METABOLIC RESPONSE TO KETOSIS MIMICKING CHALLENGE IN DAIRY HEIFERS AS MARKER FOR GENETIC MERIT

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ABSTRACT

A challenge procedure was developed aimed at simulating a ketotic stress situation in dairy heifers. Parameters of the metabolic response were analyzed for their potential as metabolic markers for milk production. The challenge consisted of 5 IU insulin per 100 kg b.w. followed by an infusion of 2.0 g D(-)betahydroxybutyrate (BHB) per 100 kg b.w.. Eight blood samples were drawn within 120 minutes and analyzed for BHB, glucose and free fatty acids (FFA). A total of 125 challenge tests were performed on 93 Holstein heifers of 6 to 13 months in 2 groups. In group 1, 26 heifers were challenged twice within 4 weeks, forming challenge series 1 and series 2, respectively. In group 2, 38 heifers were challenged in series 3 and 34 heifers in series 4 which included 6 repeated heifers from series 3.

BHB concentrations, immediately after the infusion, were 11.75±0.43, 11.92±0.44, 10.96±0.36 and 11.93±0.37 mg/100ml (mean±s.e.) in the 4 series. Plasma concentrations of glucose decreased for 30 to 35 minutes as well as free fatty acids for 25 to 30 minutes following the insulin injection. Additional analyses of 10 biochemical plasma compounds in a subset of 14 heifers showed that no other metabolites or enzymes changed significantly subsequent to the challenge. Weight of the heifers was the most important factor affecting response parameters. Parameters of the response in plasma concentrations of BHB, glucose and FFA were estimated using regression models fitted to the concentration curves and were analyzed for repeatability, heritability and correlations with breeding value for milk yield (BV). The repeatability of BHB and glucose response varied between the two groups but the different age structure did not systematically affect the results after correction for weight. Repeatability estimates were: Clearance of BHB 0.215 (group 1) and 0.758

(group 2); Half-life time of BHB 0.478 (1) and 0.578 (2); Volume of distribution of BHB 0.537 (1); Time to lowest concentration of glucose (t_{min}) 0.485 (1) and 0.112 (2); ratio of clearance to t_{min} 0.290 (1) and 0.253 (2). FFA parameters were not repeatable. The repeatability estimates were higher than 0.5 for amylase, alkaline phosphatase, creatinine, cholesterol and GOT. The repeatability was zero (negative) for protein and urea.

Genetic parameters were estimated using an iterative MIVQUE procedure with an additive sire model. Twenty-five sires with an average of 3.1 daughters were included. Heritabilities were estimable for clearance of BHB ($h^2=0.42$) and minimum concentration of glucose ($h^2=0.47$). The genetic correlation between the two parameters was $r_g=-0.41$. Correlations between response parameters and BV were generally low and not consistent in heifers for which BV was estimated using pedigree information of expected transmitting ability (ETA) of sire (62 heifers), dam (75) or both (47). In stepwise multiple regression with ETA of sire as dependent variable, clearance (C_M), the ratio of C_M with time to minimum concentration of glucose (t_{min}), biological half-life time of BHB ($t_{1/2}$) and minimum concentration of glucose (A_{min}) were the regressors chosen in the best 4-variable model, accounting for 11.4 % of the variability of ETA.

It was concluded that the challenge caused short-term changes similar to subclinical ketosis in plasma concentrations of BHB and glucose, but failed to provoke a generalized metabolic reaction. The repeatable parameters of BHB and glucose concentrations were considered possible indicators for the prospective performance of the heifers.

TABLE OF CONTENTS

List of Tables

List of Figures

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1. GENERAL INTRODUCTION AND OBJECTIVE OF THESIS

1.1. GENETIC MARKERS

Increasing efforts have been directed in the past years towards finding markers which help assess the genetic merit for milk production in dairy cattle. Markers should be measurable in juvenile animals and correlated closely with the prospective productivity of the tested animal to help evaluate the production potential long before the trait of interest can be measured directly.

The ultimate purpose of genetic markers is the application in sire selection, although this thesis deals with finding metabolic traits in heifers that are correlated with the future milk production of the tested animals. The capacity of the expensive progeny testing programs in dairy breeding is limited and bull calves are selected on the basis of pedigree information which provide a rather inaccurate estimation of the true genetic potential. Consequently, a big part of the investment is wasted on bulls that finally prove to transmit negative production effects not only relative to their positive contemporaries but with respect to the breed average. A metabolic marker would provide a means to screen bull calves before they reach reproductive age and by elimination of 'metabolically' negative individuals only bulls with real improving potential would advance to test breeding, effectively enhancing selection progress.

Markers are of two principal forms : polymorphisms which show discrete distribution and metabolic parameters which have continuous characteristics.

1.1.1. Polymorphisms

Polymorphisms expressed in iso-enzymes, blood groups and milk protein types have been investigated from the beginning of the search for markers. Recently, with new developments in recombinant techniques, genomic polymorphisms (Restriction Fragment Length Polymorphisms or RFLP's) have become a main focus in marker research.

Biochemical polymorphisms are structural characteristics, genetically determined and invariable throughout life. They relate to individual gene loci which become markers if closely linked to major loci coding for milk production traits. Blood and milk protein types have been subject to extensive studies. *Lin et al.* (1986) found effects of casein and β-lactoglobulin types on milk, fat and protein yield, explaining 5 to 9 % of the phenotypic variances in Holstein, Ayrshire and crossed lines of the two breeds. *Gonyon et al.* (1987) did not find strong association of blood or milk protein polymorphisms with milk yield in an analysis involving 14 polymorphic loci in Holsteins but found significant associations with percentage solids-non-fat and percent protein for interaction effects of the J and L blood group systems and effects of β- and κ-casein alleles. Similar results were published by *Haenlein et al.* (1987) for Guernseys, with the A blood group system and interactions involving the F system having direct effects on milk production. *Graml et al.* (1987) concluded in a study on whey proteins and casein that fewer major genes accounted for a considerable amount of the genetic variability in the serum protein content of milk than previously assumed. But for total milk yield it is doubtful whether a discrete morphological character can yield enough information to be helpful in the selection of a trait that is determined by multiple genes at the very least. And it follows

Possible Genetic Markers

Polymorphisms : structural, unchanged throughout life

Cytogenetic	Karyotypes, chromosomal patterns RFLP (Restriction Fragment Length Polymorphism)
Biochemical	Enzyme isotypes Blood proteins Milk proteins

Metabolic Parameters : functional, variable, dependent on activity

Enzymes

Metabolites

Hormones

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from this that a single polymorphic trait is unlikely to discriminate sufficiently within groups of top producing animals. Nevertheless, a combination of several polymorphic techniques could yield some means for preliminary selection. Most likely, this would prove to be very laborious and expensive.

Recombinant techniques offer greater potential, measuring not the expression of genes but characterizing the substratum of inheritance itself. RFLP application points beyond single gene actions. In characterizing and identifying chromosomal fragments, it could help identify the parts of the genome that are important for the inheritance of milk production ability. Theoretically the chromosomes of dairy bulls could be dissected into small pieces, their segregation analyzed in the offspring and related to the inheritance of milk production. Segments linked with major loci for milk production could be identified and used in selection. The contribution of any single marked segment to the genetic progress would be small, but joint contribution of a large number of segments could be effective. Accumulation of information on useful segments would eventually enable them to be treated as 'known-loci', i.e. like the gene coding the desired trait itself (*Soller and Beckman*, 1983).

Selection programs based on RFLP-evaluated sires could enhance genetic progress by as much as 25 to 50 percent by some estimates. Still, the technique is very expensive. The costs of sufficient mapping of the genome to be useful in selection were estimated at $ 100,000 per sire and, in the best case, the discounted expected return was 1.5 to 3 times the costs (*Kashi et al.* (1986); *Soller and Beckmann*, 1983). *Soller* (1978) concluded that the economic justification of marker

linkage studies depends on the likelihood of identifying the mode of action of quantitative loci to be followed, which is not very high initially but would improve with the accumulation of information about specific fragments (which then become 'known-loci'). In other words, the initial investment will have to be very high to guarantee positive indentification of 'milk producing' fragments.

1.1.2. Metabolic Parameters

A functional rather than structural approach to identifying markers is to search for physiological parameters that may vary in close relationship with the production trait itself. This leads to metabolic compounds as possible choices. The actual metabolic capacity of the animal determines the limits of its productivity. Biochemical parameters are as close as possible to the ultimate marker, the trait of interest itself, and provide the most direct approach in evaluating the physiologic capacity of animals which determines the genetic merit for animal breeders. Concentrations of metabolites or hormones and enzyme activities vary continuously and the selection procedures developed for milk production can be expanded directly to include additional metabolic criteria. The analytical techniques are often routines which are fast and comparatively inexpensive and some metabolites are relatively simple compounds and readily available for experimental applications.

So far, numerous possible links between milk production and metabolites, hormones and enzymes have been found. *Hart et al.* (1979) reported differences in the change of plasma concentrations of somatotropin, insulin and thyroxine between high and low yielding animals during lactation. Different somatotropin concentrations between selection groups were also found by *Bonczek* (1985). Significant partial

correlations of thyroxine and insulin with milk yield were found by *Flach et al.* (1984). *Joakimson et al.* (1971) reported correlations between the rates of thyroxine degradation in bulls and milk yield of their daughters, but the results were not consistent for groups of bulls from four AI centers . The close relationship of the somatotropin and thyroxine regulation with genetic merit for milk yield was confirmed by *Kazmer et al.* (1986). After administration of thyrotropin releasing hormone the response in somatrotropin concentration was 2 to 3 ng/ml higher in cows with high breeding values than in control cows.

Nevertheless, age, productive and reproductive stage of the animal and the actual environment are the main contributors to the variance in milk production and cause large changes in plasma concentrations of metabolites. Most reported correlations were inconsistent among breeds or showed up only between strains with large differences in production potential. Simple concentration levels of biochemical compounds are therefore not sufficient discriminators between individual production potentials. *Marcus et al.* (1987) found no indication that prepubertal steroid levels could be of use in selection. *Tilakaratne* (1980) had to conclude that although calves with different potentials for milk production varied in aspects of energy and nitrogen metabolism, the differences were not expressed adequately in acute plasma concentrations to promise success in the search for a metabolic marker. The inconsistency in metabolic differences between animals with differing genetic merit for milk production was confirmed by *Grainger et al.* (1985) in a nitrogen utilization experiment. Plasma levels of insulin, thyroxine and tri-iodothyronine showed no significant regression with improved contemporary comparison estimates of bulls in England (*Osmond et al.*, 1981); and *Rowland et al.* (1986) concluded that despite

promising results in previous work, metabolic-profile tests were of little value for predicting milk production.

The homeostatic concentrations of metabolic parameters cannot exceed some biological limits and the value of a single blood sample depends on a large number of cofactors, including environment, constitution and condition of the animal, and laboratory and technical variabilities which may mask genetic differences. The negative outcome of efforts to predict productivity or breeding values with sufficient accuracy from plasma concentrations of hormones and metabolites prompted suggestions that metabolic reactions to imposed stress situations might be of greater predictive power with respect to milk production. Specific relationships with genetic merit for milk production have been found in parameters responding to metabolic challenges. Not surprisingly, the focus of most challenge procedures lies on energy metabolism.

Barnes et al. (1985) investigated the effects of feeding and insulin application on metabolic parameters and found differences between animals with high and low genetic merit for milk production. *Bridges et al.* (1987) reported different responses to adrenalin and noradrenalin applications in heifers of high or low genetic merit for milk fat production. Similar results were obtained by *Mackenzie et al.* (1988) after infusion of glucose, glucagon, or insulin in young Friesian bulls with high or low breeding values. The two groups of bulls also showed different reactions to fasting and refeeding but not to injections of adrenalin or arginine. *Xing et al.* (1988), however, reported higher somatotropin concentrations in bull calves with high breeding indices than in controls after infusion of arginine. The two groups also

had differing glucose and insulin concentrations after an overnight fast. Three days of fasting and refeeding were used as a stressor by *Schwab* (1986) and the different responses of somatotropin concentrations in combination with glucose and urea levels accounted for up to 50 % of the variation in breeding values of bull calves.

Nevertheless, challenges like fasting, glucose tolerance tests or diverse hormone applications have to be considered as rather general stressors and are not specifically aimed at imitating the stress of milk production. They act on basic regulatory levels of the energy metabolism and may allow an assessment of the overall metabolic strength. The challenges will reveal differences between groups of animals with distinctively different genetic predispositions, as in cases of breed differences or selective breeding for low and high genetic dairy merit. Their power in individual selection among genetically similar animals has yet to be proven.

1.2. ENERGY METABOLISM AND GENETIC VARIATION

The maximum amount of milk a cow produces depends mainly on two systems, the energy supplying digestive tract with the liver as its central organ and synthesis and secretion of milk components by udder cells. The effectiveness of both systems is limited by the individual metabolic capacity. On the hypothesis that differences in genetic merit for milk production reflect differences in metabolic capacity, it follows that animals with different genetic dispositions should show varying responses to any challenge which produces a metabolic stress.

Ketosis is the most striking syndrome related to energetic changes during high milk production. Many cows suffer a metabolic drift towards a ketotic situation in early lactation, indicated by increased free fatty acids (FFA) and increased acetate and ketones (and decreased glucose). The ketone bodies acetoacetate (AcAc) and betahydroxybutyrate (BHB) may be used in the cells of most organs as fuel in addition to glucose. They are normally produced by the liver after an increasing demand for energy surpasses the gluconeogenic capacity of the liver. A ketotic situation results whether the energy imbalance originates in an increased utilization of glucose (e.g. milk production or fetal growth) or in a shortage of glucose precursors (fasting) or in a deregulation of glucose distribution in peripheral tissues (diabetic conditions). But forced reduction of energy intake induced by prolonged fasting does not necessarily trigger the same metabolic responses as does the energy drain through increased milk production. The metabolic characteristics are different during such a 'production' ketosis (metabolic ketosis) than under other ketogenic situations (*Kronfeld*, 1971). The site of ketone production during fasting is the liver, whereas in metabolic ketosis of well-fed cows alimentary ketogenesis and mammary acetoacetate production contribute to the ketone load.

The centre of energy metabolism is the tricarboxylic acid (TCA) cycle and a key substance is oxaloacetate. It is an essential link in gluconeogenesis from most glucose precursors as well as the main 'vehicle' for acetyl-CoA (forming citrate) to enter the TCA cycle for final oxidation. Acetyl-CoA, the end product of lipolysis, cannot be transformed directly into pyruvate in the liver which would allow its use in gluconeogenesis. During energy deficit, oxaloacetate in the liver is utilized for gluconeogenesis to provide energy to peripheral tissues and its availability in the

TCA cycle is reduced. Lipolysis is activated simultaneously and glycerol released by lipolysis enters the gluconeogenic pathway while an increasing amount of acetate is competing for oxaloacetate. Forming AcAc by combining two acetylic compounds without intermission of oxaloacetate allows the liver to gain energy through partial oxidization and to supply peripheral tissues with an energy carrier other than glucose, thereby enlarging the limited energy pool (*Krebs*, 1966; *Kronfeld*, 1971). AcAc is reduced to BHB, the reaction catalyzed by BHB-dehydrogenase. BHB is a more stable compound than AcAc but has to be transformed back to AcAc before it can be metabolized in the tissue.

A hypothesis based on physiological models developed by *Krebs* (1966) assumes that selection for high milk yield led to ketogenic adaptation of high-producing cows to sustain the energetic needs in peak lactation. Still, the question arises why some cows suffer an acute ketosis while many cows do not show any ketotic changes at all under similar circumstances. This question is of central interest and highlights the metabolic dilemma of high yielding cows: the need of energy from lipolysis (which is partially transmitted by ketone bodies) for milk production and the control of negative effects of the ketones, which are acidosis and hypoglycemia through induction of insulin secretion.

Therefore, we may assume that the best cows are those which are able to overcome the problems of the auxiliary ketotic pathway and achieve metabolic homeostasis under production stress. This best adaptation may be due to:

1. higher **gluconeogenic capacity** of liver/kidney (activity of key enzymes),
2. better **endocrine response** to lack of glucose (including the regulation of

insulin, glucagon, glucocorticoids and STH) resulting in improved management of the glucose pool.

3. higher **metabolizing rate** of ketone bodies in peripheral tissue and better excretion (urine and milk) resulting in faster clearance.
4. better **homeostatic control** in general (imbalance in the glucose compartment is better controlled and may affect other metabolic pathways to a lesser extent).

1.3. OBJECTIVE OF THESIS

It should be possible to find differences between individual responses in endocrine and metabolic (or cytologic) parameters to an imposed ketotic situation which are related to the genetic adaptation to milk production. If these differences were detectable already in calves, it would provide a means to rank heifers for dairy merit before the milk yield in first lactation is known. In bulls, the estimation of breeding value for sires could be improved long before the traits of interest are expressed by their offspring. Or it could be used to select for progeny testing the most promising individual from a group of bull calves with identical pedigree, as are likely to be produced by multiple-ovulation embryo transfer.

A lengthy protocol had to be followed to induce a 'production ketosis' even in lactating cows (*Mills et al.*, 1986). It seems impractical to induce in heifers a true ketosis similar to that of lactating cows for test purpose. But it should be possible to mimic some aspects of ketosis by a challenge procedure to assess the inherent

metabolic capacity of an individual. We decided to impose a challenge which affects the homeostatic status of glucose and BHB to mimic the ketotic stress of lactation in juveniles using a combined infusion of insulin and BHB. Differences between animals would correspond to points 2 or 3 of the list of possible adaptations on page 10. The metabolic reaction of the heifers could be followed by measuring the metabolites in samples of peripherally collected blood and the repeatability of the response parameters would be evaluated. Repeatability indicates the upper limit of the genetic determination of a trait and, as such, is a first criterion for the usefulness of a parameter as predictor. Furthermore, enough tests should be performed to allow the estimation of phenotypic correlations with milk yield in first lactation and of genetic parameters (heritability and genetic correlations). The descendance of the available heifers was not checked in advance, but a goal of about 100 challenged animals was set to allow the formation of enough half-sib groups.

A note on the organization of this thesis:

The text of this thesis is organized with chapters 2, 3 and 4 written in the form of papers, each containing the appropriate part of the description of methods and a discussion of the results.

Objective of Thesis

1. To develop a challenge procedure for juvenile dairy cattle that simulates a ketotic stress similar to milk production in early lactation.

2. To evaluate the degree of individual determination of the metabolic response to the challenge (repeatability).

3. To test the reliability of the response parameters as predictors of milk production (heritabilities and correlations).

2. CHALLENGE PROCEDURE AND PHYSIOLOGIC REACTIONS

2.1. INTRODUCTION

Interest in **Metabolic Markers** for production traits has been boosted in the last two decades by studies that showed considerable genetic variation in several metabolites and hormones and genetic correlations with important traits. Selection for high milk yield affects the metabolic regulation resulting in differing plasma concentrations of hormones among selected lines. Effects on somatotropin, insulin, prolactin and thyroxine have been reported by *Joakimson et al.* (1971), *Hart et al.* (1979), *Flach et al.* (1984), *Bonczek et al.* (1985) and *Kazmer et al.* (1986).

