produces 1mole carbon dioxide (22.4litres) as shown in equation (1), it is possible to calculate VO_2 and VCO_2 due to 90% oxidation of sodium acetate infused at 2.5mmol/min (or 0.205g/min). In calculating `c' and `f' during acetate infusion, the values of VCO_2 and VO_2 attributable to complete oxidation of 90% of the infused acetate were subtracted from total VCO_2 and VO_2 to give the `non-acetate' corrected values for these variables. These latter values were then applied to the above equations (2) and (3) to derive `c' and `f' in the presence of acetate and also to calculate a `non-acetate' RQ. The `acetate' RQ was derived from the uncorrected VO_2 and VCO_2 values obtained during acetate infusion.

It seemed clear that fat oxidation was reduced almost to zero when acetate was infused, although the rate of glucose oxidation was essentially constant in both the normal and diabetic subjects. Indeed, fat oxidation contributed only minimally to total oxygen uptake when acetate was available. Acetate accounted for about 40% of the total oxygen uptake in the diabetic and non-diabetic subjects (Fig 7.C.13) close to the findings of 50% in normal and diabetic dogs (Harper, Neal and Hlavacek 1953) and 20-30% in the rat hind-quarter (Karlsson, Fellenius and Kiessling) but lower than the 80% uptake reported for rat heart (Randle, England and Denton 1977).

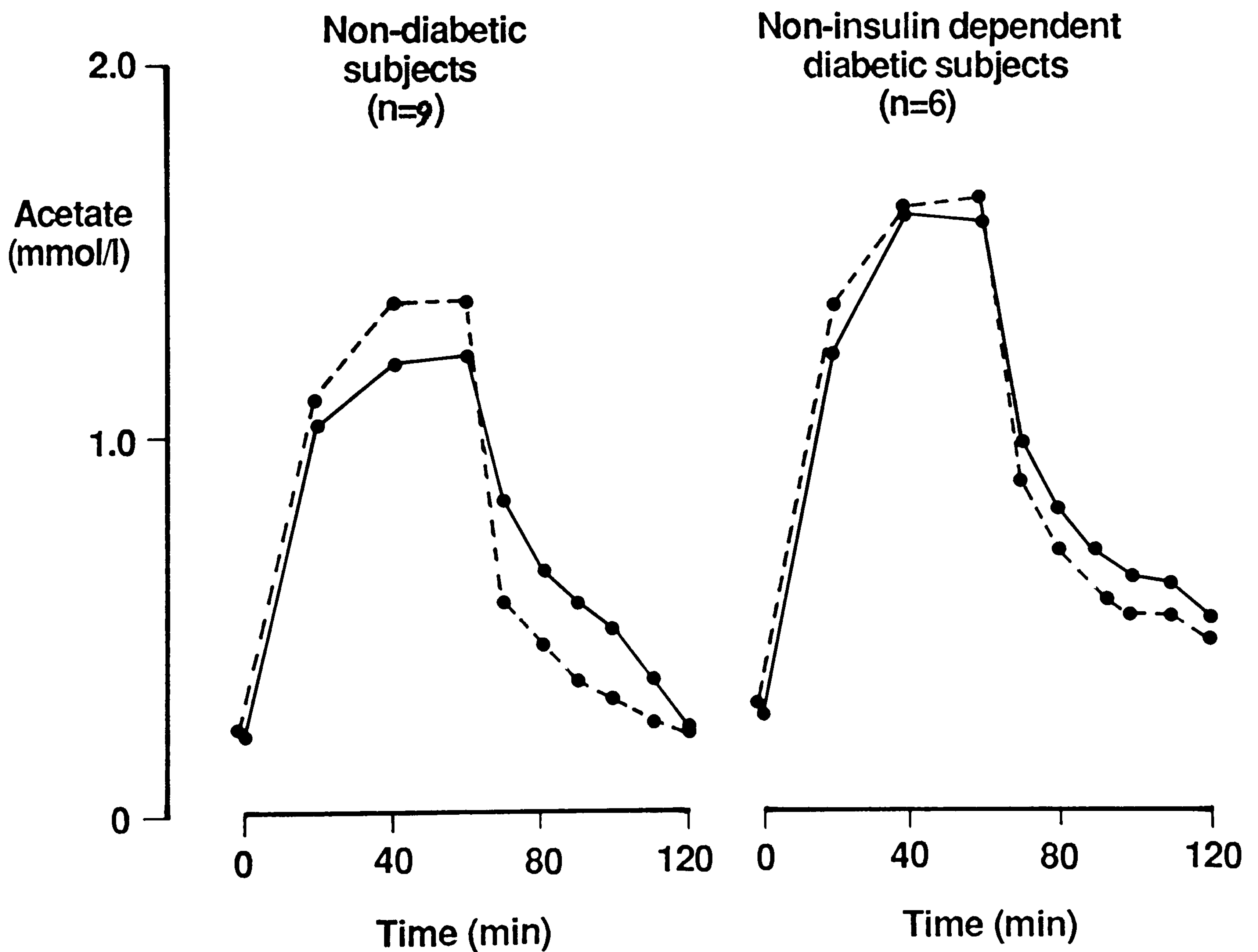
The `non-acetate' RQ in the non-diabetic subjects during the acetate infusion was increased from resting

values to 0.95 ± 0.09 (p NS), but higher than 'acetate' RQ of 0.77 ± 0.03 ($p < 0.001$). Similarly in the diabetic subjects, 'non-acetate' RQ was raised to 1.03 ± 0.13 ($p < 0.01$ compared to the rest value). These observations suggest that when acetate is available, most of the non-acetate oxidation was from carbohydrate, and furthermore that there may be net lipid synthesis from carbohydrate as judged from the high RQ values obtained.

This lipid synthesis is unlikely to be from the infused acetate because of the reduction in RQ when acetate is infused (from equation (1) of acetate oxidation above, theoretical RQ for acetate oxidation is 0.5, much lower than for fat (0.7) and glucose (1.0)), especially as lipid synthesis from acetyl CoA (and presumably acetate) has a calculated RQ of about 6.0 (Frayn 1983). The gross changes in RQ with acetate infusions in the subjects are shown in Fig 7.C.12 and Table 7.C.5.

FIG 7.C.1 :

Plasma acetate concentration during infusion of sodium acetate (150 mmol/hr) for 60 min with and without bolus glucose at 60 min.



Broken lines : iv glucose not given
Solid lines : iv glucose at 60 min
• mean value

FIG 7.C.2 :

Plasma glucose levels during acetate and bicarbonate infusions.
(means ± SD)

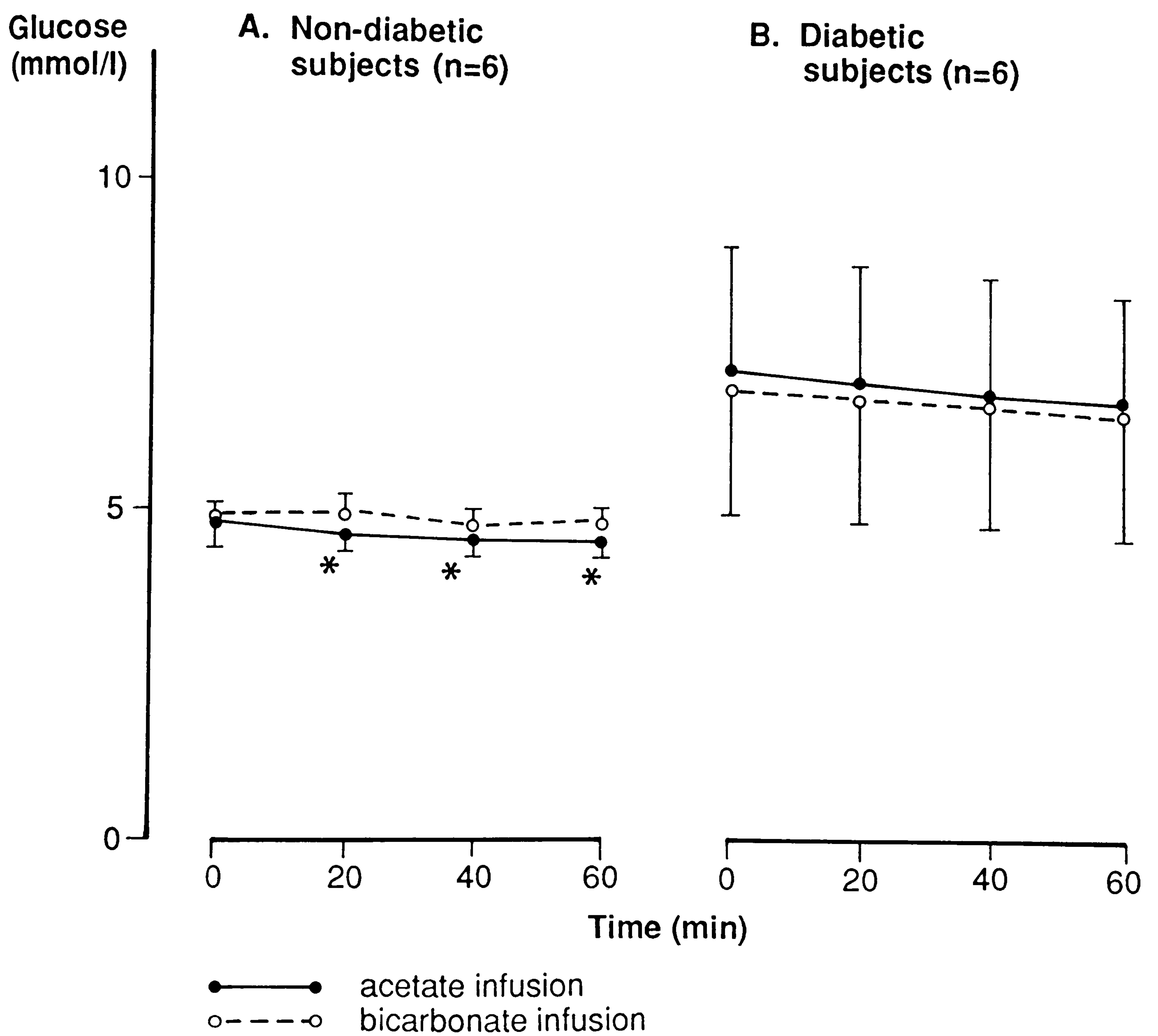


FIG 7.C.3 :

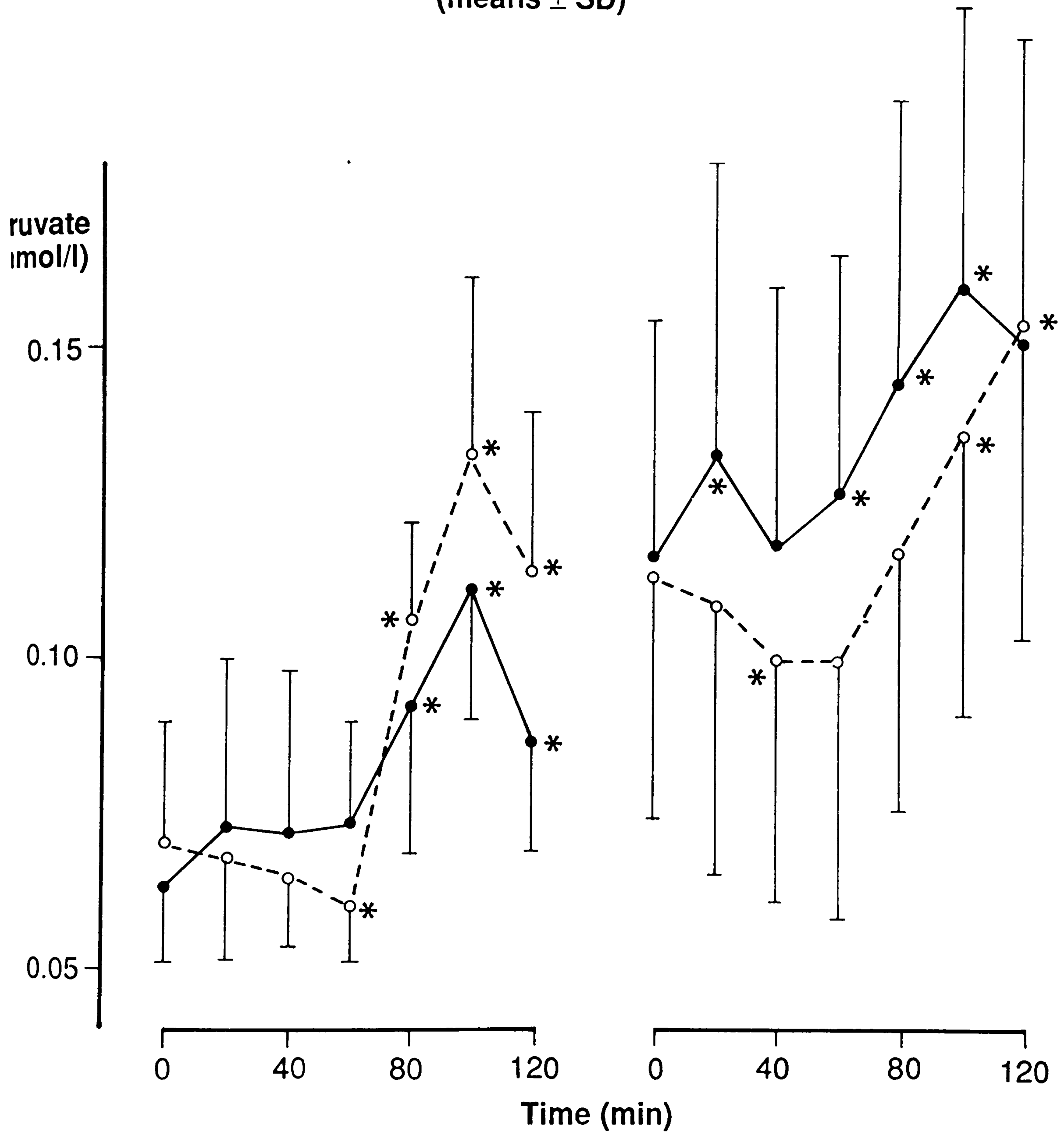


FIG 7.C.4 :

Lactate levels during acetate and bicarbonate infusions with iv glucose at 60 min.

