

Insulin Resistance Caused by Massive Degradation of Subcutaneous Insulin

ELSA P. PAULSEN, JOHN W. COURTNEY, III, AND WILLIAM C. DUCKWORTH

SUMMARY

Severe resistance to subcutaneous insulin but sensitivity to intravenous insulin persisted for 15 months in a 17-year-old diabetic girl. Heat-labile insulin-degrading activity was present in the patient's ketotic sera and in the 100,000 g fraction (soluble fraction) of adipose tissue. Serum-degrading activity was not inhibited by N-ethylmaleimide. The soluble fraction also degraded glucagon and B chain but not growth hormone or myoglobin. It was inhibited by incubation with the patient's nonketotic sera, normal sera, or Trasylol. Glutathione-insulin-transhydrogenase (GIT) activity was 66% of normal.

The biopsy of adipose tissue at remission showed a normal level of insulin- and glucagon-degrading activity. The activity was eluted from Sephadex G200 as a single peak and had properties consistent with those of the insulin-specific protease (ISP).

The increased degrading activity present during insulin resistance had properties not shared with ISP, suggesting the presence of an uncharacterized protease. DIABETES 28:640-645, July 1979.

In 1975, Schneider and Bennett¹ reported the occurrence of severe resistance to subcutaneous insulin in a 16-year-old diabetic girl who had normal sensitivity to intravenous insulin. There was no increase in the circulating antibodies to insulin or to insulin receptors. Strict control of glycemia and freedom from ketosis led to a return to normal requirements of subcutaneous insulin. Four such episodes of resistance occurred for two years before she had a complete remission. There was no explanation for

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From the Department of Pediatrics, University of Virginia School of Medicine, University of Virginia Hospital Pharmacy, Charlottesville 22908; and from the Laboratories of Endocrinology and of Metabolism, Research Service, Veterans' Administration Hospital and the Departments of Medicine and of Biochemistry, University of Tennessee Medical Units, Memphis 38104.

Address reprint requests to Elsa P. Paulsen, M.D., in Charlottesville.

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the impaired absorption of insulin from the injection site into the bloodstream.

We reported a second such case in a 17-year-old diabetic girl in whom marked resistance to subcutaneous insulin followed a severe perineal infection and persisted for about 15 months.² The use of a constant infusion system (Alza Liquid Infusion System and the Vicra Silastic Catheter) enabled the patient to live at home and to pursue normal activities.

Two further instances of marked resistance to subcutaneous insulin but normal sensitivity to intravenous insulin were recently reported by Dandona et al.³ in a 14-year-old diabetic patient and by Henry et al.⁴ in an 18-year-old student nurse.

We now present details of the studies in our patient.

CLINICAL DATA

N.G. is a 20-year-old white woman who has had diabetes since age 11; the disease was readily controlled until age 17.

At that time she was hospitalized with a perineal abscess. At surgery the abscess was found to drain anteriorly into the base of the right labia majora. It did not communicate posteriorly or with the rectum or the vagina. There was considerable gray necrotic tissue in the labia and throughout the perineum. The abscess followed the round ligament and necrotic tissue, and pus was present just below the right inguinal canal. The areas were thoroughly debrided, irrigated, and packed with iodoform gauze. The patient was placed on penicillin. The predominant organism was *Lactobacillus* spp. There was moderate growth of *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and gram-negative rods. Some *Candida albicans* and a few atypical gram-positive cocci were also found. Wound healing progressed satisfactorily, and she was discharged to take 34 U Lente insulin after 18 days of hospitalization.

At each weekly visit she had clinical and chemical evidence of poor diabetic control, and daily increases in insulin dose were made, reaching 110 U Lente daily in two weeks.

In the next two months she was hospitalized six times for ketoacidosis, with insulin requirements by the subcutaneous route rising to 1700 to 2000 U/24 hours. At this time a continuous intravenous infusion of 225 U insulin/24 hours was used to maintain good control of blood sugar concentrations. Over the next month, subcutaneous insulin requirements ranged from 3 to 5000 U/24 hours. These were administered as aliquots of a U-5000 regular pork insulin preparation every two to three hours from 8 a.m. through 10 p.m.

Antibodies to pork and beef insulin at this time, as well as one year later, were in the usual range for diabetic patients (pork, 3.4 and 4.0; beef, 2.4 and 1.4 mU/ml serum). An empiric trial of steroids merely increased the hyperglycemia and needs for the insulin. No circulating inhibitors of insulin-receptor binding were demonstrable. The patient had no clinical signs of endocrinopathies that were a basis for her insulin resistance.

