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INSULIN LEVELS**

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## RADIOIMMUNOASSAY FOR THE DETERMINATION OF LOW INSULIN LEVELS

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### ABSTRACT

The assay system was set up in such a way as to increase the sensitivity of the reaction, reducing the tracer concentration from the usual 1.000 cpm/ml to 500 cpm/ml levels of radioactivity (specific activity approximately 200 mCi/mg) and/or increasing the antiserum dilution by one thirty of the usual values in the assay of the final volume of incubation of 2,5 ml.

### RADIOIMUNOENSAIO PARA DETERMINAÇÃO DE CONCENTRAÇÃO BAIXA DE INSULINA

#### RESUMO

O sistema de ensaio foi desenvolvido para aumentar a sensibilidade da reação, reduzindo a concentração do traçador dos 1.000 cpm/ml usuais para níveis de radioatividade de 500 cpm/ml (atividade específica aproximadamente 200 mCi/mg) e/ou aumentando a diluição do anticorpo 30 vezes o valor usual no ensaio de volume final de incubação de 2,5 ml.

#### INTRODUCTION

An important aspect in radioimmunoassay for the measurement of low levels of plasma peptides with the use of ten times more dilute than for the usual RIA is the labelling of the peptide, employing to very high specific activities.

Thus, by increasing the number of substitution of radioiodine atoms in the hormone molecule we could increase the specific activity of the labelled hormone.

The specific activity depends on a number of other factors, including the sensitivity of the counting system, the volume of the incubation mixture to be counted and the concentration of hormone to be assayed. (1)

For radioiodination of polypeptide hormones the chloramine T method, combining high efficiency with simplicity is the most widely used. (2)

Purification of the labelled hormone is always necessary because of the appearance of certain labelled components distinct from those of the

main hormonal fraction i.e. contaminating proteins, altered hormonal peptides in the unlabelled (hormonal heterogeneity) and labelled (damaged component) material.

Purification method that separate on the basis of electrical charge such as starch gel electrophoresis are then required to obtain moniodoinsulin. Additional Sephadex gel filtration of the best component is necessary. With these techniques for purification of the labelled insulin, only a small degree of damage occurs even after a few weeks of storage (4).

In this paper, we report the techniques for obtaining highly purified labelled insulin with suitable specific activity and determination of the highest antibody dilution for the measurement of low insulin levels, such as after prolonged fasting.

## MATERIAL AND METHODS

### RADIOIODINATION OF PORCINE INSULIN

The standard method using chloramine T (3) was modified in our laboratory, with reduction in the amount of the oxidative agent to 1 µg of chloramine T per µg of hormone, to minimize overoxidation and iodination.

The reagents were added in the following sequence: to 20 µl of 0,2 M phosphate buffer pH 7.5 there were added successively 4 µg of porcine insulin, 1 mCi of Na<sup>125</sup>I, 4 µg of Chloramine T, 10 µg of sodium metabisulfite and 40 µl of human plasma. The total time from addition of the radioiodide until completion of the reaction and addition of plasma was generally no more than 15 seconds. The reaction was carried out at room temperature in a small glass tube.

A small aliquot of the iodination mixture, was removed for the assessment of the efficiency of the labeling by its adsorption to dextran-coated charcoal, a method that compares favorably with the standard paper chromatography-electrophoresis (8).

The efficiency is expressed as percentage of the total radioactivity incorporated into the intact hormone.

### PURIFICATION OF THE LABELLED HORMONE

After iodination, insulin <sup>125</sup>I was purified by starch gel electrophoresis (5,6) as suggested by Yalow anderson (7).

Autoradiographs of the starch gel electrophoresis of the labelled preparations, showed three radioactive zones: the most cathodal is designated as

zone 1 and the most anodal as zone 3. The middle zone (2) containing the suitable labelled hormone, because of its small proportion of unlabelled hormone and the most highly labelled molecules containing diiodothyrosyl residues, was extracted from the starch gel and purified on a Sephadex G-50 fine (1x50 cm) column. The elution was carried out with 0,02 M Veronal buffer, collecting 1 ml fractions.

#### DETERMINATION OF THE SUITABLE ANTIBODY DILUTION

##### Preliminary incubation

The labelled insulin, with two concentrations 500 and 1000 cpm/ml was incubated with the specific antiserum in dilutions of 1:700,000; 1:1,000,000, 1:1,200,000 and 1:2,000,000.