(means ± SD)

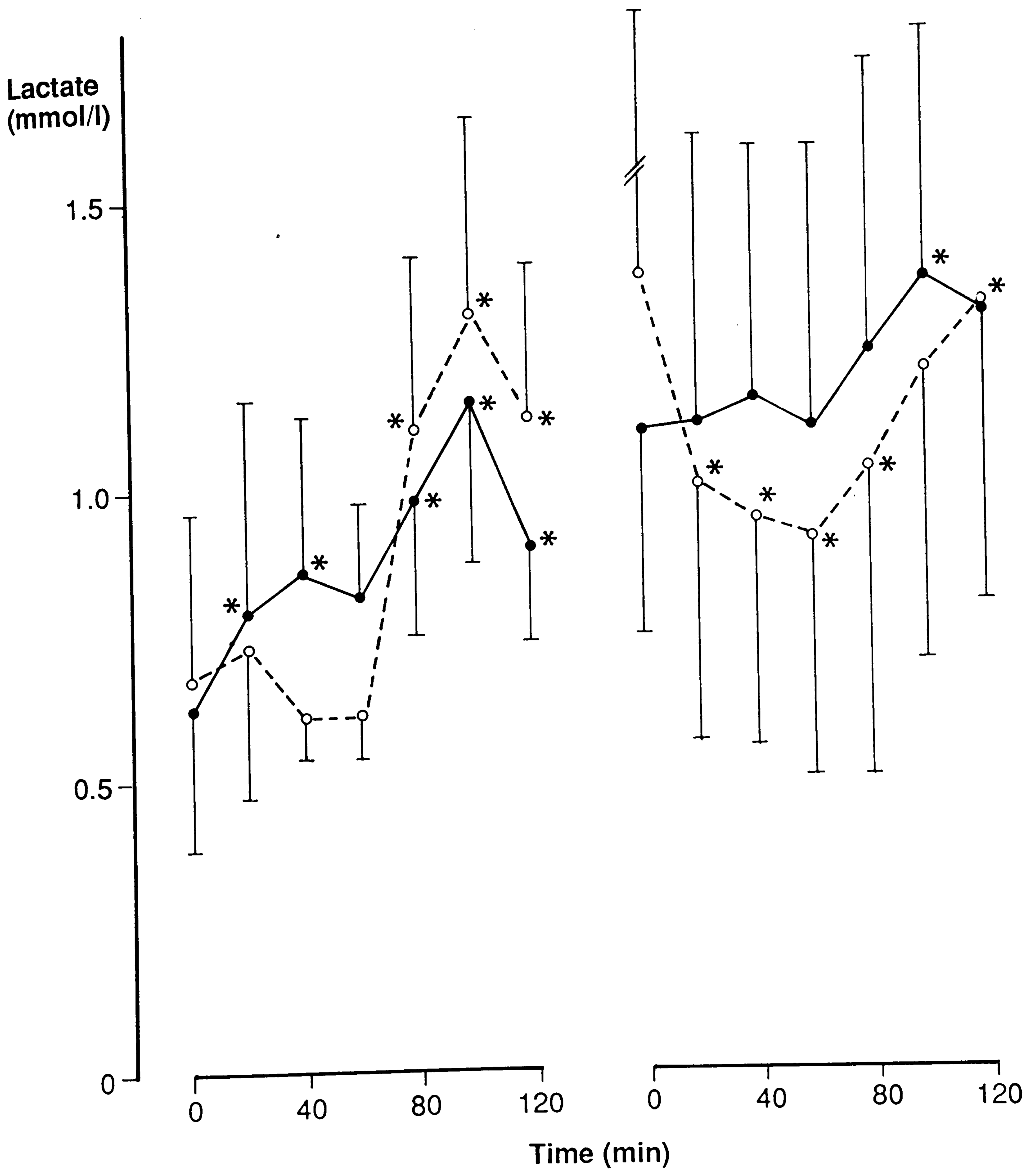
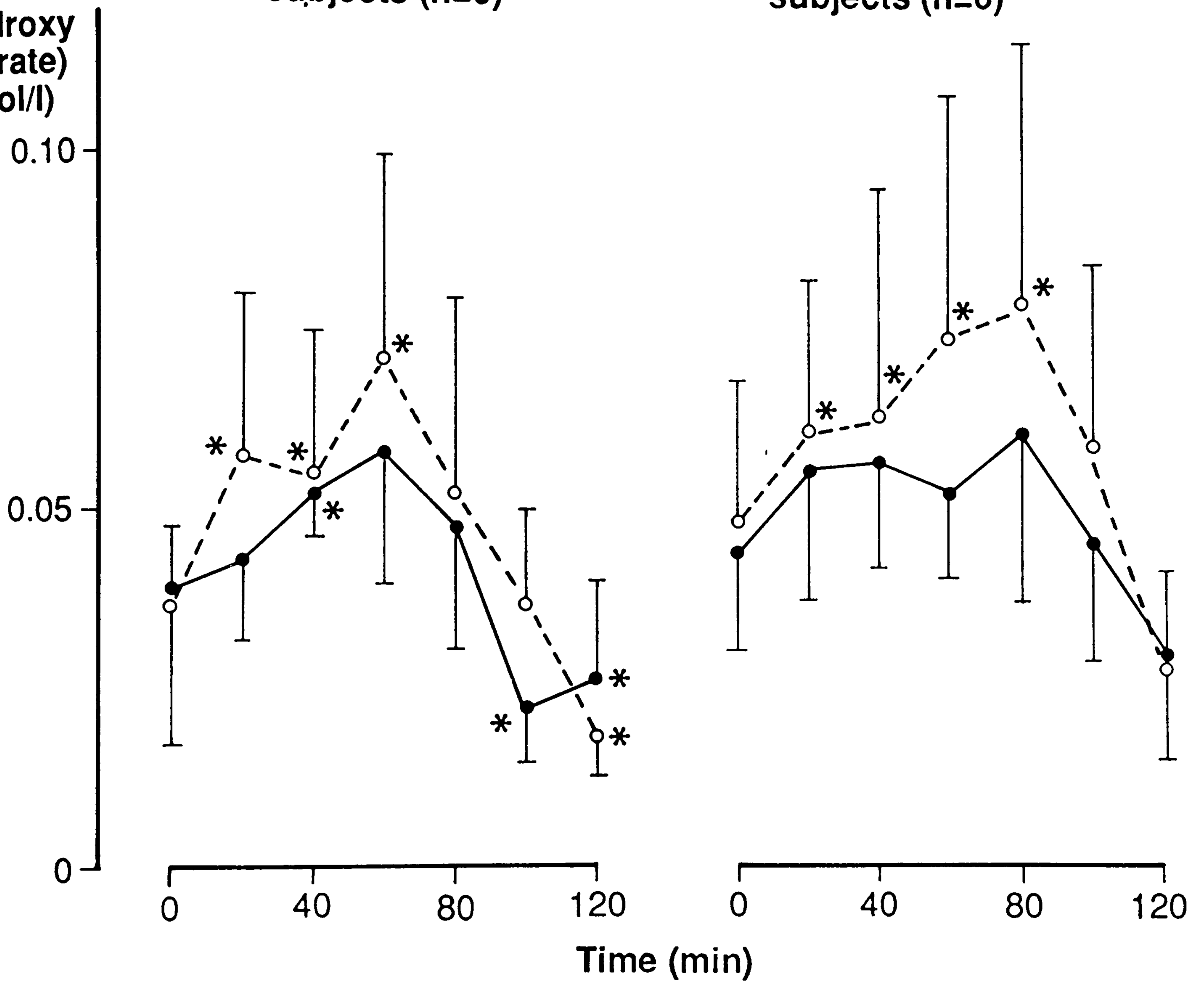


FIG 7.C.5 :

Levels of total ketone bodies during acetate and bicarbonate infusions with iv glucose at 60 min.

(means ± SD)



●——● acetate infusion to 60 min

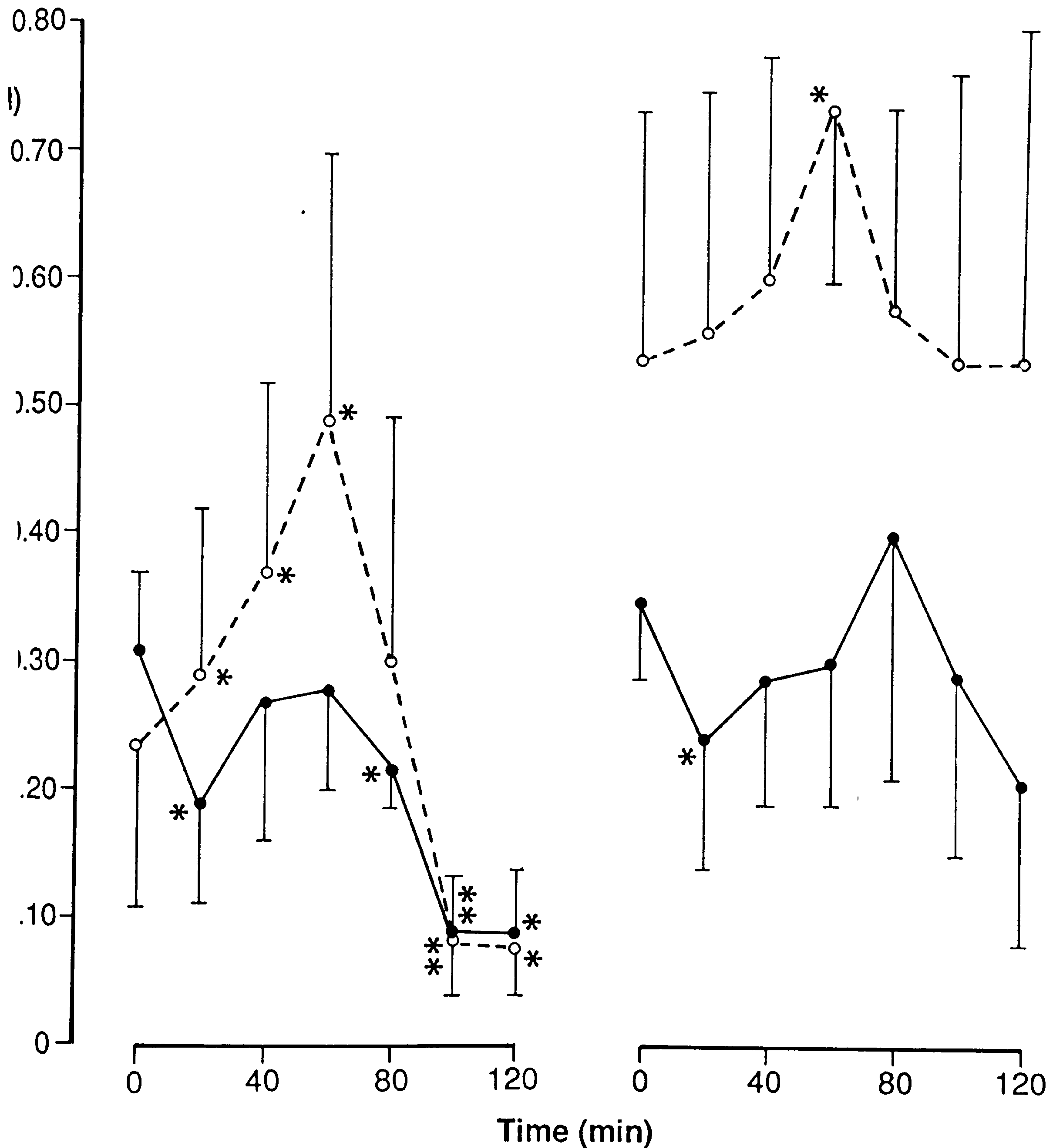
○- - -○ bicarbonate infusion to 60 min

* $p < 0.05$ compared to 0 min

FIG 7.C.6 :

NEFA levels during acetate and bicarbonate infusions with iv glucose at 60 min.

(means ± SD)



acetate infusion to 60 min

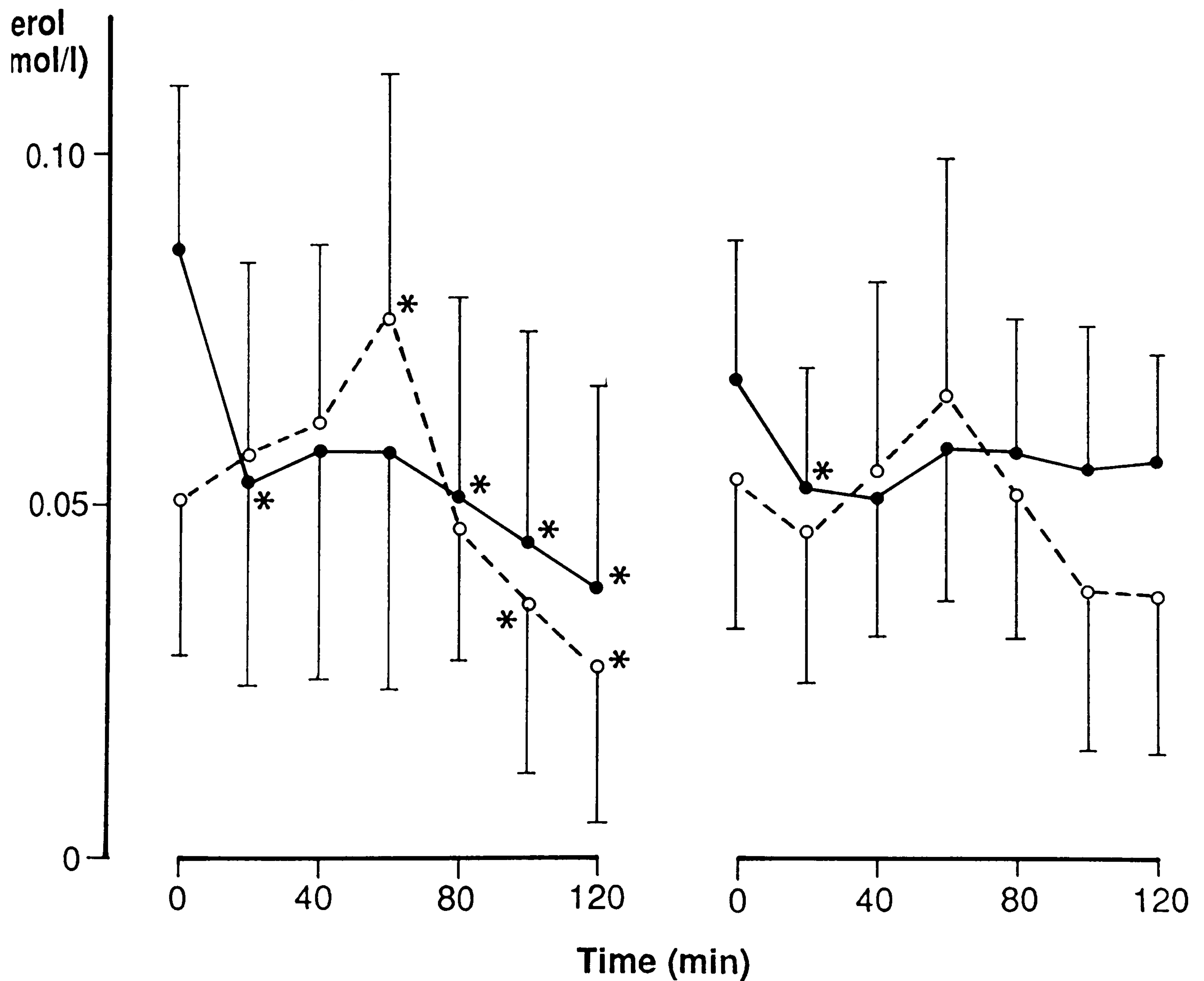
bicarbonate infusion to 0 min

* $p < 0.05$ compared to 0 min

FIG 7.C.7 :

Glycerol levels during acetate and bicarbonate infusions with iv glucose at 60 min.

(means ± SD)



acetate infusion to 60 min

bicarbonate infusion to 60 min

* $p < 0.05$ compared to 0 min

FIG 7.C.8: **Insulin levels during acetate and bicarbonate infusions**
(mean ± SD)

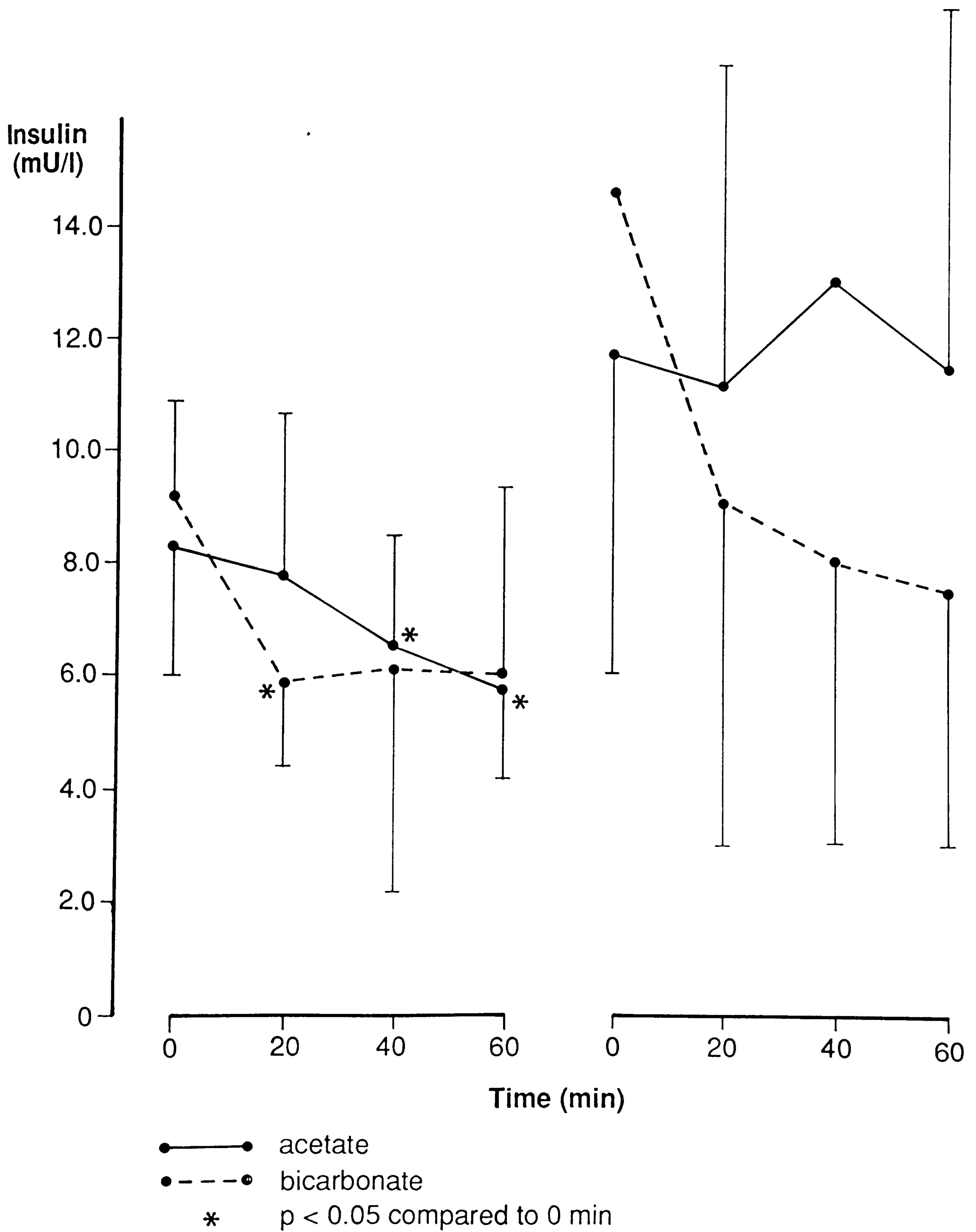


FIG 7.C.9 :

Growth hormone levels during acetate and bicarbonate infusions.

