CHAPTER 3. MATERIALS AND METHODS

1. Animals

1.1 Introduction

Ten healthy, multiparous, Holstein cows from the University of Pretoria Experimental Farm dairy herd were used in the experiment (n=10) and formed part of the data analysis. Data collection encompassed a period from the first week postpartum (week 1), to the twelfth week postpartum (week 12) for each cow and is summarized in Figure 7. In the basal period from week 1 to week 7 infrequent blood sampling occurred fortnightly, on two consecutive days with a total of 8 plasma samples (paragraph 2.1). This was followed by an experimental period from week 8 to week 12, where treatments were applied and the responses to metabolic tests were determined. These metabolic tests (described below in 3.4 and 3.5) included an insulin challenge in the morning followed by a hyperinsulinaemic euglycaemic clamp later in the day, with a total of 21 plasma samples for each of the 4 experimental periods. During the experimental period the effects of recombinant bST administration and/or intake restriction on the results of metabolic tests were determined (paragraphs 2.2.1 through 2.2.4).

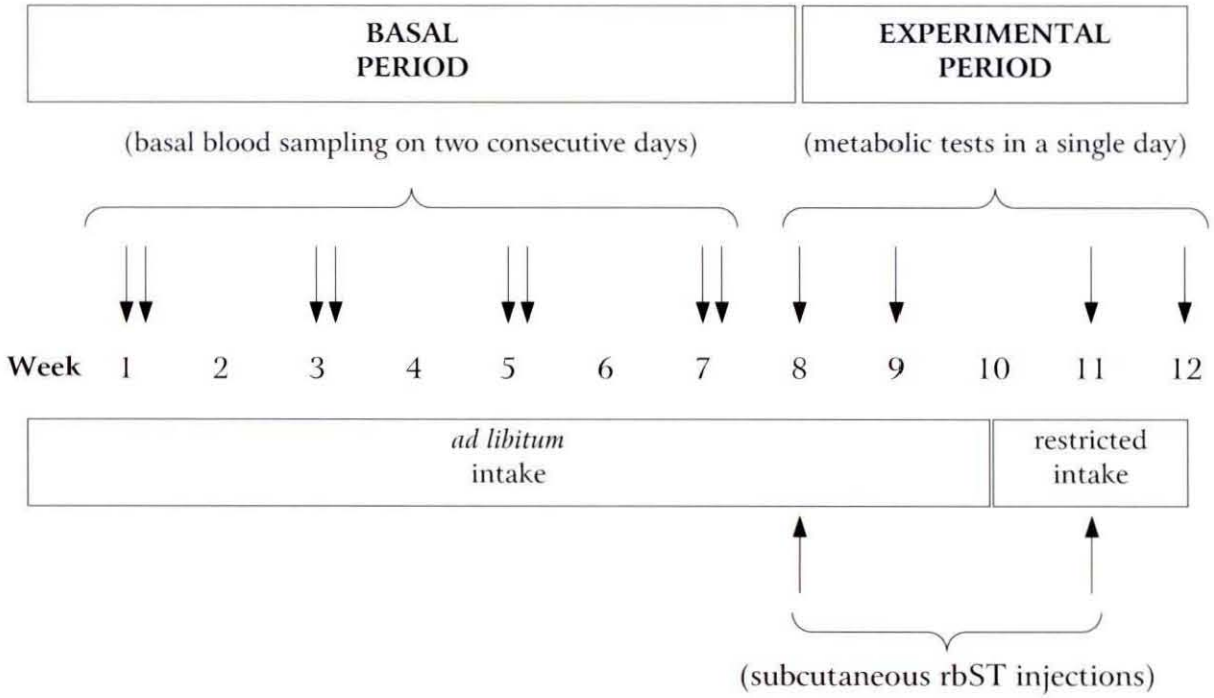

Figure 7. Diagrammatic representation of experimental protocol

1.2 Cows included in the data analysis

Cows were milked twice daily, the morning milking starting from approximately 5:00 and the afternoon milking from approximately 14:00. All cows were fed a commercial dairy ration, containing 7.6% whole cottonseed, 17.8% maize silage, 26.8% *Eragrostis curvula* hay and 47.8% high-protein concentrate (on a dry matter basis). The feed supplied an estimated 11.35 MJ ME per kg dry matter intake.

The BCS where 1 was emaciated and 5 obese, as well as age and parity of cows were used as criteria for inclusion in the experiment (Table 1). Determination of BCS, being a subjective measure of body fat, was determined in conjunction with technical personnel. All cows had a BCS between 3.0 to 3.5, were between 3.2 to 5.5 years of age and entering their second to fourth lactation. Cows in this herd were assigned three digit numbers, according to year and order of birth. For example, cow number 620 was born in 1996 and was the twentieth female calf born to the herd during that year. The data included in the data set was collected from May 2000 to April 2001.

Table 1. Description of cows included in the data set

No.	Cow	Calving Date	Sex of the Calf	Lactation	Age	BCS
1	**620**	22 May 2000	Female	Third	4.0	3.5
2	**515**	19 June 2000	Male	Fourth	5.2	3.1
3	**608**	19 June 2000	Male + Female	Third	4.3	3.0
4	**524**	30 June 2000	Male	Fourth	5.3	3.0
5	**606**	09 July 2000	Female	Third	4.5	3.3
6	**705**	21 August 2000	Female	Second	3.6	3.5
7	**631**	01 September 2000	Male	Third	4.1	3.2
8	**721**	25 November 2000	Female	Second	3.2	3.0
9	**543**	26 December 2000	Female	Fourth	5.1	3.3
10	**521**	13 January 2001	Male	Fourth	5.5	3.2

1.3 Cows excluded from the data analysis

Some cows were used to determine basal profiles of production and hormone secretion in the absence of metabolic tests and treatments, while testing the practical application of the procedures and setting up the equipment (Table 2). Cows that calved down from January 2000 to April 2000 included thirteen cows (No. 11 through 23) that were sampled according to the basal sampling procedure, up to the twelfth week postpartum. Fifteen cows were sampled, weighed and data recorded in the course of the entire experiment (from September 1999 to April 2001), but never used in further sample and data analyses. These cows were used during the planning phase of the experiment, fell ill early in experimental procedures or were included only as a safety margin, all of which lead to incomplete data collection.

Table 2. Description of cows excluded from the data set

No.	Cow	Calving Date	Sex of the Calf	Lactation	Age	BCS
11	543	17 January 2000	Female	Third	4.2	3.4
12	601	19 January 2000	Male	Third	4.0	3.5
13	405	25 January 2000	Male	Fifth	5.8	3.4
14	641	25 January 2000	Female	Second	3.1	3.3
15	535	03 February 2000	Female	Third	4.3	3.0
16	424	16 February 2000	Female	Fifth	5.6	3.4
17	421	19 February 2000	Female	Third	5.6	3.1
18	431	25 February 2000	Male	Fourth	5.5	3.4
19	439	04 March 2000	Male	Fourth	5.2	3.0
20	503	18 March 2000	Female	Fourth	5.1	3.0
21	427	28 March 2000	Male	Fourth	5.6	3.3
22	638	31 March 2000	Female	Second	3.4	3.0
23	536	01 April 2000	Male	Third	4.4	3.3
24	612	20 February 2000	Female	Third	3.7	3.1
25	527	19 June 2000	Female	Fourth	4.8	3.3
26	727	05 November 2000	Male	Second	3.0	3.0
27	643	26 November 2000	Female	Third	3.9	3.5
28	627	09 January 2001	Female (twins)	Third	4.5	3.4
29	715	26 November 2000	Female	Second	3.4	3.2
30	718	23 September 2000	Female	Second	3.2	3.4
31	710	20 October 2000	Female	Second	3.4	3.3