The incubation period lasted 5 days at 4°C, with daily checks for control of the physicochemical and immunologic behavior of the iodoinsulin.

The highest antiserum with dilution with the ratio bound/free ranging from 0,4 to 0,6 without a great increase in percentual damage to the hormone was selected.

The protocol for insulin radioimmunoassay (RIA) is given in Table 1.

##### Standard curves for the assay of insulin

For the determination of low insulin levels, three standard curves were run, spanning the range of human insulin concentration from 0,002 to 0.1 ng/ml with the antibody in the appropriate dilution (1:1,200,000) determined during the preliminary incubation with 1,000 cpm/ml of tracer and incubation time (5 days at 4°C).

Samples of plasma were collected during a standard 4 hour ethanol infusion (236 mg/min) after a 3-day fast with blood drawn every hour for 8 hr performed on four normal subjects with normal glucose tolerance test, and four obese subjects with weights more than 40% above the ideal body weight according to Life Insurance tables and normal carbohydrate tolerance.

The infusion induced in all subjects a decline in blood sugar levels with a parallel decrease in plasma insulin levels.

## RESULTS

Radioiodination: The efficiency of three labeling procedures, expressed as the percentage of the total radioactivity incorporated into the radioiodinated porcine insulin averaged 73%.

Purification of the labelled hormone: The preliminary purification of

starch-gel electrophoresis, to select the labelled hormone fraction with the highest immunoreactivity (devoid of excessive iodination and damaged molecules) i.e. fraction 2, gave a mean recovery of 73% of the radioactivity in the corresponding gel section. Its eluate gave a mean value of 88% of its total radioactivity as free labelled hormone.

Further purification in Sephadex yield with a mean for three labelled hormone corresponding to 97% of the total radioactivity present in insulin fraction.

Determination of the suitable dilutions of the antibody: The results of a typical study for the insulin are shown in Table 2. It can be seen that the highest antibody dilution after 5 days of incubation at 4°C was 1:1,200,000. These dilution is ideal because with 500 or 1000 cpm/ml the B/F was approximately the same (0,5).

Then we chose for the assay system an antiserum dilution of 1:1,200,000 with a tracer concentration corresponding 1,000 cpm/ml and incubation time of 5 days at 4°C. The tracer concentration (1,000 cpm/ml) was chosen because the values for the bound (B) and free (F) were measured in a well scintillation counter for a time not very long to accumulate about 10,000 counts.

Standard curves for RIA: Figure 1 prepared with the mean of three standard curves (Table 3).

Representation of the dose response curve with the B/F on the vertical scale and logarithm of concentration of human insulin on the horizontal scale.

Immunoassay of plasma insulin: The results of the studies performed in thirty six plasma samples from four normal subjects and thirty six plasma samples from four obese subjects are shown in table 4 and table 5. The mean of the results of blood glucose and plasma insulin in each of the fasted subjects, are indicated in table 6. The mean of blood glucose and plasma insulin levels were lower from plasma samples of normal subjects than obese subjects.

It can be seen (Fig. 2) that a significant and positive correlation occurs between blood glucose and plasma insulin in the two groups.

The statistical analysis of plasma insulin levels from plasma of normal and obese subjects is shown in table 7. The high value of the "t" calculated shows that the two measurements are statistically different at the level of  $\alpha = 0,05$ , like to hope.

In effects, obese subjects presented with similar decreases of blood glucose different changes in plasma insulin for the entire range of glucose

variation, plasma insulin levels being systematically higher than in normals, this fact might be related to the well known peripheral resistance to insulin in obesity (9).

#### CONCLUSION

These results indicated that the assay system with highest antibody dilution (1:1,200,000) and 1,000 cpm/ml (specific activity = 200 mCi/mg) levels of radioactivity for a B/F  $\approx$  0,5, was shown to be quite suitable for the measurement of low plasma insulin levels such as those obtained during ethanol - induced by hypoglycemia or prolonged fasting.



TABLE 1

## Protocol for Insulin Radioimmunoassay (RIA)

- 
1. 0,1 ml of plasma or standard sample of human insulin.
  2. 2,4 ml of standard diluent\* containing  $^{125}\text{I}$ -insulin (1000 cpm/ml) and antiserum to porcine insulin in the appropriate dilution.
  3. Incubation at 49C. Daily checks of damage of labelled hormone and its binding to the antibody.
  4. Daily checks for "incubation damage" (c) and immunoreactivity (B/F). Incubation stopped when c = 90% and B/F = 0,8 - 1,2 for usual assay and 0,4 - 0,6 for low insulin concentration.
  5. 0,2 ml of dextran-coated charcoal\*\*
  6. Centrifugation for 30' at 1,500 g and 49C.
  7. Separation of the supernatant (antibody-bound radioinsulin + free iodine) from the precipitate (free  $^{125}\text{I}$ -insulin); both are counted in well scintillation counter.