Under medical supervision at a summer health camp, she was taught to give herself insulin intravenously as boluses before meals and in the midafternoon. She was fairly well controlled on 275 to 300 U/24 hours for six weeks.

After summer camp, she returned home and continued to administer boluses of insulin intravenously. In a six-week period, however, she was hospitalized eight times because of severe acidosis. As a result of the suddenness in onset of these episodes and the failure to maintain adequate control between them, she was rehospitalized. On admission she had a recurrence of the perineal abscess, which responded promptly to cephalothin. Her liver and spleen were markedly enlarged and liver enzymes were elevated. Alpha₂, beta, and gamma globulins were elevated; albumin and alpha, antitrypsin were normal. At this time 5000 U insulin given subcutaneously did not raise her serum insulin concentrations or lower her blood glucose concentrations (Figure 1). Over the next three months she received a continuous infusion of insulin in the hospital. She was well regulated on 5 U/hour from 7 a.m. to 9 p.m. and 0.75 to 1.0 U/hour from 9 p.m. to 7 a.m., or, a total daily dose of about 80 U/24 hours. Her liver and spleen size and liver enzymes gradually returned to normal.

At this time the Alza Liquid Infusion System (LIS) became available to us (Figure 2). The elastomere balloons of the removable cartridges were filled in the hospital pharmacy under sterile conditions with insulin solutions to meet daytime and nighttime needs and were stored at 4 °C. All solutions contained 200 U heparin/ml, and flow rates ranged from 0.75 to 1.0 ml/hour. The solutions were administered through a Vicra Silastic Catheter inserted into a vein in the upper arm. The patient learned to change the cartridges and to dress the site of the insertion of the catheter daily, thus enabling her to return home. A single catheter was maintained in place for 176 days with no complications. Her insulin requirements during this period were quite stable: daytime (9 a.m. to 9 p.m.), 6.4 U/hour; nighttime (9 p.m. to 9 a.m.), 0.52 U/hour or 83 U/24 hours. During this time the patient was hospitalized once. On this occasion the LIS malfunctioned during the patient's sleeping hours. By 8 a.m. the patient was ketoacidotic and was admitted with CO₂ of 5 meq/l. Two weeks later, 14 months after the first abscess, she had a third and final perineal abscess in the same area as previously. The predominant organisms were Staphylo-

CONSTANT INFUSION AND TRIAL OF SUBQ.INSULIN
(N. G. SEPT. 1975)

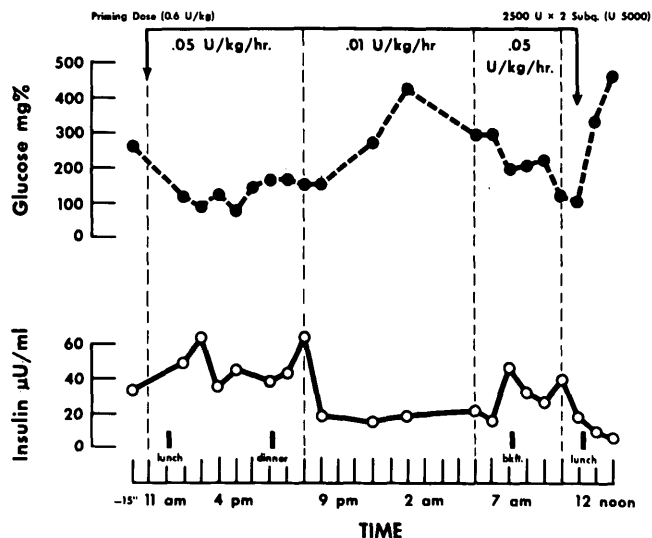


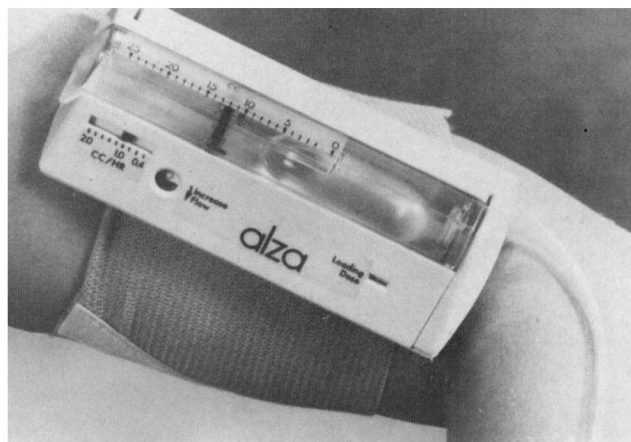
FIGURE 1. Failure of patient's plasma glucose concentration to fall or plasma insulin to rise after 5000 U of subcutaneous insulin when her insulin resistance was its highest.

coccus epidermidis, group-D Streptococcus, and alpha Streptococcus. The infection responded promptly to ampicillin and sitz baths.