Eight cows sampled for more extensive periods were excluded for assorted reasons (No. 24 through 31 in Table 2). Cow number 612 was removed from the experiment in week 8 due to a foot abscess. Cow number 527 was only sampled up to 11 weeks postpartum due to scheduling difficulties. Cow number 727 exhibited a very poor response to insulin injection (Figure 8) and, because ketosis was suspected (Sakai *et al.*, 1996), was removed from the experiment. Cow 643 refused feed, after developing severe clinical mastitis in one quarter toward the end of data collection (week 12). This cow was subsequently removed from the data analysis. Due to poor temperament and nervousness in the crate, cow number 627 was excluded in week 9, when metabolic tests required frequent handling and intensive sample collection. Cow 715 was dropped after falling ill after 7 weeks of sampling and received veterinary treatment at Onderstepoort. During the initial insulin challenges of two cows (718 and 710) extremely low baseline glucose concentrations and severe insulin resistance was observed (Figure 8). It was suspected that these cows suffered from clinical ketosis and they were removed from the experiment with no further data collection.

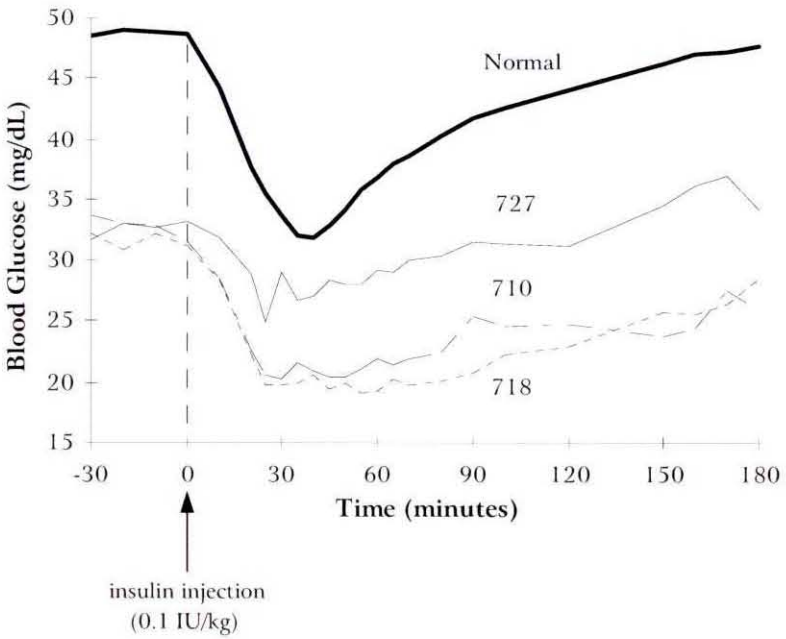

Figure 8. Insulin challenge results of cows with suspected ketosis

2. Periods and treatments

2.1 The basal period

In the basal period from week 1 to week 7 postpartum cows were exposed to standard management practices of the university. Although not subjected to any treatments, fortnightly blood samples were harvested on two consecutive days and frozen until analysis. The basal milk production was recorded with every milking and the body weight and BCS were recorded every week.

A total of 451 blood samples were collected for harvesting of plasma from all cows during the basal period. Following the basal period, insulin challenges and hyperglycaemic clamps with frequent blood sampling were initiated. A total of 64 metabolic tests were performed and 1 141 plasma samples harvested in 10 017 aliquots. Only 40 of these metabolic tests proved useful, with 4 test days for each of the 10 cows in the experiment.

2.2 The experimental period

2.2.1 The control period

The experimental period was initiated during week 8 of lactation, when samples were collected to serve as a control. Cows were subjected to insulin challenges followed by hyperglycaemic clamps during this week, while still subject to standard management practices of the herd.

2.2.2 The recombinant bovine somatotropin period (rbST)

Directly following collection of the control data (± day 56), cows received a subcutaneous injection of recombinant bST. During week 9 (± day 63, or 7 days after recombinant bST injection) metabolic tests were performed for collection of the data for the rbST period.

2.2.3 The restriction to 80% of metabolizable energy requirement period (80% ME)

During week 10 (± day 70), intake was restricted to 80% of the predicted ME requirements (NRC, 1988). From this time up to the end of the experiment, cows were isolated from the herd and individually fed a calculated amount of feed presented twice

daily. During week 11 (± day 77, or 7 days after energy restriction), metabolic tests were performed for the collection of the data for the 80% ME treatment period.

2.2.4 The 80% ME and rbST period (80% ME + rbST)

Collection of samples in the 80% ME period was immediately followed by subcutaneous injection of recombinant bST (± day 77). Cows were maintained on the restricted intake level and metabolic tests performed during week 12 (± day 84). This data was used to estimate the combined effects of the energy restriction and the recombinant bST treatments in the 80% ME + rbST period. The same experimental protocols were used in all cows and after the completion of these tests they were released back into the herd.

3. Data collection and processing

3.1 Body weight, body condition score and milk production

The nonfasting body weight and BCS of cows were recorded between 7:00 and 8:00, following the morning milkings. These observations were made on two consecutive days of every week (from week 1 to week 12 postpartum). Milk production was recorded for each cow at every milking, during the entire experiment. The weighted averages of the body weight, BCS and milk production were calculated for each week. Additionally, each cow was weighed after an overnight fast, prior to metabolic tests (weeks 8, 9, 11 and 12) to obtain the fasting body weight. These values were used to calculate the insulin injection volume and GIR.

3.2 Blood sampling

During the basal period venous blood samples were collected, occasionally following body weight and BCS measurements. The area of skin above the jugular vein was disinfected using ethanol and laboratory tissue paper. Blood was collected by jugular venipuncture into 10 mL heparinized, evacuated tubes (Becton Dickinson) that were gently inverted a few times and stored on ice-cold water until centrifugation (Hettich Zentifugen) at a gravitational force of 1 000 × g or approximately 3 200 rpm for 15 minutes. All blood

samples were centrifuged within one hour of collection, plasma transferred to seven marked 1.3 mL aliquots (DELT) and frozen at –20 °C until analysis.

During the experimental period, blood samples were collected with the aid of an indwelling catheter, using either 5 mL or 10 mL syringes (Promex). Whole-blood samples were collected into 1.3 mL aliquots containing approximately 1 mg ethylene diamine tetra-acetic acid (EDTA) disodium salt (Saarchem) per milliliter blood. These whole-blood samples were gently inverted to ensure proper mixing with anticoagulant and to prevent blood cells from settling out before analysis for glucose concentration within one minute of collection. Larger blood samples collected for harvesting of plasma were transferred to either 5 mL or 10 mL heparinized tubes (Becton Dickinson), gently inverted and placed on ice-cold water until centrifugation at 1 000 × *g* for 15 minutes. The levels of anticoagulants used were suitable for assays and the loss of glucose by glycolysis was minimized by storage on ice water, minimal delay before centrifugation (plasma) and rapid assay (whole-blood).