Calculated of bound/free ratio, corrected for damaged/hormone and free iodine.

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\*Standard diluent: 25 ml of 1% human serum albumin + 10 ml guinea pig serum + barbital buffer, 0,02 M pH 8,6 to a final volume of 1 liter.

\*\* Dextran-coated charcoal: 20 g of activated charcoal + 2 g of dextran-70/100 ml of barbital buffer. The two components are mixed at the time of use.

TABLE 2

Determination of the suitable antibody dilution and damage control for the determination of low insulin levels. Preliminary incubation (5 days at 4°C)

| Antiserum dilution | Concentration Tracer (cmp/ml) | Bound/Free *B/F | **Control (damage) % |
|--------------------|-------------------------------|-----------------|----------------------|
| 1:700,000          | 500                           | 0,85            | 5                    |
|                    | 1,000                         | 0,78            | 5                    |
| 1:1,000,000        | 500                           | 0,68            | 5                    |
|                    | 1,000                         | 0,64            | 5                    |
| 1:1,200,000        | 500                           | 0,49            | 5                    |
|                    | 1,000                         | 0,48            | 5                    |
| 1:2,000,000        | 500                           | 0,40            | 5                    |
|                    | 1,000                         | 0,30            | 5                    |

\*B/F = antibody-bound radioinsulin / free radioinsulin, the former corrected for free  $^{125}\text{I}$  and for percentage of damaged hormone.

\*\*Control = tests of the fraction of intact labelled hormone, measured by the percentage of total radioactivity bound to dextran-coated charcoal. The remainder represents "damaged" insulin.

TABLE 3

Standard curves for human insulin assay (basal values)

| Concentration Insulin |       | Bound/Free |      |      |
|-----------------------|-------|------------|------|------|
| $\mu\text{U/ml}$      | ng/ml | B/F        |      |      |
| 0,00                  | 0,00  | 0,48       | 0,49 | 0,49 |
| 1,25                  | 0,002 | 0,39       | 0,38 | 0,39 |
| 2,50                  | 0,004 | 0,35       | 0,36 | 0,37 |
| 3,75                  | 0,006 | 0,34       | 0,35 | 0,34 |
| 5,00                  | 0,008 | 0,32       | 0,33 | 0,32 |
| 6,25                  | 0,01  | 0,29       | 0,30 | 0,30 |
| 12,50                 | 0,02  | 0,27       | 0,28 | 0,28 |
| 18,75                 | 0,03  | 0,26       | 0,27 | 0,26 |
| 25,00                 | 0,04  | 0,24       | 0,25 | 0,23 |
| 37,50                 | 0,06  | 0,22       | 0,23 | 0,22 |
| 62,50                 | 0,10  | 0,21       | 0,20 | 0,21 |

Three standard curves were run, spanning the range of human insulin concentration from 0,002 to 0,10 ng/ ml corresponding to 1,25 to 62,50  $\mu\text{U/ml}$ .

TABLE 4

Blood glucose (BG) (mg/100 ml) and plasma insulin (I) ( $\mu$ U/ml) in four normal subjects in response to an alcohol infusion after 72 h - fast.

| Hours | JPP |    | EA |   | FP |   | CN |   |
|-------|-----|----|----|---|----|---|----|---|
|       | BG  | I  | BG | I | BG | I | BG | I |
| 0     | 73  | 13 | 45 | 4 | 42 | 2 | 42 | 3 |
| 1     | 48  | 7  | 32 | 1 | 23 | 1 | 27 | 2 |
| 2     | 46  | 5  | 32 | 1 | 20 | 1 | 26 | 1 |
| 3     | 44  | 7  | 30 | 1 | 20 | 1 | 25 | 2 |
| 4     | 42  | 9  | 32 | 1 | 22 | 1 | 26 | 1 |
| 5     | 58  | 6  | 40 | 6 | 26 | 1 | 25 | 1 |
| 6     | 60  | 13 | 42 | 9 | 27 | 2 | 33 | 1 |
| 7     | 62  | 9  | 42 | 9 | 29 | 1 | 35 | 2 |
| 8     | 86  | 20 | 42 | 9 | 33 | 1 | 35 | 2 |

