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Circadian Variation of Serum Glucose, C-Peptide Immunoreactivity and Free Insulin in Normal and Insulin-Treated Diabetic Pregnant Subjects*

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Summary. To examine differences among pregnant diabetic and nondiabetic subjects, serum glucose, and immunoreactivity of C-peptide, free and total insulin were measured at hourly intervals during a 24–h third trimester metabolic ward evaluation. Six normals, three mild, and four juvenile-onset type diabetics were studied. Diets were identical for all subjects. Mild diabetics differed from juvenile diabetics by having significant residual pancreatic B-cell function, as measured by C-peptide immunoreactivity. Short and intermediate acting insulins given once or twice daily to diabetics maintained serum glucose levels within the normal range throughout the 24 h. Despite wide variation in serum total insulin levels, peripheral free insulin concentrations in well-controlled diabetics fell within a relatively narrow range that was higher than in controls. Infants of the diabetic subjects were comparable to the offspring of the control women.

Key words: Twenty-four hour C-peptide, free and total insulin, normal and diabetic pregnancy.

The duration of the diabetic state prior to pregnancy has been considered previously to be the most important factor in determining the perinatal risk for the infant of a pregnant diabetic woman [1, 2]. More recently emphasis has shifted toward consideration of the excellence of blood glucose control throughout the whole period of a diabetic's pregnancy [3–5]. In fact, a number of authors have suggested that an inverse correlation between mean serum glucose and perinatal mortality exists. Despite this consideration, serum glucose levels are difficult to maintain within the normal range for all or most of the day in insulin-requiring diabetics [6, 7]. Most likely this is due to the rather limited choices available in the timing, quantity and type of exogenous insulin when compared to the rapid secretory adjustments of normal B-cells to changes in circulating nutrients. The present study was undertaken to determine whether excellent control can be achieved in insulin-requiring pregnant diabetic women and to define the factors which may underlie success or failure in accomplishing this goal.

Materials and Methods

Subjects

Six normal volunteers without biochemical evidence or family history of diabetes and seven insulin-treated diabetics (White's classes B through D) were studied in the third trimester of pregnancy (Table 1). The diabetics comprised two groups: Group I had received no insulin prior to their pregnancies and, following delivery, none of the three women required insulin therapy. The remaining four were long-term, insulin-requiring, ketosis-prone diabetics (Group II). Both control and diabetic subjects were within 20% of their

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Table 1. Clinical features of the insulin-treated pregnant diabetic subjects

Subject	Age at onset of diabetes	Age at time of study	Diabetic class	Estimated weeks of gestation	Past history	Route of delivery	Ht/cm	Wt/Kg[c]	Diet Kcal/ kg/day	Infant size kg	Maximum daily dose insulin, units/day 3rd trimester	Prior to pregnancy
BB	25	29	B	39	Gr iii P i Ab i	Vaginal	147	57.6	35	3.300	37	None
DD	18	21	C	39	Gr i P 0	Vaginal	162	67.8	30	3.410	45	None
LK	18	23	C	37	Gr ii P i	Vaginal	167	80.6	30	3.400	45	None
SH	12	24	C	38.5	Gr i P 0	C-section[a]	160	54.2	35	2.580	120	30
EM	12	19	C	39	Gr i P 0	Vaginal	168	76.5	35	3.220	180	30
ML	10	21	C	39	Gr ii P i LC 0	C-section[b]	162.5	66.2	35	3.800	144	45
KJ	8	27	D	38	Gr i P 0	Vaginal	170.5	70.4	35	3.070	120	40
										3.410		
										3.310		
						Normal Subjects						
VR		18		40	Gr i	Vaginal	170	63	35	3.330		
AW		24		42	Gr i	Vaginal	162.5	60	35	2.860		
SS		18		40	Gr i	Vaginal	160	78	30	3.570		
MH		24		42	Gr i	C-section[a]	158	63	35	3.570		
LH		25		41	Gr i	Vaginal	170	71	30	3.970		
CP		26		40	Gr ii P i	Vaginal	168	70	35	3.520		
										3.470		

[a] Breech presentation
[b] Previous neonatal death secondary to shoulder dystocia
[c] During third trimester

Table 2. Composition of a sample 2000 calorie diet[a]

Constituent	Breakfast	Lunch	Dinner	Snack	Total g	Diet %
Protein	22	40	45	16	123	25
Carbohydrate	57	57	57	19	190	37.5
Fat	18	24	28	12	82	37.5
% total calories	24%	30%	33%	13%		100%

[a] All diet prescriptions were based upon 125 g protein; fat and carbohydrate were equally divided for the remainder of calories. Calories were 30 to 35 Kcal/kg/day.

ideal body weight. All subjects were included in the study after informed consent had been obtained.

Management of Diabetes

U-100 NPH insulin (NPH) and U–100 regular insulin (Reg) (Eli Lilly & Company, Indianapolis, Indiana, USA) were administered as a single injection one-half hour before breakfast [6]. This insulin preparation is 70% beef and 30% pork. All the diabetic subjects except BB and LK received a second injection one-half hour before dinner. The total dose usually given at 0730 was twice that administered at 1730. The ratio of NPH : Reg was 2 : 1 in the morning and 1 : 1 in the evening.