3.3 Catheterization

At 8, 9, 11 and 12 weeks postpartum, jugular catheters were inserted to facilitate frequent blood sampling during insulin challenges and hyperglycaemic clamps. A large area of skin around the jugular vein was washed using warm water, disinfectant and cloth. This area was dried with laboratory tissue paper and the area of insertion cleaned using ethanol and laboratory tissue paper. A hypodermic needle (14 × 1.5") was used to gain entry to the jugular vein and an indwelling catheter inserted at least 20 cm into the vein. The catheter material used was either polythene (Portex) or pure vinyl (Dural Plastics and Engineering). The contralateral jugular vein was also catheterized according to the same protocol, where one was used exclusively for blood sampling (usually on the right), while the other was used exclusively for infusion of solutions. Catheter integrity was maintained using a 25 IU/mL heparin solution (Novo Nordisk) in physiological saline and the catheters were plugged after insertion. Both catheters were firmly fixed to the skin, just above the point of entry, while the external loops were loosely secured close to the skin, to prevent damage. The cows were confined to a crush, before being moved to a crate inside a metabolic house for the remainder of the procedure.

3.4 Insulin challenge

After catheterization, cows were taken into a metabolic house and stalled in a raised crate with adjustable neck clamp until completion of the metabolic tests. The cows were frequently offered water, as the facility would not allow for unlimited access to water. Blood sampling commenced after at least one hour of confinement in the crate, when the whole-blood glucose concentration determined by frequent sampling stabilized. An insulin challenge (0.1 IU/kg body weight, 210 minutes) was conducted, with frequent determination of glucose in whole-blood and collection of plasma. More detail on sample collection during the insulin challenge is presented in Table 5. Insulin was injected as a commercial preparation of 40 IU/mL porcine insulin (Caninsulin®, Intervet) at a dose of 0.1 IU insulin per kg fasting body weight. Insulin was in suspension as Zn-insulin (30% amorphous and 70% crystalline) and was injected in a logarithmically decreasing manner, over a period of approximately one minute. Injection started immediately following collection of the final baseline blood sample, the time designated zero (t0). All insulin injections into the infusion catheter were followed by a saline flush, at least 4-fold greater than catheter volume.

3.5 Hyperglycaemic clamp

As soon as the whole-blood glucose concentration stabilized after the insulin challenge, a hyperglycaemic clamp (+50 mg/dL whole-blood, 120 minutes) was initiated, with frequent sampling of whole-blood and intermittent collection of plasma. More detail on sampling protocols during hyperglycaemic clamps is presented in Table 6. During the first 30 minutes following baseline observations, whole-blood glucose concentration was gradually raised to 50 mg/dL above the average baseline concentration, by injection of a 60% weight per volume (% $w/_V$) glucose solution. All glucose injections into infusion catheters were followed by a saline flush. Once the desired level of hyperglycaemia was achieved a 40% $w/_V$ glucose solution was infused at a variable rate to maintain the blood glucose concentration within a 10% range (± 10%) of this value.

The 60% w/v glucose solution (Saarchem) was prepared using glucose monohydrate ($G{\cdot}H_2O$) and distilled water (H_2O). Because the molecular weight of glucose is approximately 180 g/mol and water approximately 18 g/mol, it was calculated that only 91% of the $G{\cdot}H_2O$ was effectively glucose. The 60% w/v glucose solution was therefore made up by using 66 g $G{\cdot}H_2O$ filled to 100 mL with H_2O. For each clamp, 200 mL solution was prepared on the day preceding the clamp. During the first 30 minutes of clamped glucose (t0 to t30), the 60% w/v glucose solution was injected between blood collections using a 10 mL syringe. The volume of the injected solution was recorded every 5 minutes.

A 40% w/v glucose solution was prepared using 44 g $G{\cdot}H_2O$ filled to 100 mL using H_2O. For each clamp 500 mL solution was prepared on the day preceding the clamp and this was infused during the final 90 minutes of the clamp using a peristaltic pump (Ismatec). The weight of infused solution was recorded every 5 minutes and adjusted to maintain the whole-blood glucose concentration within the specified limits. These values were also used to calculate the GIR during the hyperglycaemic clamp.

After 2 hours of sustained hyperglycaemia, the catheters were removed, the area of skin around the insertion cleaned, recombinant bST injected when required (week 8 and week 11) and the cows were lead from the metabolic house to the milking parlour for the afternoon milking. After milking, animals on *ad libitum* intake were released back into the herd, while those on the restricted intake levels were separated from the herd for individual feeding.

3.6 Recombinant bovine somatotropin injection

After the metabolic tests performed in week 8 and 11, a small area of skin (postscapular) was cleaned using a commercial disinfectant and laboratory tissue. A subcutaneous recombinant bST injection was applied following the manufacturer's instructions. A commercial preparation of 1.4 mL recombinant bST in disposable syringes was used, containing Zn n-methionyl bST (Zn sometribove), equivalent to 500 mg bST (Lactotropin®, Elanco).

3.7 Calculations

3.7.1 Glucose infusion rate

The volume of the 60% w/v glucose solution was recorded every 5 minutes and was directly used in calculating the GIR. The weight of the 40% w/v solution, recorded every 5 minutes was used indirectly, to calculate the weight of glucose infused during this period. The weight of 100 mL of the 40% w/v glucose solution was measured as 114.7 g and 34.9 g glucose was effectively infused for every 100 g of this solution. The absolute rate of glucose infusion was calculated from the recorded values.

$$\text{Grams Glucose Infused} = 0.4 \times \frac{\text{Weight Solution Infused}}{1.147}$$

After calculation of the absolute rate of glucose infusion, the total weight of glucose infused was used to calculate the GIR as the weight glucose infused per minute, adjusted for the body weight of cows (i.e. mg / kg × min).

$$\text{Glucose Infusion Rate (GIR)} = \frac{\text{Total Weight Glucose Infused}}{\text{Fasting Body Weight} \times \text{Time}}$$

3.7.2 Glucose area under the curve

To evaluate the glucose response to insulin injection the area under the glucose concentration curve was calculated using trapezial geometry (Figure 9). Briefly, the AUC was calculated between each time point, or glucose sample collected (whole-blood and plasma). The formula used to calculate the area of a trapezium is:

$$\text{Area Under the Curve } (AUC_n) = \tfrac{1}{2} (y_0 + y_n) (x_n - x_0)$$

where y_0 = the glucose concentration in mg/dL at the first time point

y_n = the glucose concentration in mg/dL at the second time point

x_0 = the time in minutes of the first time point

x_n = the time in minutes of the second time point

The sum of the individual areas between time points was used to calculate the total AUC of the baseline preceding time zero (t–30 to t0) and the initial response after insulin injection (t0 to t30) for both whole-blood and plasma. The AUC value used for comparison of the response to insulin injection (t0 to t30) was a corrected AUC (Figure 9), calculated by correcting the response AUC for the baseline AUC, using the formula:

$$\text{Corrected AUC} = \text{AUC}_{t0 - t30} - \text{AUC}_{t-30 - t0}$$

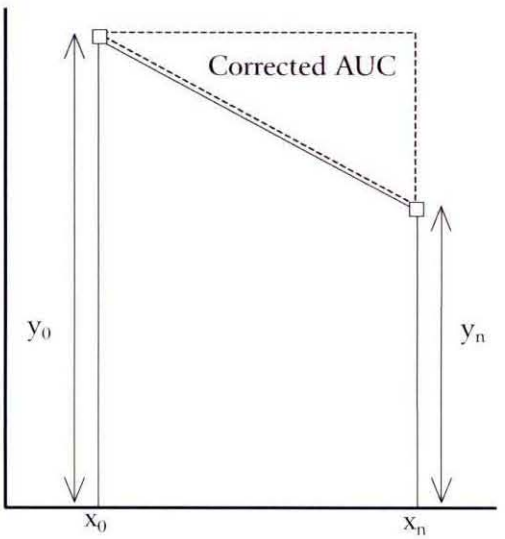

Figure 9. The area of a trapezium between two observations

3.7.3 Maximum glucose response

In addition to the AUC, the absolute change in glucose concentration from the baseline was used to compare the glucose responses between treatments. This maximum response was calculated by deducting the lowest whole-blood glucose concentration from the weighted mean of the four baseline whole-blood glucose observations, in the formula:

$$\text{Maximum Glucose Response} = \text{Baseline Glucose} - \text{Minimum Glucose}$$

4. Sampling protocols

4.1 Basal blood collection

To determine the plasma profile of cows during the basal period, 10 mL blood samples were collected in week 1, 3, 5 and 7 postpartum on two consecutive days, for harvest and storage of plasma (Figure 7). The eight samples were planned for days 7 and 8, 21 and 22, 35 and 36, as well as 49 and 50 postpartum. However, actual sampling occurred on days as close to the designated date as was practical, as presented in Table 3, where the day of collection is given as the weighted mean for all cows (n=10), ± the standard deviation (SD).