(mean ± SD)

A. Non-diabetic subjects (n=6)

B. Diabetic subjects (n=6)

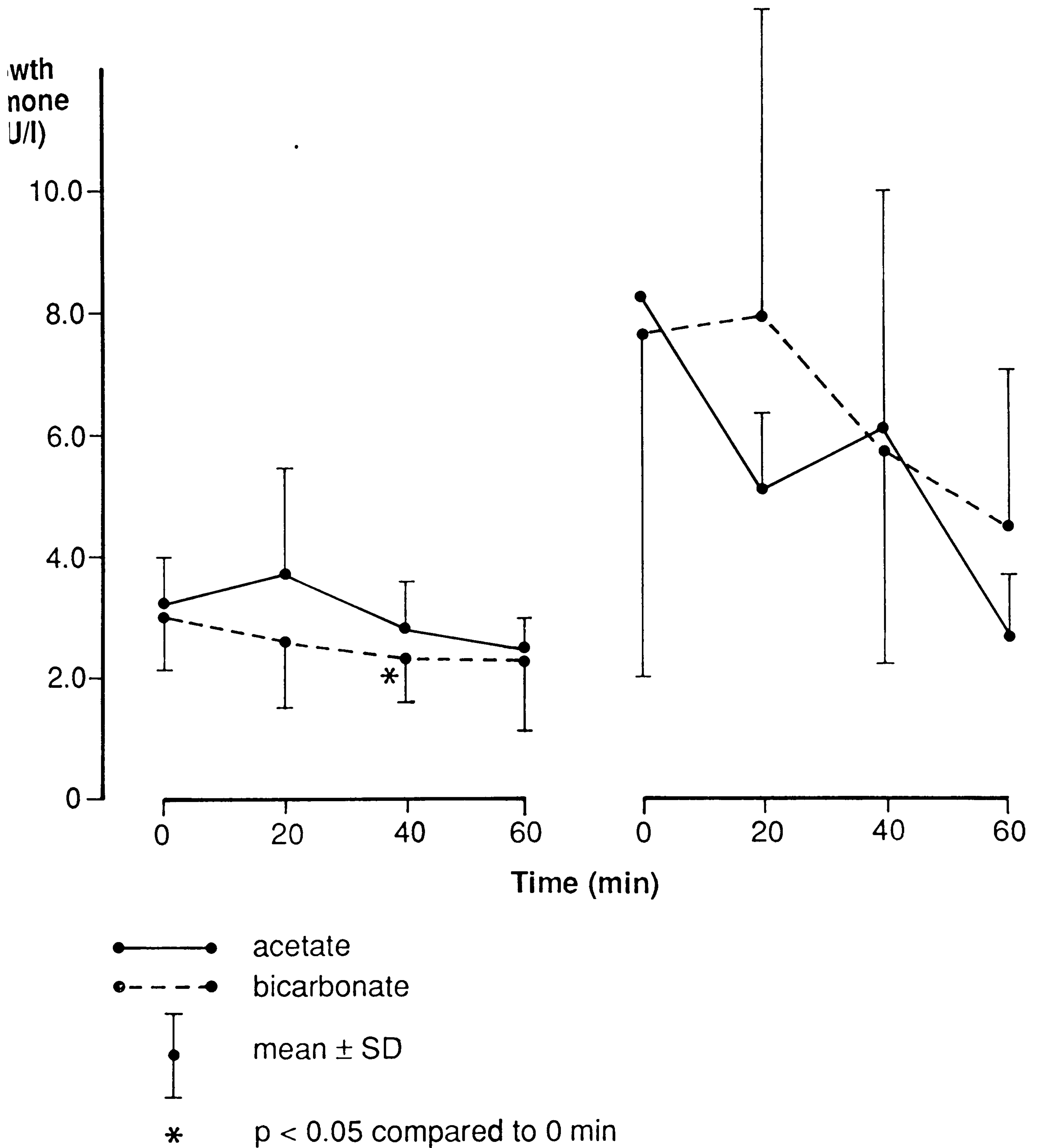
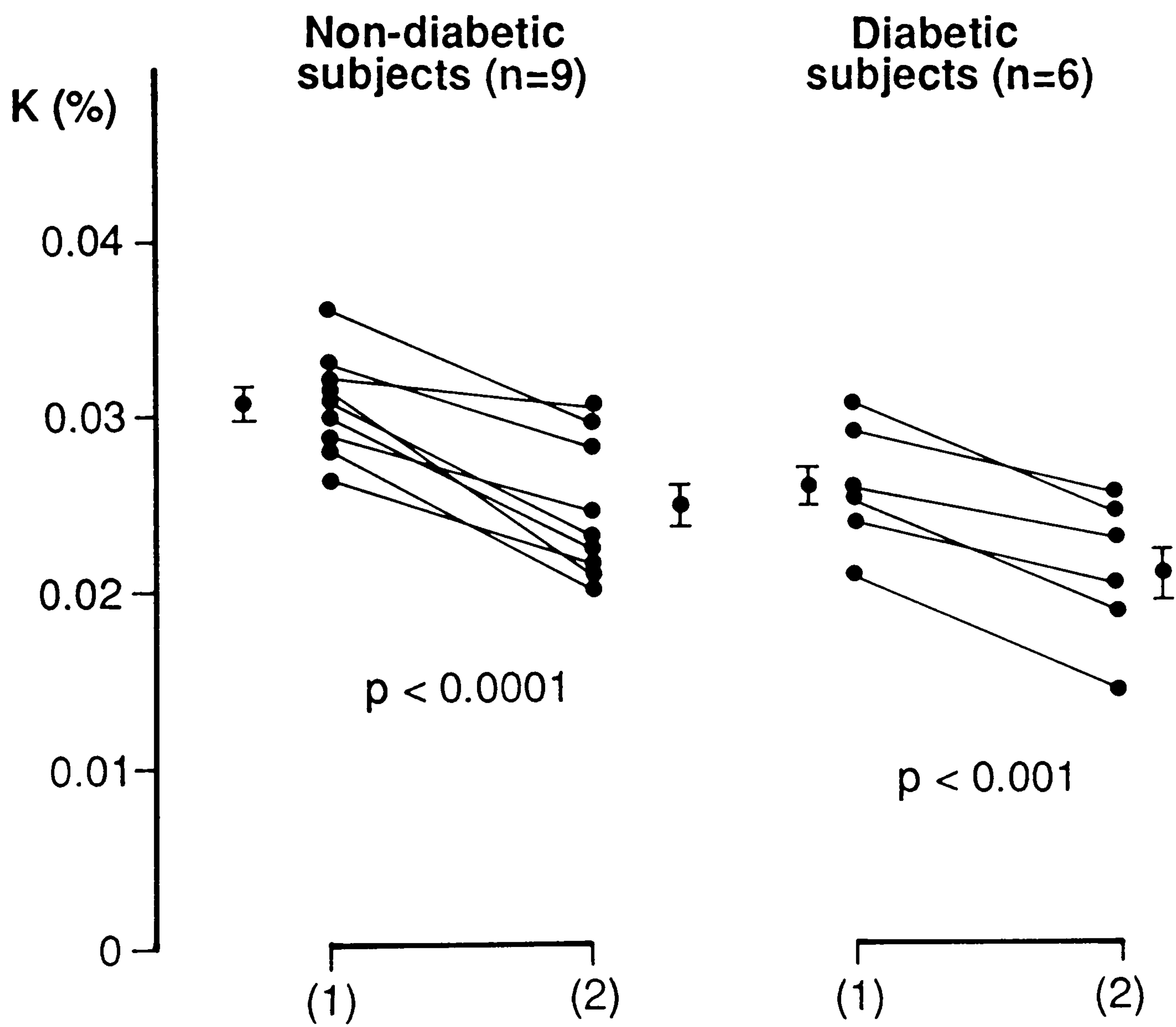


FIG 7.C.10 :

The elimination rate constant for acetate (K) with and without intravenous glucose

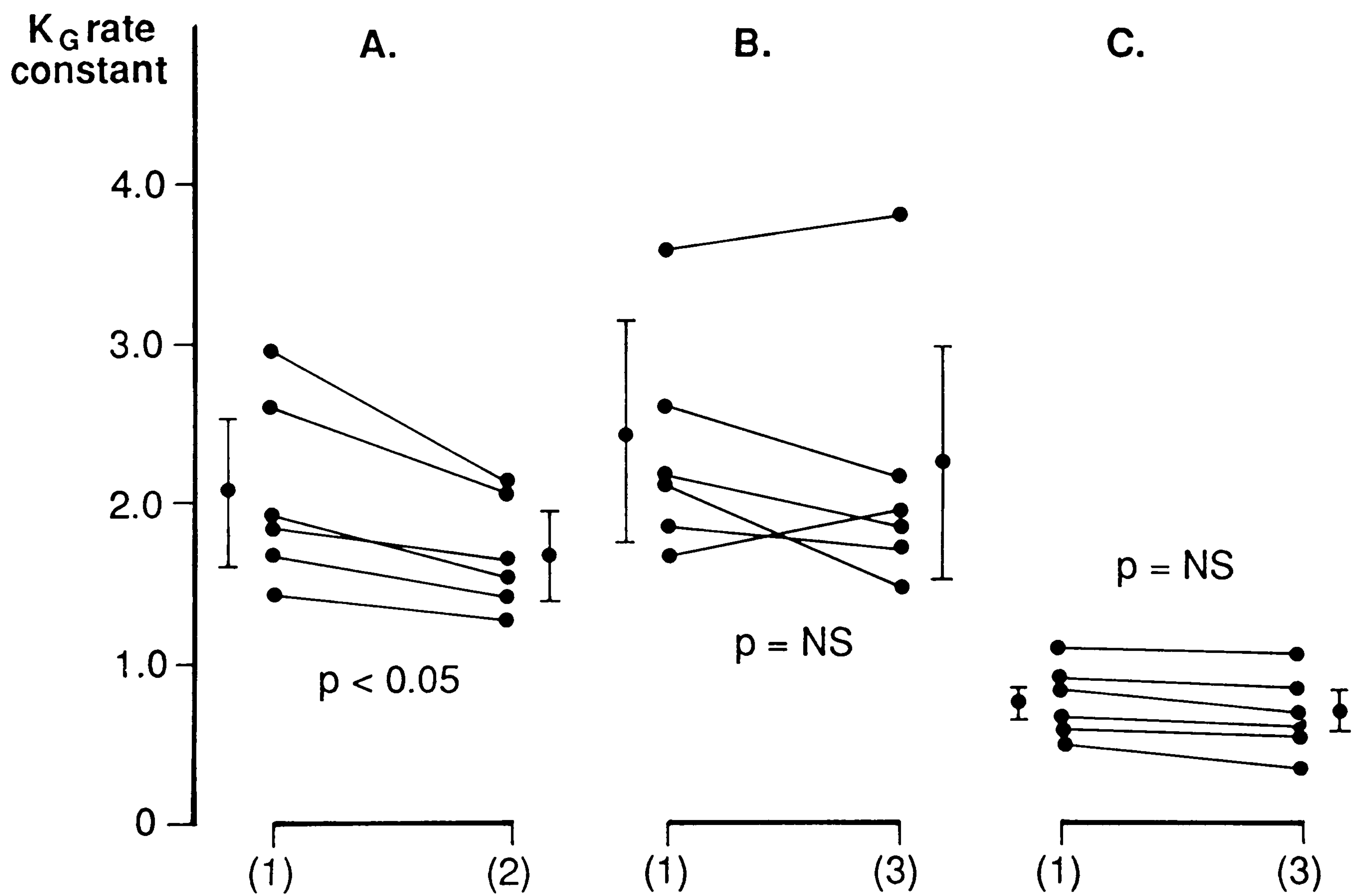


(1) Acetate only
(2) Acetate with iv glucose

mean ± SD

FIG 7.C.11 :

The elimination rate constant of glucose (K_G) with prior acetate, saline and bicarbonate infusion in non-diabetic and diabetic subjects.



A : Non- diabetic subjects (n=6)
B : Non-diabetic subjects (n=6)
C : Diabetic subjects (n=6)
(1) : Acetate infusion
(2) : Saline infusion
(3) : Bicarbonate infusion

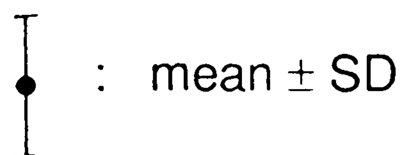
 : mean ± SD

FIG 7.C.12 :

Respiratory exchange ratio (RER) measurements in non-diabetic and diabetic subjects at rest and during acetate infusion.