The daily diet of the diabetic patients (Table 2) contained 30–35 calories/kg body weight and 125 grams of protein. The remainder of the calories came equally from carbohydrate and fat. Multivitamin and iron supplemented the diet given to all the subjects.

The diabetic women tested second-voided preprandial urine specimens for glucose with Clinitest® (Ames Division, Miles Laboratories, Inc., Elkhart, Indiana, USA) and urinary ketones with Acetest® (Ames Division, Miles Laboratories, Inc.) tablets only if glucosuria was greater than 0.5 g/100 ml. Serum glucose was measured in the fasting state and 2 h after breakfast approximately every 2 wk. In this way the low and high serum glucose values for 24 h could usually be defined [8]. If the serum glucose was elevated above 100 mg/100 ml in the fasting state or 160 mg/100 ml 2 h after breakfast, the total daily insulin dose was raised by 20 to 40%. Reassessment of serum and urine glucose responses was carried out 4 to 7 days later. Additional adjustment upward by a further 20 to 40% was undertaken if the serum glucose levels remained elevated.

Metabolic Research Unit Protocol

The diabetic pregnant women were admitted to a metabolic research ward for a 3-day period to evaluate circadian fluctuations in their serum glucose and hormone levels. The normal pregnant women who served as controls were instructed to begin a diet

similar to that of the diabetics at least 2 weeks prior to their admission.

At 0800 on the day following admission, after an overnight fast, the first blood specimens were obtained preprandially, followed by samples at 1, 2, 3, and 4½ h after each meal. The 4½ h sample served as a preprandial sample for the next meal. After completion of the bedtime snack, samples were taken after 1, 2, 3, 5, and 8½ h, the last serving as a preprandial sample. Insulin was given to the diabetics 30 minutes before breakfast and, if necessary, 30 minutes before dinner.

Analysis of Serum

Serum was separated at 4°C and frozen at −20°C for later analysis. Glucose was measured by the method of Hoffman [9]. Total C-peptide immunoreactivity (CPR) was assayed using a human C-peptide (CP) antiserum and human C-peptide standards [10]. A modification of the double-antibody method of Morgan and Lazarow [11] was used to determine serum insulin (IRI): (1) free insulin was assayed following polyethylene glycol precipitation of antibodies to insulin and insulin bound to antibodies; (2) total insulin was assayed following hydrochloric acid incubation. Both insulin determinations employed the methods of Nakagawa et al. [12]. Further analysis of CPR in the diabetic subjects was performed by fractionating acidified sera on Bio Gel P-30 columns equilibrated in 3M acetic acid [13]. Both the proinsulin and C-peptide material was read from the human C-peptide standard. This permitted CPR to be compared with the CP values.

Expression of Results and Statistical Analysis

Results are given as the mean ± standard error of the mean (SEM) for group analysis. An average value for various determinations was calculated by dividing the sum of the particular measurements by the number of determinations. The average 24–h value (called the "daily value") for each individual calculated in this manner correlated closely with the area under the curve for the 18 samples obtained. For tests of statistical significance of difference between means, Student's *t* test for unpaired analysis was employed [14].

Results

Normal Subjects

Serum Glucose, C-Peptide Immunoreactivity (CPR), and Immunoreactive Insulin (IRI). The circadian variation of serum glucose, CPR and IRI in the six normal pregnant subjects is shown in Figure 1. The average serum glucose before each of the three main meals was 78 mg/100 ml (obtained by summing the 0800, 1300, and 1800 values for each of the six subjects and dividing by 18). The greatest increase in glucose was noted between the fasting and 1–h postprandial samples. The highest average serum glucose was approximately 120 mg/100 ml 1 h after breakfast. Peak average

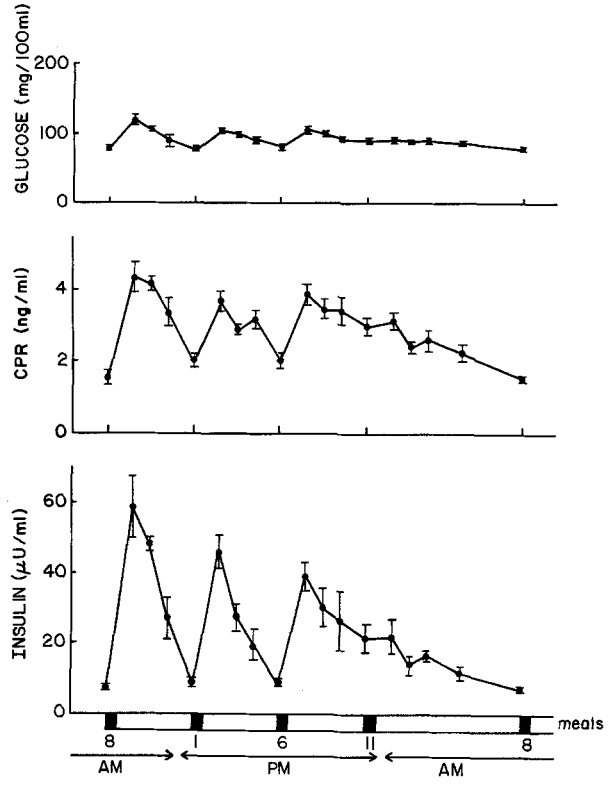

Fig. 1. Serum glucose, insulin, and CPR levels in six normal women in the 3rd trimester of pregnancy (mean ± SEM)