Systematic differences of metabolites and enzymes were found in lactating cows. *Peterson and Waldern* (1981) found high repeatabilities for alkaline phosphatase (AP) and creatinine in Holstein cows and *Peterson et al.* (1982) reported heritabilities between 0.4 and 0.5 for amylase, AP and creatinine. Lower but still considerable heritabilities for glutathione reductase and creatinine kinase were estimated by *Flach et al.* (1985) in German black pied bulls. *Peterson et al.* (1982) also found genetic correlations as high as -0.9 between milk yield and amylase, AP or creatinine. These findings imply that screening of plasma compounds in cows could be useful in predicting production potential. So far, few of these parameters analyzed in juvenile animals or in bulls have been found to be correlated with milk production consistently enough to be widely accepted as an aid to selection. Several investigators have found little or no relationship between metabolic parameters and production traits (*Marcus et al.*, 1987; *Tilakaratne et al.*, 1980; *Grainger et al.*, 1985;

Osmond et al., 1981; *Rowland et al.*,, 1986).

Those negative results were derived from experiments which involved screening of plasma compounds of animals in normal production environments. Under these circumstances, the animals presumably would be in homeostatic conditions which would tend to mask any metabolic differences. More promising results were produced after imposing a metabolic stress to force a reaction related to the genetically determined metabolic potential of the individual. Challenge procedures that provoked distinctive metabolic responses in animals of differing genetic merit for milk yield involved fasting and refeeding (*Barnes et al.*, 1985; *Schwab*, 1986) and parenteral application of glucose, insulin, glucagon, adrenalin or arginine (*Barnes et al.*, 1985; *Bridges et al.*, 1987; *Mackenzie et al.*, 1988; *Xing et al.*, 1988).

Imposing a challenge similar to some of the stresses of milk production seems to be the best approach. The foremost problem for the lactating cow is to meet the energy requirements for milk production. Up to 70 g of glucose are consumed by mammary gland cells to produce 1 kg milk (*Boekholt*, 1976) with lactose production requiring as much as 85 % of the metabolic glucose turnover (*Bergman, 1976*). During lactation the metabolism is tuned toward a reduction in peripheral glucose consumption and an increased glucose supply to the udder. The limiting factor in lactose synthesis (and therefore milk production) is the availability of glucose, although the mammary gland is able to regulate its intracellular glucose concentration independently within certain limits of the arterial glucose concentration, as demonstrated in the goat by *Faulkner* (1983). Decreasing plasma glucose concentration, therefore, will not substantially reduce milk production (and glucose

demand by mammary gland cells) before the critical energy situation results in metabolic disorder. A typical example of such a metabolic emergency is the ketosis syndrome commonly found in high yielding cows. Cows in peak lactation are not able to cover their energy demand by feed intake alone and must break down body reserves. Lipolysis results in the production of surplus acetoacetate (AcAc) and betahydroxybutyrate (BHB) by the liver. The overproduction causes mounting plasma concentrations of those ketones. Normal concentrations of BHB in cows are 2 - 5 mg/100 ml plasma. Levels above 10 mg/100 ml are often found in lactating cows without negative effects (subclinical ketosis). Symptoms of acute ketosis (acetonemia) are expected with plasma concentrations of BHB above 30 - 40 mg/100 ml. (*Kronfeld,* 1971; *Bergman,* 1976; *Schwalm et al.,* 1976; *Andersson,* 1984; *Whitaker et al.,* 1983).

Our hypothesis states that productivity is limited by the cow's ability to counteract the drain in plasma glucose and to metabolize the increasing amount of ketone bodies. The metabolic responses to a challenge which simulates those effects of the milk-production stress are more likely to yield parameters correlated strongly with production ability than other attacks on homeostasis. On this basis a challenge protocol was developed aimed at mimicking production stress in heifers, using insulin to lower plasma glucose concentrations combined with parenteral introduction of BHB.

2.2. MATERIAL AND METHODS

2.2.1. Challenge Procedure

A metabolic situation similar to milk production stress in peak lactation was simulated by a combination of **insulin** and **BHB**. The intended challenge consisted of 5.0 IU of insulin (SIGMA No I-5500) and 2.0 g of D(-)BHB (5.9 g of DL-BHB Na-salt, 40% D(-)-enantiomer; SIGMA No H-6501) per 100 kg body weight. The actual BHB doses attained in each series are given in Table 3.

Prior to the infusion the heifers were fasted overnight for 15-18 hours. A catheter was inserted into the jugular vein in the morning and was used for both the infusion of insulin and BHB and withdrawal of blood samples. All challenges were started between 9 a.m. and 1 p.m., at the earliest one hour after catheter setting. The challenge was initiated with the insulin injection after an initial blood sample had been taken. Five minutes after the insulin injection, passive infusion of BHB was started. An infusion of 100 ml saline solution (NaCl 0.9 %) was prepared and a 50 % solution of BHB injected into the infusion bag. The infusion time was a nominal 10 minutes, but in practice the time varied between 4.5 and 16 minutes. Subsequently, 7 blood samples were drawn over a two-hour period, the first immediately after the infusion was finished, followed by samples at 20, 25, 30, 45, 75 and 115 minutes after the start of infusion.

After the application of insulin and the infusion of BHB the catheter was flushed with 5 to 10 ml of saline solution and filled with heparinized saline between

Table 1. Challenge Protocol.

	Minutes
Fasting overnight (15-18 h)	
Initial blood sample 1	
Insulin 5 IU/100kg : injection	-5
D(-)BHB 2 g/100kg : start infusion	0
Blood sample 2 (1^{st} sample after infusion)	(10)†
Blood samples 3 to 8	20, 25, 30, 45, 75, 115

† Sample taken immediately after infusion.

manipulations. The first 5 ml of blood drawn for each sample was discarded, then blood was drawn in a 10 ml Vacutainer tube containing 20 mg K-oxalate and 25 mg NaF additive. The tubes were kept in ice water and centrifuged within 3 hours of drawing. The plasma was collected in two 5 ml Pyrex tubes and stored at -20° C.

2.2.2. Test Animals

A total of 97 Holstein heifers were challenged, some heifers twice, resulting in a total of 125 stress tests on 93 animals after exclusion of five irregular tests. The test animals included all heifers of the appropriate age range of 6 to 13 months available in the UBC[1] Oyster River herd at the time of testing. All animals had been kept in a sheltered free stall barn and fed similar diets of silage and hay prior to all four test series.

The challenges were done in 4 series, 4 to 8 animals per day. The 27 animals challenged in June '87 formed series 1 in group 1 and were repeated after 4 weeks in July '87 forming series 2 in group 1. The 38 animals challenged in December '87 formed series 3 in group 2 and the 34 animals challenged in May '88 formed series 4 in group 2. Six animals in series 4 were repeated from series 3. The animals in this second repeated group were 6 to 7 months and 11 to 12 months old at the time of testing. Table 2 gives the number of animals, ranges in age and dates of tests.

[1] University of British Columbia

Table 2. Test Animals.

Series	Date	Number of Animals	Age *(days)*
1	June '87	27	209-369
2	July '87	26 (26)†	237-396
3	Dec '87	38	201-414
4	May '88	34 (6)‡	206-380

† number of animals repeated from series 1

‡ number of animals repeated from series 3

2.2.3. Metabolites

Plasma samples from group 1 (53 challenges on 27 animals) were analyzed for glucose, BHB and free fatty acids (FFA). Samples from group 2 (72 challenges on 66 animals) were analyzed for BHB and glucose. Glucose was analyzed using Kit No 510 of SIGMA (*SIGMA Technical Bulletin No 510,* 1980). FFA were determined with the method of *Duncombe* (1963) following the procedure of *Itaya and Ui* (1965) for the extraction of long chain fatty acids from plasma, using chloroform as the organic solvent.

BHB for group 1 was analyzed following the colorimetric method of *Kientsch-Engel and Siess* (1983). Due to the inconsistency of the results obtained by this method (intra-assay and inter-assay coefficients of variability both being greater than 20 % for normal plasma concentrations) the procedure was abandoned for the analysis of subsequent samples of group 2. The samples of group 2 were analyzed by the tris-hydrazine method introduced by *Williamson et al.* (1962) and modified by *Chandrasekaran et al.* (1972). The tedious step of plasma precipitation with perchloric acid was omitted following a modification indicated by *Barnouin et al.* (1986). (For details see Appendix 2).

2.2.4. Statistical Analysis

Least squares means were estimated with a hierarchical analysis of variance model including groups and series within groups as independent factors. PROC GLM of *SAS* (Statistical Analysis Systems) was used for the analysis. Pearson product moment correlations were estimated with PROC CORR of *SAS*. The error probability for tests of significance was set at $p \leq 0.05$.

2.3. RESULTS AND DISCUSSION

2.3.1. Uncontrolled Systematic Effects

Table 3 gives the means and standard errors for age, weight, infusion time (inf.time), amount of BHB infused per 100 kg b.w. (BHBdose), infusion rate per minute (inf.rate) and insulin dose per 100 kg b.w. (ins.dose) separately for each series. Series 1 and 2 concerned group 1 and group 2 included the remaining two series. The age difference between series 1 and 2 reflected the interval of 4 weeks between the repeated challenges in group 1, also resulting in higher average weight in series 2. The higher weight in series 4 was probably due to seasonal effects. It was not possible to keep the experimental conditions constant over all 4 series. BHBdose and infusion rate were different for the two groups. The differences between series within group were small but also significant because of very small standard errors which indicates that within each series a high degree of uniformity was attained. The heifers in series 2 received a lower dose of insulin than in the other three series. The average infusion time was the same for all 4 groups.

2.3.2. Changes in Mean Concentrations of Glucose and FFA

One hundred and twenty-five challenges were performed using the standardized procedure described above. The mean plasma concentrations of glucose and BHB of the two groups at each time point sampled are plotted in Fig.1. The least squares means of glucose, BHB and FFA for each series are shown in Table 4 to Table 6.

The prechallenge concentration of glucose (mean$\pm$s.e.) was 77.5$\pm$1.2 mg/100 ml

Table 3. Experimental Conditions.

Least squares means with standard errors.

	Group 1		Group 2	
	Series 1	Series 2	Series 3	Series 4
No heifers	27	26	38	34
Age *(day)*	309.0^{a} ± 9.8	335.5^{b} ±10.0	304.3^{a} ± 8.3	309.0^{a} ± 8.7
Weight *(kg)*	294.9^{a} ± 7.6	319.4^{b} ± 7.9	289.5^{a} ± 6.5	317.2^{b} ± 6.9
Inf.time *(min)*	9.1 ± 0.3	8.9 ± 0.3	8.7 ± 0.2	8.4 ± 0.2
BHBdose *(g/100kg)*	1.86^{a} ± 0.02	1.77^{b} ± 0.02	2.42^{c} ± 0.01	2.37^{d} ± 0.01
Inf.rate *(g/min)*	0.62^{a} ± 0.03	0.64^{a} ± 0.03	0.82^{b} ± 0.02	0.92^{c} ± 0.02
Ins.dose *(IU/100kg)*	5.01^{a} ± 0.004	4.61^{b} ± 0.004	5.01^{a} ± 0.003	5.00^{a} ± 0.004

Means with common superscript in same row are not different ($p \leq 0.05$).

Inf.time = Infusion time; BHBdose = BHB dose; Inf.rate = Infusion rate; Ins.dose = Insulin dose.

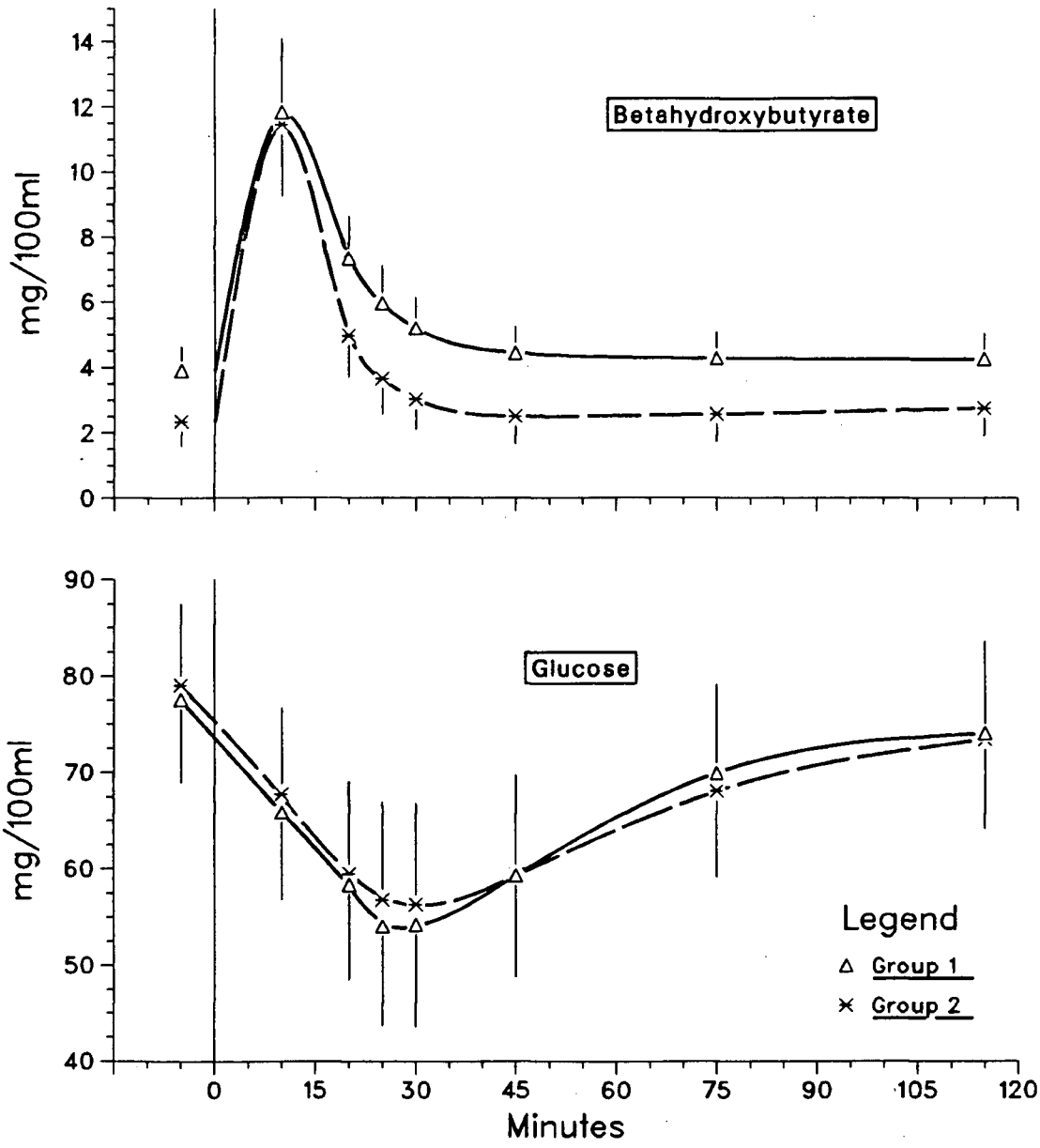

Figure 1. Glucose and BHB concentrations. Group 1 and 2.

L.S. means with S.D. (vertical bars).
Plasma concentrations after insulin (at -5 min) and BHB challenge (infusion started at 0 min).

plasma in group 1, ranging from 59.6 to 99.5 mg/100 ml, and 79.0±1.0 mg/100 ml plasma in group 2 (59.4 to 93.7 mg/100 ml). The lowest level was reached at 30 minutes (35 minutes after insulin injection) in both groups (54.2 mg/100 ml in group 1 and 56.3 mg/100 ml in group 2) (Fig.1). While the mean glucose concentrations of the groups were similar, the means between series within each group showed significant differences (Table 4). The concentrations in series 2 and 4 were higher than for series 1 and 3. The differences are more pronounced in the lower concentrations under the challenge effect. The animals in series 3 were challenged in December and in the other series in May to July, resulting in the external conditions being different. This could explain the differences in group 2, especially the slower normalization of the glucose level in series 3, indicated by significantly lower concentrations at 45 and 75 minutes.

In group 1, the initial level of glucose was the same in both series (Table 4). Although the concentrations were consistently higher in series 2, differences are only significant at 20 and 30 minutes. The decrease in glucose concentration in series 2 was slower and shorter than in series 1 and the lowest concentration was reached at sample time 25 minutes (30 minutes after insulin injection), 5 minutes earlier than in the other series.

FFA concentrations for group 1 (Table 5) followed the same pattern as glucose. The concentrations in series 2 were higher than in series 1 most of the time but with the exception of the sample at 25 minutes (30 minutes after insulin injection), none of the differences between the FFA concentrations were significant. The initial values of FFA were 110.1±7.4 μeq/l and 116.8±7.8 μeq/l for series 1 and series 2,

Table 4. Glucose Concentration. Series 1 to 4.

Least square means with standard errors of plasma concentrations after insulin (at -5 min) and BHB challenge (infusion start at 0 min).

	Group 1		Group 2	
Sample time (min)	Series 1	Series 2	Series 3	Series 4
-5	77.0^{a} ± 1.7	78.1^{ab} ± 1.7	76.9^{a} ± 1.4	81.2^{b} ± 1.4
10	64.3^{a} ± 1.7	67.4^{ab} ± 1.8	63.9^{a} ± 1.4	71.5^{b} ± 1.5
20	55.3^{a} ± 1.9	61.4^{b} ± 1.9	55.0^{a} ± 1.6	63.8^{b} ± 1.7
25	51.6^{a} ± 2.0	56.6^{ab} ± 2.0	52.8^{a} ± 1.7	60.8^{b} ± 1.7
30	50.7^{a} ± 2.1	57.7^{b} ± 2.1	52.3^{a} ± 1.7	60.4^{b} ± 1.8
45	56.7^{ab} ± 2.0	62.0^{ac} ± 2.1	54.7^{b} ± 1.7	64.1^{c} ± 1.8
75	70.4^{a} ± 1.8	69.5^{a} ± 1.8	64.3^{b} ± 1.5	71.8^{a} ± 1.5
115	73.7^{ab} ± 1.8	74.3^{ab} ± 1.9	70.1^{a} ± 1.5	76.6^{b} ± 1.6

Means with common superscript in same row are not different ($p \leq 0.05$).

Table 5. Free Fatty Acids Concentration. Series 1 and 2.

Least square means with standard errors of plasma concentrations after insulin (at -5 min) and BHB challenge (infusion start at 0 min).

	Group 1	
Sample time (min)	Series 1	Series 2
-5	110.1 ± 7.4 [a]	116.8 ± 7.8 [a]
10	72.7 ± 6.7 [a]	87.5 ± 6.7 [a]
20	59.7 ± 5.7 [a]	70.3 ± 5.7 [a]
25	52.6 ± 5.3 [a]	73.1 ± 5.7 [b]
30	68.8 ± 6.0 [a]	86.1 ± 6.4 [a]
45	100.3 ± 7.4 [a]	105.2 ± 7.8 [a]
75	125.7 ± 9.5 [a]	134.5 ± 9.5 [a]
115	140.1 ± 7.8 [a]	132.4 ± 7.8 [a]

Means with common superscript in same row are not different ($p \leq 0.05$).

respectively. The lowest values were reached at sample time 25 minutes and 20 minutes with 52.6 µeq/l and 70.3 µeq/l, respectively. As with glucose, the minimum FFA concentration in series 2 was higher and occurred 5 minutes earlier than in series 1 resulting in divergent tendencies of the concentration curves between 20 and 30 minutes (25 and 35 minutes after insulin injection).

The marked decrease in the plasma concentrations of glucose and FFA for 25 to 35 minutes following the challenge corresponds to known insulin effects and was also found by *Mackenzie et al.* (1988) after application of 1.0 mg insulin per 100 kg b.w. and *McCann and Reimers* (1985) after 2 IU insulin per 100 kg. *Barnes et al.* (1985), in contrast, found an increase in glucose concentration after injection of 0.6 IU/100kg b.w.. The animals were injected 2 hours after feeding and the effect of such a low insulin dose was probably masked by metabolic reactions due to the post-feeding situation.