TABLE 5

Blood glucose (BG) (mg/100 ml) and plasma insulin (I) ( $\mu$ U/ml) in four obese subjects in response to an alcohol infusion after 72 h - Fast.

| Hours | NV |    | MPS |    | LS |    | RM |   |
|-------|----|----|-----|----|----|----|----|---|
|       | BG | I  | BG  | I  | BG | I  | BG | I |
| 0     | 60 | 18 | 54  | 12 | 52 | 9  | 45 | 7 |
| 1     | 50 | 11 | 54  | 11 | 43 | 8  | 37 | 4 |
| 2     | 50 | 12 | 50  | 9  | 42 | 7  | 29 | 3 |
| 3     | 52 | 11 | 49  | 9  | 36 | 7  | 26 | 4 |
| 4     | 45 | 12 | 42  | 8  | 30 | 5  | 24 | 3 |
| 5     | 43 | 7  | 36  | 7  | 36 | 6  | 20 | 5 |
| 6     | 39 | 4  | 34  | 7  | 43 | 8  | 20 | 4 |
| 7     | 46 | 7  | 36  | 6  | 47 | 11 | 25 | 4 |
| 8     | 48 | 5  | 44  | 8  | 48 | 15 | 27 | 5 |

Fig. 2 Correlations between blood glucose and plasma insulin in normal subjects (A) and in obese subjects (B).

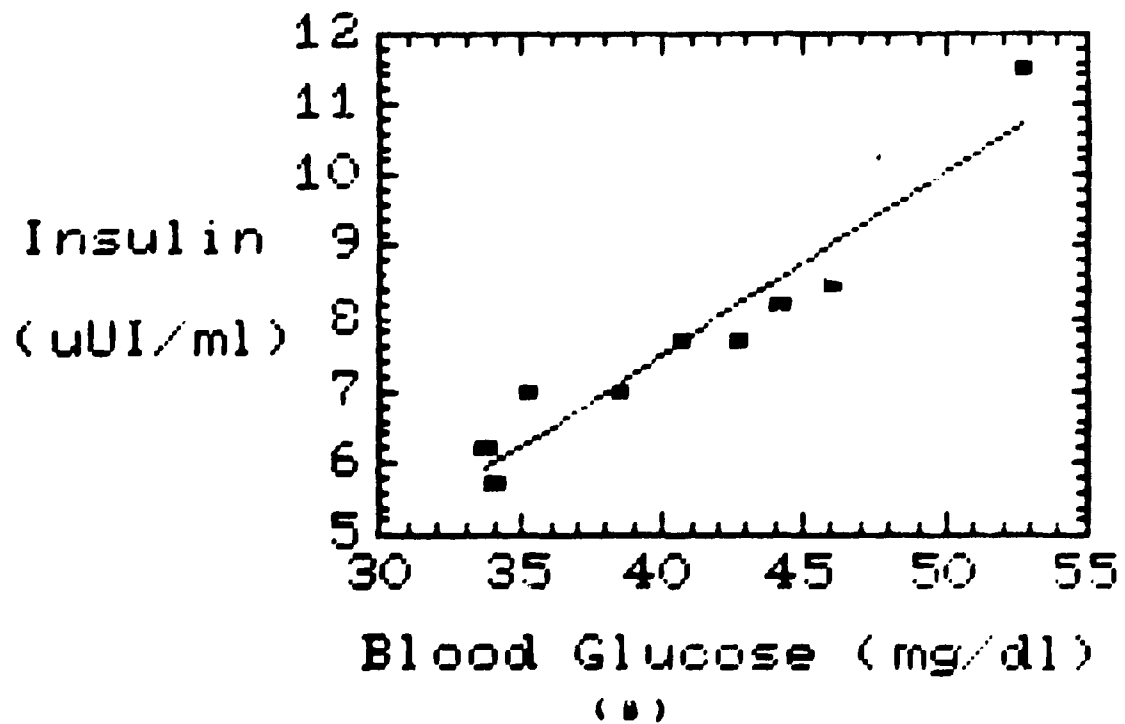
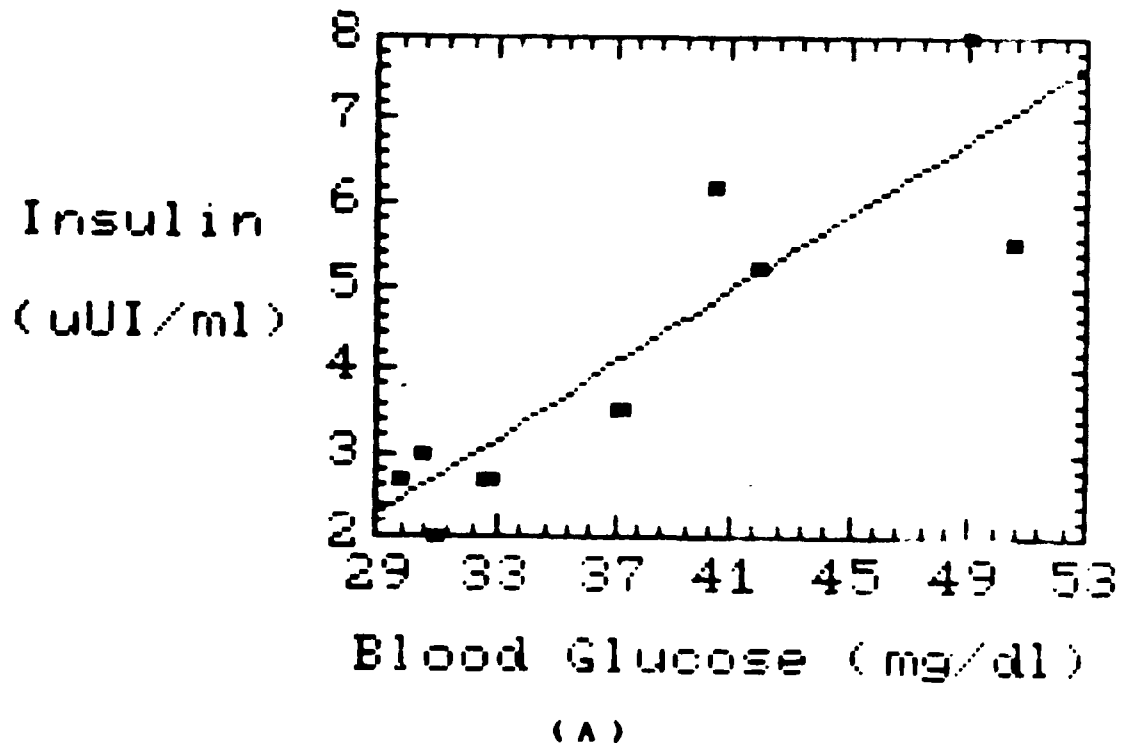