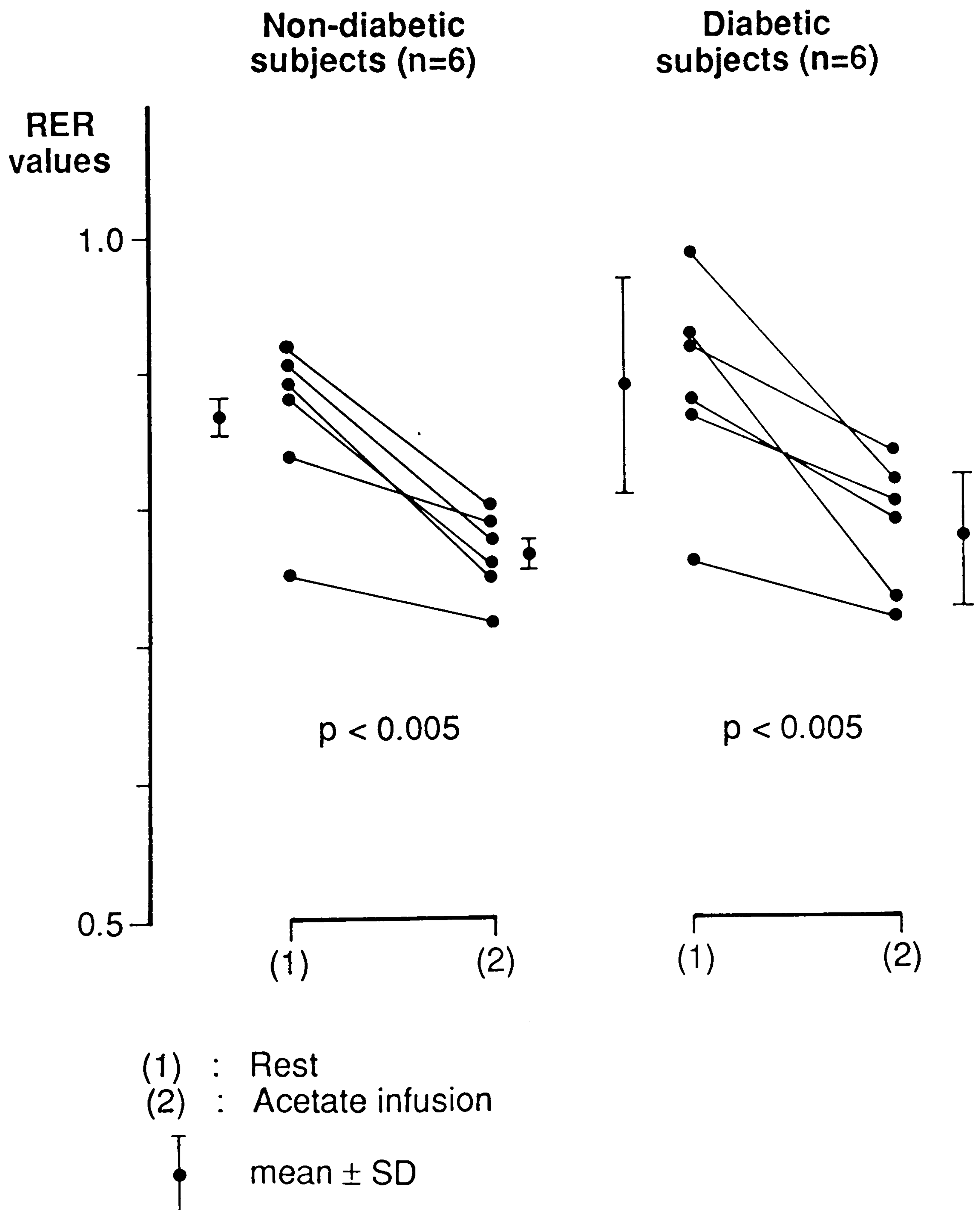
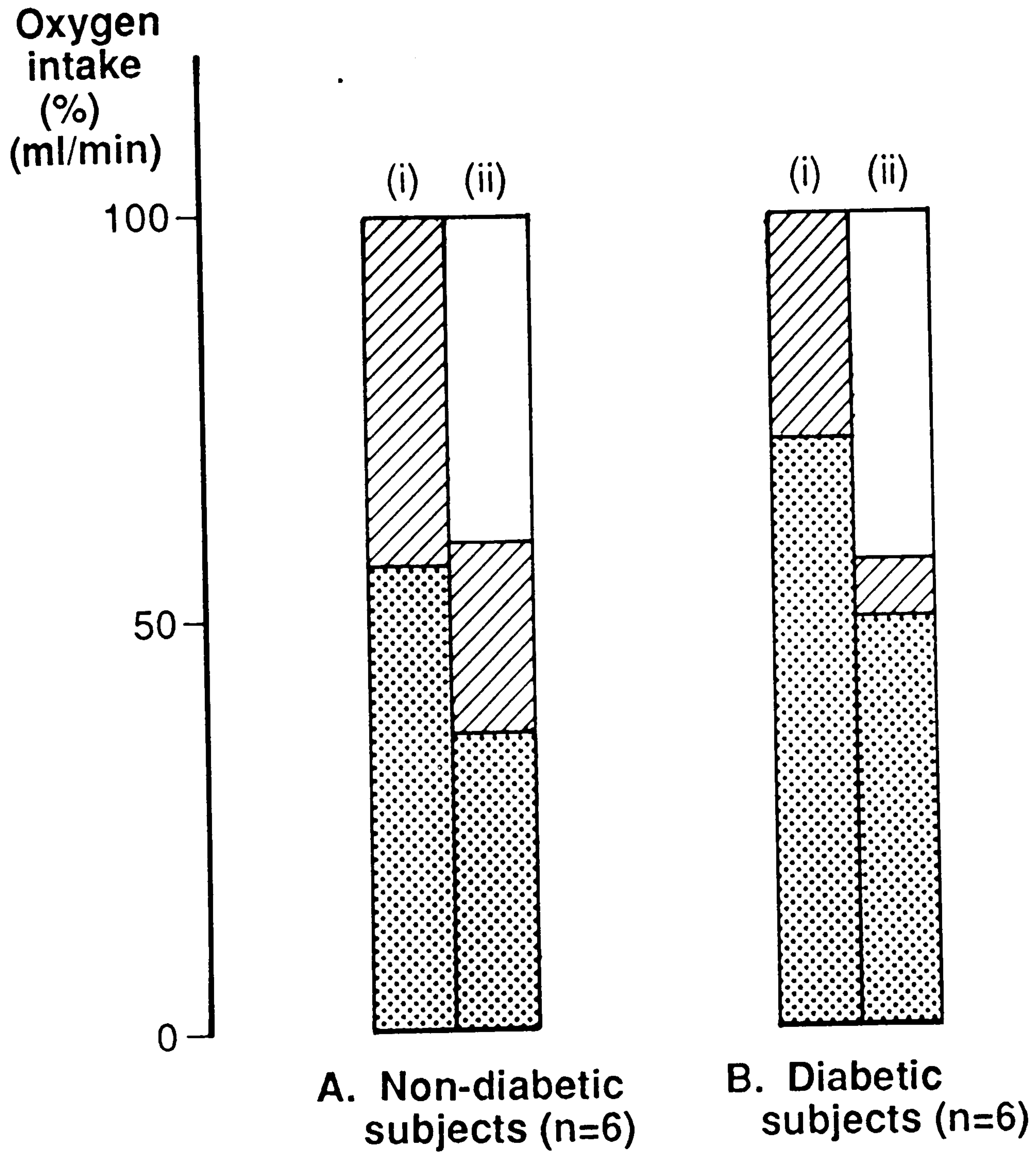
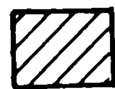


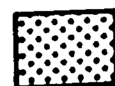
FIG 7.C.13 :

The contribution of glucose and fat to oxygen uptake before and during acetate infusion.



acetate

 fat

 glucose

(i) - basal
(ii) - during acetate infusion

DISCUSSION

Both the acetate and bicarbonate infusions caused a metabolic alkalosis, with similar increases in plasma bicarbonate levels and probably blood pH. These changes did not cause any untoward effects in the volunteers, and the alkalosis was apparently well compensated as there were no changes in arterialised venous blood pCO_2 and pO_2.

Metabolic Effects

The slight rise in the levels of the glycolytic products, lactate and pyruvate, during acetate infusion with tendency towards reduced levels with bicarbonate suggests that glycolysis was probably enhanced with the former. In the latter, there may have been increased gluconeogenesis from lactate to make glucose immediately available for energy needs. Acetate might thus have replaced lactate as the source of oxidative fuel, as has been reported with the perfused rat liver (Snoswell, Trimble, Fishlock, Storer and Topping 1982). These findings on lactate and pyruvate partially agree with those of Huckabee (1958, 1961) in dogs infused with bicarbonate or hyperventilating hospitalized patients; in both groups, the associated alkalosis caused increased blood lactate and pyruvate levels, even with bicarbonate infusion, and this was attributed to increased glycolysis as a homeostatic mechanism of the body to quickly reduce body bicarbonate in severe alkalosis. The rise in the levels of the KBs with both infusates suggests the effect as due to alkalosis, as has been described previously

in rats (Mackay, Barnes, Carne and Wick 1940, Mackay, Wick, Carne and Barnum 1941) and normal human subjects (Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982).

There was also a consistent fall in the plasma levels of NEFAs and glycerol in the non-diabetic and diabetic subjects, suggesting some degree of inhibition of adipose tissue lipolysis. This has been reported previously (Crouse, Gerson, DeCarli and Lieber 1968), and is probably a true finding especially as the control bicarbonate infusion caused a rise in the levels of those two metabolites. There was no associated increase in peripheral insulin release as insulin levels decreased, if anything, with the acetate infusions. It may have been a direct effect, analogous to the antilipolytic function of `ketone bodies' (Robinson and Williamson 1980, Balasse 1986).

There was no consistent change in the plasma levels of insulin and growth hormone and the scatter of responses was wide. These findings with insulin are in keeping with earlier observations in isolated rat (Montague and Taylor 1968) and bovine (Manns, Boda and Willes 1967) islets that acetate is ordinarily not insulinogenic, unlike propionate and butyrate. The lack of change in growth hormone values differs from the report (Schmitz, Hansen, Hansen, Orskov and Alberti 1982), albeit in uraemic patients, that acetate suppresses growth hormone release in humans.

Kinetics of Acetate Utilization

The results indicate that acetate is rapidly oxidised

in humans. The MCR in non-diabetic and diabetic subjects is similar to results reported by Richards, Vreman, Zager, Feldman, Blaschke and Weiner (1982); the turnover rate as determined by this non-isotopic infusion technique in non-diabetic subjects is also similar to the value of 8.3μmol/min.kg derived by an isotopic dilution method (Skutches, Holroyde, Myers, Paul and Reichard 1979). The utilization of acetate, expressed as MCR, turnover rate, elimination rate constant (Kac) or half-time, was lower in the diabetic subjects. This provides some gross and quantitative evidence for a defect in acetate metabolism in diabetes, which has been reported previously in sheep (Ballard 1972, Jarrett and Potter 1957), rats (Buckley and Williamson 1977), dogs (Ciaranfi and Fonnessu 1953, Harper, Neal and Hlavacek 1953) and tentatively in humans (Smith, Humphreys and Hockaday 1986), and which may account for the increased acetate levels seen in that condition.

The observations made in the diabetic patients were probably not due to the age difference with the non-diabetic volunteers, although Skutches, Holroyde, Myers, Paul and Reichard (1979) demonstrated that acetate turnover rate and plasma levels decreased with increasing age. Other parameters of acetate use were changed in the diabetics in this study, and observations on a large number of normal and diabetic subjects (Chapter 5.C) did not demonstrate any correlation between plasma acetate and age. Also, those various parameters did not correlate significantly with age

or BMI in the non-diabetic or diabetic subjects studied here.

Tolerance Studies

All the subjects, diabetic and non-diabetic, showed impaired acetate tolerance when glucose was simultaneously available. This observation conflicts with evidence in ruminants (Jarrett and Filsell 1960, 1961, Bartos, Skarda and Dolezel 1968, Ballard 1972, Forsberg, Baldwin and Smith 1984) that glucose has a 'permissive' role in acetate utilization, as the source of the NADPH (from the pentose phosphate shunt) required for lipogenesis. This difference from humans (and presumably, other non-ruminant mammals) may therefore reflect the metabolic fate of acetate in the two groups of animals. Acetate acts as the major lipogenic substrate in ruminants which derive little glucose from their diet, and are mostly supplied with it by gluconeogenesis from propionate. Non-ruminants derive most of their glucose from dietary sources, and in some of them, glucose is a major substrate for long-chain fatty acid synthesis (whether in liver or adipose tissue) as well as supply of immediate energy needs. Hence in this latter group of mammals, any acetate present is likely to be oxidised. This oxidation will be in competition for the 'respiratory chain' with any other substrate, according to its concentration, and particularly with glucose during the early part of an ivGTT. This may account for the reduced acetate disposal rates seen with available glucose in this

study.

Also, acetate might be expected to worsen glucose tolerance, acting as a competitive substrate. This does not appear to be the case. Glucose tolerance (K_G) did not change with immediately previously infused acetate or bicarbonate. Also, the increase in insulin release in response to glucose was no different between the infusates. Since acetate provides additional calories (200 kcal/mole), and yet glucose tolerance and insulin levels remained unchanged from values obtained with the control study, it is possible that acetate might improve tissue insulin sensitivity. However, since the 150mmol acetate infused could produce only 30kcal on oxidation and the acetate infusion ended as the glucose (equivalent to about 150kcal) was injected, it is probable that any acetate effect would be swamped by the larger glucose change.

Previous workers have reported variable effects of acetate on plasma glucose levels. These include both reduced plasma glucose in sheep (Asplund, Orskov, Hovell and Macleod 1985), anaesthetized dogs (Ward, Warthen, Harding and Thompson 1985) and diabetic humans (Lipsky, Alper, Rubini, Van Eck and Gordon 1954) or unchanged plasma glucose levels in non-diabetic humans (Lipsky, Alper, Rubini, Van Eck and Gordon 1954, Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982). Here, plasma glucose levels fell on acetate infusion only in the non-diabetic subjects. Despite the widely varying amounts of acetate infused in the various studies, including this, there has been no report of

elevated plasma glucose levels in whole-body studies, although many were inadequately controlled. In some earlier experiments, we found that the K_G in 6 non-diabetic subjects was increased significantly after acetate infusion compared to values after a saline infusion, although the values after acetate and bicarbonate infusions were similar (Fig 7.C.11). It is therefore possible that the described effects of acetate on blood glucose levels merely reflected alkalosis, rather than the acetate *per se*, especially as it has been shown in diabetic patients that alkalosis, from acetate or bicarbonate, reduced splanchnic glucose output and peripheral glucose levels (Lipsky, Alper, Rubini, Van Eck and Gordon 1954).

Energy Expenditure

During acetate infusions, the RQ fell to values to be expected were acetate the principal substrate oxidized. Energy expenditure was unchanged during acetate infusion, suggesting that the oxidation of acetate replaced that of an endogenous substrate. The displaced substrate was most likely NEFA, in that while the calculated rate of carbohydrate oxidation remained essentially unchanged, that of fat decreased almost to zero. This qualitative effect of acetate has been described previously in rat heart (Randle, England and Denton 1970) and the perfused rat hind-quarter (Karlsson, Fellenius and Kiessling 1975, 1976, 1977) but not quantitatively in whole-body studies and in humans. Those earlier studies also showed, as here, that the acetate

effect did not alter energy expenditure and total oxygen uptake. The mobilisation of fat as NEFAs and glycerol from adipose tissue stores has been shown in this study to be reduced when acetate is available, and this may be the basis for the reduced fat oxidation rate. The specific molecular mechanistic details remain unknown.

The assumptions made in calculating the substrate oxidation rates with infused acetate seem reasonable. There is evidence from previous studies (given under the `Results' section) that infused acetate is predominantly immediately oxidized in humans. Further support is provided by the findings of a rapid acetate elimination rate (Kac) and reduced RQ in this study. While a value of 90% oxidation is probably about right for the non-diabetic subjects, it is likely that the percentage oxidation is less in diabetic subjects. Nonetheless, as these latter subjects were metabolically well controlled as adjudged from fasting plasma glucose and HBAlc levels, and were assessed at steady state plasma acetate levels, the difference from normal values will probably not significantly affect the trend of the observations. Indeed, if acetate oxidation in the diabetic subjects is assumed to be 75% of the amount infused at steady state, `c' is calculated as $0.14 \pm 0.04$g/min (p NS compared to fasting value and value obtained when 90% oxidation was assumed) and `f', 0.01 ± 0.02g/min ($p < 0.05$ compared to fasting but p NS compared to value obtained with an assumed oxidation rate of 90%). This pattern was

maintained with acetate oxidation rates assumed as low as 60%.

Urinary loss of acetate was insignificant being always less than 3% of the total infused acetate (all subjects (mmol/l): fasting urinary acetate 0.06 ± 0.01; post-infusion urinary acetate 4.50 ± 1.30); an observation described by earlier workers (Ciaranfi and Fonnesu 1954, Richards, Vreman, Zager, Feldman, Blaschke and Weiner 1982) even with amounts of infused acetate higher than used here. Furthermore, substrate cycling between acetate and acetyl CoA was probably not important, since that cycle contributes less than 0.5% of the total heat produced even in a ruminant e.g. the sheep (Crabtree, Marr, Anderson and MacRae 1987).

Similarly, the assumption of 8g/day urinary nitrogen excretion was probably adequate to calculate the protein oxidation rate in all the subjects who were clinically in zero nitrogen balance as assessed by the maintained constant body weight over the study period. Previous estimates of nitrogen excretion vary from 4g/day (Rafelson, Hayashi and Bezkorovainy 1980) in normal people on the usual Caucasian diet, to 9g/day again in normal individuals after 4 days of complete starvation (Elia, Zed, Neale and Livesey 1987). The overnight urinary urea levels (mmol/l) in the subjects studied : non-diabetic 150 ± 21, diabetic 152 ± 25, correspond to a daily nitrogen excretion of about 6.5g. Furthermore, the calculation of 'c' and 'f' is not very sensitive to the value used for N excretion. As an example, consider substrate oxidation in an individual with VO_2 of

240ml and VCO_2 of 200ml (RQ 0.83) with differing N excretion rates of 4, 8 and 12g/day. The calculated values for `c' at these different rates are respectively (g/min) 0.131, 0.123 and 0.117; and for `f' respectively (g/min) 0.061, 0.055 and 0.052, which are not much different from one another despite the extreme values for the nitrogen excretion rate. The choice of 8g/day was therefore probably adequate. Nitrogen excretion rate was not influenced by acetate infusion, as assessed from the post-acetate infusion urinary urea levels in this study and as has been demonstrated in sheep, in which intraruminal administration of acetate (144kJ/kg $W^{0.75}$) over 3days did not influence urinary N or creatinine excretion (Asplund, Orskov, Hovell and Macleod 1985).