Table 3. Days of basal sample collection

Sample	Day Planned	Day Collected ± SD
1	7	7.5 ± 1.18
2	8	8.5 ± 1.18
3	21	21.5 ± 1.18
4	22	22.7 ± 1.12
5	35	35.7 ± 1.34
6	36	36.9 ± 1.27
7	49	49.4 ± 1.35
8	50	50.4 ± 1.35

* All samples were designated numbers in order of collection. A total of 92 samples were collected from each cow during the entire experiment (week 1 to week 12).

4.2 Challenges, clamps and intake restriction

On selected days (Figure 7), cows were exposed to insulin challenges and hyperglycaemic clamps, where all treatment periods followed each other sequentially. The control and rbST period were in week 8 and week 9 respectively, where all cows were on *ad libitum* intake. The feed restriction to 80% of the predicted ME requirement was initiated during week 10, followed by the 80% ME period in week 11 and the combination of recombinant bST and 80% ME treatments in week 12 (80% ME + rbST period). The control period was planned for day 56 postpartum, rbST for day 63, feed restriction for day 70, 80% ME for day 77 and the 80% ME + rbST period for day 84 postpartum. Overlapping of the dates for metabolic tests in the schedule and practical considerations

were taken into account, to allow tests on days as close as possible to the designated date (Table 4).

Table 4. Days of treatment periods and intake restriction

Period	Day Planned	Day Performed ± SD
Control	56	57.5 ± 1.96
rbST	63	64.4 ± 1.84
Intake restriction	70	71.4 ± 1.84
80% ME	77	78.7 ± 1.89
80% ME + rbST	84	85.4 ± 1.96

4.3 The insulin challenge protocol

After the cows had become accustomed to the metabolic house and whole-blood glucose stabilized (at least 60 minutes after entering), three 10 mL blood samples were collected at the time designated minus thirty minutes (t–30), t–20 and t–10 minutes, for the harvest of plasma of the baseline period (Table 5). At the time designated zero (t0), the final 10 mL baseline sample was collected, followed immediately by injection of insulin at 0.1 IU/kg body weight. Small blood samples for analysis of glucose concentration in whole-blood were also collected at t–30, t–20, t–10, t0, t10, t20, t25, t30, t35, t40, t45, t50, t55, t60, t65, t70, t80, t90, t100, t120, t150, t180 and t210 (or until blood glucose concentration stabilized). Blood samples (5mL) for the storage of plasma were simultaneously collected at t30, t60, t90, t120 and t150.

Table 5. Timing of samples in the insulin challenge

Time	10 mL Sample	5 mL Sample	1 mL Sample
t –30	✓		✓
t –20	✓		✓
t –10	✓		✓
t 0	✓		✓
Insulin Injection			
t 10			✓
t 20			✓
t 25			✓
t 30		✓	✓
t 35			✓
t 40			✓
t 45			✓
t 50			✓
t 55			✓
t 60		✓	✓
t 65			✓
t 70			✓
t 80			✓
t 90		✓	✓
t 100			✓
t 120		✓	✓
t 150		✓	✓
t 180			✓
t 210			✓

4.4 The hyperglycaemic clamp protocol

After stabilization of the blood glucose concentration following insulin challenge, another three 10 mL baseline samples (for hyperglycaemic clamp) were collected at the times designated t–30, t–20 and t–10 (Table 6). At the time designated zero (t0), another baseline sample was taken, followed immediately by injection of the 60% w/v glucose solution. Small blood samples for the determination of whole-blood glucose concentration were collected at t–30, t–20, t–10, t0, t5, t10, t15, t20, t25, t30, t40, t50, t60, t70, t80, t90, t100, t110 and t120. Blood samples (10 mL or 5 mL) for plasma storage were collected at t20, t40, t60, t80, t90, t100, t110 and t120.

Table 6. Timing of samples in the hyperglycaemic clamp

Time	10 mL Sample	5 mL Sample	1 mL Sample
t –30	✓		✓
t –20	✓		✓
t –10	✓		✓
t 0	✓		✓
Glucose Injection			
t 5			✓
t 10			✓
t 15			✓
t 20	✓		✓
t 25			✓
t 30			✓
t 40	✓		✓
t 50			✓
t 60	✓		✓
t 70			✓
t 80	✓		✓
t 90		✓	✓
t 100	✓		✓
t 110		✓	✓
t 120	✓		✓

5. Sample analyses

5.1 Basal samples

5.1.1 Plasma glucose concentration

Individual basal samples were analyzed for the concentration of glucose in plasma (mg/dL), utilizing an enzymatic technique (YSI 2300 STAT Plus Glucose and Lactate Analyzer). Each collected plasma sample was analyzed and recorded individually.

The individual variation in plasma glucose (within cow, between the two consecutive samples) in the basal period was generally below 10%, but high in one instance (19.7%). Some variation was to be expected, as these values represent nonfasting plasma glucose concentrations (Table 7). Both the individual plasma glucose concentrations and the

fortnightly weekly average plasma glucose concentrations were recorded. The weighted average of the two consecutive samples was used to calculate the plasma glucose concentration for that week (e.g. 57.6 mg/dL ± 11.1%, week 1).

Table 7. Variation in consecutive basal period plasma glucose samples

Cow	Week 1	Week 3	Week 5	Week 7
620	67.3 ± 5.6%*	66.3 ± 2.6%	66.4 ± 4.7%	62.9 ± 5.6%
515	64.0 ± 10.2%	56.9 ± 1.6%	62.8 ± 1.4%	59.6 ± 2.0%
608	54.2 ± 0.8%	51.7 ± 4.0%	64.1 ± 7.5%	59.6 ± 3.0%
524	58.5 ± 4.1%	57.4 – #	61.8 – #	61.8 ± 0.0%
606	60.7 ± 2.1%	56.5 ± 0.4%	67.6 ± 7.5%	64.2 ± 6.1%
705	51.8 ± 19.7%	61.7 ± 0.6%	58.0 ± 2.0%	59.2 ± 6.6%
631	57.7 ± 2.7%	63.6 ± 0.7%	60.9 ± 0.8%	56.9 ± 4.2%
721	61.3 ± 9.6%	59.2 ± 4.3%	61.8 ± 0.9%	60.5 ± 6.3%
543	55.9 ± 3.8%	62.5 ± 2.5%	64.1 ± 2.0%	62.3 ± 0.1%
521	45.0 ± 9.3%	66.8 ± 1.3%	59.4 ± 7.9%	64.9 ± 5.2%
Mean	**57.6 ± 11.1%**	**60.2 ± 8.0%**	**62.7 ± 4.7%**	**61.2 ± 4.0%**

* All values are presented as a weighted average ± coefficient of variation (CV), or the SD as a percentage of the mean.
\# Samples not available for analysis, due to inability to sample or tube breakage.