There is an obvious similarity in the differences of the glucose and the FFA concentration curves between series 1 and 2 in group 1 (Fig. 2). In both series the same animals were challenged under similar conditions but the insulin dose was lower by 0.4 IU/100kg in series 2. The shorter response in series 2 after a relatively small change in the amount of insulin injected contrasts to the results of *McCann and Reimers* (1985) who found a comparatively modest difference in the response of glucose concentrations after a tenfold increase of the insulin dose in obese and lean heifers, but they also found that the return to normal concentrations was slower in lean than in obese heifers after injection of 20 IU insulin. It becomes evident that the condition of test animals, energy status and

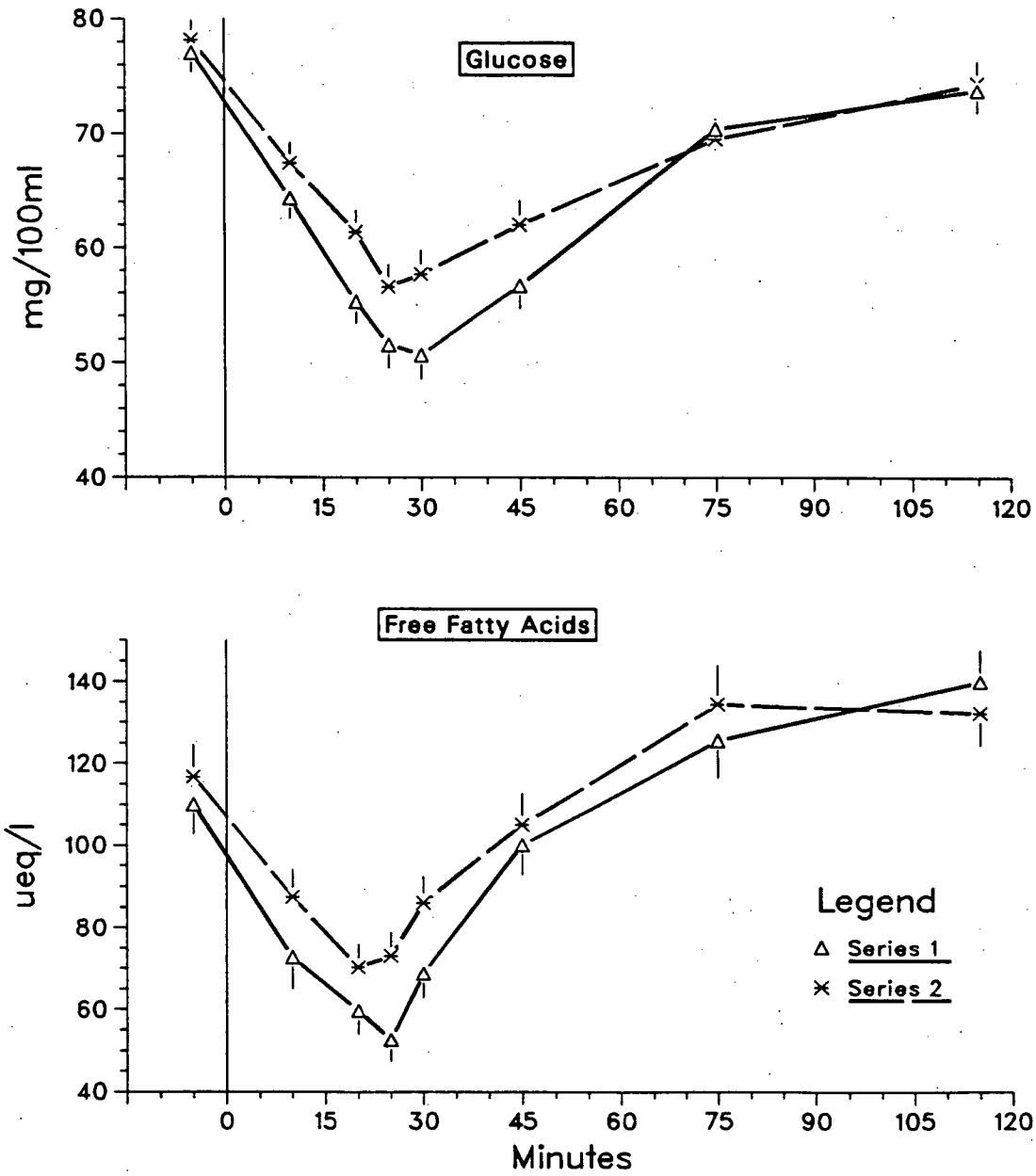

Figure 2. Glucose and FFA concentrations. Series 1 and 2.

L.S. means with S.E. (vertical bars).
Plasma concentrations after insulin (at -5 min) and BHB challenge (infusion started at 0 min).

insulin dose have to be carefully balanced and standardized to allow individual variability in glucose response to be expressed with as little confounding as possible. Fasting overnight of 15 to 18 hours as applied in this study is aimed at equalizing the test animals in a state of a slight energy deficit, but it does not remove differences in body condition which is also a source of variation.

2.3.3. Changes in Mean Concentrations of BHB

The prechallenge plasma concentrations of BHB were 3.9±0.1 mg/100 ml in group 1 and 2.3±0.1 mg/100 ml in group 2 (Figure 1). Concentrations immediately after the infusion showed high variability among animals, ranging from 7.8 to 16.4 mg/100 ml and 7.5 to 19.5 mg/100 ml for group 1 and 2, respectively. Mean BHB concentrations measured in the two groups were different throughout the entire sampling period with the exception of the peak value immediately after infusion (Fig.1). The means in the two series of group 1 were not different at most sample times, but the two series in group 2 differed slightly from each other.

The average concentration of 11 to 12 mg BHB/100 ml plasma reached after the infusion is equivalent to ketone concentrations usually referred to as 'subclinical ketosis' (*Bergman* 1971, *Whitaker et al.* 1983). Concentrations above normal levels are often found in cows during peak lactation but clinical symptoms of ketosis are only expected with ketone levels above 40 mg/100ml (*Payne* 1977). The goal of the challenge procedure to produce subnormal glucose and elevated BHB concentrations was achieved in all heifers, but the ketotic stress of the challenge was of modest nature only.

Table 6. Betahydroxybutyrate Concentration. Series 1 to 4.

Least square means with standard errors of plasma concentrations after insulin (at -5 min) and BHB challenge (infusion start at 0 min).

	Group 1		Group 2	
Sample time (min)	Series 1	Series 2	Series 3	Series 4
-5	4.11^{a} ±0.17	3.69^{b} ±0.15	2.16^{c} ±0.12	2.57^{d} ±0.13
10	11.75^{a} ±0.43	11.92^{a} ±0.44	10.96^{a} ±0.36	11.93^{a} ±0.37
20	7.20^{a} ±0.25	7.45^{a} ±0.25	4.61^{b} ±0.21	5.28^{c} ±0.21
25	5.87^{a} ±0.22	6.04^{a} ±0.22	3.26^{b} ±0.18	4.03^{c} ±0.19
30	5.07^{a} ±0.19	5.29^{a} ±0.19	2.67^{b} ±0.16	3.36^{c} ±0.16
45	4.47^{a} ±0.16	4.41^{a} ±0.16	2.15^{b} ±0.13	2.93^{c} ±0.14
75	4.49^{a} ±0.15	4.03^{b} ±0.15	2.24^{c} ±0.13	2.87^{d} ±0.13
115	4.27^{a} ±0.15	4.22^{a} ±0.16	2.42^{b} ±0.13	3.08^{c} ±0.13

Means with common superscript in same row are not different ($p \leq 0.05$).

2.3.4. Effects of Systematic Factors

The factor found to be of greatest importance was weight of the heifers, which itself was correlated with age ($r=0.862$ and $r=0.776$ in group 1 and 2, resp.). Insulin dose (IU/100kg b.w.) in series 2 was lower than in the other series because of accidental loss of material but within series the dose was nearly constant. Some variation in BHB dose was introduced in series 1 and series 3 with the intention to include as many animals as possible despite supply problems. BHB doses in series 1 varied between 1.7 g and 1.9 g per 100 kg b.w. and in series 3 between 2.2 g and 2.5 g per 100 kg b.w.. The dose was almost constant in series 2 and series 4 at 1.8 g and 2.4 g per 100 kg b.w., respectively. The challenge dose is therefore confounded with group.

The correlations between plasma concentrations at various sampling times after infusion or of the differences with normal concentrations (amplitude of response) and systematic effects are listed in Table 8 and Table 9 for the two groups. BHB concentrations (Table 8) and amplitudes of response (Table 9) showed consistent correlations with weight in group 2 only. The apparently differing effect of BHB dose on amplitude of response in the two groups was probably due to confounding of BHB dose with weight, the two variables being negatively correlated in group 1 and positively correlated in group 2.

Heavier animals received higher amounts of BHB in only slightly larger infusion volumes because infusion dose was standardized by unit body weight. The resulting higher infusion rates were reflected in correlations between BHB concentration after infusion and body weight of $r=0.326$ in group 1 and $r=0.455$ in group 2 (Table

Table 7. Correlations between weight and initial concentrations of glucose, BHB and FFA.

	Group 1 N=53	Group 2 N=72
Glucose initial value	-0.503	-0.233
BHB initial value	-	-0.249
FFA initial value	-	n.a.

Correlations different from zero are included ($p \leq 0.05$).

- = not significant n.a. = not available

Table 8. Correlation of weight, BHB dose and insulin dose with concentrations immediately after infusion (10 minutes) and at 30 minutes after start of infusion.

	Weight *(kg)*		BHB *(g/100kg)*		Insulin *(IU/100kg)*	
Group	1	2	1	2	1	2
Sample time						
			Glucose			
10 min	-0.460	-	-	-0.290	-	-
30 min	-	-	-	-	-0.329	-0.236
			Betahydroxybutyrate			
10 min	0.326	0.455	-	-	-	-
30 min	-	0.320	-	-	-	-0.246
			Free Fatty Acids			
10 min	-	n.a.	-	n.a.	-	n.a.
30 min	0.336	n.a.	-0.334	n.a.	-	n.a.

Correlations different from zero are included ($p \leq 0.05$).

- = not significant n.a. = not available

Group 1 : N = 53
Group 2 : N = 72 ; N = 70 for BHB 30 min

8). Equilibration in the body resulted in the plasma concentration of BHB being independent from body weight in group 1 at 30 minutes and considerably reduced to r=0.320 in group 2.

To avoid this weight-dependent effect, either the infused volume (and time) or the amount BHB per unit body weight had to be adjusted. The best solution would be to challenge all animals at the same weight. In comparisons of parameters of BHB concentration among animals of different weight classes, this weight effect has to be taken into account.

The negative correlation between weight and initial glucose concentration (Table 7) diminished after the infusion (10 min) and disappeared at the time of lowest glucose level at 30 minutes in both groups (Table 8). The amplitude of glucose response, the difference between glucose concentrations after the challenge and the prechallenge concentration, also was not correlated with weight (Table 9).

Infusion of BHB was shown to depress glucose concentrations in dogs (*Madison et al.* 1964, *Mebane and Madison* 1964) and had similar hypoglycemic effects in ruminants (*Bergman* 1971). However, no consistent effects of BHB dose on glucose concentration were found after the challenge because the variation in BHB dose was small within group and the dominant factor affecting glucose concentration was insulin. BHB also inhibits lipolysis, lowering the concentration of its breakdown products, the fatty acids, in the blood stream. The anti-lipolytic effect was demonstrated by *Hellman et al.* (1969) in vitro, but the negative correlation of FFA concentration 30 minutes after infusion with BHB dose (r=-0.334, Table 8) was

Table 9. Correlations of weight, BHB dose and insulin dose with amplitude of response (differences between plasma concentrations and initial concentrations) at 20 minutes and 30 minutes after BHB infusion.

	Weight *(kg)*		BHB *(g/100kg)*		Insulin *(IU/100kg)*	
Group	1	2	1	2	1	2
Sample time						
			Glucose			
20 min	-	-	-	-	-0.315	--
30 min	-	-	-0.334	-	-0.332	-
			Betahydroxybutyrate			
20 min	-	0.576	-	0.356	-	-
30 min	-	0.522	-	0.352	-	-
			Free Fatty Acids			
20 min	-	n.a.	-	n.a.	-	n.a.
30 min	0.276	n.a.	-	n.a.	-	n.a.

Correlations different from zero are included ($p \leq 0.05$)

- = not significant n.a. = not available

Group 1 : N = 53
Group 2 : N = 72 ; N = 70 for BHB 20 min ; N = 69 for BHB 30 min

confounded with the effect of weight in group 1. BHB dose was negatively correlated with weight (r=-0.458) and FFA was positively correlated with weight (r=0.336, Table 8). It is therefore impossible to evaluate the short term effect of BHB dose on FFA from the data of the challenge experiment.

2.3.5. Effects of Initial Plasma Concentrations

Initial concentrations of all three metabolites included in this study were inconsistently correlated with uncontrolled systematic factors with the exception of initial glucose concentration which was negatively correlated with weight (Table 7). The correlation coefficient between initial glucose and weight was r=-0.503 in group 1 and r=-0.233 in group 2, confirming that plasma glucose levels are higher in young animals. The lower effect in group 2 is probably due to interaction of weight with season, the two series of group 2 being performed in December and May. A low negative correlation was also found between BHB concentration and weight in group 2.

Concentration differences in FFA were dependent on the initial concentration for at least 30 minutes (Table 10). The concentration of fatty acids begins to rise in ruminants within 12 hours of fasting as a consequence of lipolysis (*Athanasiou*, 1978). The high negative correlation between initial concentration and amplitude of response indicates that in heifers with stronger metabolic reaction to the overnight fast (i.e. higher FFA levels), the inhibitory effect of the challenge on lipolysis was less effective.

In the case of glucose, the correlation between the amplitude of response and the

Table 10. Correlations between initial concentrations and amplitude of response (differences with initial value) at 20 minutes, 30 minutes and 75 minutes after BHB infusion.

Sample	20 minutes		30 minutes		75 minutes	
Group	1	2	1	2	1	2
Glucose	-0.313	0.242	-	-	-	-
BHB	-0.458	- (70)	-0.466	-0.349 (69)	-0.579	- (69)
FFA	-0.699	n.a.	-0.644	n.a.	-	n.a.

Correlations different from zero are included ($p \leq 0.05$)

- = not significant n.a. = not available

Group 1 : N = 53
Group 2 : N = 72, unless indicated (N)

initial concentration was inconsistent in direction and low in both groups, indicating that no systematic effect of initial value on the amplitude of glucose response exists.

The negative correlation between initial concentration and the amplitude of response of BHB is lower in the second group than in the first group at 30 minutes and not significant at 20 and 75 minutes, i.e. the differences with the initial sample are independent of the initial sample itself in group 2 but not in group 1. The heifers of the first group had on average almost twice the initial concentrations of the second group and the same difference of about 2 mg/100 ml plasma persisted after the normalization of the BHB concentration resulting in different shapes of the curves (Fig. 1). The two groups not only received different amounts of BHB infused (1.8 g/100kg vs. 2.4 g/100kg) but the analysis was done using two different methods. The highly negative correlation between initial value and the amplitude of response found throughout the sampling period in group 1 indicates a systematic bias and leads to the conclusion that the values of the initial samples do not reflect true basal concentrations in group 1. The method used for analyzing the samples from this group was obviously not sensitive enough to accurately assess BHB at low concentrations. The differences in experimental conditions and analytical procedure render the BHB parameters of the two groups not directly comparable.

2.4. CONCLUSIONS

Since the goal of the challenge procedure was the derivation of response characteristics with individual repeatability, the preceding description of physiological

reactions should allow factors which affect response parameters to be detected. The concentration curves of glucose, FFA and BHB in general followed the expected pattern with a somewhat higher variability than anticipated in the BHB concentration following the infusion. Most important is weight as function of the developmental stage of the juvenile, growing organism. Weight is highly correlated with age and affects almost all parameters considered in the study. Season also could have an effect on glucose and BHB concentrations. The shapes of the concentration curves of glucose (and FFA) around the minimum concentration reflected small differences in insulin dose between the two series of group 1.

Inconsistencies in protocol among the four test series caused small but significant differences in the response. Different methods for the analysis of BHB resulted in different basal concentrations in the two groups that will make direct comparisons of BHB parameters between the groups difficult.

The proposed challenge with insulin and BHB causes deviations from normal metabolism of glucose and BHB that are comparable to the effects of a mild subclinical ketosis. Effects on other metabolites may not be similar to ketosis, e.g. FFA concentration is decreased after the challenge but increased during ketosis.

3. EVALUATION OF RESPONSE PARAMETERS

3.1. INTRODUCTION

Concentrations of metabolites and hormones or enzyme activities are a possible source of information about the prospective performance of dairy heifers or the genetic merit of bulls. Individual differences are accentuated in the metabolic response to a stress induced by standardized procedures and such metabolic differences between animals could be used in the prediction of breeding values. However, the difficulty lies in identifying suitable parameters of metabolic compounds with a high enough genetic component of variance and correlation with milk yield.

Some metabolites, especially of energy metabolism, react readily to a challenge. *Athanasiou and Phillips* (1978) demonstrated highly significant changes in glucose, free fatty acids (FFA) and betahydroxybutyrate (BHB) concentrations of lactating cows during a fasting period of two days. *Barnes et al.* (1985) compared plasma concentrations of various compounds between selected groups of Holstein cows and heifers in response to feeding and insulin injection. The concentration differences of prolactin, insulin and FFA were affected by selection. Recently, *Mackenzie et al.* (1988) reported significant differences between 'high' and 'low' breeding-index bulls in pre- and post-challenge levels of several metabolites and hormones. They concluded that differences were most evident in the metabolism of glucose and insulin. Similarly, *Sejrsen et al.* (1984) reported differences between two groups of bulls in reaction to fasting. The correlations of plasma concentrations of FFA, glucose, insulin and thyroxine at the age of 3.5 and 7 months with breeding values

were evaluated and some found significant .

Comparing concentrations at discrete sampling times is not the most efficient way of investigating a dynamic system; although, simple differences in metabolites or enzymes between samples taken before and at fixed times after a challenge are most widely used. But samples are subject to sampling errors (which may be reduced by averaging samples over a given period) and autocorrelation makes comparisons of neighbouring points troublesome. Information about the dynamic aspects of the metabolic reaction may be blurred in multiple comparisons or reduced to a static 'before - after' alternative.

Condensing as much information about the shape of concentration curves in as few parameters as possible makes comparisons more straightforward. In an attempt to parameterize the whole concentration curve, *Bridges et al.* (1987) used a graphical method to calculate the area under the concentration curve. They found area differences in the response of glucose concentration in two groups of heifers with high and low breeding value after injection of adrenalin. Coefficients of regression models fitted to the sample data probably provide the simplest parameterization. To avoid problems due to autocorrelation, *Flux et al.* (1984) compared regression coefficients after fitting polynomials to some of their data. However, multiple regression coefficients are difficult to interpret biologically but the functions can also be used to estimate theoretical curve characteristics such as minimum and maximum values or areas which will have smaller sampling errors than point estimates. These estimated values of the concentration curves are integrating the information contained in all data points.

Response parameters estimated using regression models fitted to the plasma concentration curves of some metabolites in dairy heifers will be discussed here. They were used to describe the individual reaction of heifers infused with insulin and betahydroxybutyrate to provoke an individual metabolic response related to future productive capacity. The challenge procedure was aimed at simulating a ketogenic situation typically found in lactating cows during early lactation.

3.2. MATERIAL AND METHOD

3.2.1. Test Animals and Test Procedure

Test animals and challenge procedure are described in detail in chapter 2.