Thus, with carbohydrate oxidation unaffected by acetate, glucose tolerance should be uninfluenced by available acetate, as was seen in this study, although admittedly the excess glucose may be diverted into metabolic pathways other than oxidation such as glycogenesis and lipogenesis. Reduced fat mobilization and possibly increased fat synthesis (from observations of raised `non-acetate' RQ) and deposition in adipose tissue, might, by reducing NEFA levels, improve tissue insulin sensitivity and therefore glucose tolerance (Randle, Garland, Hales and Newsholme 1963). This may indeed be the case, especially as the additional, but small,caloric load from infused acetate did not worsen glucose tolerance. It would thus be of interest to investigate glucose tolerance with control

bicarbonate infusions in which the amount of glucose injected is increased to control for the caloric load due to acetate.

CONCLUSION

This study shows that:

i. acetate infusions produce a metabolic alkalosis similar to that observed with almost equimolar amounts of bicarbonate;

ii. the most consistent change in blood metabolites during acetate infusions is reduction in levels of NEFAs and glycerol, and our findings suggest that adipose tissue lipolysis was suppressed;

iii. an acetate infusion did not appear to worsen glucose tolerance, despite the additional albeit minor caloric burden;

iv. glucose impaired acetate tolerance, contrary to observations in ruminant mammals;

v. acetate is rapidly metabolised in humans, although at a slower rate in diabetic subjects, probably due to the reduced activity of acetyl CoA synthetase described in that disease;

vi. acetate suppresses fat oxidation, while not disturbing glucose oxidation, thus maintaining resting energy expenditure; this reduction in fat oxidation is probably

secondary to reduced NEFA supply from adipose tissue.

D. THE METABOLIC EFFECTS OF ACETATE DURING URAEMIC HAEMODIALYSIS IN DIABETIC AND NON-DIABETIC SUBJECTS.

AIMS

During haemodialysis against acetate containing fluids, a large amount of free acetate enters the circulation, and may be metabolised. It can contribute up to 40% of the total energy expenditure during dialysis (Skutches, Sigler, Teehan, Cooper and Reichard 1983).

Previous studies on the metabolic effects of acetate during haemodialysis (Gonzalez, Pearson, Garbus and Holbert 1974, Wathen, Keshaviah, Hommeyer, Cadwell and Comty 1978, Avram, Lipner, Sadiqali, Iancu and Gan 1976) were not adequately controlled, and it was therefore difficult to separate any effect of acetate from that due to alkalosis or even prolonged fasting.

This study attempts to compare the metabolic changes in stable uraemic patients on both acetate and bicarbonate dialysis in order to control for the possible effects of changes in anionic metabolism. The effect of the high acetate load on the metabolism of intravenously administered glucose in the non-diabetic and diabetic subjects was also investigated.

SUBJECTS AND METHODS

5 male non-diabetic uraemic subjects (aged 50.0 ± 13.0yr, BMI 24.5 ± 3.7kg/m^2, HBAlc 7.9 ± 2.0%), and 5 (3 male) diabetic uraemic subjects (aged 53.0 ± 6.6yr, BMI 23.0 ±1.3kg/m^2, HBAlc 11.5 ± 2.7%, all insulin-treated) were

studied. They all had established chronic end-stage renal failure and had been regularly on haemodialysis for at least 6months. Their haematocrit was 32.0 ± 2.6% and average pre-dialysis urea and creatinine values were for the non-diabetic subjects : 27.6 ± 4.0 mmol/l and 930 ± 224nmol/l respectively, and for the diabetic subjects : 30.2 ± 5.4mmol/l and 971 ± 258nmol/l. All were clinically anephric with creatinine clearance values less than 2ml/min, and were on thrice weekly haemodialysis at the Renal Unit, Churchill Hospital, Oxford. None was on steroids or any other drug (except insulin) known to influence glucose metabolism.

The number of patients in the study was relatively small because there was considerable difficulty in recruiting subjects who were in stable glycaemic control, free of intercurrent infections, well enough to tolerate overnight fasting and who did not suffer hypotensive and hypoglycaemic episodes during the studies.

The studies were performed during each patient's routine treatment programme. The type of dialyser (COBE Centry 2Rx Dialysis Control Unit, Allegro Hollow Fibre), blood flow rate (200ml/min), dialysate flow rate (500ml/min), duration of each dialysis session (5hr) and frequency of dialysis (thrice per week) were not changed. Vascular access in each patient was by forearm arteriovenous fistulae. Blood samples were withdrawn from the arterial end of the fistula, while glucose and heparin infusions were into the venous end.

Each subject had isosmolar acetate and bicarbonate

dialyses at 1week interval, in a random fashion. The respective compositions of the dialysates (Macarthy's Laboratories Ltd, Romford, U K) were (mmol/1):

Acetate dialysate :	Na	131
	K	1.57
	Mg	0.5
	Ca	1.54
	Cl	96.9
	Glucose	0
	Acetate	38.0
Bicarbonate dialysate :	Na	130
	K	2.0
	Mg	0.38
	Ca	1.5
	Cl	105
	Glucose	0
	Acetate	2.0
	Bicarbonate	30.0

Each patient was anticoagulated during dialysis with 10-15,000 units of heparin.

The patients arrived in the Renal Unit after an overnight fast (with water drunk as wished) at about 8.30 am. After a 30min rest, two initial blood samples were taken at 10min interval before the dialysis and heparin infusions were commenced. Another specimen was taken at the end of the first hour of dialysis and then the study proceeded as for a standard ivGTT after intravenous injection of glucose, 10 g/m^2 body surface area. The study was usually completed by the end of the second hour of dialysis, after which the patient was fed. Blood pressure and blood glucose (BM-Stix, Boehringer Mannheim) measurements were done at 30min intervals throughout each study period to detect the onset of any hypotension or hypoglycaemia, both complications

usually necessitating discontinuation of the procedure.

An initial pilot study was done using two `dummy plasma' solutions to assess diffusive losses of plasma constituents into the dialysate, since that was technically impossible to detect with the dialysis set-up for the patients. These `dummy plasma' solutions respectively contained:

	A	B
albumin(g/l)	40	40
NEFA (linoleic acid) mmol/l	1.0	0.5
3-hydroxybutyrate mmol/l	0.5	0.25
acetoacetate mmol/l	0.25	0.125
lactate mmol/l	1.0	0.5
pyruvate mmol/l	0.2	0.1
glycerol mmol/l	0.25	0.1
insulin mU/l	50	20
glucose mmol/l	10	5

and were considered to approximate to the levels of the various substrates in diabetic (A) and non-diabetic (B) plasma. It was observed that the fractional losses of these metabolites were identical during acetate and bicarbonate dialysis at the same dialyser settings. The losses were detectable only for glucose in (A) (about 6% at levels > 7.0 mmol/l) while for the other substrates, albumin and insulin, fractional losses were minimal. It was therefore assumed during the experiments that the arterial plasma values fairly accurately reflected the true values, and that, in the case of glucose, the diffusive loss into the dialysis fluid was not sufficient significantly to affect the qualitative trend of the observations.

The results were compared by a 3-factor repeated measures design ANOVA with one (non-diabetic v diabetic)

between-, and two (treatment-acetate v bicarbonate dialysis, and time) within-subject variables. The p values given are for the main effect of treatment. Paired t tests were used for assessing differences between treatment in the same subjects.

RESULTS

The changes in the levels of the various metabolites and insulin are shown in Table 7.D.1. and Figs 7.D.1 - 7.D.10.

During acetate dialysis, plasma acetate levels (mmol/l) rose within the first hour to 1.87 $\pm$ 0.16 and 2.08 $\pm$ 0.19 respectively in the non-diabetic and diabetic subjects, and was maintained at about this level for the second hour of the study (Fig 7.D.1). With bicarbonate dialysis, plasma acetate also increased slightly, perhaps due to the small amount of acetate (2 mmol/l) present in the bicarbonate dialysis fluid. This increase was not considered great enough to affect the trend of the observations.

The changes in the different blood metabolites, insulin and growth hormone in the two subject groups during the two hours of dialysis with intravenously administered glucose in the second hour are shown in Figs 7.D.2 to 7.D.10.

i. Glucose and Insulin (Figs 7.D.2 - 7.D.3)

In both groups of subjects, but more in the diabetics, plasma glucose levels fell slightly during both acetate and bicarbonate dialysis, probably due to haemodilution and

TABLE 7.D.1 : THE LEVELS OF BLOOD METABOLITES BEFORE AND AFTER 60MIN DIALYSIS WITH ACETATE OR BICARBONATE IN NON-DIABETIC AND DIABETIC SUBJECTS(mean± SD).

	ACETATE		BICARBONATE	
	before	after 60min	before	after 60min
A.Non-diabetic (n=5)				
Glucose	5.0 ± 0.4	4.4 ± 0.6	5.0 ± 0.7	4.6 ± 1.0
Insulin	12.3 ± 3.8	10.2 ± 5.0	11.3 ± 2.8	12.0 ± 2.8
Lactate	0.64 ± 0.38	0.40 ± 0.16	0.55 ± 0.08	0.57 ± 0.16
Pyruvate	0.079 ± 0.015	0.060 ± 0.028	0.060 ± 0.029	0.055 ± 0.028
Aceto-acetate	0.045 ± 0.010	0.068 ± 0.015*	0.043 ± 0.010	0.056 ± 0.021
3-hydroxy butyrate	0.034 ± 0.019	0.153 ± 0.100*	0.048 ± 0.051	0.087 ± 0.042
NEFA	1.68 ± 1.13	2.15 ± 0.84*	2.17 ± 1.08	2.29 ± 0.71*
Glycerol	0.073 ± 0.033	0.084 ± 0.028	0.081 ± 0.012	0.071 ± 0.022
Acetate	0.18 ± 0.11	1.87 ± 0.16*	0.17 ± 0.07	0.29 ± 0.19*
B.Diabetic (n=5)				
Glucose	8.1 ± 3.1	6.2 ± 1.1	10.0 ± 5.3	8.0 ± 4.3
Free Insulin	14.4 ± 4.0	15.4 ± 4.9	17.4 ± 7.0	16.0 ± 7.8
Lactate	0.92 ± 0.46	0.71 ± 0.31	0.67 ± 0.11	0.70 ± 0.36
Pyruvate	0.076 ± 0.015	0.074 ± 0.010	0.089 ± 0.033	0.079 ± 0.031
Aceto-acetate	0.089 ± 0.021	0.132 ± 0.028*	0.088 ± 0.020	0.069 ± 0.017
3-hydroxy butyrate	0.153 ± 0.130	0.243 ± 0.150*	0.195 ± 0.147	0.183 ± 0.131
NEFA	1.14 ± 0.93	1.91 ± 0.58*	1.21 ± 0.88	2.32 ± 0.44*
Glycerol	0.127 ± 0.045	0.097 ± 0.052	0.108 ± 0.031	0.105 ± 0.052
Acetate	0.28 ± 0.06	2.08 ± 0.91*	0.31 ± 0.23	0.38 ± 0.26*

* $p < 0.05$ compared to fasting value.
11
Unit of measurement for all the metabolites is mmol/l; and for insulin mU/l.

diffusive loss into the dialysate. There was no significant difference in glucose disposal (K_G) during acetate and bicarbonate dialysis in the same subjects (K_G acetate v bicarbonate : non-diabetic 1.34 ± 0.08 v 1.39 ± 0.09, diabetic 0.66 ± 0.13 v 0.69 ± 0.19, both p NS). Although diffusive loss of glucose into the dialysate probably occurred especially post-glucose injection, it was reckoned from observations in the pilot studies described under the `Subjects and Methods' section that such losses were similar between both acetate and bicarbonate dialysates, and did not exceed 6% of the plasma values.

Plasma total insulin (non-diabetic) and free insulin (diabetic) level was essentially unchanged during dialysis in both groups of subjects during the first hour of dialysis, whichever mode of dialysis was employed. Also the glucose induced rise in insulin levels was similar with both dialysates in the two groups of subjects.

ii. Lactate and Pyruvate (Figs 7.D.4 - 7.D.5)

Acetate did not have any significant effects different from bicarbonate on levels of pyruvate during dialysis and with available glucose. However, in all the subjects, lactate levels fell slightly, although not significantly on acetate, while remaining essentially unchanged on bicarbonate. Post-glucose injection lactate levels rose only slightly in both subject groups with acetate or bicarbonate dialysis.

iii. NEFA and Glycerol (Figs 7.D.6 - 7.D.7).

Plasma NEFA increased on both dialysates in both groups of subjects, and fell, as expected after glucose injection. The diabetics however had a greater increase over time than the normal subjects ($p < 0.005$). The absolute rise during dialysis, and fall with glucose were similar with the two dialysates. There was no significant difference in glycerol responses with the two treatments.

These different effects on blood NEFA and glycerol levels suggest triglyceride hydrolysis from a post-heparin increase in lipoprotein lipase activity rather than adipose tissue lipolysis as the cause of the increased NEFA level.

iv. `Ketone Bodies' (Figs 7.D.8 - 7.D.9)

There was a rise in blood acetoacetate and 3-hydroxybutyrate levels during acetate dialysis in both the non-diabetic and diabetic subjects. During bicarbonate dialysis, the levels of these metabolites did not change significantly. The rise over time in levels of these ketone bodies was highly significant ($p < 0.005$). The glucose-induced suppression of these KB levels was almost absent during acetate, unlike bicarbonate, dialysis. These trends were essentially similar in both the non-diabetic and diabetic subjects.

v. Growth Hormone (Fig 7.D.10a,b).

With both forms of dialysis, growth hormone values fell over time and continued into the post-glucose injection period. The trends were similar in the diabetic and non-diabetic,

FIG 7.D.1 :

Change in acetate levels during uraemic haemodialysis with iv. glucose at 60 min.