5.1.2 Plasma insulin concentration

The basal samples were analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA), developed for the analysis of bovine insulin (DRG, EIA-2340). Variation between the two consecutive samples occurred because the insulin concentration will partly be determined by the time after meal consumption. Individual assay of the fortnightly samples (Table 8) in a single randomly selected cow revealed large variation between the two samples (CV between 8% and 34%), where no bulking of samples occurred. All other basal samples analyses were conducted with bulking, which involved mixing of equal volumes from each sample into a single aliquot, which was assayed as a "single sample", to incorporate the expected variation of the nonfasting plasma insulin concentrations into the fortnightly values.

Due to the nature of the experiment, samples varied greatly in period of storage. Although initial planning clearly stated very little risk, it was later confirmed that storage time did have a significant effect on the insulin concentration (µg/L) in plasma. Although the basal, fortnightly plasma insulin concentrations of all cows were assayed, comparison between the two runs (as well as the samples of varying age on the same plate) could not be done due to unacceptably high variation and variable loss of peptide. The control sample run with the experiment samples in the second run was greatly decreased (at –239% of the first run), with an undetectable insulin concentration. Two additional results of samples previously analyzed revealed inconsistent reductions of –12.2% and –118% of the values obtained in the first assay run and no further use was made of this data.

Table 8. Samples assayed in the basal period

Sample *	SINGLE COW			OTHER COWS		
Number	Glucose	Insulin	IGF-I	Glucose	Insulin	IGF-I
1	✓	✓	✓	✓	①	⑤
2	✓	✓	✓	✓	①	⑤
3	✓	✓	✓	✓	②	⑥
4	✓	✓	✓	✓	②	⑥
5	✓	✓	✓	✓	③	⑦
6	✓	✓	✓	✓	③	⑦
7	✓	✓	✓	✓	④	⑧
8	✓	✓	✓	✓	④	⑧

* Samples were designated numbers in order of collection. Samples that were analyzed individually and not bulked with other samples are designated ✓. Samples that were bulked together are designated by the same circled number, for example ① where sample number 1 and 2 were bulked, mixed and analyzed as "a single sample" for insulin concentration.

5.1.3 Plasma IGF-I concentration

Plasma IGF-I concentrations (µg/L) were analyzed using a sandwich ELISA developed for the analysis of human IGF-I (IDS, AC-27F1). The basal samples of an individual cow were assayed separately, while the samples for other cows were bulked similar to the protocol used for insulin assay (Table 8).

The IGF peptide in plasma is prone to destruction and it became clear that samples could not be stored for long periods of time and should have been assayed shortly after collection. Although we were assured that analysis protocols would be in order, the first run of the assay clearly indicated otherwise. Loss of peptide was already evident and further comparison of samples of varying age was clearly not advisable. Although the full data of four cows was recorded, no IGF-I concentrations were included in the data set.

5.2 The insulin challenge

5.2.1 Whole-blood glucose concentration

Once the cows became used to the conditions of the metabolic house, small whole-blood glucose samples were assayed to establish the stability of the blood glucose concentration. The whole-blood samples were analyzed using the same technique as the plasma assay (YSI), which rapidly made the results available. All the whole-blood samples collected during the insulin challenge (as set out in Table 5) were analyzed for glucose concentration. During the baseline of the control period the average whole-blood glucose concentration within each cow exhibited little variation, as these observations represent the fasting plasma glucose concentrations of the control period. The CV of the control period baseline was 1.5% (range = 0.5% to 3.2%), with an average concentration for all cows of 48.7 mg/dL. Similar results were obtained for variation within cow in the rbST period (CV 1.5%, from 0.4% to 2.4%), the 80% ME period (CV 1.3%, from 0.5% to 2.0%) and the 80% ME + rbST period (CV 1.6%, from 0.5% to 3.8%). There was no significant difference ($P < 0.70$) between the individual baseline observations, therefore the whole-blood AUC was used to compare the effects of treatments on the baseline concentrations.

After insulin injection, frequent whole-blood samples were collected and analyzed for blood glucose concentration. These values were used to plot and monitor blood glucose concentrations during the insulin challenge. The AUC response was calculated by correcting the AUC from t0 to t30 for the baseline AUC and this corrected value was used to compare the glucose responses to insulin injection between treatments.

5.2.2 Plasma glucose concentration

Each individual baseline plasma sample collected was analyzed for the concentration of glucose in mg/dL for all cows in the control period (Table 9). As with the whole-blood glucose concentrations, there was very little variation between the four baseline samples for the insulin challenge (samples 9 to 12), which were not significantly different ($P < 0.14$). The average CV within cows for the four time points was 1.8% with a range between 0.5 to 3.5% in the control period. Similar results were obtained for the rbST period (CV 1.8%, range = 0.8% to 3.0%), the 80% ME period (CV 2.1%, range = 0.9% to 2.8%) and the 80% ME + rbST period (CV 2.1%, range = 0.6% to 4.2%). For the other treatment periods, each baseline plasma sample was separately analyzed for glucose concentration (Table 10). The individual plasma glucose concentrations and the plasma AUC from t–30 to t0 were used to determine the effects of treatments.

All the plasma samples of the insulin response were likewise analyzed for the plasma glucose concentration, which included samples 13 to 17 in Table 9 and Table 10. The corrected plasma AUC was used to determine the glucose response to insulin injection of treatments.

Table 9. Samples assayed in the control period (insulin challenge)

		SINGLE COW			OTHER COWS		
Sample *	Time	Glucose	Insulin	IGF-I	Glucose	Insulin	IGF-I
9	–30	✓	✓	✓	✓	×	①
10	–20	✓	✓	✓	✓	×	①
11	–10	✓	✓	✓	✓	×	①
12	0	✓	✓	✓	✓	×	①
13	30	✓	×	✓	✓	×	×
14	60	✓	×	×	✓	×	×
15	90	✓	×	✓	✓	×	×
16	120	✓	×	×	✓	×	×
17	150	✓	×	×	✓	×	×

* Samples 9 - 12 refer to the baseline period prior to insulin challenge, with samples 13 - 17 obtained between t30 and t150 minutes after insulin injection. Samples that were individually analyzed are designated ✓, with samples not analyzed designated ×. Samples that were bulked are indicated with the same circled number.

5.2.3 Plasma insulin concentration

The individual plasma samples for a single cow were analyzed for insulin concentration during the initial baseline of the control period (Table 9). For other cows, no samples were assayed for the insulin challenge in any of the treatment periods (Table 9 and Table 10). The insulin concentration during the insulin challenge was not included in the data set, for any of the cows sampled (see paragraph 5.1.2).

5.2.4 Plasma IGF-I concentration

The individual baseline plasma samples from the control period (fasting IGF-I concentration), as well as two samples during the response phase were analyzed in a single cow (Table 9). In the other treatment periods only the fasting plasma IGF-I concentrations were determined by bulking the individual plasma samples (Table 10). No IGF-I values were used during the statistical analysis of the data after completion of only a single assay run (see paragraph 5.1.3).