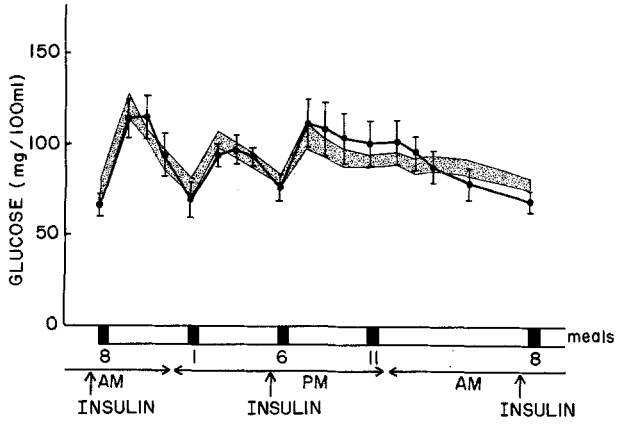

Fig. 2. Comparison of circadian variation of serum glucose in seven diabetic pregnant women and six normal subjects. The values (m ± SEM) in the diabetics are indicated by the solid line while the normals are depicted by the stippled area (m ± SEM)

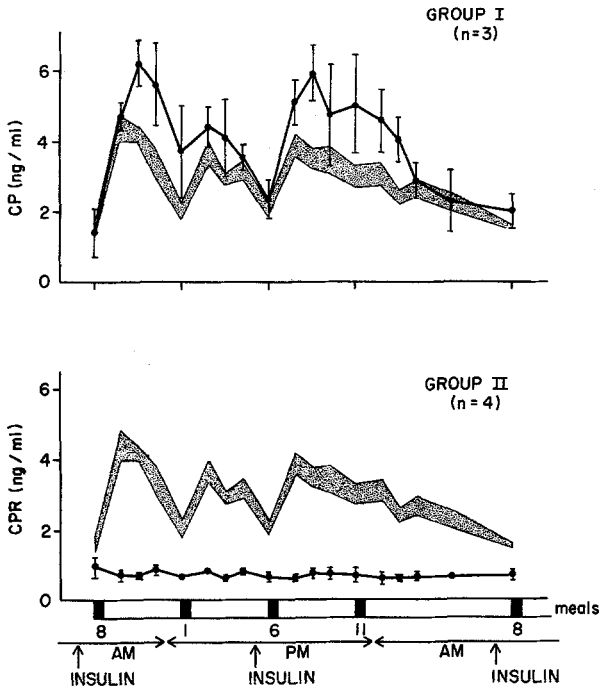

Fig. 3. CPR and CP levels in Group I and Group II diabetic women and control subjects. The stippled area represents CPR (m ± SEM) in normals. The continuous line (m ± SEM) in the upper panel depicts the CP concentration in Group I diabetics and in the lower panel the CPR concentration in Group II diabetics. CP was derived by subtracting proinsulin immunoreactivity from CPR