Four weeks later the patient was brought in for periodic evaluation to determine whether she were still resistant to subcutaneous insulin. A dose of 100 U of regular insulin effectively lowered her blood glucose (Figure 3). She was then regulated on prebreakfast and presupper subcutaneous insulin beginning at 80 U Lente plus 35 U regular and 50 U Lente plus 35 U regular. Over the ensuing year and a half the requirement fell to the present dose of 30 U Lente plus 15 U regular before breakfast and 15 U Lente plus 10 U regular before dinner. She still becomes ketoacidotic easily, however, and recently required admission 12 hours after she omitted the evening Lente dose.

She was amenorrheal since the onset of her insulin resistance. Her plasma FSH, LH, and prolactin concentrations are within normal limits. She responds only minimally to estrogen-progesterone treatment. She has recurrent

FIGURE 2. Alza Liquid Infusion System. The expanded elastomere balloon contains insulin in a heparin solution.



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RECOVERY OF RESPONSE TO SUBQ INSULIN

(N.G. JUNE 9, 1976)

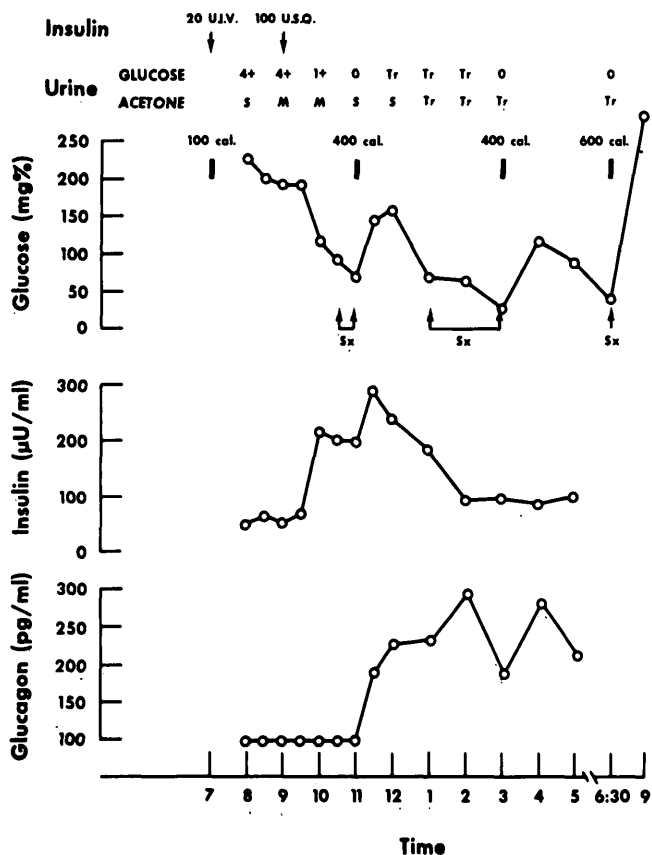


FIGURE 3. Effective absorption of 100 U insulin administered subcutaneously, reflected in a rise in plasma insulin and a fall in plasma glucose. Note normal plasma glucagon response to insulin-induced hypoglycemia. Induction of hypoglycemia is a warning to test patient for remission of insulin resistance with small amount of insulin.

monial vaginitis and was totally anergic to a delayed hypersensitivity skin-test panel of candida, trichophyton, and streptokinase-streptodornase antigens.

During her first hospitalization for the problem of insulin resistance, fasting cholesterol and triglyceride concentrations were normal (140 mg/dl and 124 mg/dl, respectively) after the ketoacidosis was corrected. Fasting glucagon concentrations were suppressed in the presence of hyperglycemia (55 pg/ml with a glucose of 509 mg/dl). At the time of her recovery, she was able to suppress glucagon secretion when serum glucose values ranged from 200 down to 64 mg/dl (Figure 2) and she responded appropriately to hypoglycemia by stimulating glucagon secretion.

MATERIALS AND METHODS

Materials. Porcine insulin, A and B chains, was a gift of Doctor Mary Root of Eli Lilly and Co. Proinsulin was provided by Doctor Donald A. Steiner and human growth hormone by the National Pituitary Agency. Myoglobin was obtained from Sigma. These substances were radiolabeled with ¹²⁵I by the method of Hunter and Greenwood.⁵ Specific activities ranged between 150 and 250 µC/µg.