3.2.2. Parameters of BHB, Glucose and FFA Response

Multiple regression models were fitted and evaluated by an R^2-analysis for the concentration curves of glucose, FFA and BHB. The multiple regression model is defined as follows:

$$Y_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + \ldots + b_n \cdot X_{ni} + e_i$$

The elements in the model are:

Y_i = i^{th} observation of dependent variable,

X_{ni} = corresponding value of n^{th} independent variable (regressor),

b_0 = intercept,

b_n = partial regression coefficient,

e_i = error term $N(0,\sigma^2_{(e)})$

Plasma concentration (conc) and its logarithmic transformation (ln(conc)) were entered as dependent variables. Sampling time (min), its inverse (min^{-1}) and logarithm (ln(min)) and the squared and cubic (min^3) values of time were entered as independent variables. The model with the highest coefficient of determination (R^2) after fitting over all data was thought to be the best approximation of the biologically true curve. The analysis was then done for each challenge individually. The average R^2 of the individual curves gives an indication of the individual fit of the model.

Five different parameters describing the change in concentration were estimated individually for each curve from the regression coefficents for glucose and FFA. Parameters of foremost interest derived for BHB were biological half-life time and clearance. These parameters were then used for further comparisons.

3.2.3. Other Plasma Compounds

Samples from 8 heifers of group 1 (covering the whole age range) and of all 6 repeatedly challenged heifers of group 2 taken at -5, 10, 25, and 45 minutes during both challenge tests were additionally analyzed for cholesterol, urea, glutamate-oxaloacetate transaminase (GOT), lactate dehydrogenase (LDH), alkaline phosphatase (AP), protein, albumin, bilirubin, creatinine and amylase. These analyses were all done in a commercial biomedical laboratory using standard procedures[1].

[1] B.C. Bio-Medical Laboratories, Burnaby, B.C.

3.2.4. Statistical Analysis

Procedure RSQUARE of Statistical Analysis System (*SAS, Release 5.08 and Release 6.03 PC version*) was used for the R^2-analysis of the best models describing the response in BHB, glucose and FFA. Procedure STEPWISE was used to evaluate uncontrolled systematic effects (weight, age, BHBdose, ins.dose, inf.time, inf.rate) on response parameters.

The effect of the challenge on the plasma concentrations of cholesterol, urea, SGOT, LDH, AP, protein, albumin, bilirubin, creatinine and amylase was tested in an analysis of variance (PROC GLM of SAS) with following hierarchical model:

$$y_{ijkl} = \mu + G_i + A(S)_{ij} + S(S,A)_{ijk} + e_{ijkl}$$

The effects in the model are defined as follows:

G_i = effect of group,

$A(S)_{ij}$ = effect of animal within group,

$S(S,A)_{ijk}$ = effect of sample time within animal and group.

e_{ijkl} = error term $N(0,\sigma^2_{(e)})$.

Correlations were estimated using Procedure CORR of SAS. Differences between group means of response parameters were compared in a t-test (PROC TTEST, SAS). The error probability in significance tests was set at $p \leq 0.05$.

3.3. RESULTS AND DISCUSSION

3.3.1. Model Comparison

Table 11 lists the coefficients of determination (R^2) of the best fitting models for the concentration curves of BHB, glucose and FFA for group 1. For group 2 an analogous fit was achieved. In the case of BHB, the comparison of the hyperbolic ($R^2 = 0.725$) with the logarithmic model ($I^2 = 0.351$) showed a better fit of the former. The differences between the two-variable model involving time and the inverse of time (min, min^{-1}) and the simple model with the inverse of time (min^{-1}) alone was small. Therefore, the simple hyperbolic model was chosen for further analysis.

The coefficients of determination were low even for the best fitting models fitted over all data for the concentration curves of FFA ($R^2 = 0.288$) and glucose ($R^2 = 0.290$). A considerably improved fit was achieved after fitting the models to each individual curve with an average coefficient of determination of $R^2 = 0.604$ for FFA and $R^2 = 0.719$ for glucose. This indicates a high variability in the plasma concentrations at each sampling point and individual response to the challenge. The differences between the models involving one or two regressor variables were relatively large. The model chosen as best fit for glucose included time and the logarithm of time (min, ln(min)), and for FFA time and its inverse (min, min^{-1}).

Table 11. R^2-comparison of best fitting models for concentration curves of BHB, glucose and FFA in group 1.

One- and two-variable regressions with highest R^2's (best fit).

	Model		R^2 (I^2)†	
	Dependent	Regressor	Overall	Individual‡
BHB	Conc.	min^{-1}	0.725	0.909 ± 0.061
	Conc.	min,min^{-1}	0.767	0.957 ± 0.033
	ln(conc.)	min	0.375 (0.351)†	0.473 ± 0.170
Glucose	Conc.	min^{-1}	0.121	0.323 ± 0.188
	Conc.	min,ln(min)	0.290	0.719 ± 0.124
FFA	Conc.	min^2	0.189	0.403 ± 0.254
	Conc.	min,min^{-1}	0.288	0.604 ± 0.220

† R^2 values for transformed data (e.g. logarithmic transformation of BHB concentration) are not directly comparable with those of models fitted on the original data. To make the coefficients of determination comparable, the squared deviations from the expected values in the model after backtransformation have been used (SS'_{res}). The value comparable with R^2 then becomes

$$I^2 = 1 - (SS'_{res} / SS_{tot}).$$

‡ Mean ± standard deviation

N = 53 for BHB and glucose; N = 50 for FFA

3.3.2. Model Parameters

BHB Model $y = a + b \cdot 1/x$

In this simple regression model y denotes BHB concentration (mg/100 ml) and x is time (min). The parameters describing the concentration curve are derived from this hyperbolic model similarly to the usually employed logarithmic derivation of kinetic parameters underlying an open one compartment model (*Ritschel*, 1982). The model is a stark simplification of true ketone kinetics, which involve at least 4 important compartments: plasma BHB, plasma AcAc, liver tissue and extrahepatic tissue (*Cobelli et al.*, 1982; *Wastney et al.*, 1984).

This simplification seemed justified since the goal of this investigation was the estimation of parameters reflecting overall metabolic performance, not biochemical pathways. In addition, although the same terminology was used, the estimated kinetic parameters were not exactly the same as those derived with the logarithmic model, reflected in 'clearance' becoming **mass** instead of **volume** per unit time.

The model was fitted over the 7 samples drawn after the infusion of BHB and used for the estimation of following parameters :

Concentration after infusion of BHB $A_{10} = a+b/10$ in [mg/100ml].

Assuming a standardized infusion time of 10 minutes, A_{10} is estimated at $x = 10$.

Mean fractional removal rate $k = -b/200$ in [mg/(100ml • min)].

The slope of the tangent of the concentration curve is found as $y' = \delta y/\delta x = -b/x^2$. If $y'=0$, the tangent is horizontal and the concentration of BHB

on its normal value A_{norm}.

As an estimator for k, y' moves continuously from $-b/x^2$ to zero. The mean value of k is found as $k = {}^1/_2(-b/x^2+0) = -b/2x^2$. For the calculation of the decrease from A_{10} to A_{norm}, x will be set to $x_0=10$ and k becomes -b/200.

Volume of distribution $V_d = D_{init} / A_{10}$ in $[l \cdot 10^{-1}]$.

With D_{init} = initial dose of BHB in [mg]. V_d estimates the volume in which the infused drug becomes diluted.

Mean Clearance $C_M = V_d \cdot k$ in [mg/min]

Clearance of BHB is a measurement of the removal speed of BHB from blood plasma. Because of different properties of the hyperbolic model, 'Mean Clearance' denotes the mean amount of BHB that is removed from the compartment per unit time, not the volume cleared as in the derivation from the logarithmic model.

Biological half-life time $t_{1/2} = 2 \cdot b/(b/10+a)$ in [min].

Estimates the time needed to reduce by half the plasma concentration of BHB after infusion (standardized at 10 minutes).

The logarithmic model is commonly used in analyzing kinetics of drugs (*Ritschel, 1982*), supposedly because it fits best, which stays in apparent contrast to the results of this analysis of the BHB concentration. But further comparison of parameters of the two models shows that the better fit of the hyperbolic model is due to confounding of infused with endogenous BHB. The logarithmic model yields an estimated plasma clearance of $C=-0.517$ liters per minute and a 'mean

Table 12. Comparison of the logarithmic and hyperbolic BHB models.

Parameters estimated for group 1.

	Logarithmic	Hyperbolic
V_d *(l)*	71.69±12.29	48.95± 7.67
Clearance	-0.517±0.155 *(l/min)*	-216.79±25.64 *(mg/min)*

clearance' of $C_M = -216.8$ mg/min is estimated with the hyperbolic model used to compare BHB parameters (Table 12). The biological half-life time estimated with the hyperbolic model is $t_{1/2} = 34.8$ minutes from which 10 minutes have to be subtracted because all estimates of the BHB model refer to 10 minutes as initial value. After 25 minutes, 12.9 l plasma are cleared or 5.4 g BHB removed from the system, as calculated with C (logarithmic model) or C_M (hyperbolic model), respectively. Those values correspond to 18 % of the estimated volume of distribution (average of $V_d = 71.7$ l with logarithmic model) or 89 % of the amount of BHB infused (average amount of BHB = 6.12 g/heifer). The percentage difference arises from the fact that the plasma concentrations of BHB have not been corrected for the basic (normal) plasma content prior to parameter estimation (average normal BHB concentration in group 1 was 3.9 mg/100ml plasma). Therefore, even after eliminating all of the infused BHB, the clearance of the system would be far from being complete. The poor fit of the logarithmic model may be explained by this discrepancy. The simple logarithmic model expects mathematically the complete elimination of the drug to approach zero asymptotically while the hyperbolic model allows an intercept different from zero and the interference with endogenous BHB therefore does not affect the parameter estimation.

Glucose Model $\qquad y = a + b_1 \cdot \ln(x) + b_2 \cdot x$

The variable y is glucose concentration (mg/100 ml) and x is time (min).

The model was fitted over all 8 samples per challenge with the prechallenge sample time initialized to 1 minute. The 5 parameters describing the glucose curve are :

Prechallenge (initial) concentration $A_i = a+b_2$ in [mg/100ml].

Time to minimum concentration $t_{min} = -b_1/b_2$ in [min].

At t_{min} the first derivative $y' = \delta y/\delta x = b_1 x^{-1} + b_2 = 0$, therefore, $x = -b_1/b_2$.

Minimum concentration $A_{min} = a+b_1 \cdot \ln(t_{min}) + b_2 \cdot t_{min}$ in [mg/100ml].

By definition, the lowest y-value occurs at t_{min} and

$A_{min} = y|_{x_{min}} = a + b_1 \cdot \ln(-b_1/b_2) + b_2 \cdot (-b_1/b_2)$.

Decrease in concentration $A_{diff} = A_i - A_{min}$.

Decrease in percent of initial concentration $A_{perc} = A_{diff} / A_i$.

An index combining the response in BHB and glucose in a single variable was defined based on a combination of clearance of BHB and t_{min} of glucose. Fast return to homeostatic conditions following the challenge depends on high clearance and short t_{min} and the animal with the fastest return to homeostasis is supposed to have the highest metabolic capacity. The metabolic coefficient (MC) was therefore defined as

$$MC = |\text{clearance} / t_{min}|,$$

the units of which are in mg/min^2.

FFA Model $\qquad y = a + b_1 \cdot (1/x) + b_2 \cdot x$

The variable y is FFA concentration (μeq/l) and x is time (min).

The model was fitted over all 8 samples per challenge with the time variable of the first sample set to 1 minute. For the concentration curve of FFA the

parameters then are estimated as follows :

Prechallenge (initial) concentration $A_i = a+b_1+b_2$ in [μeq/L].

Time to minimum concentration $t_{min} = +\sqrt{(b_1/b_2)}$ in [min].

At t_{min} the first derivative $y' = \delta y/\delta x = -b_1 x^{-2}+b_2 = 0$.

Minimum concentration $A_{min} = a+b_1/t_{min}+b_2 \cdot t_{min}$ in [μeq/L].

$A_{min} = y|t_{min} = a+b_1/\sqrt{(b_1/b_2)}+ b_2 \cdot \sqrt{(b_1/b_2)}$.

Decrease in concentration $A_{diff} = A_i - A_{min}$.

Decrease in percent of initial concentration $A_{perc} = A_{diff} / A_i$.

3.3.3. Parameter estimation

The means of the estimated parameters for BHB as well as the variances were different in the two groups as a result of using different methods of analysis (Table 13). The estimates for both groups were within biologically reasonable limits but the structural differences required separate treatment of the parameters of the two groups in further analysis.

The distribution coefficient, defined as $\Delta = V_d$ / (body weight), was Δ=0.160 ml/g for group 1 and Δ=0.237 ml/g for group 2. Both values lie within the limits of 0.15 to 0.27 ml/g given by *Ritschel* (1982) as indicative of a drug being distributed in the **extracellular** fluid. At the end of the infusion (at 10 minutes) most infused BHB was therefore still in the extracellular space. BHB has to pass the cell membranes to be metabolized. Clearance as measured with the proposed model is determined mainly by the short-time drop in plasma BHB concentration over the 20 to 30 minutes following the infusion. Differences between animals may reflect the

Table 13. Parameters of BHB and glucose curves.

Means ± standard deviations.

		Group 1	Group 2
Weight (*kg*)		306.2± 34.2	302.6±47.1
BHB	A_{10} (*mg/100ml*)	11.81± 2.00	10.53± 2.21†
	V_d (*l*)	48.95± 7.67	71.79±13.57‡
	Clearance (*mg/min*)	-216.79±25.64	-341.04±39.29‡
	$t_{1/2}$ (*min*)	34.87±14.45 (N=48)	22.39± 3.39‡
	MC (mg/min^2)	0.788±0.171	1.138± 0.219†
Glucose	A_i (*mg/100ml*)	76.09± 9.62	79.24± 7.41
	A_{min} (*mg/100ml*)	54.02± 9.32	58.64±10.05
	A_{diff} (*mg/100ml*)	20.83± 7.35	20.79± 5.14
	A_{perc} (%)	26.59± 8.60	26.43± 7.25
	t_{min} (*min*)	28.65± 7.07	30.73± 5.60

† Differences between groups significant ($p \leq 0.05$).

‡ Variances unequal; differences significant after adjustment for unequal variances ($p \leq 0.05$).

Group 1 N=52
Group 2 N=72

further distribution (exchange rates with intracellular space and excretion) rather than metabolic transformation and elimination, which occurs at a slower pace (*Wastney et al.*, 1984).

Glucose parameters were comparable and not different between the two groups. From an initial (normal) level of 75 to 80 mg/100ml, the concentration fell over 30 minutes to between 50 and 60 mg/100ml. These 'theorethical' values were in complete agreement with the actual concentrations discussed in chapter 2. A similar response in glucose concentration was reported by *McCann and Reimers* (1985) and *Mackenzie et al.* (1988) after application of insulin. The metabolic coefficient (MC) which combined the effects on glucose and BHB concentrations was different between the two groups, reflecting the differences in the clearance estimates.

3.3.4. Challenge Effects on Other Metabolic Compounds

The effect of sample time was not significant for creatinine, urea, cholesterol, amylase, AP, protein, albumin, bilirubin, LDH and GOT analyzed in 14 heifers (Table 14 and 14a). Therefore, the challenge procedure did not provoke a change in the plasma concentrations. Significant animal effects found in all 10 plasma compounds indicate a strong inividual component in the variability of the concentrations. The effect of group was significant only in protein, bilirubin and LDH which could reflect environmental differences between groups or differences in challenge procedure.

The animal effect was confounded with age in group 1 which involved 8 animals ranging from 6 to 12 months of age but not in group 2 with 6 animals first

Table 14. Challenge effects on metabolites.
Analysis of variance tables for creatinine, urea, cholesterol, amylase and alkaline phosphatase.

Source	DF	SS	F	
Creatinine				
Group	1	2502.6	2.33	
Animal(G)	12	12890.8	42.44	*
Sample(A*G)	42	1063.1	0.31	
Error	56	4598.5		
Urea				
Group	1	4.538	3.72	
Animal(G)	12	14.628	33.96	*
Sample(A*G)	42	1.508	0.08	
Error	56	24.390		
Cholesterol				
Group	1	5.300	3.08	
Animal(G)	12	18.016	253.49	*
Sample(A*G)	42	0.249	0.09	
Error	56	3.665		
Amylase				
Group	1	61236.0	4.36	
Animal(G)	12	168662.2	520.2	*
Sample(A*G)	42	1134.9	0.05	
Error	56	262101.6		
AP				
Group	1	43.57	0.01	
Animal(G)	12	46365.04	439.33	*
Sample(A*G)	42	369.38	0.03	
Error	56	14744.50		

Hierarchical model includes effects of group, animal within group (animal(G)) and sample time within animal and group (sample(A*G))

* = effect significant ($p \leq 0.05$)

Table 14a. Challenge effects on metabolites (ctd).
Analysis of variance tables for protein, albumin, bilirubin, LDH and GOT.

	Source	DF	SS	F	
Protein					
	Group	1	254.63	4.75	*
	Animal(G)	12	643.48	30.90	*
	Sample(A*G)	42	72.88	0.12	
	Error	56	808.5.5		
Albumin					
	Group	1	14.792	1.84	
	Animal(G)	12	96.422	35.52	*
	Sample(A*G)	42	9.500	0.11	
	Error	56	118.000		
Bilirubin					
	Group	1	26.298	8.27	*
	Animal(G)	12	38.167	4.14	*
	Sample(A*G)	42	32.250	1.59	
	Error	56	27.000		
LDH					
	Group	1	483817.0	11.57	*
	Animal(G)	12	501682.8	21.87	*
	Sample(A*G)	42	80290.0	0.22	
	Error	56	478861.7		
GOT					
	Group	1	751.51	4.29	
	Animal(G)	12	2103.36	113.92	*
	Sample(A*G)	42	64.63	0.10	
	Error	56	829.5		

Hierarchical model includes effects of group, animal within group (animal(G)) and sample time within animal and group (sample(A*G))

* = effect significant ($p \leq 0.05$)

challenged at the age of 6 to 7 months and repeated at 11 to 12 months. The insignificant effect of group in creatinine, urea, albumin, cholesterol, amylase, AP and GOT leads to the conclusion that the significant animal effect is due to individual metabolic differences which are independent of age.

3.3.5. Factors Affecting Response Parameters

Correlations with factors of experimental conditions were calculated separately for the two heifer groups. **Weight** of the heifer was the only variable with consistently significant correlations with the parameters of the concentration curves of BHB, glucose and FFA in both groups (Table 15). Significant correlations were also found between weight and creatinine, AP and LDH (Table 16).

Since the challenge series were performed in different seasons, external conditions were not constant. The challenge conditions were also not absolutely constant between the test series. BHB dose (g/100kg) and insulin dose (IU/100kg) varied little within each series but mean values of BHB dose were higher in group 2 and insulin dose was reduced in series 2 of group 1. The variability of infusion time was considerable among animals but the average infusion time was uniform over the 4 series as was infusion rate of BHB (g/min), which was dependent simultaneously on dose and infusion time. BHB and insulin dose, infusion time and infusion rate affected response parameters, but the correlations were not consistent in the 4 series. Furthermore, those factors were insignificant when fitted in stepwise multiple regression together with weight which indicates confounding with weight. Therefore, the parameters were corrected for effect of weight only before analyzing them for individual effects.

Table 15. Correlations between response parameters and weight.