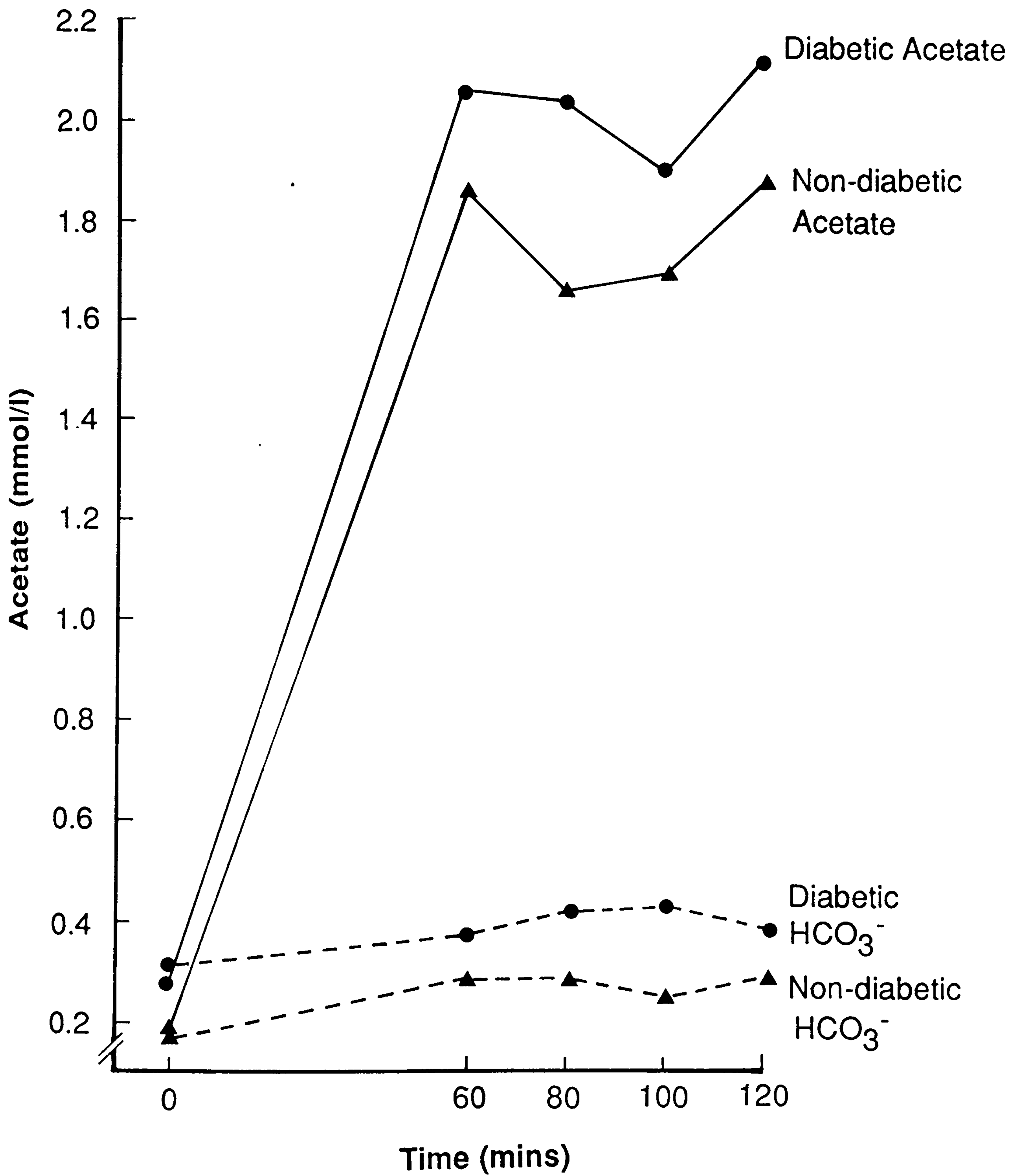


FIG 7.D.2 :

Change in glucose levels during uraemic haemodialysis with iv. glucose at 60 min.

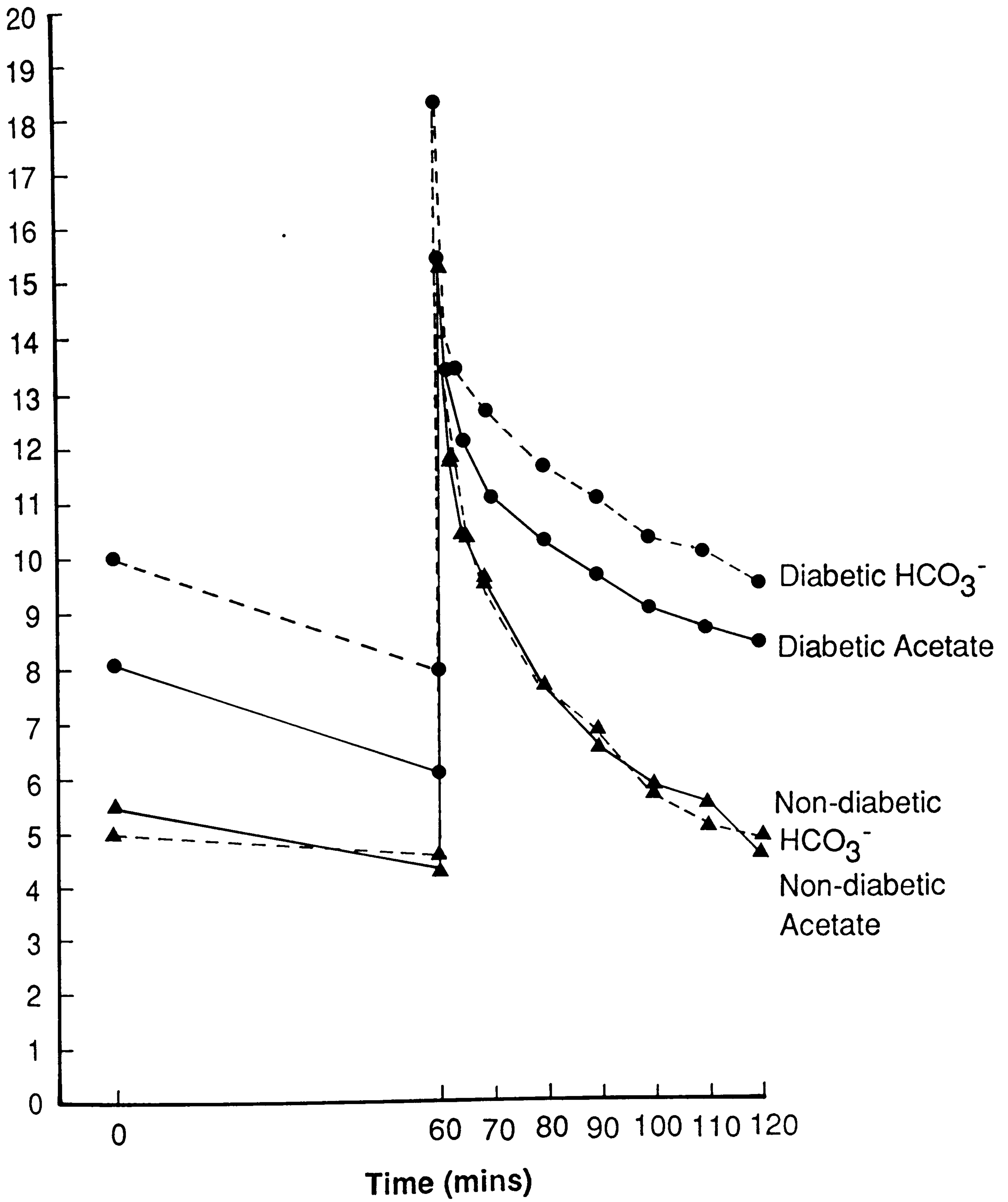


FIG 7.D.3 :

Change in insulin levels during uraemic haemodialysis with iv. glucose at 60 min.

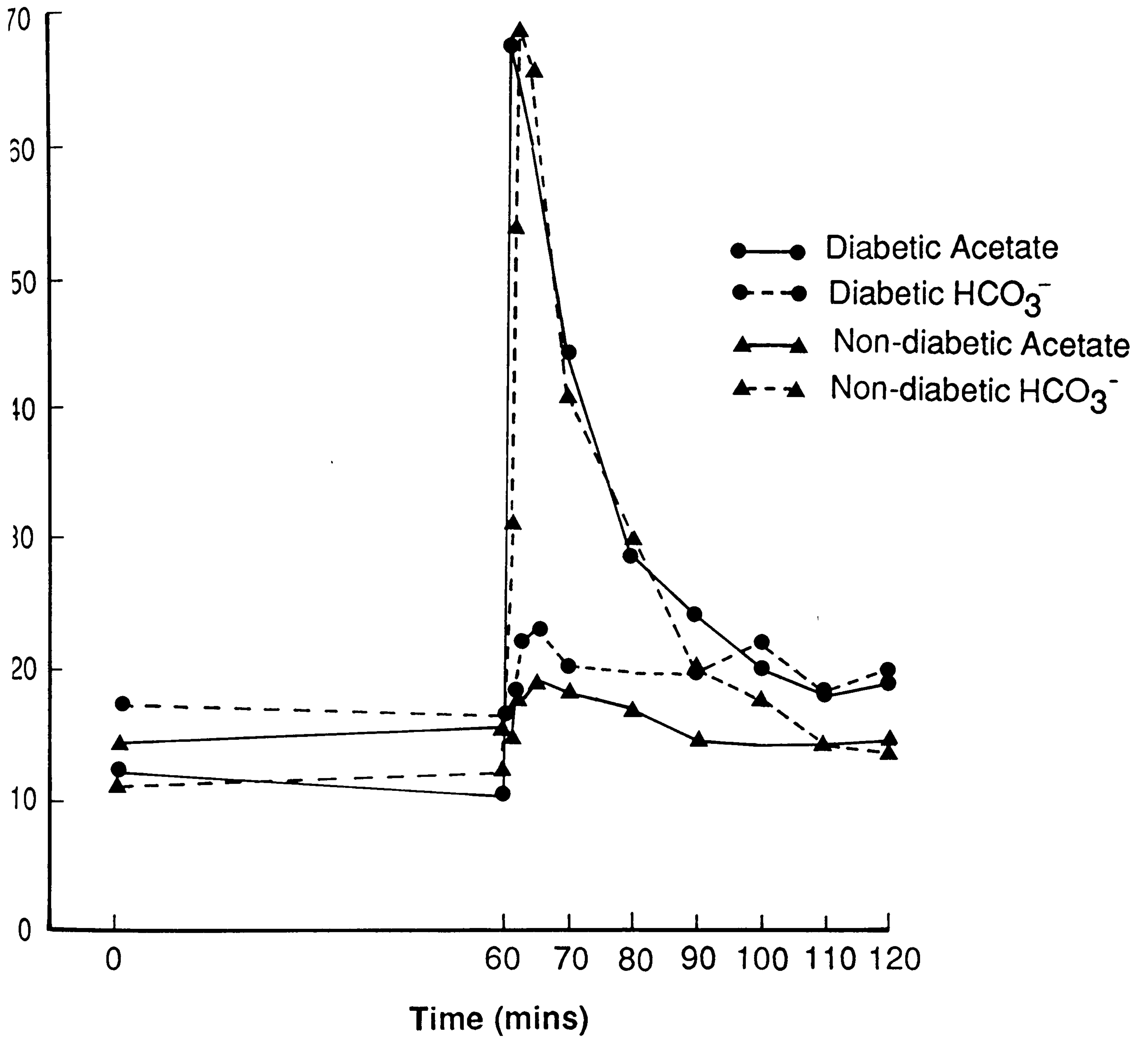


FIG 7.D.4 :

Change in lactate levels during uraemic haemodialysis with iv. glucose at 60 min.

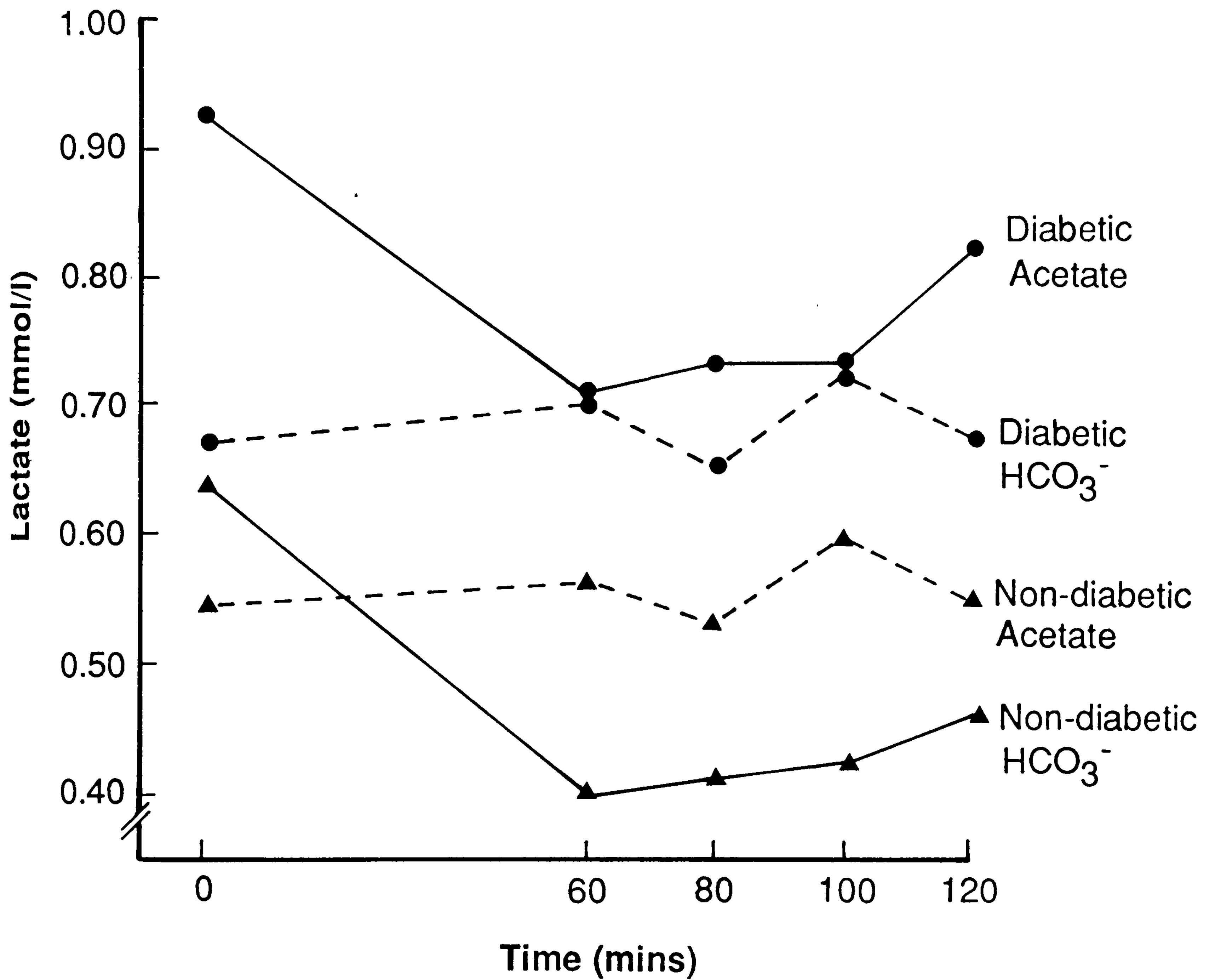


FIG 7.D.5 :

Change in pyruvate levels during uraemic haemodialysis with iv. glucose at 60 min.

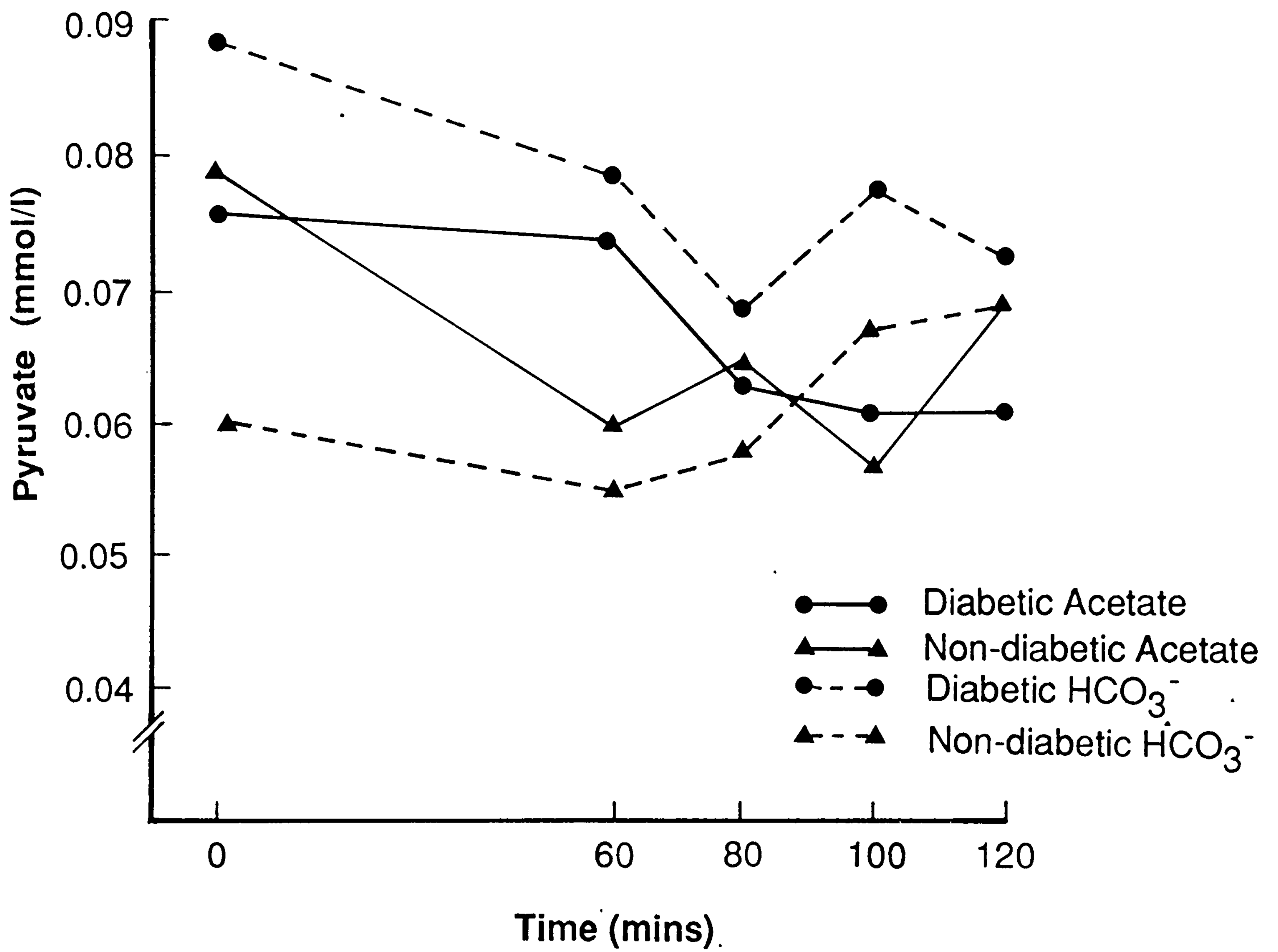


FIG 7.D.6 :

Change in NEFA levels during uraemic haemodialysis with iv. glucose at 60 min.