TABLE 6

Mean blood glucose (BG) and plasma Insulin (I) from four normal and four obese subjects (Table 4 - 5)

| Hours     | Normal subjects |        | obese subjects |        |
|-----------|-----------------|--------|----------------|--------|
|           | BG              | *I     | BG             | *I     |
| 0         | 50,5            | 5,5    | 52,7           | 11,5   |
| 1         | 32,7            | 2,7    | 46,0           | 8,5    |
| 2         | 31,0            | 2,0    | 42,7           | 7,7    |
| 3         | 29,7            | 2,7    | 40,7           | 7,7    |
| 4         | 30,5            | 3,0    | 35,2           | 7,0    |
| 5         | 37,2            | 3,5    | 33,7           | 6,2    |
| 6         | 40,5            | 6,2    | 34,0           | 5,7    |
| 7         | 42,0            | 5,2    | 38,5           | 7,0    |
| 8         | 49,0            | 8,0    | 44,2           | 8,2    |
| $\bar{X}$ | 38,1000         | 4,3111 | 40,8556        | 7,7222 |
| s         | 7,9407          | 2,0090 | 6,2959         | 1,6791 |
| r         | 0,8824          |        | 0,9560         |        |

TABLE 7

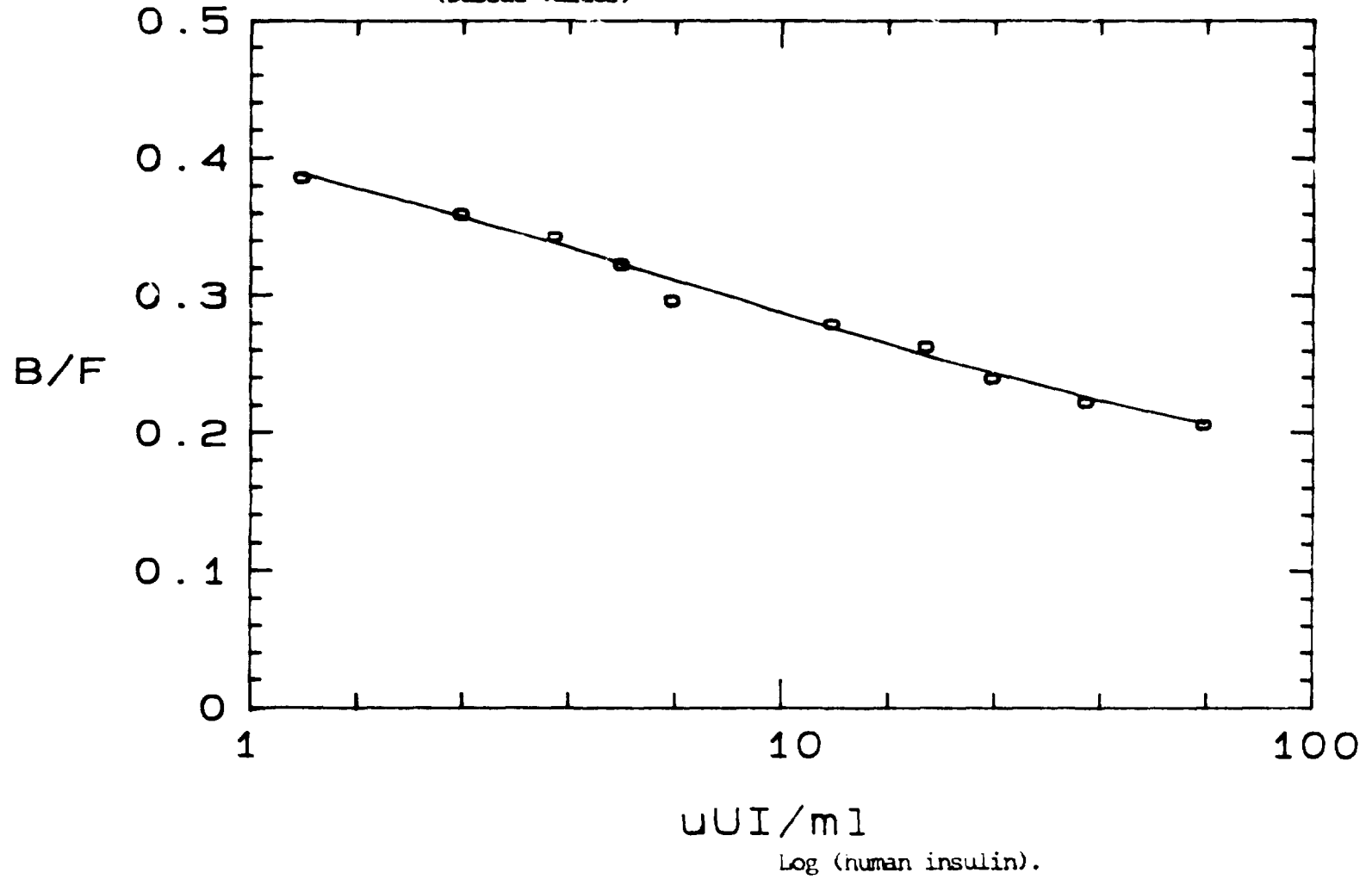
Statistical analysis of the mean insulin concentrations from four normal and four obese subjects in response to an alcohol infusion after a 72 h - fast .

| Hours                     | Insulin concentration $\mu\text{U/ml}$ |                |
|---------------------------|--|----------------|
|                           | Normal Subjects                        | obese subjects |
| 0                         | 5,5                                    | 11,5           |
| 1                         | 2,7                                    | 8,5            |
| 2                         | 2,0                                    | 7,7            |
| 3                         | 2,7                                    | 7,7            |
| 4                         | 3,0                                    | 7,0            |
| 5                         | 3,5                                    | 6,2            |
| 6                         | 6,2                                    | 5,7            |
| 7                         | 5,2                                    | 7,0            |
| 8                         | 8,0                                    | 8,2            |
| number of obs.            | 9                                      | 9              |
| average                   | 4,31111                                | 7,72222        |
| variance                  | 4,03611                                | 2,81944        |
| Std deviation             | 2,00901                                | 1,67912        |
| median                    | 3,5                                    | 7,7            |
| computed "t"<br>statistic | -3,90637                               |                |



Fig. 1 Standard curve for human insulin assay  
(basal values)

Porcine  
Insulin  $^{125}\text{I}$



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