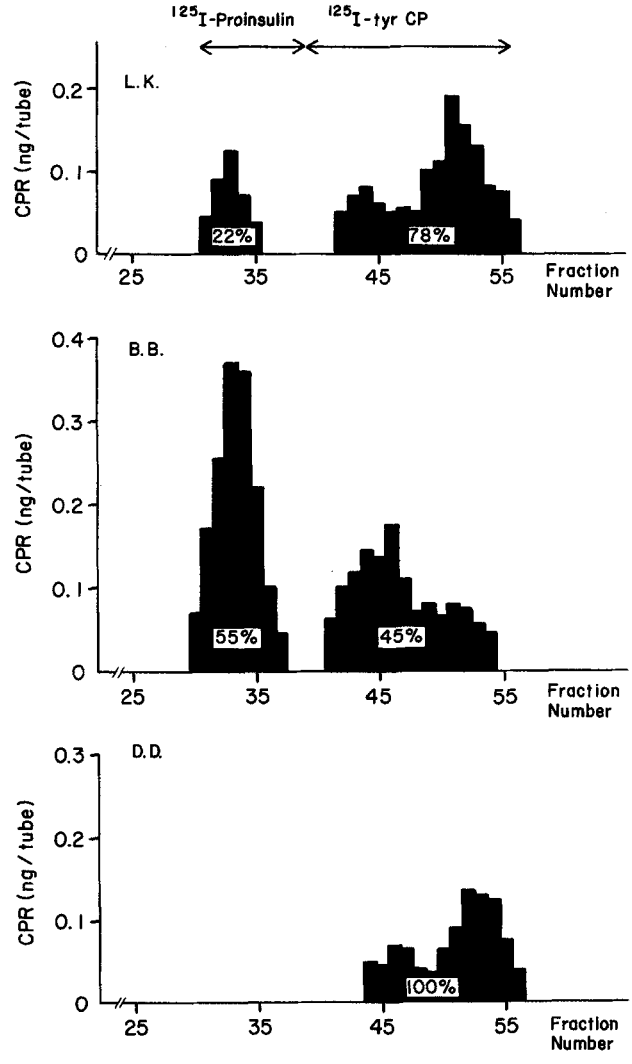

Fig. 4. Bio gel P–30 gel filtration patterns of serum of Group I subjects. Proinsulin, which crossreacts in the connecting peptide immunoassay, is located in fractions 29–37; 0.2 ml of serum was applied to the column

glucose concentrations after lunch and dinner were 105 mg/100 ml. This diminution in maximal postprandial glucose concentration occurred even though the carbohydrate content was equal in the three major meals and calories in each subsequent meal were increased over the previous meal (Table 2). The daily 24–h glucose levels in the six women ranged from 83 to 102 mg/100 ml.

CPR and IRI levels paralleled the glucose excursions. The highest peaks occurred 1 h after breakfast while the peaks 1 h after lunch and dinner were lower. Following the 1800 meal, the gradual diminution in glucose was paralleled by declines in CPR and IRI. The 0800 preprandial values were similar to those observed 24 h earlier. The daily values of serum CPR and IRI in the six individuals ranged from 2.53 to 3.81 ng/ml and 18.6 to 31.4 μU/ml respectively.

Diabetic Subjects

Glucose. The means of the daily 24–h glucose levels were 110.7 ± 8.4 mg/100 ml for the three Group I subjects and 79.5 ± 8.5 mg/100 ml for Group II. As illustrated in Figure 2, the circadian variation of serum glucose in the seven diabetics was comparable to the controls. However, this was fortuitous because of substantially greater upward and downward deviations from normal in some diabetic subjects.

CPR. To assess B-cell secretory function in these seven insulin-treated diabetics, serum CPR was measured. In Group II diabetics, serum CPR was significantly lower ($p<0.05$) than in normal subjects at 17 of the 18 sampling times and did not vary during the day (Fig. 3, lower portion). The mean daily CPR value for the four subjects studied was 0.69 ± 0.11 ng/ml, which is just above the detection limit of the method (0.5 ng/ml). In contrast, CPR in Group I diabetic subjects was markedly higher (6.70 ± 1.50 ng/ml) than in the controls (3.0 ± 0.14 ng/ml), $p<0.01$, and increases in CPR after food intake were clearly evident.

The C-peptide antiserum used for the C-peptide radioimmunoassay cross-reacts with human proinsu-

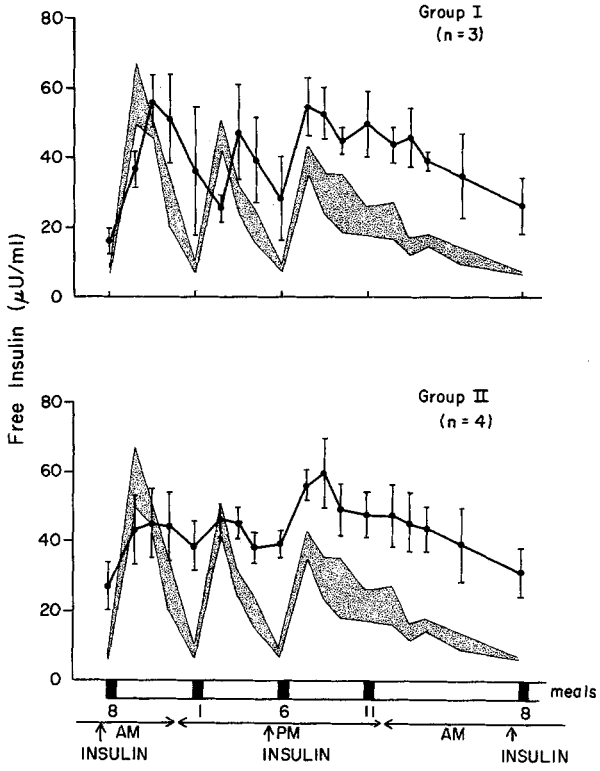

Fig. 5. Serum free insulin (m ± SEM) in Group I and Group II pregnant diabetics. The stippled area represents the m ± SEM of the normal subjects