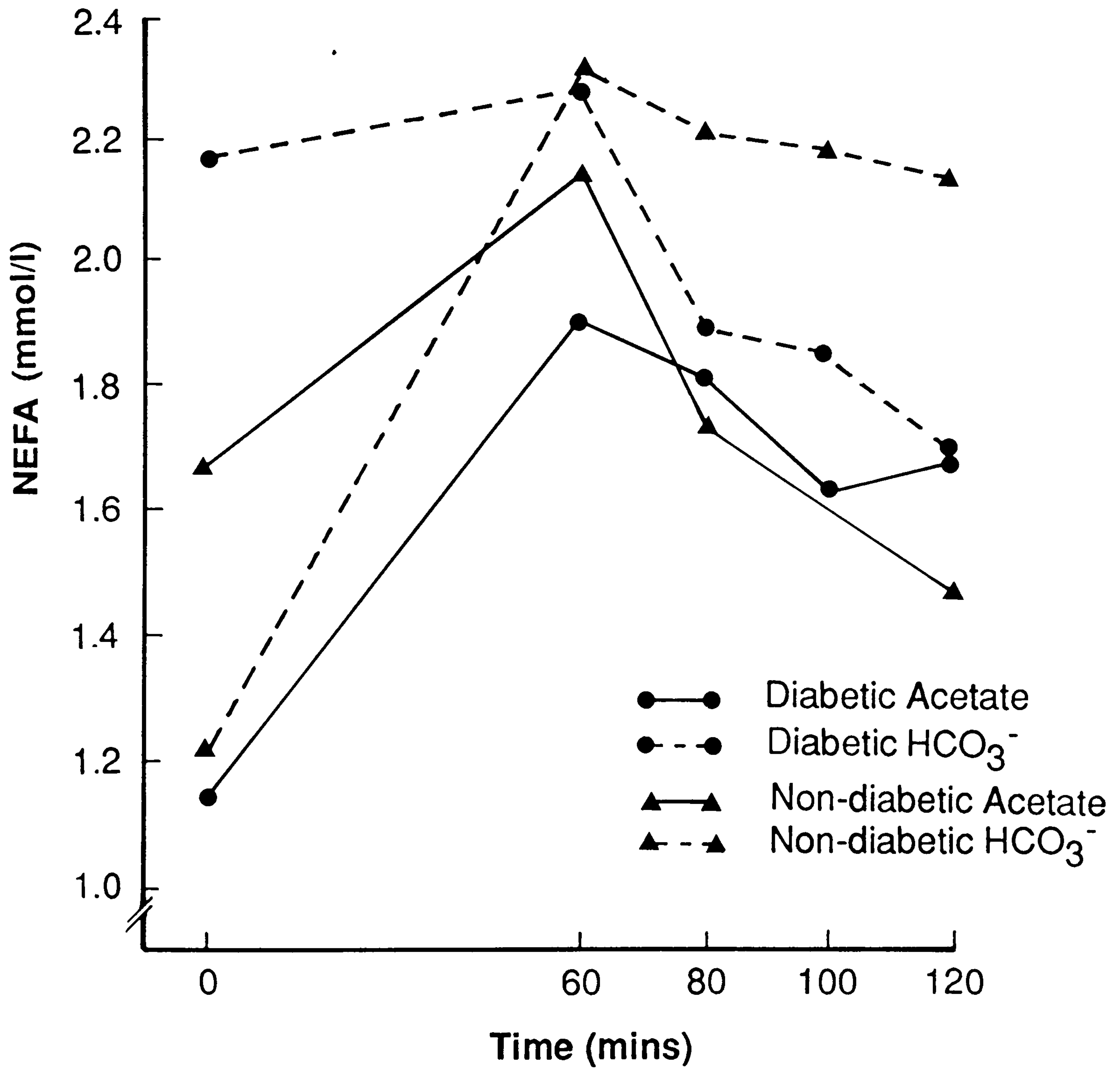


FIG 7.D.7 :

Change in Glycerol levels during uraemic haemodialysis with iv. glucose at 60 min.

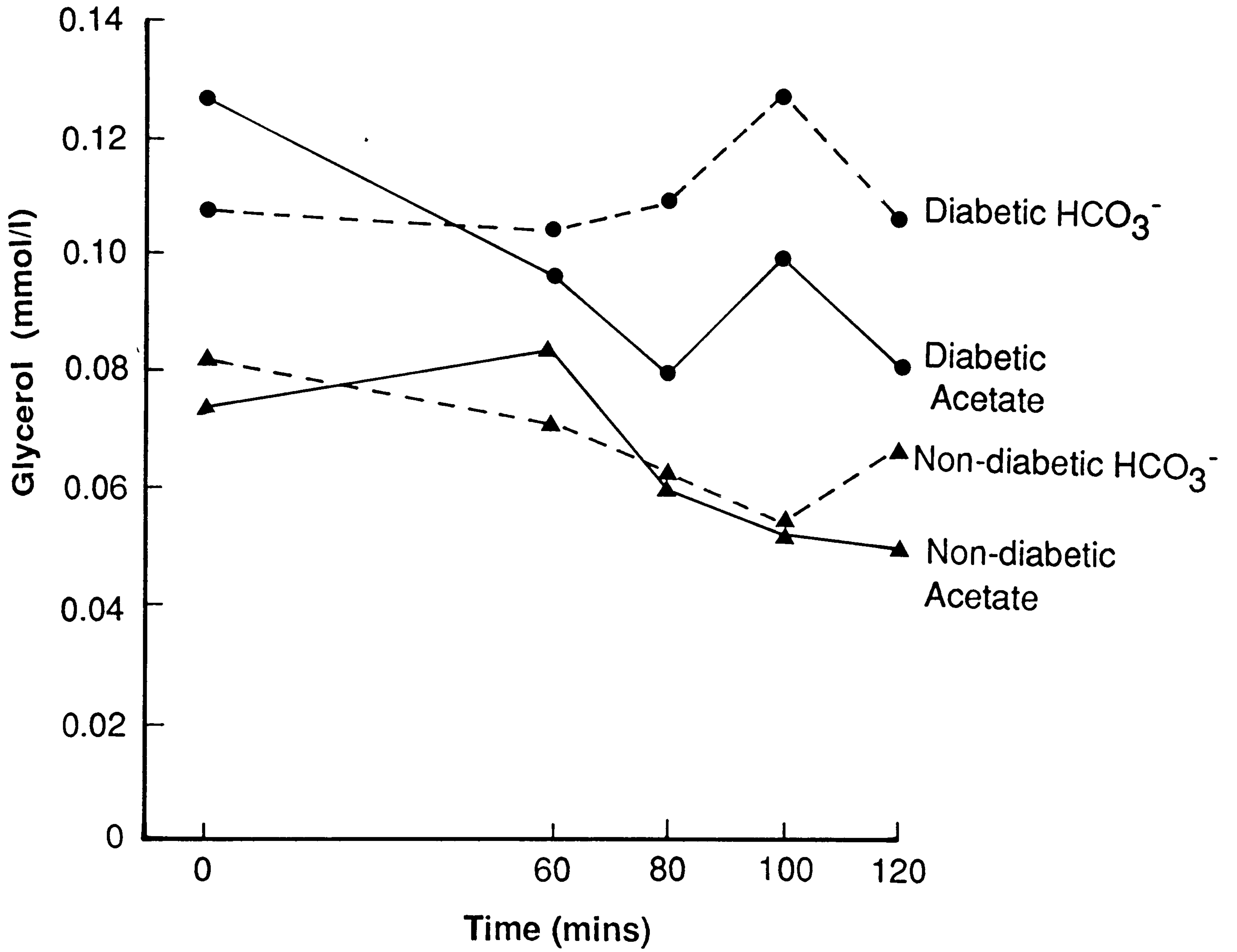


FIG 7.D.8 :

Change in acetoacetate levels during uraemic haemodialysis with iv. glucose at 60 min.

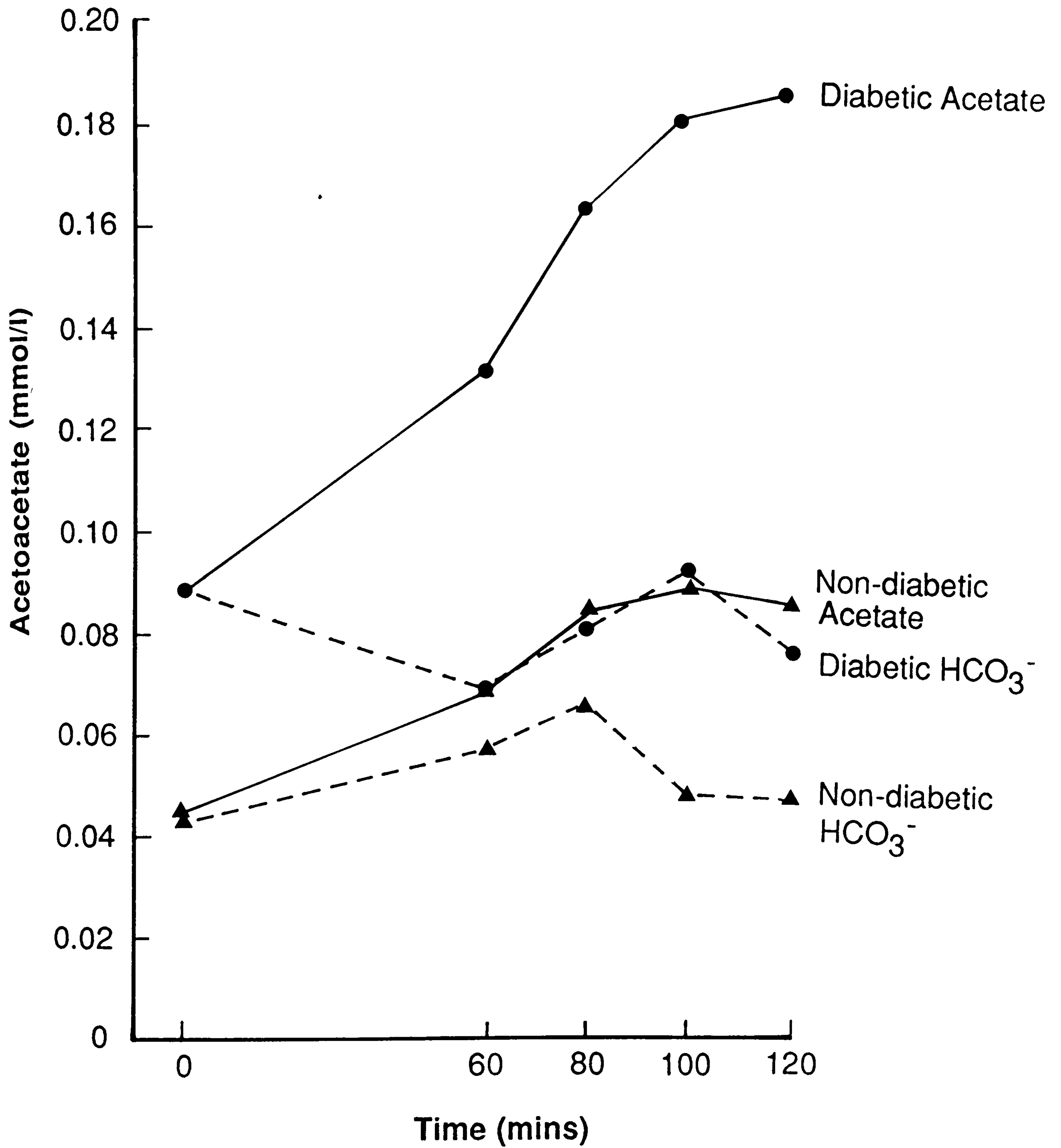


FIG 7.D.9 :

Change in 3-hydroxybutyrate levels during uraemic haemodialysis with iv. glucose at 60 min.