Table 10. Samples assayed in rbST and/or 80% ME periods (insulin challenge)

		SINGLE COW			OTHER COWS		
Samples	Time	Glucose	Insulin	IGF-I	Glucose	Insulin	IGF-I
30, 51, 72	–30	✓	①	②	✓	×	③
31, 52, 73	–20	✓	①	②	✓	×	③
32, 53, 74	–10	✓	①	②	✓	×	③
33, 54, 75	0	✓	①	②	✓	×	③
34, 55, 76	30	✓	×	×	✓	×	×
35, 56, 77	60	✓	×	×	✓	×	×
36, 57, 78	90	✓	×	×	✓	×	×
37, 58, 79	120	✓	×	×	✓	×	×
38, 59, 80	150	✓	×	×	✓	×	×

* The baseline samples for insulin challenge include samples 30 - 33 (rbST), 51 - 54 (80% ME) and 72 - 75 (80% ME + rbST). The symbol ✓ indicates samples assayed individually, × indicates samples not assayed, while bulked samples are represented by a circled number.

5.3 The hyperglycaemic clamp

5.3.1 Whole-blood glucose concentration

Once the whole-blood glucose concentration stabilized after the insulin challenge, the hyperglycaemic clamp was initiated. All the 1 mL whole-blood samples collected during the hyperglycaemic clamp (as set out in Table 6) were immediately analyzed and plotted during the clamp for the control period (Table 12) and treatment periods (Table 13). Once the glucose infusion commenced, whole-blood glucose concentrations were plotted and monitored to maintain hyperglycaemia. The average whole-blood glucose concentration was 47.5 mg/dL in the control period, with little individual variation between the four baseline observations. The CV was 2.0%, with a range between 1.0 to 3.1% in the control period, with similarly low values in the rbST period (CV 1.7%, range from 0.3% to 3.1%), the 80% ME period (CV 1.6%, range 0.4% to 3.4%) and the 80% ME + rbST period (CV 1.8%, range 0.5% to 3.5%). Although there was an effect of the time of sampling (t–30, t–20, t–10 and t0), this was generally very small. This included significant ($P < 0.05$) differences for 7 of the 24 observations, which were related to differences of between 1.8% and 2.4% of the average baseline glucose concentration (Table 11). Only the whole-blood glucose concentrations were used in the data set.

Table 11. Variation in individual baseline samples (hyperglycaemic clamp)

Time Points	*Control*	*rbST*	*80% ME*	*80% ME + rbST*
t–30 *vs.* t–20	–	–	–	–
t–30 *vs.* t–10	1.8%	–	–	1.8%
t–30 *vs.* t0	2.4%	2.0%	2.0%	–
t–20 *vs.* t–10	–	–	–	–
t–20 *vs.* t0	1.9%	–	–	2.4%
t–10 *vs.* t0	–	–	–	–
Average	**47.5 mg/dL**	**41.5 mg/dL**	**45.5 mg/dL**	**44.5 mg/dL**

During the final 40 minutes of hyperglycaemia the system stabilized, as confirmed by the stable GIR of this phase. There was no difference between the individual GIR values of t90, t100, t110 and t120 ($P < 0.16$) and this represented a steady-state (SSGIR). The average concentration increment of the plateau phase was +48.3 mg/dL for the control period, +51.7 mg/dL for the rbST period, +49.4 mg/dL for the 80% ME period and +52.6 mg/dL for the 80% ME + rbST period. The target level of hyperglycaemia was +50.0 mg/dL (± 10%) and was achieved in every instance (i.e. for all cows in all clamps).

5.3.2 Plasma glucose concentration

Each individual baseline plasma sample collected during a hyperglycaemic clamp was analyzed for all cows in the control and treatment periods (Table 12 and Table 13 respectively). The four baseline samples from the control period exhibited little variation with a CV within cows of 1.6% of the 65.0 mg/dL (range of 0.5% to 3.0%), with similar values for the rbST period (CV 3.3%, range of 1.5% to 4.7%), 80% ME period (CV 3.3%, range of 1.0% to 5.2%) and the 80% ME + rbST (CV 2.4%, range of 1.6% to 3.9%). There was no difference between the glucose concentrations of the four baseline samples ($P < 0.52$). The individual concentrations were therefore used to calculate the weighted mean concentration, which was used to compare the baseline values for the hyperglycaemic clamp between treatments.

From t90 to t120 of the hyperglycaemic clamp (the plateau phase) the average plasma glucose concentration increased from the baseline (65.0 mg/dL) to plateau (131.4 mg/dL) was +66.4 mg/dL for the control period. The plasma glucose increment was +71.2 mg/dL for the rbST period, +66.9 mg/dL for the 80% ME period and +70.2 mg/dL for the 80% ME + rbST period.

Table 12. Samples assayed in the control period (glucose clamp)

		SINGLE COW			OTHER COWS		
Sample *	Time	Glucose	Insulin	IGF-I	Glucose	Insulin	IGF-I
18	–30	✓	✓	✓	✓	①	③
19	–20	✓	✓	✓	✓	①	③
20	–10	✓	✓	✓	✓	①	③
21	0	✓	✓	✓	✓	①	③
22	20	✓	×	×	✓	×	×
23	40	✓	✓	✓	✓	×	×
24	60	✓	×	×	✓	×	×
25	80	✓	×	×	✓	×	×
26	90	✓	✓	✓	✓	②	④
27	100	✓	✓	✓	✓	②	④
28	110	✓	✓	✓	✓	②	④
29	120	✓	✓	✓	✓	②	④

*The hyperglycaemic clamp baseline samples included samples 18 - 21 for the control period. Hyperglycaemia was reached and clamped at +50 mg/dL by 30 minutes, or before sample 23. The plateau period encompassed samples 26 - 29. Individual samples that were analyzed are designated ✓ and those not analyzed as ×. Samples that were bulked together have the same circled number.

5.3.3 Plasma insulin concentration

For a single cow, the baseline samples and plateau samples (during SSGIR) of the control period was analyzed separately (Table 12), together with one sample of the rising phase of circulating blood glucose concentration. For all other cows and in the other treatment periods (Table 13) samples collected during the baseline and plateau phases were bulked. The insulin increment between baseline and plateau (or MPII) estimates the degree of reaction of the pancreas to a supraphysiological glucose concentration, where gluconeogenesis should not contribute to the glucose pool in blood. Due to the conditions described in paragraph 5.1.2, no insulin values were included in the data set.

5.3.4 Plasma IGF-I concentration

For a single cow, the baseline and plateau samples during the control period were analyzed separately (Table 12), while a single sample between the baseline and plateau was also assayed. For the treatment periods all the samples assayed were bulked for the baseline and plateau phases (Table 13). No further assays of plasma IGF-I samples were conducted (see paragraph 5.1.3).

Table 13. Samples assayed in rbST and/or 80% ME periods (glucose clamp)

		SINGLE COW			OTHER COWS		
Samples	Time	Glucose	Insulin	IGF-I	Glucose	Insulin	IGF-I
39, 60, 81	–30	✓	①	③	✓	⑤	⑦
40, 61, 82	–20	✓	①	③	✓	⑤	⑦
41, 62, 83	–10	✓	①	③	✓	⑤	⑦
42, 63, 84	0	✓	①	③	✓	⑤	⑦
43, 64, 85	20	✓	×	×	✓	×	×
44, 65, 86	40	✓	×	×	✓	×	×
45, 66, 87	60	✓	×	×	✓	×	×
46, 67, 88	80	✓	×	×	✓	×	×
47, 68, 89	90	✓	②	④	✓	⑥	⑧
48, 69, 90	100	✓	②	④	✓	⑥	⑧
49, 70, 91	110	✓	②	④	✓	⑥	⑧
50, 71, 92	120	✓	②	④	✓	⑥	⑧

* Baseline samples include samples 39 - 42 (rbST), 60 - 63 (80% ME) and 81 - 84 (80% ME + rbST). The plateau period samples include samples 47 - 50 (rbST), 68 - 71 (80% ME) and 89 - 92 (80% ME + rbST). Samples analyzed separately (✓), not analyzed (×) and bulked (circled numbers) are indicated.