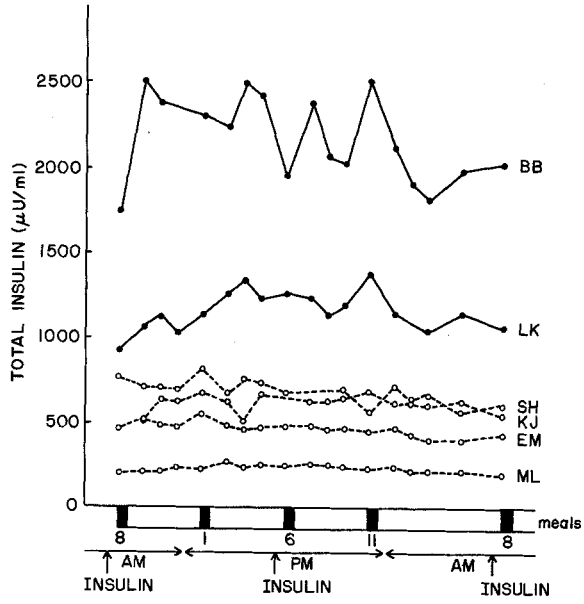

Fig. 6. Serum total insulin in Group I (closed circles) and Group II (open circles) diabetic women

lin [10]. However, under most circumstances serum CPR represents mainly the C-peptide, because the serum proinsulin concentration is much lower than that of C-peptide (less than 1/20th) and proinsulin reacts only one-third as well as C-peptide with this particular antiserum. In insulin-treated diabetic patients, on the other hand, circulating insulin antibodies bind endogenously secreted proinsulin and retard its clearance from the blood [13]. Under these circumstances proinsulin may become a major determinant of the CPR level. In order to correct for this interfering effect in Group I subjects, the contribution of proinsulin to the high levels of CPR was determined by gel filtration. As shown in Figure 4, proinsulin comprised 22% of CPR in LK and 55% in BB. DD, who had been treated with insulin for only 5 days, did not have insulin-binding antibodies, and her CPR and C-peptide levels were equivalent. The absolute C-peptide levels in each specimen were calculated by subtracting the proinsulin concentration from that of the total CPR. Because of the possibility that antibody-bound proinsulin might vary during the course of the 24–h study, an additional ten samples from each patient were checked by an independent method using polyethylene glycol to separate free C-peptide from antibodybound proinsulin (unpublished observation).

The results of the two methods were comparable. The upper portion of Figure 3 shows the mean C-peptide levels in Group I diabetics were higher than those of the controls at most sampling times during the day.

Free Insulin. The circadian variation of free insulin in Groups I and II diabetic subjects is compared to that of the controls in Figure 5. Group I received an average of 42 units of insulin per day (range 37–45 units) while Group II averaged 142 units of insulin per day (range 84–157 units). The mean daily value was 40.8 μU/ml in Group I and 43.7 ± 7.2 μU/ml in Group II compared to a mean daily value of 24.4 ± 2.0 μU/ml in the six normal subjects ($p<0.05$ compared to both Groups I and II). The higher circulating free insulin levels in the diabetics occurred mainly in the 1800 through 0800 interval.

Total Insulin. DD had not developed circulating insulin antibodies and her total and free insulin levels were therefore equivalent, as is the case in normal subjects. The two other Group I diabetics had higher total insulin values than those in Group II, and no definite circadian pattern was evident in any of the subjects (Fig. 6). The daily total insulin concentration ranged from 230 μU/ml for ML to 2165 μU/ml for BB.

Repeat Studies. Examples of subjects who were

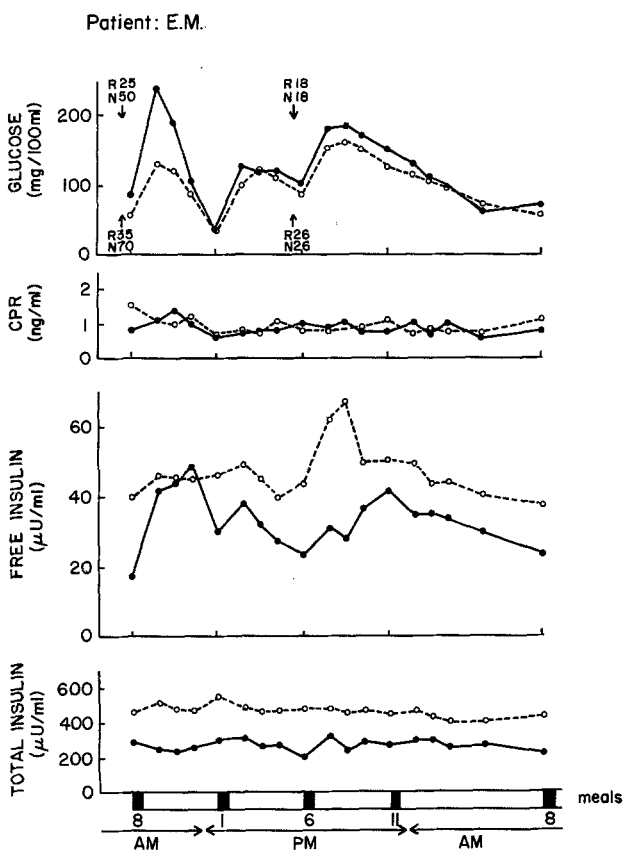

Fig. 7. Glucose, CPR, free and total insulin concentrations in subject EM during the 2nd (closed circles) and 3rd trimester (open circles)