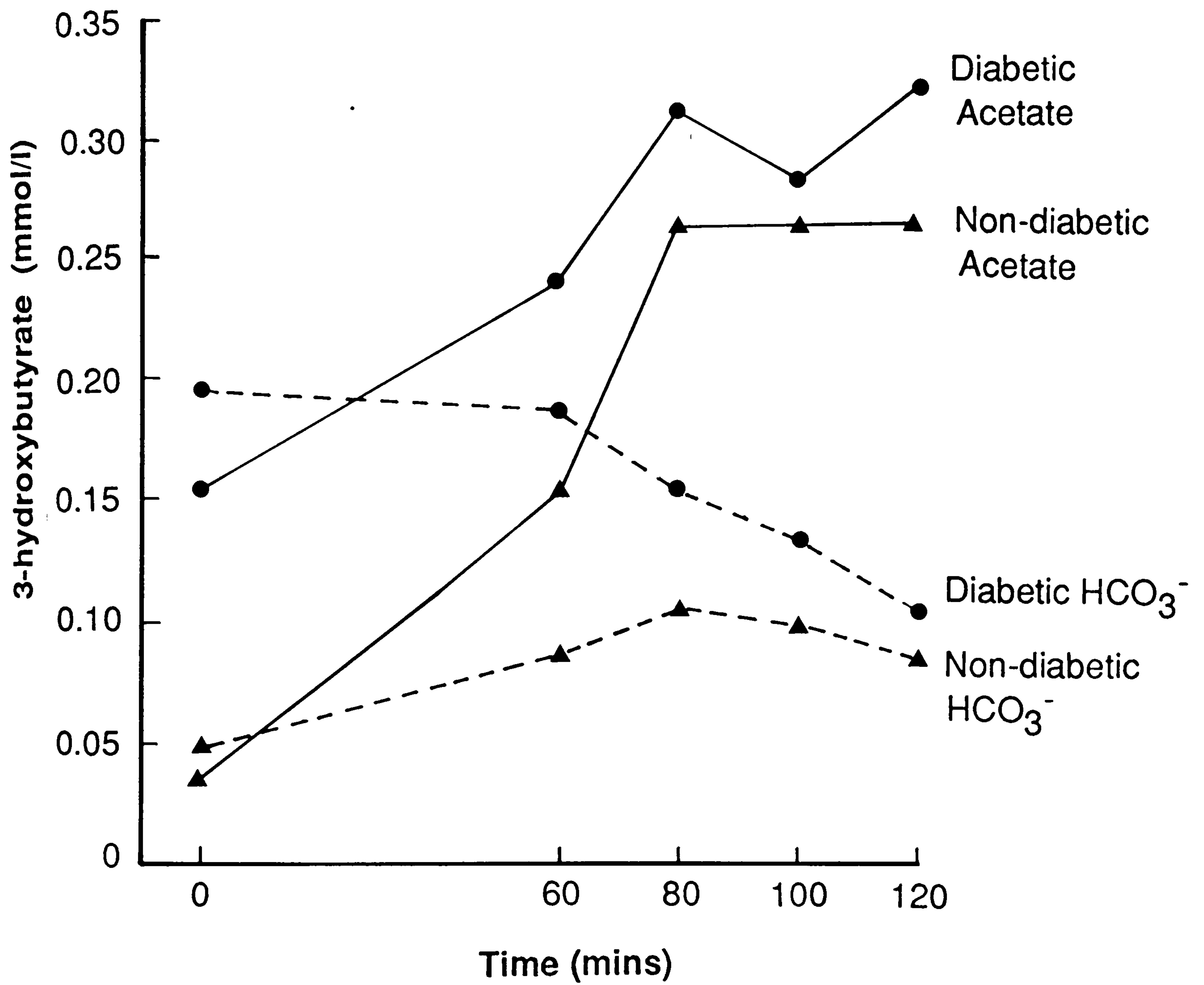
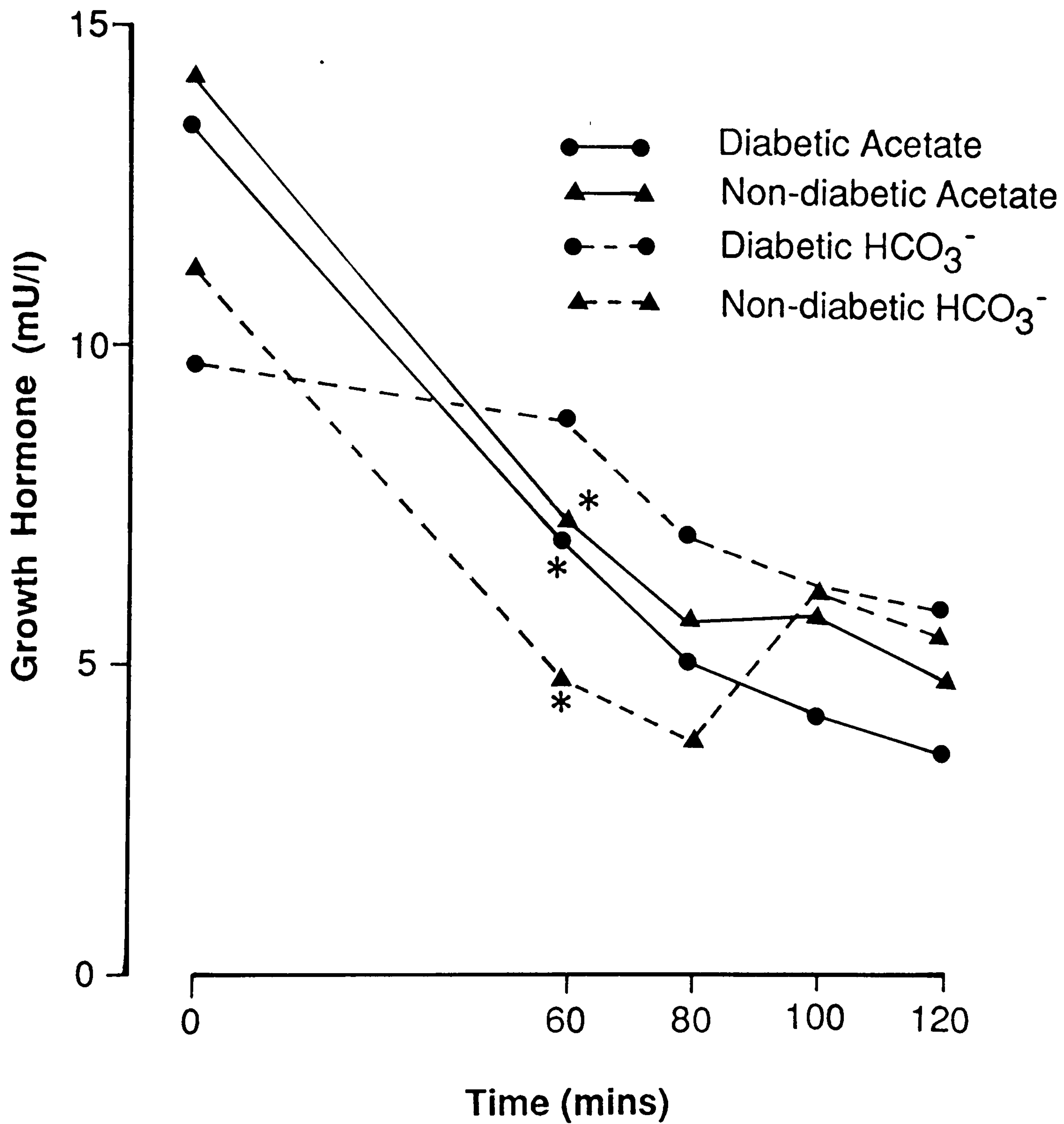


FIG 7.D.10a :

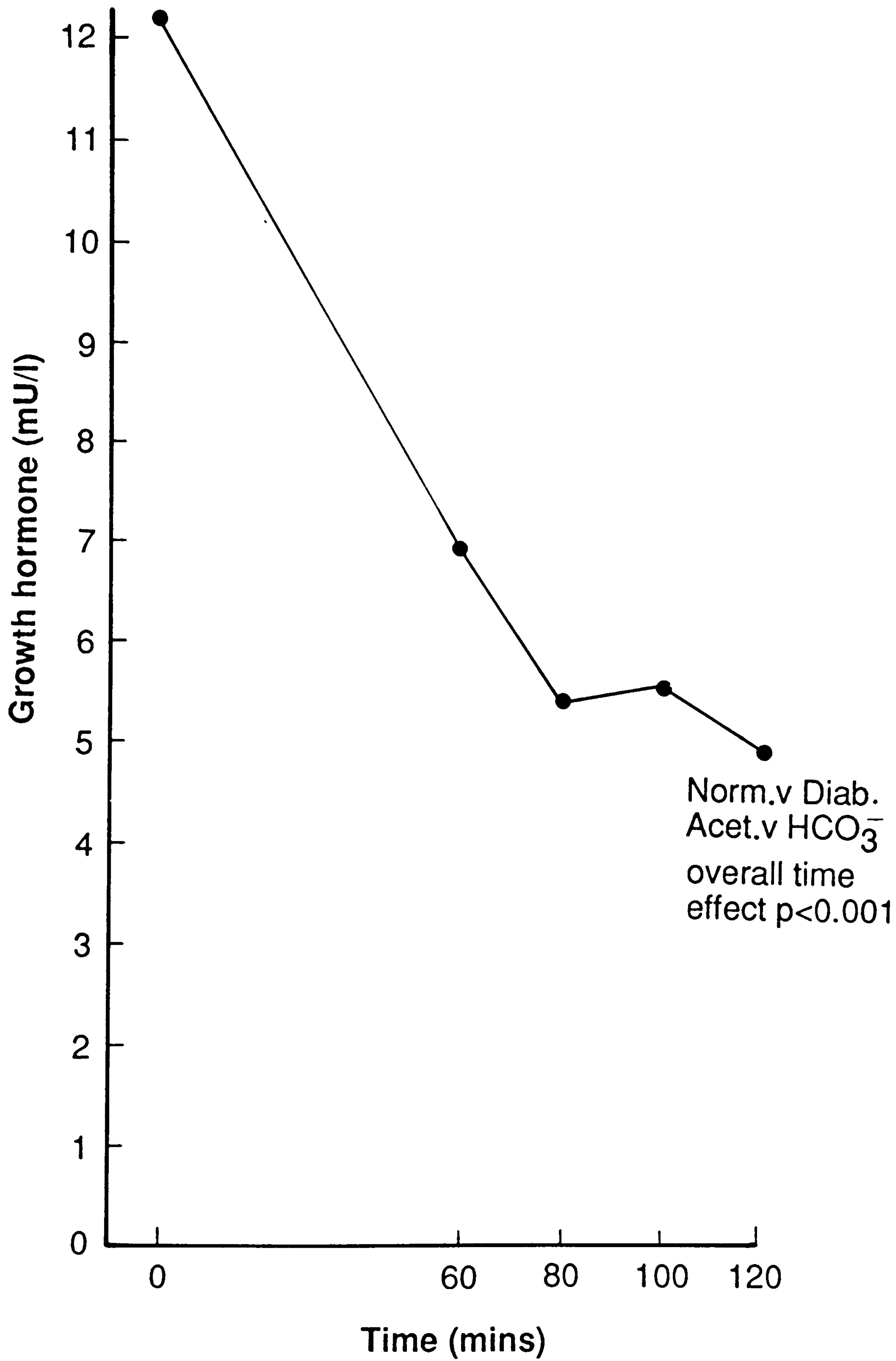
Change in growth hormone levels during uraemic haemodialysis with iv. glucose at 60 min.



* $p<0.05$ compared to 0 min value

FIG 7.D.10b :

Change in growth hormone levels during uraemic haemodialysis with iv. glucose at 60 min.



and with both dialysates. Only the time effect was significant ($p < 0.001$), as shown in Fig 7.D.10b.

DISCUSSION

The use of acetate as a buffer in dialysis fluids has been suggested as one possible cause of some of the morbidity (nausea, vomiting, hypotension, increased atherosclerotic vascular disease) associated with that form of uraemic haemodialysis, prompting recent attempts at replacing it with bicarbonate which is considered to have less morbidity (Mansell and Wing 1983). Since acetate occupies a prime position in metabolic pathways, being converted via a single step to acetyl CoA, it is possible that this presumed effect of acetate has a metabolic basis. Wathen, Keshaviah, Hommeyer, Cadwell and Comty (1978) reported raised blood levels of NEFA and KBs and decreased values for lactate and pyruvate during acetate haemodialysis without glucose, findings similar to those observed here, but the likelihood of these changes being due solely to the procedure itself or otherwise to alkalosis caused by the infused acetate was not assessed by adequately controlled studies. As an example, the rise in NEFA and ketone bodies may merely reflect increased triglyceride hydrolysis by lipoprotein lipase released from the capillary endothelium by the high doses of heparin used during dialysis. However, there may indeed be a metabolic basis, since Liang and Lowenstein (1978) demonstrated in anaesthetized dogs that acetate caused increased cardiac output and coronary blood

flow due to enhanced production of AMP from its activation to acetyl CoA, an observation confirmed clinically in uraemic humans (Mehta, Ahmad and Dubose 1983).

The findings in this study suggest that acetate has only minimal direct effects during haemodialysis, which cannot be ascribed either to alkalosis (as effects also seen with bicarbonate) or to post-heparin lipolytic activity. Although findings elsewhere in this thesis (Chapter 7.C) suggest an inhibition of adipose tissue lipolysis with available acetate, the marked rise in NEFA levels seen with both acetate and bicarbonate dialysis, often to the same degree, would suggest that the acetate effect was not powerful enough to counteract that of heparin. Fasting NEFA levels were rather high in all the subjects, and this may merely reflect random variation and not a heparin effect, as basal samples were always taken before any heparin was administered..

The levels of the KBs were however higher with acetate treatment despite the similar NEFA values with bicarbonate treatment, raising the possibility of either a direct conversion of acetate to ketones at the high plasma levels seen in dialysis, or otherwise an enhancement of fatty acid catabolism to ketones by acetate. The level of total ketone bodies reached with acetate dialysis (about 0.5mmol/l) was probably not great enough significantly to depress myocardial function.

Despite all these competitive substrates, glucose tolerance remained unchanged. Further evidence that

glycolysis remained unaffected by acetate *per se* is provided by the lack of a difference in values of the glycolytic products, lactate and pyruvate, between acetate and bicarbonate treatments. Although these were blood concentrations and may not strictly reflect true production and utilization rates, this observation agrees closely with the quantitative data adduced in Chapter 7.C that acetate does not affect glucose oxidation, and that, in not worsening glucose tolerance despite providing an immediately available energy source, acetate may act by improving tissue insulin sensitivity (especially as insulin values remained unchanged).

It would also appear from this study that the described effect of acetate in lowering growth hormone levels (Schmitz, Hansen, Hansen, Orskov and Alberti 1982) is due to alkalosis and not specifically to acetate. The mechanisms of such an effect are unknown and will be the subject of further investigation.

CONCLUSION

It is concluded that the major metabolic effect of acetate during dialysis is an increase in blood ketone bodies in non-diabetic and diabetic subjects. This is the only effect that cannot be ascribed to either the technical aspects of the procedure (such as use of high doses of heparin) or to alkalosis (effect of this controlled by bicarbonate dialysis). Glucose oxidation, as assessed by K_G

values or blood levels of glycolytic products (lactate and pyruvate), was unchanged with acetate.

CHAPTER 8

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The following general conclusions were reached :

i. the acetate kinase based enzymatic spectrophotometric method of acetate assay as here modified is sensitive and specific enough for the acetate levels in human plasma, and gives reproducible values comparable to those reported from other enzymatic methods; values tend to be higher than those determined by gas chromatography and the basis for such difference remains uncertain;

ii. acetate is present in measurable amounts in human plasma and appears to be rapidly metabolised as earlier documented in ruminant and other non-ruminant mammals;

iii. glucose and fatty acid metabolism constitute the major endogenous sources of circulating acetate as plasma levels rise on infusion of glucose or fat and conversely decrease when NEFA levels fall after insulin administration during the euglycaemic clamp;

iv. the fasting plasma glucose level consistently correlates with that of acetate; and the indices of glucose disposal after a glucose load correlate with the associated change in acetate levels in such a manner as to suggest that an impaired glucose disposal is associated with impairment of acetate disposal;

v. acetate utilization is impaired in diabetes, with higher fasting plasma levels, slower metabolic clearance rate, smaller elimination rate constant and longer half-life of infused acetate as compared to non-diabetic subjects; this

probably reflects the reportedly reduced activity of acetyl CoA synthetase in diabetes;

vi. glucose tolerance was not affected by the additional caloric burden imposed by a 150mmol/hr acetate load;

vii. as reported in ruminants, human plasma acetate levels rise on a high fibre diet in diabetics and after oral lactulose in non-diabetics; levels after the latter correlate with changing breath hydrogen excretion, another indicator of colonic fermentation;

viii. the fall in glycerol and NEFA levels during acetate infusions accords with previous observations that acetate suppresses adipose tissue lipolysis; in a group of diabetic subjects, fasting acetate correlated inversely with fasting NEFA levels; the rise in levels of blood ketone bodies during acetate infusions and also during haemodialysis in spite of reduced NEFA levels also confirms previous observations of possible direct conversion of acetate to ketone bodies;

ix. acetate tolerance was worsened in humans when glucose was simultaneously available unlike observations in ruminants, probably reflecting its metabolic fate in the different mammals; it is predominantly oxidised in non-ruminants but is the major lipogenic substrate in ruminants;

x. when substrate oxidation rates were assessed from respiratory exchange measurements and assumptions based on reported rates of acetate oxidation, it could be demonstrated that NEFA oxidation was consistently suppressed

while glucose oxidation remained unchanged when acetate was available; this was thought to be due to the reduced NEFA supply from suppression of adipose tissue lipolysis.

xi. the conversion of ethanol to acetate by human liver does not appear to be influenced by prior intake of chlorpropamide, a sulphonylurea antidiabetic drug reported to inhibit hepatic aldehyde dehydrogenase activity.

It would therefore be of further interest to :

(a) explore the clinical utility of plasma acetate measurements in humans as a means of assessing compliance to high fibre diets and as an alternative or complement to measurement of post-lactulose breath hydrogen excretion in malabsorptive disorders associated with colonic bacterial overgrowth;

(b) analyse arterio-venous differences in acetate levels across various human tissues and organs (especially skeletal muscle, liver, brain and adipose tissue) after glucose and lipid infusions to assess the relative role of each organ in producing and utilizing acetate, and furthermore to assess any changes in pattern that may be due to diabetes;

(c) explore the molecular mechanisms of the effect of acetate in reducing fat oxidation and mobilization by assessing its effect on endothelial lipoprotein lipase or otherwise via any influence on the Na-K ATPase activity which, for instance, has been shown to influence colonic acetate absorption;

(d) quantitate the effect of acetate on glucose tolerance

by estimating insulin sensitivity during the euglycaemic clamp with simultaneously infused glucose, acetate and insulin; control clamps with only glucose and insulin should then indicate any change in insulin sensitivity due to acetate;

(e) assess the possible role of acetate derived from colonic fermentation of dietary fibre as a source of extra calories in high fibre diets, since hitherto the energy contribution from this source has been neglected;

(f) further attempt to resolve the quantitative differences in GLC or enzymatic method - derived plasma acetate levels.

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