6. Sample assay techniques

6.1 Glucose concentration

The glucose concentration in whole-blood and plasma was determined by a method utilizing an enzymatic technique that determined the concentration of glucose in samples against a known standard of 180 mg/dL (YSI 2300 STAT Plus). Briefly, glucose diffuses across a 3-layered immobilized membrane (Figure 10) that contains enzyme, *glucose oxidase* and a cellulose acetate layer that protects a platinum anode from other oxidizing substrates and contamination (YSI, 1997). Enzymatic glucose oxidation results in hydrogen peroxide (H_2O_2) production, which undergoes non-enzymatic oxidation at the platinum anode, resulting in electron ($e-$) production. The glucose concentration is proportional to the steady-state hydrogen peroxide concentration, which in turn is proportional to $e-$ flow. The difference in $e-$ flow in nanoampere (nA) resulting from the known calibration standard diluted into 600 µL buffer (steady-state plateau current – baseline current) is used to determine the glucose concentration in controls or samples. These results were automatically corrected for temperature differences.

Because of the instability of glucose concentration in whole-blood, control samples were not available for the assays of glucose concentration in whole-blood. During the analysis of plasma, two control samples that were collected and frozen with the experiment samples were used as controls. The average concentration of control 1 was 48.8 mg/dL with an average SD calculated within each run, of 0.50 mg/dL (or 1.0%, range = 0.0% to 3.3%). The difference between runs was also small, with a between-assay SD of 0.57 m/dL, or 1.16%. The average concentration of control 2 was 52.0 mg/dL, with a within-assay SD of 0.62 mg/dL (or 1.2%, range = 0.1% to 3.4%) and between-assay SD of 0.59 mg/dL (or 1.1%).

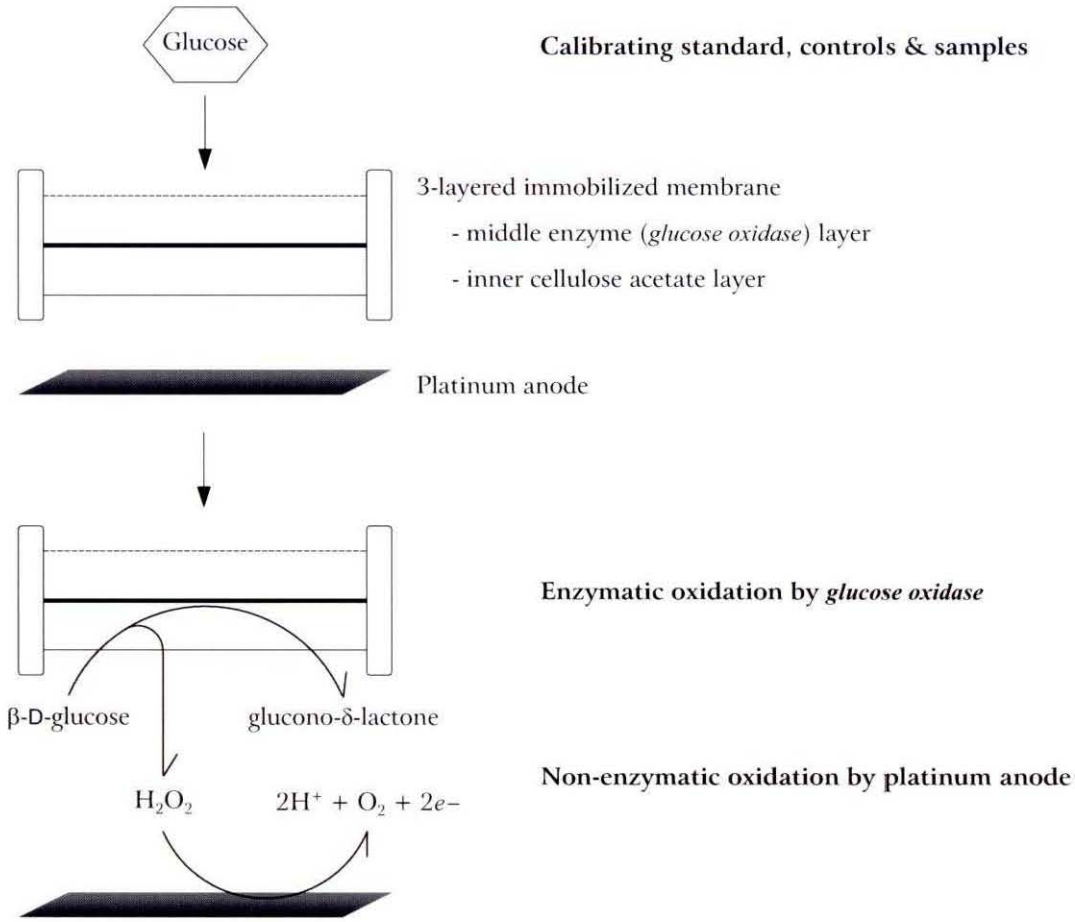

Figure 10. Diagrammatic representation of glucose assay (YSI, 1997)

6.2 Plasma insulin concentration

The plasma insulin concentration was determined using a bovine insulin ELISA kit (DRG, EIA-2340). This ELISA technique (DRG, 2001) utilized solid phase, 2-site binding of the insulin in the samples with tetramethylbenzidine (TMB) as a chromogenic substrate (Figure 11). The kit supplied a 96 well plate, with 7 standards (0, 0.25, 1.0, 2.0, 4.0 and 6 μg/L) run in duplicate to generate the standard curve from which sample concentrations were calculated. A control sample collected and frozen during the experiment was also included in the assays.

The control, together with the standards, allowed for the analysis of 80 samples per kit. During the first run, a total of 21 samples were analyzed separately for a single cow, with 59 more observations as bulked samples. The total number of plasma samples, regardless of bulking, that were used during the first run was 225 that included samples from 6 cows (5 complete analyses + 1 partial). During the second run, 70 bulked samples were analyzed, which included 232 samples from 6 cows (5 complete analyses + 1 partial). The insulin concentration analysis was therefore completed in two runs, utilizing 457 samples. Due to peptide instability with long-term storage (decay of control and samples), insulin data was not included in the statistical analyses (paragraph 5.1.2).