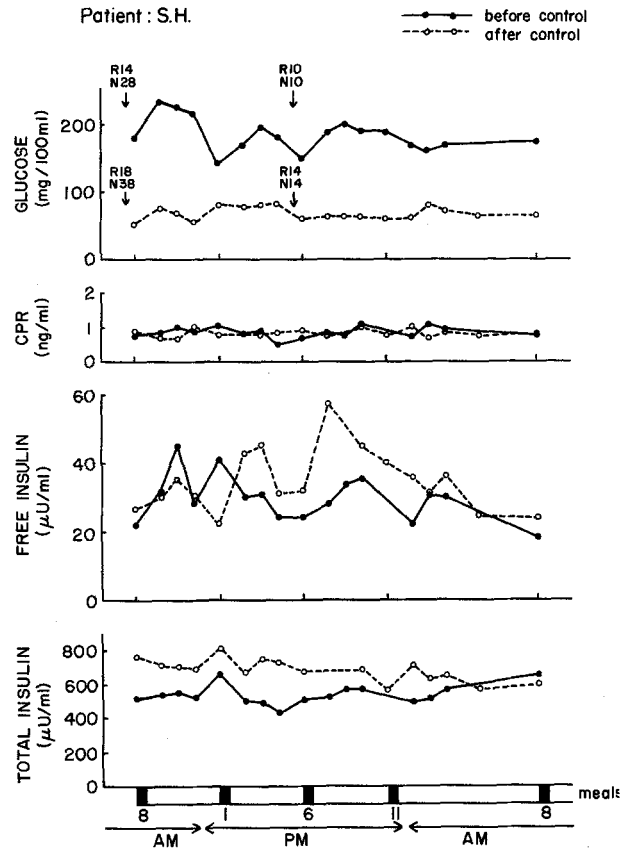

Fig. 8. Glucose, CPR, free and total insulin concentrations before and 2 wk after her insulin dosage was increased to achieve improved metabolic control

studied on the metabolic ward on more than one occasion are shown in Figures 7 and 8. EM was evaluated in the 2nd and 3rd trimesters (Fig. 7). The total dose of insulin in the first study was 111 units. With the exception of the initial three morning samples, her glucose levels were only slightly higher than those observed during the 2nd study in which 157 units were administered. CPR was similar at both times. On the other hand, free insulin increased from a daily value of 31.1 μU/ml to 46.8 μU/ml and total insulin from 267 μU/ml to 464 μU/ml.

Two separate studies of SH are displayed in Figure 8 to compare poor control with improved control when diet was unchanged and total insulin dosage was increased. In the initial investigation, while receiving 62 units insulin daily, the daily value of serum glucose was 184 mg/100 ml and urinary glucose 2 g/100 ml. The daily free insulin was 29.7 μU/ml and total insulin 538 μU/ml. After raising the insulin dosage to 84 units per day for 2 weeks, the serum glucose concentration fell into the normal range (daily value 68 mg/100 ml). Glucosuria was not detected and hypoglycemic symptoms did not occur. Daily free and total insulin levels rose to 34.6 μU/ml and 680 μU/ml, respectively.

Pregnancy Outcome. The normal and diabetic women's histories and gestational age at the time of delivery, together with routes of delivery and infant weights, are shown in Table 1. No respiratory distress syndrome or hypoglycaemia (blood glucose < 40 mg/100 ml) occurred in the newborn. The diabetic subjects required no insulin for approximately 48 h following delivery, even though some subjects were receiving in excess of 100 units on the day prior to delivery.

Discussion

Specific information regarding the factors which may be important in achieving excellent control of the blood glucose level in pregnant, insulin-requiring diabetics is limited [15–18]. Studies performed in Europe [3, 8], which have utilized lengthy periods of hospitalization and multiple injections of short-acting

insulin for strict metabolic control, have resulted in a low incidence of infant mortality and morbidity. The present study was specifically designed to provide a general approach to diet and to insulin dosage in the outpatient management of pregnant diabetic women. In addition, the results indicate the daily variation in blood glucose and insulin concentrations in nondiabetic pregnant women in the 3rd trimester, and demonstrate the feasibility of maintaining the blood glucose concentration of diabetic women within the normal range in an outpatient setting.

Since NPH and regular insulin are not altered by mixing, they can be combined in a single injection. The timing of the morning and evening regular insulin was chosen to permit its activity to peak when absorption from breakfast and dinner was maximal. Similarly, morning NPH should peak when absorption from the midday meal was greatest, while the evening dose of NPH insulin was administered to cover the bedtime snack and to ensure adequate insulin activity through the night. The advantages of this regimen have been described by Oakley et al. [7].