		Group 1	Group 2
BHB	A_{10}	0.258	0.531 *
	V_d	0.288 *	0.280 *
	Clearance	-0.778 *	-0.874 *
	$t_{1/2}$	-0.540 *	-0.453 *
	MC	0.497 *	0.744 *
Glucose	A_i	-0.513 *	-0.227
	A_{min}	-0.330 *	-0.207
	A_{diff}	-0.251	0.068
	A_{perc}	-0.083	0.128
	t_{min}	0.078	-0.002
FFA	A_i	0.040	-
	A_{min}	0.367 *	-
	A_{diff}	-0.248	-
	A_{perc}	-0.366 *	-
	t_{min}	0.103	-

- = not available

* = correlation coefficient significant ($p \leq 0.05$)

Group 1 N=52
Group 2 N=72

Table 16. Correlations between other metabolic compounds and weight.

Parameter		Mean ± S.D.	Correlation with weight [1]
Creatinine	(*µmol/l*)	81.81 ±13.77	0.727 *
Urea	(*mmol/l*)	3.23 ± 0.64	0.093
Cholesterol	(*mmol/l*)	2.31 ± 0.50	-0.023
Protein	(*g/l*)	63.88 ± 3.88	0.015
Albumin	(*g/l*)	30.39 ± 1.39	-0.176
Bilirubin	(*µmol/l*)	2.86 ± 0.87	0.039
AP	(U/l)	108.38 ±23.7	-0.387 *
LDH	(U/l)	780.11 ±111.4	0.491 *
GOT	(U/l)	37.31 ± 5.80	-0.061
Amylase	(U/l)	110.56 ±48.99	0.189

* = correlation coefficient significant ($p \leq 0.05$)

[1] N = 28 (means of 4 samples per challenge)

3.3.6. Between-Challenge Correlation of Response Parameters

The correlations between repeated challenges of the same animal for the weight-corrected response parameters are listed separately for each group in Table 17. Significant correlations were found in group 1 for the concentration after infusion (A_{10}, r=0.504), the biological half-life time ($t_{1/2}$, r=0.847) and the volume of distribution (V_d, r=0.467) of BHB and the time to lowest concentration (t_{min}, r=0.468) of glucose. The correlations between challenges for clearance of BHB and for MC (combining clearance and t_{min} of glucose), were not significant and no other parameter of the glucose or FFA curve had a significant correlation coefficient. The highest correlation between challenges for FFA parameters was found for the initial concentration (A_i) with r=0.201. Therefore, the parameters of FFA were excluded from further analysis. The animals in group 1 were of variable age which ranged from 6 to 13 months and the interval between repeated challenges was 4 weeks. The question arose whether the individual effect in the response to the challenge was due to confounding with age (or developmental stage of the heifers) although the observations were corrected for weight. This led to the second group of repeated challenges in 6 heifers of the same age of 6 to 7 months at the first challenge and 11 to 12 months at the second challenge 5 months later. The twice challenged heifers of group 2 were the youngest animals in test series 3 and consequently the oldest in test series 4.

Correlations between repeated challenges in group 2 were generally high for the parameters of BHB but the only significant value was found for $t_{1/2}$ with r=0.887. Clearance (r=0.811) and V_d (r=0.731) were close to significant ($p \leq 0.05$) which indicated a strong individual tendency in the 6 animals. A_i of glucose (r=0.756)

Table 17. Between-challenge correlations of response parameters after weight correction.

		Group 1	Group 2
BHB	A_{10}	0.504 *	0.651
	V_d	0.467 *	0.731
	Clearance	0.360	0.811
	$t_{1/2}$	0.847†*	0.887 *
	MC	0.320	0.557
Glucose	A_i	0.103	0.756
	A_{min}	0.151	0.224
	A_{diff}	0.174	0.017
	A_{perc}	0.217	-0.101
	t_{min}	0.468 *	0.227
FFA	A_i	0.201	-
	A_{min}	0.017	-
	A_{diff}	0.157	-
	A_{perc}	-0.057	-
	t_{min}	0.000	-

* = correlation coefficient significant ($p \leq 0.05$)

Group 1 N=25 (BHB); N=26 (Glucose,FFA).
(† N=22 for $t_{1/2}$ of BHB)

Group 2 N=6

was the only glucose parameter with a high estimate in group 2. The small number of the twice challenged animals (6) in group 2 requires very high values for correlations to be significant but the conformity in the correlation coefficients of the two groups led to the conclusion that after removing the effect of weight, age does not affect the individual response in BHB systematically.

The between-challenge correlations for glucose parameters were generally lower than for BHB parameters and not consistent between the two groups which leads to the conclusion that the glucose reaction was more affected by uncontrolled factors.

3.4. CONCLUSIONS

The combination of insulin and BHB used as stressor to provoke a metabolic reaction in heifers had the expected effects on plasma concentrations of glucose, FFA and BHB but failed to provoke a response in other metabolites or plasma enzymes. The metabolic effects were limited to the 3 compounds most closely related to the energy metabolism.

The individual response in the elimination of BHB and the changes in glucose could be characterized by kinetic parameters after fitting a suitable regression model. Concentration after infusion, volume of distribution, clearance and biological half-life time of BHB and initial concentration and time to minimum concentration of glucose were the parameters with individual differences expressed in significant correlations between repeated challenges in the same individual. Parameters derived from the best fitting model for FFA concentration showed no significant correlations

between repeated challenges.

Plasma concentration of creatinine, cholesterol, urea, protein, and bilirubin and activity of AP, LDH, GOT and amylase also showed significant individual effects. Whether the parameters derived for the BHB and glucose response are better indicators for the metabolic capacity than those metabolites cannot be decided based on the data.

Weight was a significant covariable affecting response parameters. With the age of the tested heifers ranging from 6 to 13 months, the heaviest animal weighed more than twice as much as the lightest one at the time of testing (390 kg vs. 174 kg). The correlations with weight probably reflected the metabolic changes in the phase of rapid growth. Reduction of the variability of age could be achieved by performing challenge tests within narrow age limits to facilitate comparisons between the animals.

4. GENETIC PARAMETERS AND CORRELATIONS WITH BREEDING VALUE.

4.1. INTRODUCTION

Evaluating animals in a breeding program at an early age is genetically and economically advantageous. The earlier selection decisions are made, the better available resources can be directed selectively towards the chosen individuals. In dairy breeding, the traits of interest are measurable in females only and after the animals have reached reproductive age and completed at least a portion of their first lactation. This current method means that important decisions about early selection are based on pedigree information alone. The breeding index, as derived from the pedigree is a statistical value expressing the expected mean of the actual genetic merit for milk yield of all animals with the same index. Two calves having the same breeding indices may differ considerably in their true genetic and metabolic potential even if the pedigree is identical. Enhancing preselection of bulls for the expensive progeny testing programs or being able to discriminate between animals with identical breeding values is difficult without additional information about the 'actual' metabolic capacity of the individual.

Endocrinologic and metabolic parameters, if correlated with production potential and measurable in juveniles, could contribute to improved accuracy of selection indices from pedigree information. To qualify as a **Metabolic Marker** for the genetic merit of milk production, a trait should be genetically determined itself and correlated with milk yield. A variety of plasma compounds have been reported to be correlated with milk yield, at least when determined in lactating cows. They are

found among metabolites (e.g. glucose, FFA, urea, bilirubin, protein, creatinine) and among plasma (or serum) enzymes (e.g. GOT, GLDH, AP, GPT, LDH) (*Gibson et al.*, 1987; *Adam et al.*, 1985; *Sejrsen et al.*, 1985; *Baur*, 1984; *Flach et al.*, 1984; *Peterson et al.*,1982; *Graf et al.*,1978).

It will be more difficult to find characteristics in juvenile individuals that are clearly related to prospective milk production. The juvenile physiology is tuned toward growth and development and genetic traits important to the lactating cow may not be expressed during this stage. But the basic metabolic mechanisms are the same at any stage of development and any individual differences are of interest as possible markers for milk production. *Allaire et al.* (1981) found that a combination of sexual steroids (estrogens and progesterone) estimated at 150, 200 or 300 days of age in heifers accounted for up to 20 % of the variation in subsequent first lactation milk yield. But in a larger study, *Marcus et al.* (1987) could not confirm the usefulness of sexual steroids in predicting first lactation performance.

Physiological considerations as well as experimental evidence lead to parameters of the energy metabolism as possible markers (*Tilakaratne et al.*, 1980; *Land et al.*, 1983). The productive ability of milking cows, especially in early lactation, is determined by their ability to buffer deviations from metabolic equilibrium and to sustain energetic homeostasis. It follows that metabolic differences among animals are most likely found under production stress. Under the assumption that differences in reaction to production stress are genetically determined, the appropriate challenge procedure should bring them into expression in the juvenile animal. Differences in the response parameters to the challenge could then be used in the prediction of

genetic merit for milk production.

4.2. MATERIAL AND METHOD

4.2.1. Test Animals and Challenge Procedure

Animals and experimental procedure are described in chapter 2.

4.2.2. Response Parameters

Multiple regression models were fitted to the concentration curves of BHB and glucose and the regression coefficients were used to estimate curve parameters. **Clearance** (C_M), biological **half life time** ($t_{1/2}$), **concentration** after infusion (A_{10}) and **volume of distribution** (V_d) were estimated for BHB. Parameters derived for the glucose concentration curve were **initial concentration** (A_i), **minimum concentration** (A_{min}), **decrease in concentration** (A_{diff}), **relative decrease** (A_{perc}) and **time** until the minimum concentration was reached (t_{min}). In addition, a **metabolic coefficient** (MC) was derived as the ratio of C_M to t_{min}. All parameters were corrected for weight.

Creatinine, cholesterol, urea, protein, albumin, bilirubin, AP, LDH, GOT and amylase were analyzed in 4 samples per challenge for 14 animals. The means of those variables were used in the repeatability analysis since the challenge procedure had no apparent effect on the values.

4.2.3. Repeatability

Repeatabilities for the parameters of FFA, glucose and BHB were estimated with the random model (*Pirchner,* 1983):

$$Y_{ij} = \mu + H_i + e_{ij}.$$

The elements in the model are defined as follows:

Y_{ij} = j^{th} measurement of variable Y in heifer i,

H_i = effect of heifer i,

e_{ij} = error term $N(0,\sigma^2_{(e)})$.

Repeatability is the ratio of heifer variance (σ^2_H) to total variance (σ^2_T) and corresponds to the intraclass correlation. It gives an upper limit of the genetic determination of a trait. The numerator includes additive and non-additive components of the genetic variance and permanent environmental variations and the denominator is the total phenotypic variance. Heifer and total variance were estimated from the Least Squares estimates of variance within and between heifers using analysis of variance methods.

The response parameters of BHB and glucose were preadjusted for weight but for the metabolic plasma compounds analyzed in 14 heifers the model was modified by adding weight as covariable to the model. The model was therefore:

$$Y_{ij} = \mu + H_i + b \cdot (W_i - \overline{W}) + e_{ij}.$$

The elements denoting the covariance effect are defined as follows:

Y_{ij} = mean of 4 samples of variable Y in j^{th} challenge of heifer i,

b = regression coefficient of variable Y on weight,

W_i = weight of heifer i,

$\overline{W}$ = mean of weight.

4.2.4. Variance Components

With the exception of A_{10}, means and variances of the BHB parameters were unequal between the two groups. All parameters with unequal variances were normalized within each series as follows:

$$Y_{Ai} = (A_i - \overline{A})/\sigma_A$$

The elements in the formula are:

Y_{Ai} = normalized value of A_i,

A_i = i^{th} value of variable A,

$\overline{A}$ = mean of variable A,

σ_A = standard deviation of variable A.

The 92 heifers were descendant from 39 sires. Eighteen of the sires had multiple progeny groups including 71 heifers overall. The size of those half-sib groups ranged from 2 to 10 heifers with a mean of 3.9 heifers per group. Components of variance were first estimated for the 18 sires having multiple progeny using Henderson's Method 3 and underlying following model:

$$y_{ik} = \mu + S_i + e_{ik}$$

The elements in the model are:

y_{ik} = observation in k^{th} daughter of sire i,

S_i = random effect of sire i,

e_{ik} = error term $N(0, \sigma^2_{(e)})$.

The calculation was done with PROC GLM of SAS (*SAS PC Version 6.03*, 1986). These variance components were then used as priors in an approximate iterative MIVQUE analysis for a single trait using an additive sire model (*Henderson*, 1984). The matrix calculations were done with PROC MATRIX of SAS (*SAS Version 5.08*, 1985). Twenty-five sires with more than 1 progeny or which were related to other sires in the set were included in the MIVQUE calculations, resulting in an average of 3.1 heifers per half-sib group.

Heritability was estimated as follows:

$$h^2 = 4 \cdot \sigma^2_{sire} / (\sigma^2_{sire} + \sigma^2_{error}).$$

Genetic correlations between response parameters were estimated as follows:

$$r_g = \sigma_{(A,B)s} / \sqrt{(\sigma^2_{A(s)} \cdot \sigma^2_{B(s)})}.$$

The covariance and variance components in the formula are defined as follows:

$\sigma_{(A,B)s}$ = sire component of covariance between A and B,

$\sigma^2_{A(s)}$ = sire component of variance of A,

$\sigma^2_{B(s)}$ = sire component of variance of B.

The sire component of the covariance between A and B was derived from the sire variance component of the sum of A and B as follows:

$$\sigma_{(A,B)s} = [\sigma^2_{(A+B)s} - \sigma^2_{A(s)} - \sigma^2_{B(s)}] / 2.$$

Since lactation results of the tested animals were not yet available to calculate

correlations with response parameters, breeding values (BV) for milk production were estimated from pedigree information. Estimated transmitting ability (ETA) for milk in BCA units[1] (BCA (milk)) of the sires were available for 62 heifers. Seventy-five dams had cow indices for 1988 (which also are ETA's in BCA units) but only 47 heifers had information on both parents. Correlations between BV's and response parameters were calculated separately for the three heifer groups with sire, dam and combined estimates ignoring the unknown intercept between sire and dam values of BCA (milk).

Maximum R^2 stepwise multiple regression (*SAS*, PROC STEPWISE) was used to evaluate the relative importance of the response parameters as predictors of breeding value. All eight response parameters of BHB and glucose were entered as regressors and the best 2-variable and 4-variable models derived separately for BCA (milk) of sire and BCA (milk) of dam and the combined BCA (milk) estimate. For 7 heifers with BCA estimates of sire and for 12 heifers with BCA estimates of dam, it was possible to compare the effects of the kinetic parameters of BHB and glucose with those of the concentrations of amylase, cholesterol, creatinine, AP, GOT, LHD and urea. Stepwise multiple regressions of BCA (milk) of sire and dam was run first with all seven metabolites as independent regressors. Then the 3 metabolites identified by the best fitting model were combined with the 3 best fitting parameters of glucose and BHB and the new set of regressors entered in stepwise regression.

[1]Agriculture Canada, Sire Appraisal Direct Comparison, Feb. 1989

4.3. RESULTS AND DISCUSSION

4.3.1. Repeatability of Response Parameters

The repeatabilities of the response parameters of BHB, glucose and FFA are given in Table 18. The most consistent result for BHB parameters was obtained for $t_{1/2}$ with repeatabilities of R=0.478 and R=0.578 in group 1 and group 2, respectively. All parameters of BHB showed a repeatability higher than 0.5 in at least one group. Clearance (C_M) had a high repeatability in group 2 only (R=0.758); whereas, in group 1 it had the lowest value of all BHB parameters. C_M is a function of the volume of distribution (V_d) and the fractional removal rate (k). V_d is defined by the peak plasma concentration of BHB after infusion and the infused amount of the drug and k is dependent on the form of the descending curve. The 'normal' concentrations after elimination of the infused BHB are important in estimating k. But the method used for the analysis of BHB concentration in group 1 was not sensitive enough to determine the true basal concentrations and possible differences between animals were not detected (see chapter 2). The individual component in k therefore is underestimated in group 1 which could explain the low repeatability of C_M as a function of k. This conclusion is supported by the fact that in group 2, C_M was the best repeatable BHB parameter.

Glucose parameters were not as repeatable as BHB parameters, as indicated by lower between-challenge correlations in chapter 3. Only t_{min} showed substantial repeatability for glucose in group 1 (R=0.485) but the value was not confirmed in the results of group 2. The repeatability of the 'synthetic' metabolic coefficient (MC) was consistently low with R=0.290 and R=0.253 in group 1 and 2, respectively.

Table 18. Repeatabilities of response parameters. (R ± S.E.)

		Group 1	Group 2
BHB	A_{10}	0.521 ± 0.149	0.293 ± 0.409
	V_d	0.537 ± 0.145	0†
	C_M	0.215 ± 0.195	0.758 ± 0.190
	$t_{1/2}$	0.478 ± 0.164 (N=23)	0.578 ± 0.298
	MC	0.290 ± 0.187	0.253 ± 0.418
Glucose	A_i	0†	0.303 ± 0.406
	A_{min}	0†	0.096 ± 0.443
	A_{diff}	0.153 ± 0.195	0.063 ± 0.445
	A_{perc}	0.080 ± 0.199	0†
	t_{min}	0.485 ± 0.153	0.110 ± 0.441
FFA	A_i	0.199 ± 0.196	-
	A_{min}	0†	-
	A_{diff}	0.133 ± 0.200	-
	A_{perc}	0†	-
	t_{min}	0†	-

† = value negative

Group 1 N=25 for FFA and BHB ; N=26 for Glucose
Group 2 N=6

MC combines the respective accuracy of the two factors clearance and t_{min}, resulting in a value for repeatability close to the poorer estimate for the original variables.

Although the results of the repeatability estimations for the two groups were not very consistent for BHB and glucose, they indicate potential usefulness of clearance and $t_{1/2}$ of BHB and t_{min} of glucose in genetic evaluation. Regarding repeatability as the upper limit of heritability, we might expect intermediate h^2's for these parameters.

Tilakaratne et.al (1980) found repeatabilities for BHB concentration of R=0.051 in fed calves and R=0.268 in fasted calves. The corresponding repeatabilies for glucose concentration were R=0.368 and R=0.201, respectively (N=42). *Rowlands* (1986) found a repeatability for glucose concentration in young bulls of R=0.47 (N=428) and in adult bulls of R=0.62 (N=214). The kinetic parameters obtained here for plasma glucose and BHB showed repeatabilities of the same magnitude, although the values are less accurate because of the smaller number of animals involved.

4.3.2. Repeatability of Other Metabolites

The challenge procedure did not affect the plasma concentration of metabolites analyzed additionally for 8 heifers of group 1 and all 6 heifers of group 2 with repeated challenges. Therefore, the mean of the four samples analyzed per challenge was used to estimate repeatability. The effect of group was also not significant for most variables. The repeatability between challenges in the same animal for cholesterol, creatinine, urea, albumin, AP, GOT, and amylase was estimated for the

Table 19. Repeatabilities of metabolites and enzymes.

	R ± S.E. (N=14)
Amylase *(U/l)*	0.883 ± 0.061
AP *(U/l)*	0.595 ± 0.179
Bilirubin *(mmol/l)*	0.447 ± 0.222
Cholesterol *(mmol/l)*	0.756 ± 0.119
Creatinine *(mmol/l)*	0.667 ± 0.154
GOT *(U/l)*	0.745 ± 0.123
LDH *(U/l)*	0.297 ± 0.253
Protein *(mg/l)*	0†
Albumin *(mg/l)*	0.075 ± 0.275
Urea *(mmol/l)*	0†

† = value negative

combined data of the two groups. For the repeatability estimation of protein, bilirubin and LDH the effect of group was added to the analysis of variance model.

The plasma concentrations of some metabolites showed more differences between individuals than the response parameters of BHB or glucose (Table 19). High repeatabilites were found for amylase ($R>0.8$), GOT and cholesterol ($R>0.7$), creatinine ($R>0.6$) and AP ($R>0.5$). Urea, protein and albumin were the only metabolites of low or non-estimable repeatability.