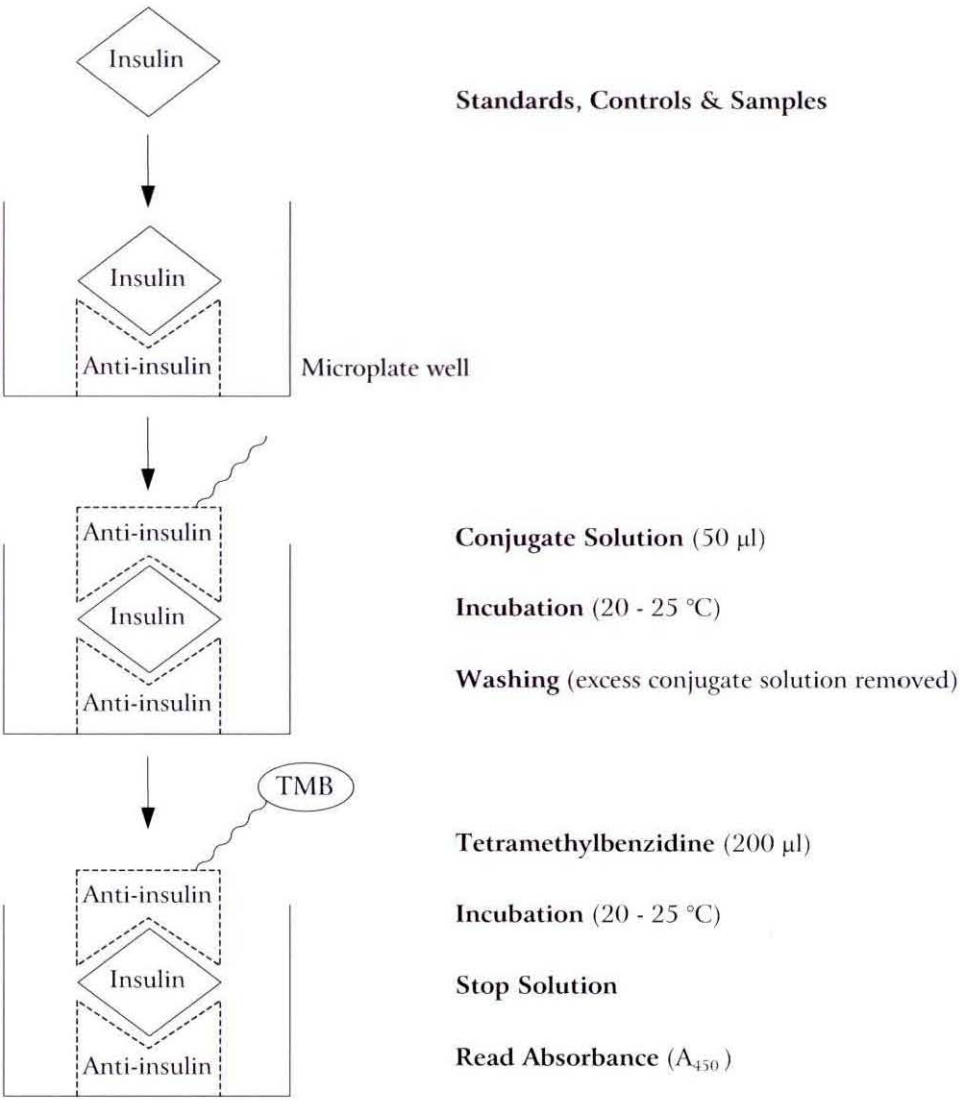

Figure 11. Diagrammatic representation of the insulin ELISA (DRG, 2001)

6.3 Plasma IGF-I concentration

Plasma IGF-I concentration was determined using an IGF-I ELISA kit (IDS, AC-27F1). The technique (IDS, 2001) utilized solid phase, 2-site binding of the total IGF-I in the samples, after release from binding proteins (Figure 12). The technique allows for the analysis of 80 samples, once the 6 standards used to generate a standard curve (0, 15, 34, 119, 360 and 1201 µg/L), a control supplied with the kit and an own control have been added to the 96 well plate.

During the first run, a total of 23 samples were analyzed separately for a single cow, with 57 more observations as bulked samples. The total number of plasma samples, regardless of bulking, that were used during this assay was 227, which included samples from 4 cows. Due to peptide decay in samples and controls and the varying age of stored plasma samples, no IGF-I data was included in the data set (paragraph 1.5.3).

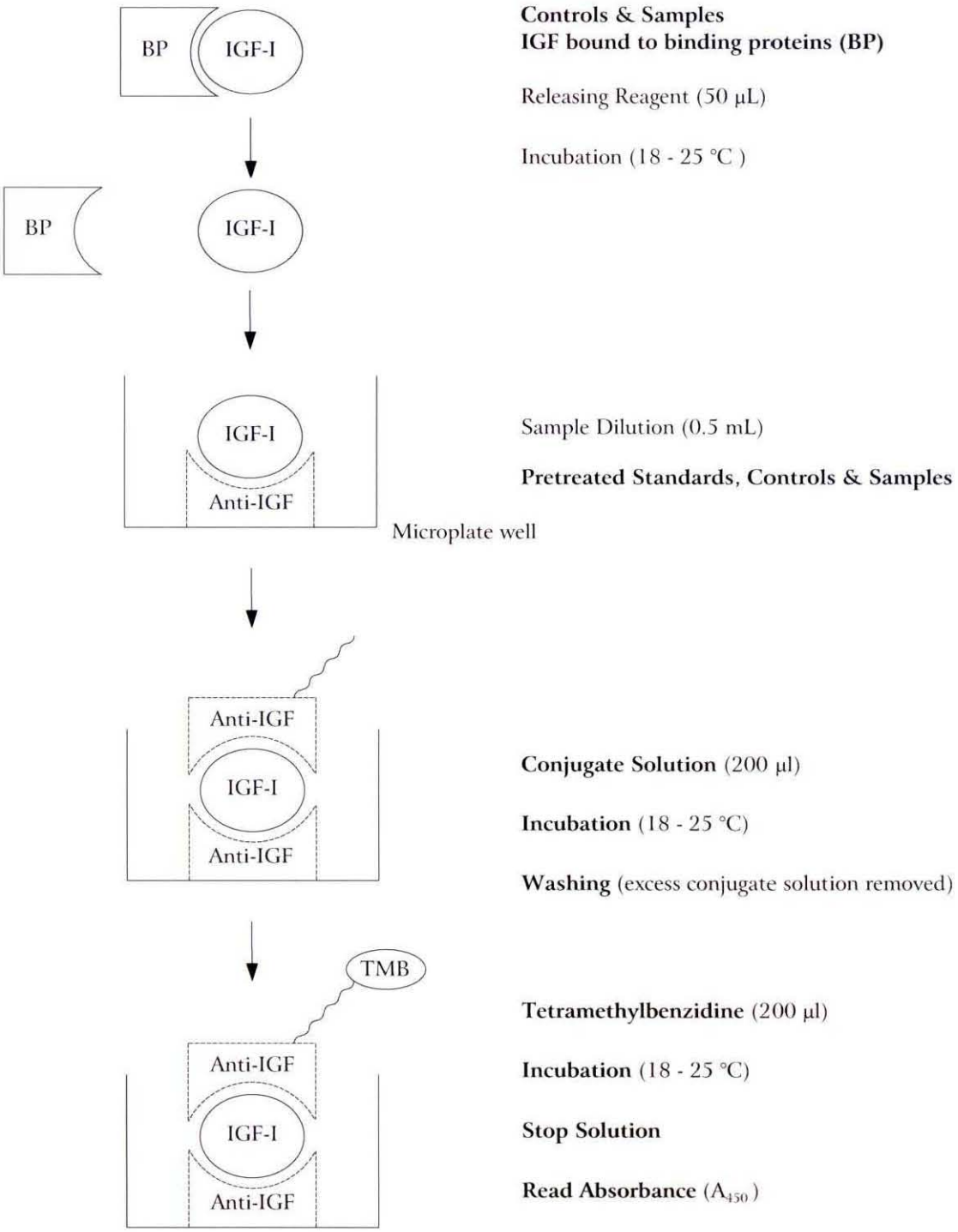

Figure 12. Diagrammatic representation of the IGF-I ELISA (IDS, 2001)