Diets were carefully designed for both the control and diabetic women. Total caloric intake was calculated according to weight. The protein content was maintained at 125 g per day and the carbohydrate was divided equally among the three major meals. Intervals between onset of meals were kept constant at 5 h except for a 9–h span between the bedtime snack and breakfast. A progressive decrease in the areas under the glucose and insulin curves was noted in the control subjects. This failure to observe diminished carbohydrate tolerance as the day progresses is similar to the results obtained previously by Malherbe et al. in nonpregnant individuals [19].

After institution of an appropriate diet and adjustment of insulin dose, subjects were studied over 24 h on the metabolic ward under conditions designed to resemble their usual daily schedule, as far as possible. Serum glucose patterns for the seven diabetics were similar to those in normals and their mean daily glucose concentration did not differ significantly from that of the controls. Although it should be noted that a number of individual glucose values did fall outside the range established in the nondiabetic subjects, these results indicate the feasibility of achieving tight metabolic control in highly motivated patients with presently available therapy. Furthermore, although one might have anticipated that these patients would have suffered frequent and/or severe hypoglycaemic episodes, only three of the 126 glucose values obtained from the seven diabetic patients were below 50 mg/100 ml. Similar approaches for the assessment of metabolic control in diabetic women were recently described by Persson [20] and Gillmer et al. [21, 22].

The development of a radio-immunoassay for human C-peptide has facilitated the study of B-cell secretory capacity in patients treated with insulin. The high serum CPR level observed in two Group I subjects (BB, LK) was explained in part by high levels of endogenous proinsulin bound to circulating insulin antibodies. Their free C-peptide concentrations, obtained after correcting for this interfering effect, were comparable to the normal values. This finding thus raises the question as to why these women developed carbohydrate intolerance in the face of adequate endogenous insulin secretion. Amongst the possibilities that must be considered are increased peripheral resistance to the biological action of insulin, delayed secretion of the hormone in the initial minutes after ingesting food, or that the correlation of serum C-peptide and insulin differ in these diabetics compared to control pregnant women. These findings regarding C-peptide modulation in normals and Group I subjects in response to meals contrasted with long-term insulin-requiring, ketosis-prone diabetics, Group II subjects, whose C-peptide was unresponsive to meals and was uniformly low during the metabolic ward study. This discrimination between types of diabetes is similar to the recent findings of Heding and Rasmussen [23].

Both endogenous and exogenous insulin contributed to the changes in circulating free insulin concentrations in Group I subjects who had significant B-cell secretory function, as determined by the C-peptide assay. On the other hand, in Group II subjects serum-free insulin was derived exclusively from exogenous insulin. The serum-free insulin pattern in both diabetic groups resembled that observed in normal subjects, in that increased levels occurred following the main meals. These findings, taken in conjunction with the normal plasma glucose excursions, suggest the appropriateness of the manner in which insulin was administered. However, the mean daily free insulin concentration in these well-controlled diabetics was approximately 70% higher than in controls, and the peak level after meals tended to be delayed. The need for higher insulin levels to effect normal blood sugars in these patients might reflect the unphysiological route of insulin administration or increased insulin resistance. Recent studies by Liljenquist et al [24] have indicated that glucagon-stimulated hepatic glucose production is restrained by insulin. However, as fasting portal vein insulin levels are approximately 2- to 3-fold higher than that in a peripheral vein [25], it may be necessary for insulin-requiring diabetics to have higher than normal systemic concentrations of insulin to ensure that the liver is exposed to insulin concentrations comparable to those in the portal vein of normal subjects

Total insulin, which is the sum of free and antibody-bound insulin, did not vary in parallel with

serum-free insulin or glucose concentrations. In the two Group I diabetics with circulating insulin antibodies, total insulin levels fluctuated during the day. However, in the Group II diabetics with relatively low concentrations of total insulin, serum total insulin levels did not change significantly from hour to hour, even though large doses of insulin were administered at two specific times of the day. Similar observations have been made recently by Rasmussen et al. [26]. Furthermore, it is interesting to note that all diabetics demonstrated daily free insulin levels within a narrow range despite the wide variation in administered insulin doses and their serum total insulin concentrations. Obviously, additional studies will be necessary to elucidate the interplay of factors which determine the serum-free insulin concentration and its relationship to antibody-bound insulin.

The infants of these diabetic pregnancies provided an in vivo assessment of the effect of normalization of maternal serum glucose levels. All deliveries occurred vaginally except for two Caesarean sections, which were indicated obstetrically. Infant birth weight, and 1- and 5-min Apgar scores of the diabetic mothers did not differ from those observed in the nondiabetic pregnancies. It is tempting, therefore, to attribute the success of these diabetic pregnancies to the maintenance of relatively normal maternal blood glucose levels.