The high repeatabilities of amylase, AP, GOT and creatinine are comparable to the estimates of *Peterson and Waldern* (1981). Evidence of the considerable magnitude of the individual determination of enzymes and metabolites in lactating cows was also found by *Graf et al.* (1978) and by *Peterson et al.* (1982). Correlations with milk yield were significant for (among others) the levels of GOT and glucose. *Adam et al.* (1985) derived heritabilities in the broad sense for liver specific enzymes in lactating twins. For GOT and AP, he found estimates of $h^2=0.21$ and $h^2=0.52$, respectively, which compare to the repeatabilities 0.745 and 0.595 found here.

The non-estimable repeatability of urea which indicates that the urea concentration had no individual significance, is in contrast to findings of *Rowlands* (1986) who reported a repeatability of $R=0.30$ in young bulls and $R=0.47$ in adult bulls. *Tilakaratne* (1980) found even higher repeatabilities in calves. *Peterson and Waldern* also estimated a repeatability for urea concentration of $R=0.30$ in dry cows but in lactating cows the estimates were low with $R=0.16$ in pregnant and $R=0.01$ in non-pregnant cows. High urea levels are linked with increased protein catabolism.

Evidence exists that cattle with high genetic merit for milk production rely relatively more on fat catabolism than on protein catabolism compared with unselected animals (*Tilakaratne,* 1980), but those genetic differences among the heifers were not expressed under the kind of stress applied by the challenge procedure imposed in the present study.

4.3.3. Heritabilities and Genetic Correlations

Clearance of BHB (C_M, $h^2=0.42$) and minimum concentration of glucose (A_{min}, $h^2=0.47$) were the only two parameters for which heritabilities were estimable (Table 20). Estimates for A_{10}, V_d, $t_{1/2}$, MC, A_i and t_{min} lay outside the parameter space for heritability. The estimates suffer from the insufficient number of animals. The average sire group size was 3.1 animals. The relatively high estimates for C_M and A_{min} indicate a genetic component in those parameters and emphasize a possible value in genetic evaluations.

Only the result for C_M was consistent with the repeatability estimated previously if we accept that the value of $R=0.215$ for C_M in group 1 was underestimated because of analytical problems. A_{min} was poorly repeatable ($R=0.096$ in group 2, non-estimable in group1) and the h^2 estimate is inconsistent with the expectation that repeatability indicates the upper limit of heritability. Repeatabilities between 0.1 and 0.6 was estimated in the previous analysis for $t_{1/2}$, A_{10}, MC, A_i and t_{min} which showed non-estimable heritabilities here. Probable reasons for these inconsistencies are chance effects and the unfavorable structure of the data set for h^2 estimation with 18 of the 25 sires included having progeny groups of less than 4 heifers. Furthermore, different subgroups of heifers used in the estimations of

Table 20. Heritabilities of BHB and glucose parameters.

Parameter		h^2
BHB	A_{1o}	$0^{\dagger}$
	V_d	$0^{\dagger}$
	C_M	0.416 ± 0.503
	$t_{1/2}$	$0^{\dagger}$
MC		$0^{\dagger}$
Glucose	A_i	$1^{\ddagger}$
	A_{min}	0.471 ± 0.507
	t_{min}	$1^{\ddagger}$

† = not estimable (value < 0)
‡ = not estimable (value > 1)

repeatability and heritability were only partially overlapping.

Baur (1984) found an intermediate heritability for plasma glucose concentration in heifers after first calving ($h^2 = 0.30$) as did *Rowlands* (1986) in bulls ($h^2 = 0.41$). These values are comparable to $h^2 = 0.47$ found here for A_{min}, the minimum glucose concentration following the challenge but *Peterson et al.* (1982) reported a near-zero heritability for glucose ($h^2 = 0.02$) in lactating cows. No other heritability estimates are known for C_M of BHB. Further investigations will have to clarify the amount of genetic determination of parameters of plasma glucose and BHB concentrations and the response parameters to stress tests.

The genetic correlation between C_M and A_{min} was $r_g = -0.42$. Clearance is numerically negative, denoting the elimination of the substance from the system. The negative correlation therefore describes a physiologically positive relationship between the elimination of BHB and the minimum concentration of glucose. High clearance of BHB is associated with high minimum glucose concentration after the challenge. Since ketones are metabolized in most tissues and act as energy carrier from the liver to the periphery in case of glucose deficiency (*Aiello et al.*, 1984; *Bergman*, 1971), the estimated correlation fits the metabolic hypothesis that animals with a higher rate of ketone consumption are less prone to suffer a dramatic drop in plasma glucose. *Gibson et al.* (1987) found that cows with high genetic merit for milk yield had higher peak ketone levels but did not have more treatments for clinical ketosis which indicates that high-merit cows are either more ketone-tolerant or able to metabolize ketones at a higher rate so that high concentrations do not overload the metabolic system. If these differences are accurately measurable in the

individual, they could form a metabolic marker for genetic merit.

4.3.4. Correlations with Breeding Value for Milk

Correlations between breeding values estimated from BCA sire or BCA dam and the response paramters of BHB and glucose are given in Table 21. None of the correlations were significant. However breeding value estimates from pedigree information have had low accuracy and high correlations were therefore not expected.

The highest values were found between BCA (milk) of sire and C_M of BHB ($r=0.148$), A_i of glucose ($r=0.194$) and A_{min} of glucose ($r=0.200$). Correlations with BCA (milk) of dam were of almost the same magnitude for C_M ($r=-0.140$), $t_{1/2}$ ($r=-0.143$) and t_{min} ($r=0.144$). It should be noted that the correlations between BCA sire or BCA dam and response parameters were not consistent, even pointing in opposite directions.

The positive correlation between BCA sire and A_{min} of glucose, indicating a better glucose homeostasis of animals with high breeding values, is in apparent contradiction to the negative correlations between milk yield and glucose concentration found in lactating cows (*Bauer,* 1984; *Graf et al.,* 1978). But the negative correlation is a result of high glucose consumption for milk production which could cause a lower glucose level in high-producing cows even though they have better homeostatic regulation than genetically inferior cows.

Table 21. Correlations between BCA of sire or dam and response parameters.

Parameter		BCA Sire	N	BCA Dam	N
BHB	A_{1o}	0.052	63	-0.012	76
	V_d	-0.103	62	0.118	76
	C_M	0.148	63	-0.140	76
	$t_{1/2}$	0.034	63	-0.143	75
Glucose	A_i	0.194	63	0.020	77
	A_{min}	0.200	63	-0.100	77
	t_{min}	-0.037	63	0.144	77
MC		-0.094	63	0.034	76

No correlation significant ($p \leq 0.05$).

4.3.5. Relative Importance of Parameters

Table 22 includes the best 2-variable and 4-variable models of the multiple regression of BCA (milk) sire and BCA (milk) dam on C_M, A_{10}, V_d, $t_{1/2}$, MC, A_{min}, A_i and t_{min} as regressors. The 4 parameters C_M, $t_{1/2}$, MC, and A_{min} accounted for 11.7 % of the variability of BCA (milk) sire. In the best 2-variable model C_M and A_{min} accounted for 7.6 % of the variability of BCA (milk) sire. Only 6.5 % of the variability of BCA (milk) dam were removed by the best 4-variable model. In any case, a combination of parameters of BHB and glucose response described best the variability found in breeding values. The higher R^2's in the multiple regression on BCA (milk) sire were consistent with the expected higher accuracy of breeding values estimated from progeny testing. In confirmation of the diverging correlations between BCA (milk) sire or dam and response parameters (Table 21), different variables were included in the best fitting regression models for BCA (milk) sire or dam. Multiple regression on the combined estimates of BCA (milk) resulted in considerable loss of fit (Table 22). The 4 best fitting variables accounted for only 3.8 % of the variability in the combined BCA (milk) estimates compared with 11.7 % in BCA of sire.

In a joint stepwise regression on the parameters of BHB and glucose and the 10 metabolites analyzed in a subset of the heifers, 94.9 % of the variability of BCA (milk) sire in 7 heifers was explainable by the effect of clearance of BHB and urea (Table 23). In the 4-variable model, clearance was replaced by $t_{1/2}$ of BHB and A_{min} as well as t_{min} of glucose were entered, accounting for 99.8 % of the variability . BCA estimates of dams again included different parameters and were less predictable. Only 47.3 % of the variability in 12 heifers were explained by amylase

Table 22. Stepwise multiple regression of BCA (milk) sire, dam or combined on parameters of BHB and glucose concentrations.

Best 2-variable and best 4-variable model.

No of variables in model	Parameters	Significance	Model R^2
	BCA Sire (N=62)		
2	C_M	-	0.076
	A_{min}	-	
4	C_M	*	0.117
	A_{min}	-	
	$t_{1/2}$	-	
	MC	-	
	BCA Dam (N=75)		
2	A_{min}	*	0.041
	A_i	-	
4	A_{10}	-	0.065
	V_d	-	
	A_{min}	-	
	A_i	-	
	BCA combined (N=47)		
4	C_M	-	0.038
	$t_{1/2}$	-	
	A_i	-	
	t_{min}	-	

* = Variable effect significant ($p \leq 0.05$)

Regressors included are: C_M, A_{10}, V_d, $t_{1/2}$, MC, A_{min}, A_i and t_{min}.

Table 23. Stepwise multiple regression of BCA Sire and BCA dam on response parameters and metabolites.

Best 2-variable and best 4-variable model.

No of variables in model	Parameters	Significance	Model R^2
	BCA Sire (N=7)		
2	C_M	*	0.949
	Urea	*	
4	Urea	*	0.998
	$t_{1/2}$	*	
	A_{min}	*	
	t_{min}	*	
	BCA Dam (N=12)		
2	Amylase	*	0.473
	Urea	-	
4	Amylase	*	0.619
	A_i	*	
	Cholesterol	-	
	GOT	-	

* = Variable effect significant ($p \leq 0.05$)

Regressors included in the model are: C_M, $t_{1/2}$, MC, A_i, A_{min}, urea, amylase, GOT, alkaline phosphatase, creatinine, cholesterol, LDH.

and urea, the best 2-variable model. Amylase, cholesterol, GOT and A_i of glucose provided the best fit with 4 variables, accounting for 61.9 % of variablility in BCA dam. The estimation of breeding values from pedigree information used in these evaluations was not very accurate and offered only an approximation to the true genetic merit of the heifers. In combination with the low number of animals it follows that these results have to be considered as general indications of possible relationships only. But the fact that the strongest relations were found between (kinetic) response parameters and BCA (milk) sire which constitutes the more accurate estimation of the true breeding value than BCA (milk) dam, indicates that kinetic parameters possess predictive potential.

4.4. CONCLUSIONS

Parameters derived from the metabolic response to a challenge affecting BHB and glucose metabolism have an individual component. The repeatabilities of kinetic parameters of the concentration curves of BHB and glucose were not consistent in the two heifer groups but volume of distribution, clearance and biological half life time ($t_{1/2}$) of BHB and t_{min} of glucose had a repeatability higher than 0.5 in at least one group. The age structure of the groups had no systematic influence on the repeatability estimation after correction of the variables for weight.

Analyzed in a subset of the animals only, plasma levels of amylase, AP, cholesterol, creatinine and GOT were as well or better repeatable than the parameters of glucose or BHB. All of those metabolites had previously been found by others as of possible genetic determination in lactating cows.

Estimable heritabilities for clearance of BHB (C_M) and minimum concentration of glucose (A_{min}) were in the range of intermediate values, but only the result for C_M was consistent with repeatability estimates.

Correlations with breeding values were not significant, but they indicated trends compatible with physiological considerations about metabolic effects on milk production. Response parameters of BHB and glucose were evaluated in multiple stepwise regression together with additional metabolites of which correlations with milk yield in lactating cows have been reported. In the 2-variable or 4-variable model accounting for the highest amount of variability in breeding value, C_M or $t_{1/2}$, A_{min} and t_{min} were included, respectively. The prevalence of response parameters of BHB and glucose plasma concentrations over other metabolites in the BCA (milk) sire multiple regression model indicates that those parameters are probably better predictors of genetic merit and are possible markers for the metabolic potential of dairy heifers. These conclusions are preliminary due to the small number of heifers in the model and the inconsistencies found in the genetic analysis, especially the disagreement between the results of the heritability and repeatability estimation.

Individual metabolic differences similar to those found in cows under production stress are already detectable in juvenile cattle expressed in the repeatability of response parameters of glucose and BHB or the concentrations of metabolites and enzyme activities. Whether these individual differences are consistent during the growth period and relate to the later milk production remains an open question at this stage of the investigation.

5. GENERAL DISCUSSION

The challenge procedure was aimed at 'mimicking' in heifers ketotic changes which occur in lactating cows to detect individual differences in the metabolic response of the heifers. This goal was achieved for a short time in general but the effects on BHB and glucose concentration were not timed adequately to invoke 'synchronized' ketosis. The BHB concentration was at its highest level immediately after the infusion at 10 minutes while the glucose concentration reached the minimum value at 25 to 35 minutes when BHB was almost back to normal concentrations. A delay of the start of BHB infusion by 15 minutes instead of 5 minutes after insulin injection would force the main effects in both metabolites to occur at the same time. The important phase with respect to response parameters was the first 30 minutes during which elimination of infused BHB was completed and the minimum level of glucose concentration reached.

Parameters describing the change in FFA concentration failed to be repeatable although an individual reaction was observed in the same time period as in glucose. This may be due to the challenge having an opposite effect on the regulation of fat metabolism than the factors resulting in metabolic ketosis during lactation. Lipolysis increases in response to energy deficit which leads to increased plasma concentration of FFA but insulin and BHB both have antilipolytic effects. Their use in the challenge resulted in 'symptomatic' ketosis with respect to glucose and BHB concentrations but not a functional ketosis. Ketosis in lactating cows develops over several days or weeks and it seems unlikely that a practical short-time test procedure could be devised which simulates all aspects of ketoses in

heifers. It remains open whether the metabolic differentiation between animals could be enhanced by maintaining the ketotic conditions for a longer period to provoke a stronger overall metabolic reaction.

The inconsistencies and contradictions in the repeatability and heritability estimates are disappointing insofar as no clear inference is possible about the genetic determination of the several kinetic response parameters. The most consistent repeatability results in the two groups were found for clearance and biological half life time of BHB, and the heritability of clearance was also in agreement with repeatability. Plasma concentration of glucose was more affected by uncontrolled factors resulting in lower repeatability estimates or results that lay outside the parameter space of repeatability and heritability. Glucose metabolism is a fast reacting regulatory system which responds also to psychological stressors which are difficult to control in unsedated animals.

Whether the response parameters offer some potential as predictors of milk production cannot be judged based on the available data. Estimates of correlations with breeding value for milk production will give a general indication of possible relations with productivity. The correlations are expected to be low because the breeding value estimates based on pedigree information are rather inaccurate. The reliability of expected transmitting ability (ETA) for milk of sire based upon progeny testing is much higher than of the dam's estimate. The finding that the response parameters of BHB and glucose account for about twice as much of the variability of ETA sire than of ETA dam is a positive indication of actual predictive usefulness of the response parameters. A further test for the reliability of the metabolic

response parameters is possible only after the results of the first lactation of the tested heifers will be known.

6. CONCLUSIONS

1. The challenge procedure consisting of infusion of insulin and BHB induced a short-term metabolic reaction with decreased glucose and increased BHB concentrations similar to the effects of subclinical ketosis. The concentration of FFA was also decreased which was in contrast to what we expect during ketosis.
2. Other metabolites (creatinine, cholesterol, urea, protein, albumin, bilirubin) and plasma enzymes (amylase, AP, GOT, LDH) remained unchanged during the monitoring period. The effects of the challenge were limited to BHB, glucose and FFA and the test procedure did not provoke a generalized metabolic reaction.
3. Parameters derived from regression models fitted to the concentration curves of BHB (clearance, biological half life time, volume of distribution) and glucose (initial and minimum concentration, time to minimun concentration) were sufficiently repeatable to be of interest for the evaluation as markers for milk yield. For clearance of BHB and initial concentration of glucose, the heritability estimation yielded an intermediate value.
4. FFA concentration showed a response to the challenge but the parameters were of low repeatability.
5. Other metabolic compounds with high repeatability (of mean values of 4 sample per challenge) were amylase, AP, cholesterol, creatinine and GOT.
6. The kinetic parameters of BHB were relatively the best predictors of breeding value although in general the reduction of the variability of breeding value was small.

7. A last test of the reliability of the response parameters as predictors of milk production will be possible only after the yield of the first lactation of all tested heifers becomes known.

7. APPENDIX 1 : EFFECTS OF INSULIN AND BHB AND INDIVIDUAL RESPONSE

7.1. EFFECTS OF INSULIN

The individual effects of insulin on the three metabolites of foremost interest - glucose, BHB and FFA - are exemplified in Fig.3 which shows the plasma concentrations after insulin application in one animal from a preliminary study. Glucose concentration shows a tendency to decrease starting five to ten minutes after the injection of insulin. The concentration then drops continuously for approximately 30 minutes, reaching a minimun level between 50 and 60 mg/100ml which is about two thirds of the initial concentration. Normal plasma concentration is restored 100 to 150 minutes after injection.

The decrease in free fatty acids after insulin injection shows a similar pattern to glucose response. After a sharp decline in the first 30 minutes the concentration of FFA remains at about two third of the initial level for 60 to 120 minutes. In general, the variability in FFA concentration is larger than in glucose concentration.

Betahydroxybutyrate does not show a clear trend over the entire monitoring period of 3 hours with the exception of two lower values 30 to 60 minutes after injection. Ketones usually follow the trend of FFA concentration with a delay of 30 to 60 minutes.

The infusion of 100 ml saline solution after 20 minutes did not seem to affect the trends of the concentration curves.

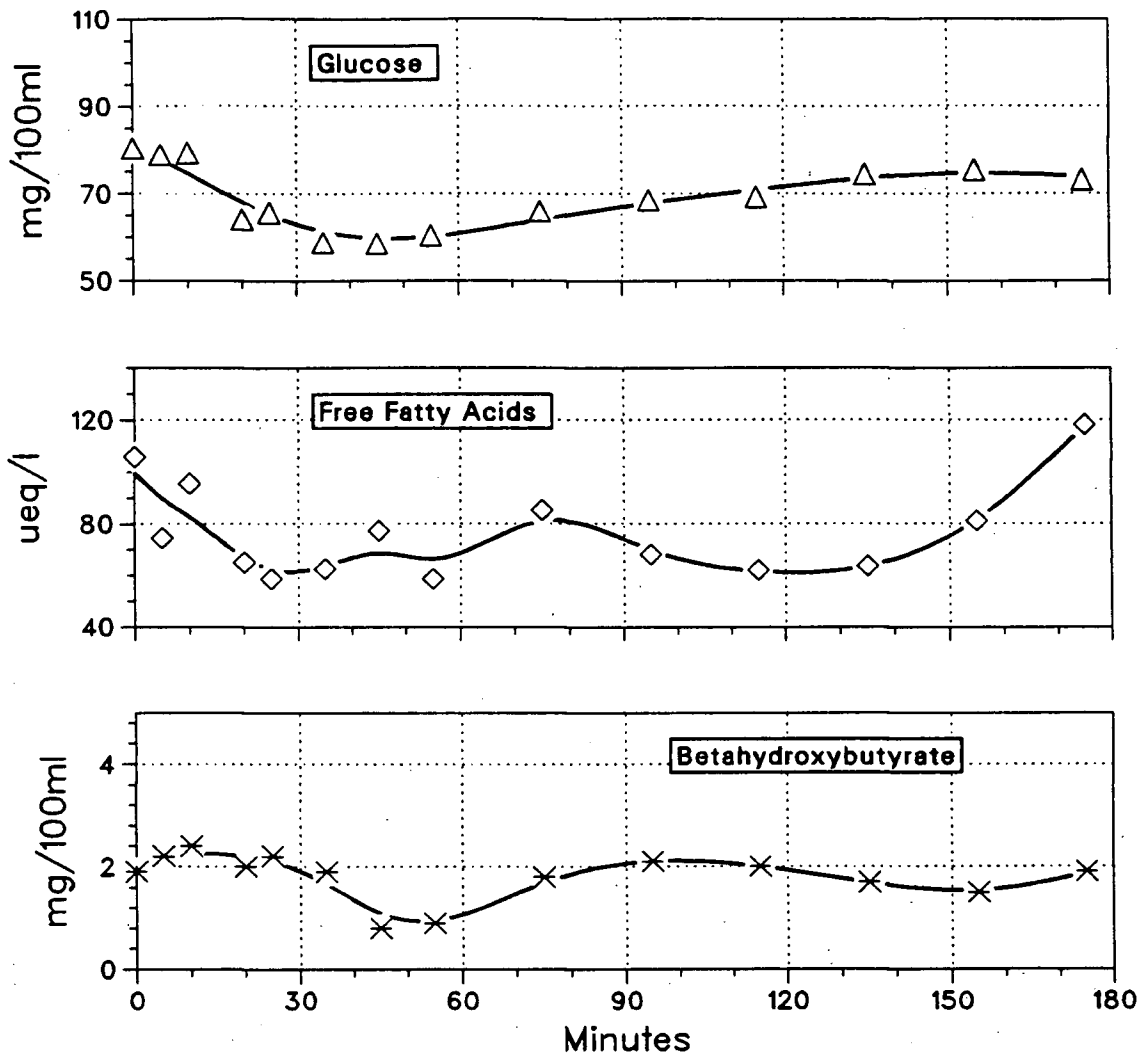

Figure 3. Effect of insulin.

Plasma concentrations of glucose, FFA and BHB after injection of Insulin (5 IU/100 kg) at time 0 minutes; infusion of NaCl 0.9 % from 20 to 25 minutes.

7.2. EFFECTS OF BHB

Fig.4 shows the plasma concentrations of glucose, FFA and BHB of one animal from the preliminary study receiving an infusion of 1.2 g D(-)BHB per 100 kg body weight. The infusion started 20 minutes after initiating the challenge procedure by injecting saline solution (corresponding to the insulin injection in Fig.1) and run over 20 minutes. The concentration of BHB immediately after the infusion is twice the initial level and decreases to pretreatment levels within 15 minutes. Neither Glucose nor FFA seem to be affected by the infused amount of BHB, which corresponded to about half the dose used in the challenge experiments and was infused over a period of 23 minutes. The resulting peak concentration BHB of 4.9 mg/100 ml plasma was too low to have a detectable effect.

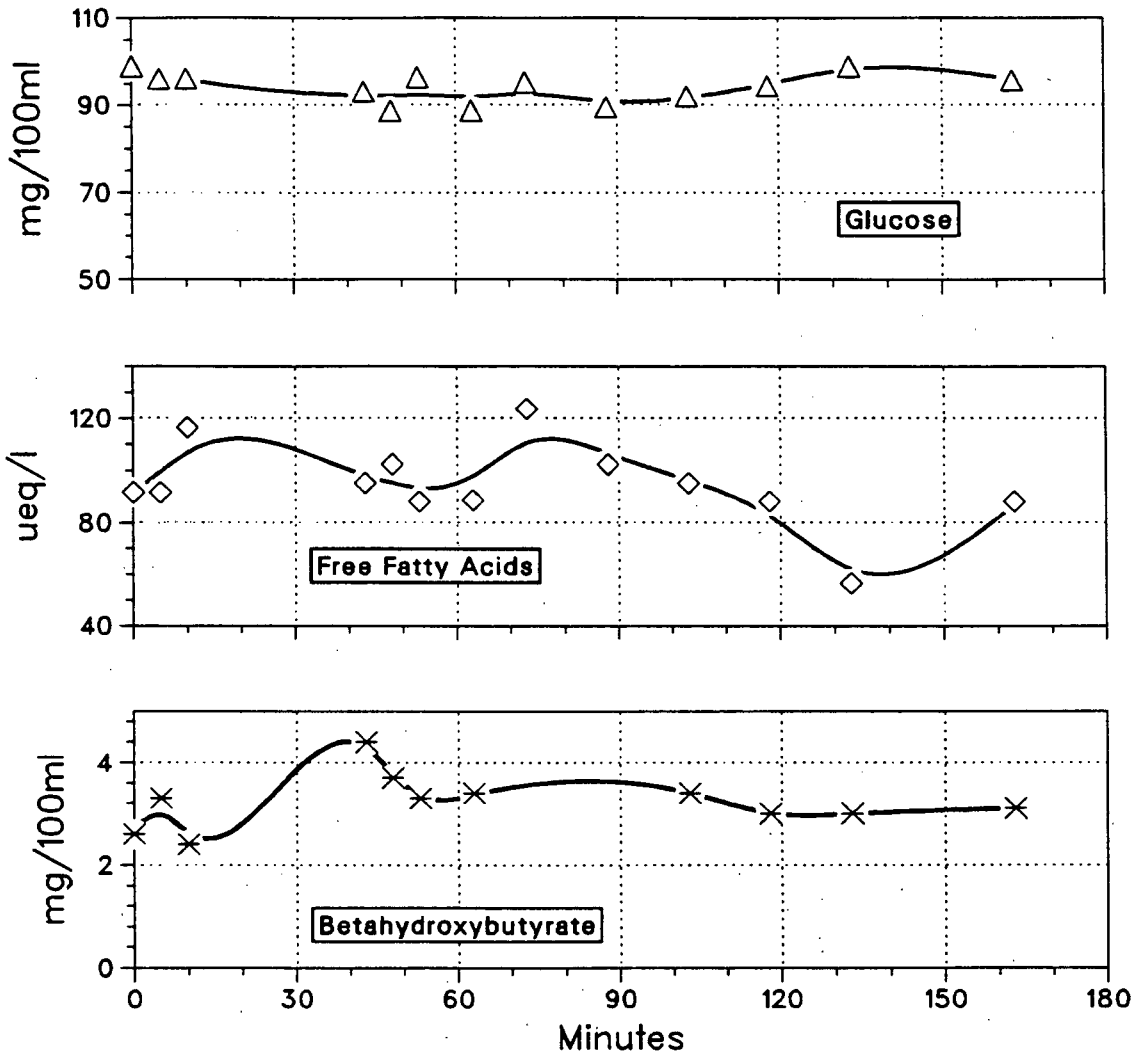

Figure 4. Effect of betahydroxybutyrate.

Plasma concentrations of glucose, FFA and BHB after infusion of BHB (1.2 g/100 kg) from 20 to 43 minutes.

7.3. INDIVIDUAL RESPONSE TO THE CHALLENGE PROCEDURE

Seven animals were sampled during the infusion period through a second catheter inserted into the opposite jugular vein to monitor the development of plasma concentrations during the infusion. The individual concentration curves during and after infusion of BHB are given in Fig.5 to Fig.8. Glucose and BHB concentrations of all 7 heifers are overlaid on each other in Fig.9 and Fig.10, resp., to facilitate the comparison of the individual reactions in each metabolite.

The response during the challenge was highly variable. The highest concentration of BHB was generally reached at the end of the infusion and immediately after the infusion the concentration started to decrease. Infusion time varied from 4.5 to 11 minute but the duration of the infusion did not appear to systematically affect the peak or the shape of the BHB concentration curve. Glucose concentration during infusion showed in general a declining trend in the first 30 minutes and was pulsatile in some animals (H711, H703, H690). No systematic effects of infusion time, amount BHB infused, weight or age of animal or initial concentrations on the shape of the curves were detectable among the seven heifers. The number of animals (7) is too small to allow conclusive statements about the reactions in glucose and BHB concentrations but the correlations between curve parameters and systematic effects discussed in chapter 3 confirm the 'visual' impression insofar as only weight of the animal was found to affect the parameters consistently.

Animal H683 had by far the highest BHB peak 5 minutes after start of the infusion. The BHB solution for the infusion of this animal was injected in less than

half of the usual volume of 100 ml NaCl 0.9% and the infused solution was therefore higher concentrated. The fact that the early peak concentration was already down on 'normal' levels at the end of the infusion and that the infusion run over the normal time period (11 minutes) indicates a slow down of the infusion flow towards the end probably because of the higher viscosity of the saline solution than had it been diluted in 100 ml.

Two facts are of interest concerning BHB. First, the increase in concentration is not uniform in all heifers (extreme examples are H722 and H685), but the highest plasma concentration is usually reached at the end of the infusion. Irregular flow of the infusion or different distribution rates in body compartments are possible sources of variation in the build up of the plasma concentration. *Wastney et.al.* (1984) concluded in a tracer study that at least 4 compartments were of importance in the human ketone metabolism, plasma BHB, plasma AcAc, liver tissue and peripheral tissues, which should apply to ruminants, too. The exchange rates vary between the compartments and individual differences of those exchange rates could affect the measurable amount in plasma. Second, plasma concentrations of BHB at the end of the infusion vary widely. The lowest concentration was 9.1 mg/100ml (H690) and the highest concentration was 17.7 mg/100ml (H683) in the last sample before the end of infusion. Concentrations between 8.0 mg/100 ml (H722) and 15.6 mg/100ml (H711) were found among the 7 heifers immediately (1 minute) after the infusion.

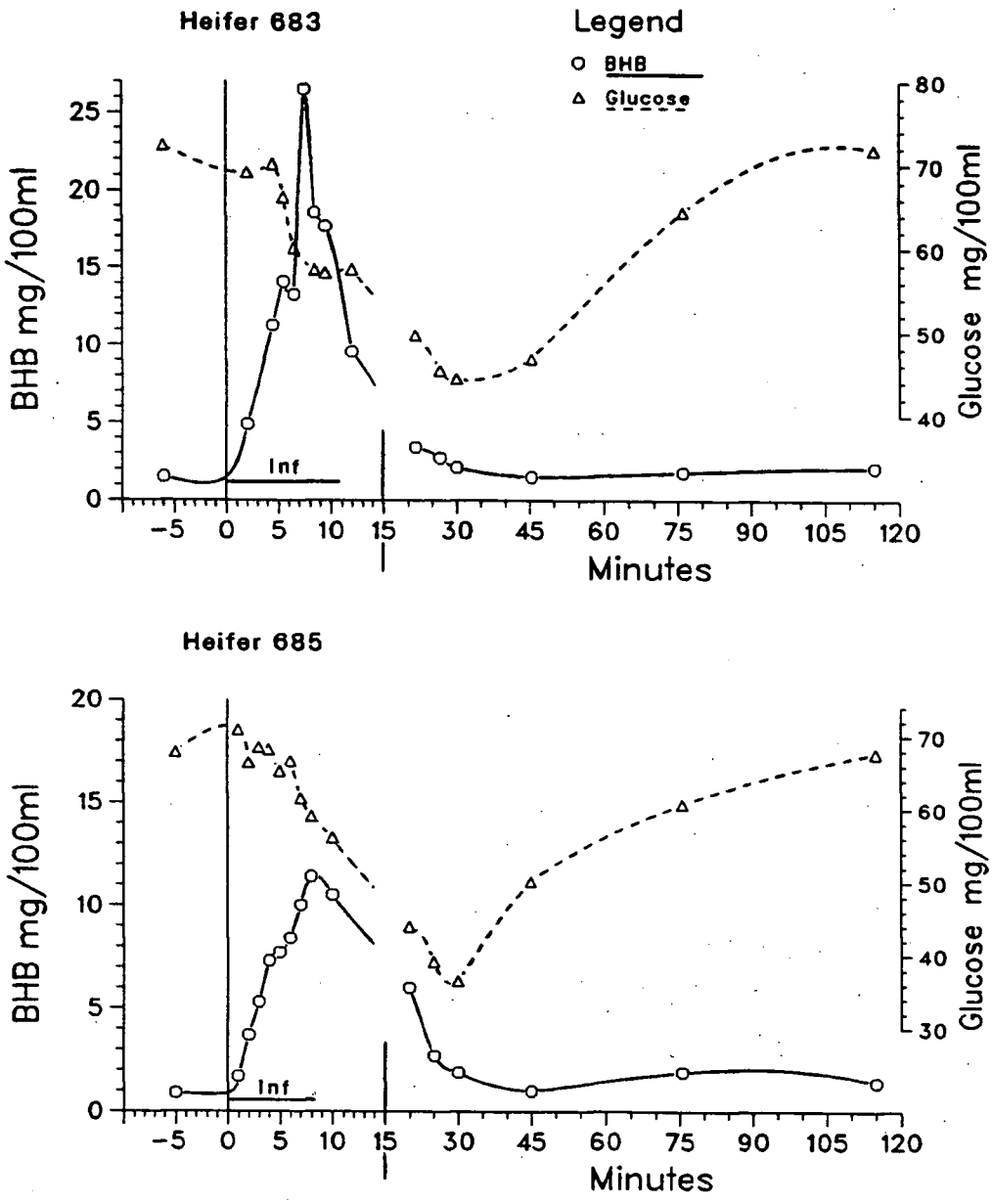

Figure 5. BHB and glucose concentrations. Individual response. Heifers 683, 685.

Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

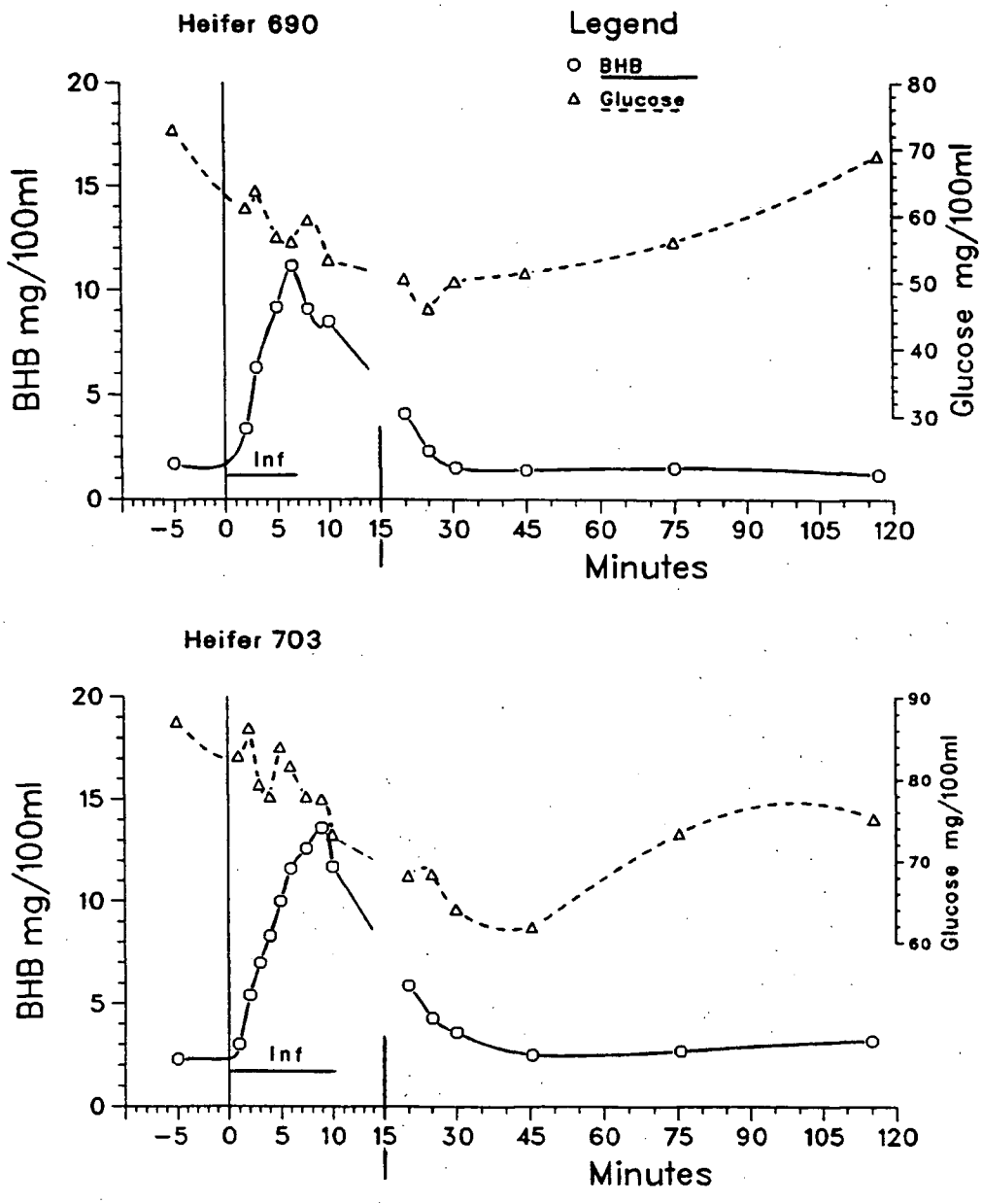

Figure 6. BHB and glucose concentrations. Individual response. Heifers 690, 703.

Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

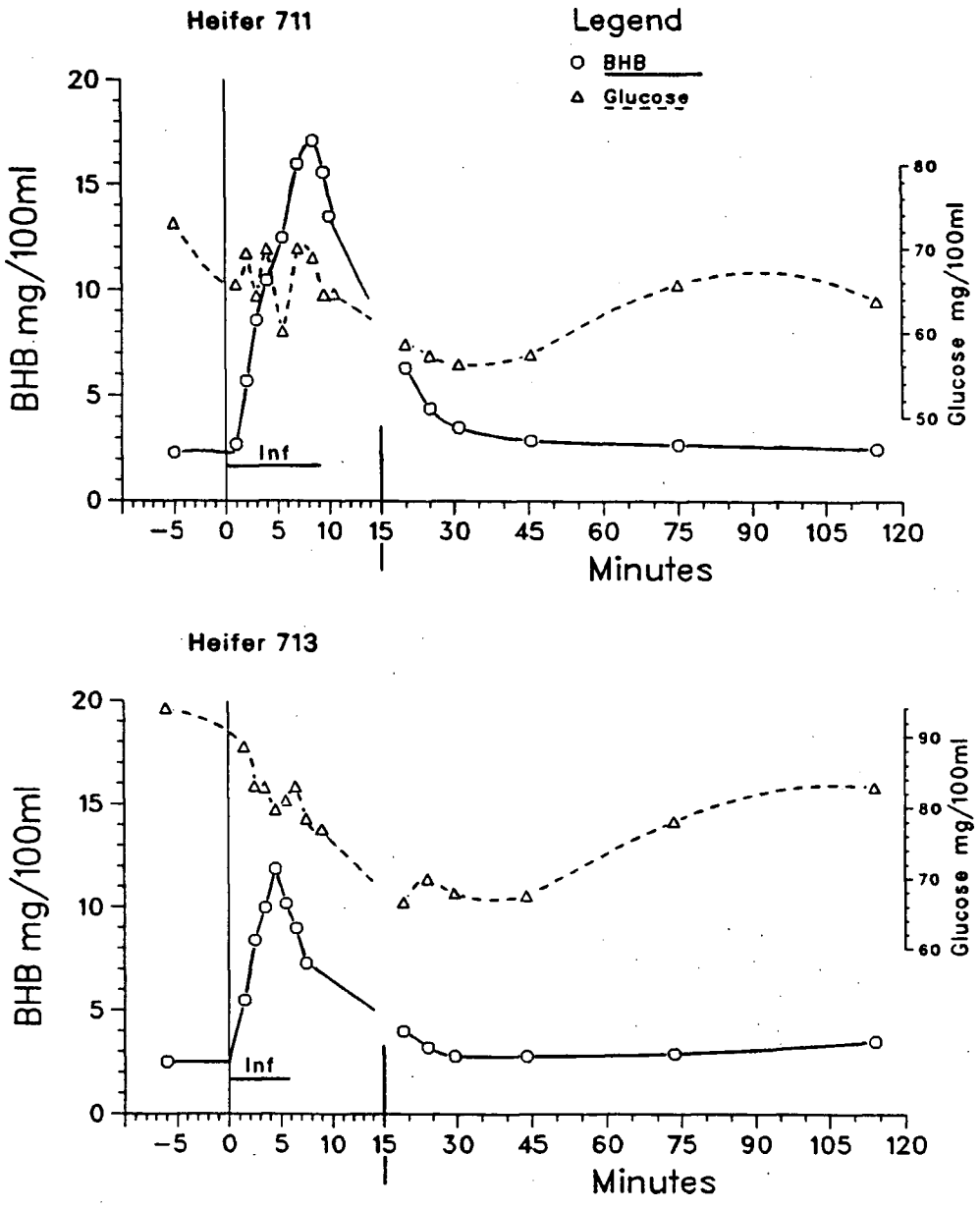

Figure 7. BHB and glucose concentrations. Individual response. Heifers 711, 713. Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

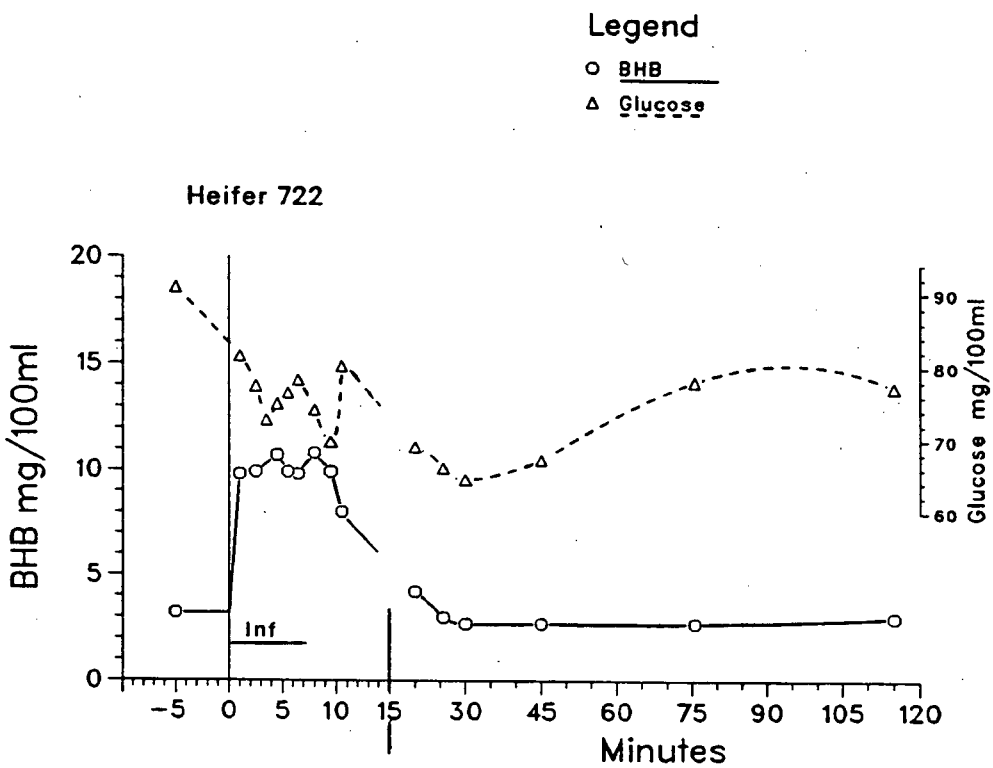

Figure 8. BHB and glucose concentrations. Individual response. Heifer 722. Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

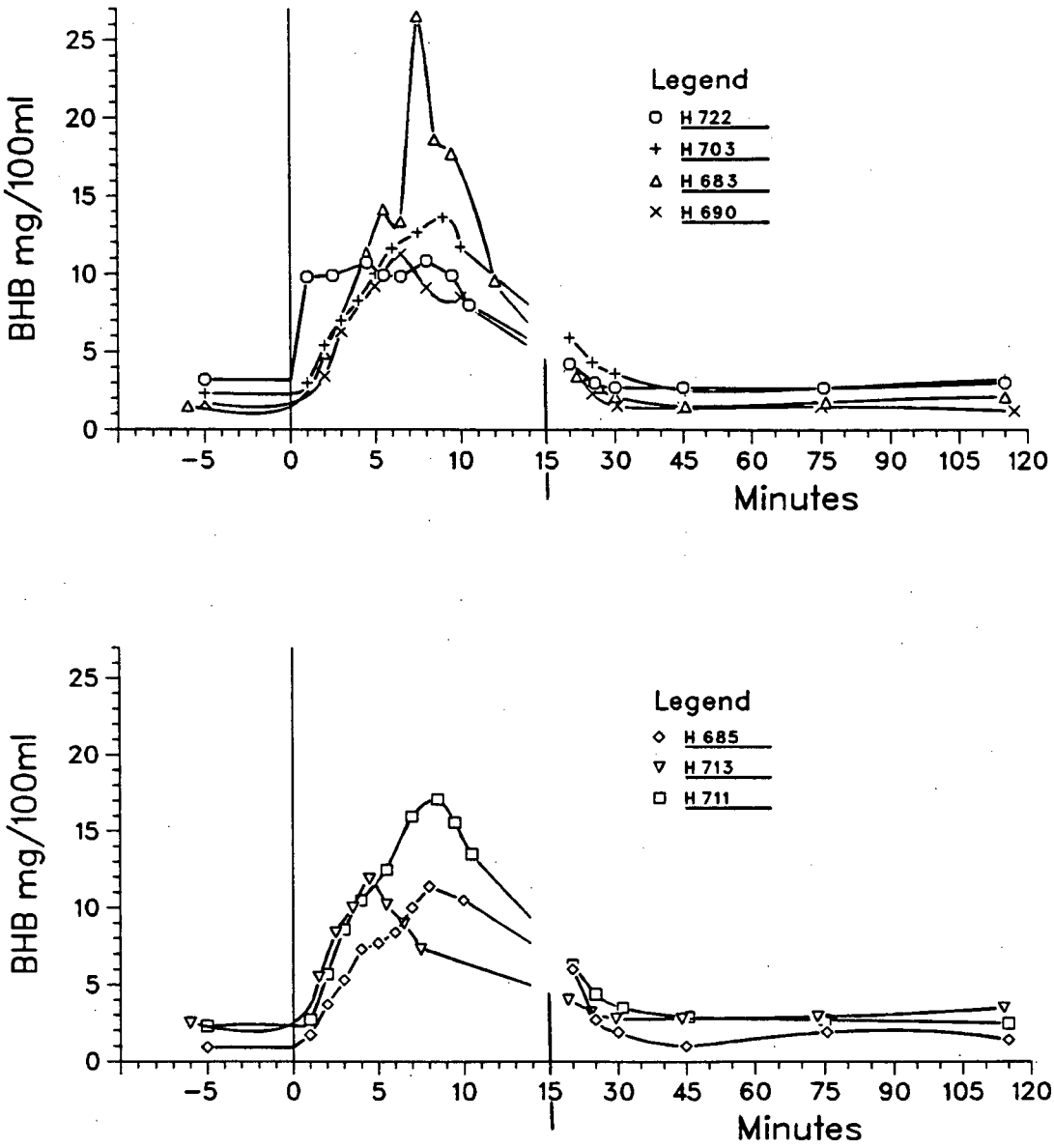

Figure 9. BHB concentration. Comparison of individual response.

Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

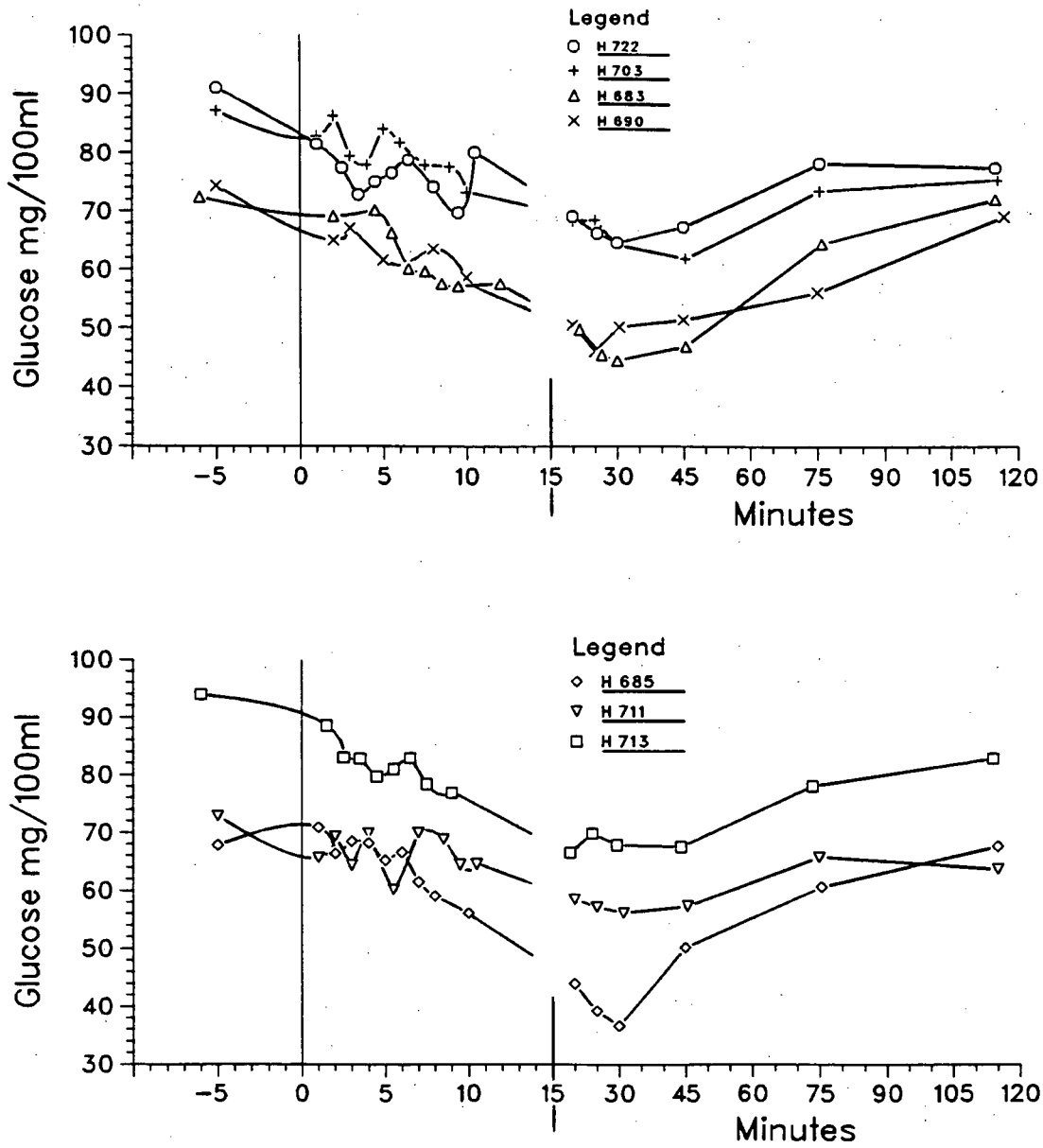

Figure 10. Glucose concentration. Comparison of individual response.

Insulin (5 IU/100kg b.w.) at -5 minutes; BHB (2 g/100kg b.w.) infusion started at 0 minutes.

APPENDIX 2: ANALYTICAL METHODS

Three colorimetric methods were used for the analysis of plasma concentrations of glucose, D(-)betahydroxybutyrate (BHB) and free fatty acids (FFA). The absorption was measured with the *Gilford Stasar II* photometer for wave lengths in the visible spectrum and with the *Shimadzu UV-160* spectrophotometer for the UV spectrum (< 400 nm).

1. GLUCOSE ANALYSIS

Kit No 510 of SIGMA was used to analyze plasma glucose (SIGMA, Technical Bulletin 510). Plasma was deproteinized using $ZnSO_4$ 0.5 N and NaOH 0.5 N and centrifuged. The clear supernatant was analyzed following the procedure indicated for Kit No 510. The extinction was measured at 450 nm.

the test principle is based upon following enzymatic reactions:

Glucose Oxidase

1. Glucose + 2 H_2 + O_2 ----------> Gluconic Acid + 2 H_2O_2

Peroxidase

2. H_2O_2 + o-Dianisidine ----------> oxidized o-Dianisidine

(colorless) (brown)

The brown color developing in the reaction is proportional to the original glucose concentration. Dextrose 100mg/100ml was used as standard and the standard curve

interpolated linearly from 0 to 100mg/100ml. The intra-assay and inter-assay coefficients of variability were 3.4 % and 4.3 %, respectively.

2. FREE FATTY ACIDS ANALYSIS

Procedure (*Duncombe*, 1962; modified *Itaya and Ui*, 1965):

Four ml chloroform, 1 ml phosphate buffer (ph 6.6) and 0.3 ml plasma are added to a 15 ml test tube. One drop of octanol added to each tube prevents emulsification during the extraction. The tubes are shaken slowly for 90 seconds using rubber stoppers. After 15 minutes, the upper layer together with the protein precipitate is aspirated with a pipette and discharded. Three ml Cu-triethanolamine solution[1] is added to the chloroform layer and the solution is shaken for 60 seconds. After 15 minutes, the Cu-triethanolamine solution is aspired and discharded. The Chloroform layer is then filtered (Whatman No 2) and 1 ml of Na-diethyldithiocarbamate solution[2] added. The brownish colour is measured at 440 nm.

Oleic acid (SIGMA No O-7501) in chloroform at concentrations of 5.0 µM and 50.0 µM were used as standards. The standard curve was interpolated linearly from 0 to 50 µM. A recovery rate of 108.7 % and 93.7 % was found after supplementing plasma with 100 µmol/l and 50 µmol/l oleic acid.

[1] Triethanolamine 1 M : acetic acid 1 N : $Cu(NO_3)_2 \cdot 3H_2O$ 1 M 9:1:10 v/v/v
[2] 0.1 % Na-diethyldithiocarbamate in n-butanol

3. BETAHYDROXYBUTYRATE ANALYSIS

BHB for group 1 was analyzed following the colorimetric method of *Kientsch-Engel and Siess* (1983). The method relies on following reactions :

$$\text{BHB} \xrightarrow{\text{BHB-DH}} \text{AcAc}$$

$$\text{NAD}^+ \xrightarrow{\text{PMS}} \text{NADH}$$

$$2\text{Fe}^{2+}\text{-BPS} \longleftarrow 2\text{Fe}^{3+}\text{-BPS}$$

The reaction forming acetoacetic acid (AcAc) from BHB is catalyzed by BHB-dehydrogenase (BHB-DH) with reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. NADH is oxidized to NAD^+ by the second reaction which reduces Fe^{3+} to Fe^{2+} releasing H^+ and is mediated by phenazine methosulphate (PMS). The iron ion forms a chelate with bathophenanthroline disulphonic acid (BPS) in the reaction solution. The reactions occur in stochiometric proportions. The absorbance of the Fe^{2+}-BPS compound measured at 546 nm is proportional to the initial amount of BHB.

The inconsistency of the results obtained by this method, intra-assay and inter-assay coefficients of variability both being greater than 20 % for normal plasma concentrations, forced the abandonment of the method for the analysis of the subsequent samples of group 2.

The samples of group 2 were analyzed by the tris-hydrazine method introduced by *Williamson et al.* (1962) and modified by *Chandrasekaran et al.* (1972). The tedious

step of plasma precipitation with perchloric acid could be omitted following a modification indicated by *Barnouin et al.* (1986). This method is based on the same reaction principle as the method of *Kientsch-Engel and Siess* but NADH formed is measured directly at 340 nm. BHB and AcAc are interconvertible, the reaction catalyzed by BHB-DH works in both directions. An equilibrium is reached depending on the pH of the reaction milieu. To carry the oxydation of BHB to its endpoint, AcAc has to be removed. Hydrazine traps and inactivates AcAc. Addition of oxamic acid inhibits possible interference from lactate dehydrogenase present in non-precipitated plasma.

Procedure :

In a 10-ml tube, 0.1 ml plasma are added to 2 ml of a Tris buffer solution containing hydrazine hydrate, oxamic acid and NAD. After 5 minutes of equilibration at 37°C, initial absorbance is measured. Then 0.1 ml BHB-DH in buffer (SIGMA No H-5132 : 1 IU/ml in Tris) are added. The mixture is thoroughly swirled and incubated for 90 minutes at 37°C. The final measurement is made at 340 nm immediately after incubation. The difference in absorbance is proportional to the amount of BHB.

Two standard solutions equivalent to 5.0 and 25.0 mg D(-)-BHB/100 ml (SIGMA No H-6501) were interpolated linearly from 0 to 25 mg for the standard curve.

Preparation of buffer solution :

Tris-HCl 0.5 M and Tris base 0.5 M and hydrazine hydrate 0.5 M are mixed 4:2:1 v/v/v. After adjustment of the pH to 8.5, 25 mmol oxamic acid are added (2.26 g of oxamic acid per 1000 ml of Tris-hydrazine). NAD buffer (NAD 25 mM in Tris 0.5 M) and Tris-hydrazine-oxamate buffer and H_2O are mixed 1:9:10 v/v/v. All preliminary solutions are kept refridgerated and the final solution prepared freshly

every day.

The coefficients of variability between and within assay were 8.6 % and 4.6 %, respectively. A recovery experiment yielded 87.0±7.0 % for low-concentration additive and 95.5±7.2 % for high-concentrated additive when the BHB content of the plasma was high (10 -15 mg/100ml). In low-BHB plasma (2 - 5 mg/100ml) the recovery rate was 96.3±2.9 % and 103±7.8 %, respectively (N=4).

4. OTHER METABOLITES AND ENZYMES

Creatinine, urea, cholesterol, protein, albumin, bilirubin, AP, LDH, SGOT and amylase were analyzed in a commercial laboratory (B.C. Bio-Medical Laboratories, Burnaby, B.C.) using a 'Roche' Cobas B_{10} Centrifugal Analyzer for amylase and a Boehringer Mannheim (BMC) Hitachi 737 Analyser for other metabolites and enzymes. Methods and references are listed on page 109.

	Method	Reference
Creatinine	Jaffe method (kinetic assay)	
Urea		Neumann and Ziegenhorn (1977). XVI Nordisca Kongressen Klinsk Kemi Och Klinsk Fysiologi. Oulu, Finland.
Cholesterol		Siedel et al. (1981). J. Clin. Chem. Biochem. 19:838
Bilirubin		Wahlefeld et al. 1972. Scand. J. Clin. Lab. Invest. 29 (Suppl. 126) Abstr. 11.12.
Protein	Biuret reaction (mod)	
Albumin		Dumas et al. 1987. Clin. Chim. Acta 31.
AP	p-Nitrophenylphosphate method	Commission Enzymologie 1977. Ann. Biol. Clin. 35:371
LDH	UV (P-L) method	Deutsche Gesellschaft Klin. Chemie
SGOT	UV (P-L) method	Deutsche Gesellschaft Klin. Chemie
Amylase	Pantrak 'Behring'	Wallenfels et al. 1978. Carbohydrate Research 61:359.

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