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References

1. White, P.: Pregnancy and diabetes. In: Joslin's diabetes mellitus (eds. A. Marble, P. White, R. F. Bradley, L. P. Krall), pp. 581–598. Philadelphia: Lea & Febiger 1971
2. Delaney, J. J., Ptacek, J.: Three decades of experience with diabetic pregnancies. Amer. J. Obstet. Gynec. **106**, 550–556 (1970)
3. Karlsson, K., Kjellmer, I.: The outcome of diabetic pregnancies in relation to the mother's blood sugar level. Amer. J. Obstet. Gynec. **112**, 213–220 (1972)
4. Essex, N. L., Pyke, D. A., Watkins, P. J., Brudenell, J. M., Gamsu, H. R.: Diabetic pregnancy. Brit. med. J. **1973 IV**, 89–93
5. Yssing, M.: Long-term prognosis of children born to mothers diabetic when pregnant. In: Early diabetes in early life (eds. R. A. Camerini-Davalos, H. S. Cole), pp. 575–586. New York: Academic Press Inc. 1975
6. Molnar, G. D., Taylor, W. F., Langworthy, A. L.: Plasma immunoreactive insulin patterns in insulin-treated diabetics. Studies during continuous blood glucose monitoring. Mayo Clin. Proc. **47**, 709–719 (1972)
7. Oakley, W., Hill, D., Oakley, N.: Combined use of regular and crystalline protamine (NPH) insulins in the treatment of severe diabetes. Diabetes **15**, 219–222 (1966)
8. Persson, B.: Assessment of metabolic control in diabetic pregnancy. In: Size at birth. American Elsevier Publishing Company, Inc. Ciba Foundation Symposium **27**, 247–267 (1974)
9. Hoffman, W. S.: A rapid photoelectric method for determination of glucose in blood and urine. J. biol. Chem. **120**, 51–55 (1937)
10. Block, M. B., Mako, M. E., Steiner, D. F., Rubenstein, A. H.: Circulating C-peptide immunoreactivity. Studies in normals and diabetic patients. Diabetes **21**, 1013–1026 (1972)
11. Morgan, C. R., Lazarow, A.: Immunoassay of insulin, two antibody systems. Diabetes **12**, 115–126 (1963)
12. Nakagawa, S., Nakayama, H., Sasaki, T., Yoshino, K., Yu, Y. Y., Shinozaki, K., Aoki, S., Mashimo, K.: A simple method for the determination of serum free insulin levels in insulin treated patients. Diabetes **22**, 590–600 (1973)
13. Fink, G., Cresto, J. C., Gutman, R. A., Lavine, R. L., Rubenstein, A. H., Recant, L.: Plasma proinsulin-like materials in insulin treated diabetics. Horm. Metab. Res. **6**, 439–443 (1974)
14. Snedecor, G. W.: Statistical methods. 5th ed., p. 534. Ames: Iowa State College Press 1956
15. Kalkhoff, R., Schalch, D. S., Walker, J. L., Beck, P., Kipnis, D. M., Daughaday, W. H.: Diabetogenic factors associated with pregnancy. Trans. Ass. Amer. Phycns **27**, 270–280 (1964)
16. Freinkel, N.: The effect of pregnancy on insulin homeostasis. Diabetes **13**, 260–267 (1964)
17. Spellacy, W. N., Goetz, F., Greenberg, B. Z., Ells, J.: Plasma insulin in normal "mid" pregnancy. Amer. J. Obstet. Gynec. **92**, 11–15 (1965)
18. Kalkhoff, R. K., Richardson, B. L., Stoddard, F. J.: Defective plasma insulin response during prednisolone glucose tolerance tests in subclinical diabetic mothers of heavy infants. Diabetes **17**, 37–47 (1968)
19. Malherbe, C., de Gasparo, M., de Hertogh, R., Hoet, J. J.: Circadian variations of blood sugar and plasma insulin levels in man. Diabetologia **5**, 397–404 (1969)
20. Persson, B.: Treatment of diabetic pregnancy. Israel J. med. Sci. **11**, 609–616 (1975)
21. Gillmer, M. D. G., Oakley, N. W., Brooke, F. M., Beard, R. W.: Metabolic profiles in pregnancy. Israel J. med. Sci. **11**, 601–608 (1975)
22. Gillmer, M. D. G., Beard, R. W., Brooke, F. M., Oakley, N. W.: Carbohydrate metabolism in pregnancy. Brit. med. J. **1975 III**, 399–402
23. Heding, L. G., Rasmussen, S. M.: Human C-peptide in normal and diabetic subjects. Diabetologia **11**, 201–206 (1975)
24. Liljenquist, J. E., Bomboy, J. D., Lewis, S. B., Sinclair-Smith, B. C., Felts, P. W., Lacy, W. W., Crofford, O. B., Liddle, G. W.: Effect of glucagon on net splanchnic cyclic AMP production in normal and diabetic men. J. clin. Invest. **53**, 198–204 (1974)
25. Blackard, W. G., Nelson, N. C.: Portal and peripheral vein immunoreactive insulin concentrations before and after glucose infusion. Diabetes **19**, 302–306 (1970)
26. Rasmussen, S. M., Heding, L. G., Parbst, E., Velung, Aa.: Serum IRI in insulin-treated diabetics during a 24-hour period. Diabetologia **11**, 151–158 (1975)

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