Specimens. Nonhemolyzed sera were obtained from the

patient during ketoacidosis and after its correction and from age-matched nondiabetic and nonketotic diabetic subjects.

A biopsy of adipose tissue was taken both during the time of marked insulin resistance and at the onset of its remission. In both instances, insulin was withheld for 24 hours until the biopsy was completed. Both samples were taken from the abdominal area. Control adipose tissue was obtained from a nondiabetic woman undergoing an appendectomy. Informed consents were obtained.

Incubation studies. Adipose tissue was homogenized in 0.25 sucrose in a Potter-Elvehjem Teflon-glass homogenizer at room temperature. The homogenate was centrifuged at 4 °C at 10,000 g for 20 minutes. The fat layer and pellet were removed and each was resuspended in sucrose. The soluble extract was centrifuged at 100,000 g for 50 minutes. The 100,000 g pellet was resuspended in sucrose.

Sera, whole homogenates of adipose tissue, and adipose tissue extracts were incubated with labeled proteins to measure degrading activity.

Sera in triplicate aliquots of 0.25 ml were incubated in a 1:1 mixture with 0.25 ml Krebs-Ringer buffer at pH 7.4 in 1% human serum albumin containing 3 to 5000 cpm of labeled hormone. After two hours at 37° the tubes were plunged into ice and 0.5 ml 10% TCA was added. The tubes were vortexed and spun at 4 °C at 5000 rpm for five minutes. Radioactivity was counted in the pellets and the supernates. The percent of hormone degraded was calculated from the increase in radioactivity in the supernate above control tubes incubated with human serum albumin.

Patient's sera (0.25 ml) showing insulin-degrading activity was incubated in a 1:1 mixture with either Trasylol (10,000 U/ml) in 1% human serum albumin, the patient's ketotic or nonketotic sera, or nondiabetic sera and was incubated with 1% human serum albumin (1:1) at 50 °C for 30 minutes to determine heat lability of the degradative activity.

The resuspended pellets and fat layer and the 100,000 g supernate from the adipose tissue biopsies were assayed for insulin- and glucagon-degrading activity by the production of TCA-soluble products from ¹²⁵I-labeled hormones. Briefly, the assay consisted of incubating the test material, labeled and unlabeled hormones, and 0.5% bovine serum albumin in 0.05 M Tris HCl at pH 7.0 in a total volume of 1 ml at 37 °C in a Dubnoff shaker for two hours. The reaction was terminated by the addition of 1 ml 10% TCA and the mixture was centrifuged at 4 °C. Radioactivity in the supernate and

TABLE 1
Insulin-degrading activity of patient's sera

	Net degradation of I-125-insulin above controls* (%/ml/h)
Nonketotic sera	2.8
Ketotic sera plus	9.4
Heat (50 °C, 15 min)	0
Normal serum (1:1)	4.5
n-Ethylmaleimide 15 mM (1:1)	9.3
Tryptophan 15 mM (1:1)	9.7
B Chain (5 µg)	8.5

* Control subjects were nondiabetic and diabetic individuals, the latter of whom were in good control (N = 6). None of these sera contained degrading activity.

TABLE 2
Insulin- and glucagon-degrading activities in adipose tissue extract*

	Insulin-degrading activity (%/mg protein/min)	Glucagon-degrading activity (%/mg protein/min)
First biopsy	1.93	2.27
Second biopsy	0.31	0.35
Control (N = 7)	0.38 ± 0.11	0.40 ± 0.16

* 100,000 g supernate (no activity in the pellet).

pellet was measured and the percent of degraded hormone calculated as noted above.

The ¹²⁵I-labeled proteins incubated with the 100,000 g supernate of adipose tissue extract were porcine insulin, proinsulin, A chain, B chain, human growth hormone, and myoglobin. Aliquots of the TCA supernate containing degradation products of ¹²⁵I-insulin were preserved for gel filtration studies.

Analysis of the first biopsy of adipose tissue for glutathione-insulin-transhydrogenase (GIT) activity was kindly carried out by Dr. P. T. Varadani.

Gel filtration studies. The 100,000 g supernate of the adipose tissue extract from the second biopsy was dialyzed against 0.02 M acetate buffer, pH 6.2, with 0.1 mM dithiothreitol and applied to a Sephadex G-200 column measuring 0.9 × 58 cm. The fractions were assayed for insulin- and glucagon-degrading activity using low substrate concentrations (1 × 10⁻¹⁰ M) and for GIT activity using insulin concentrations of 2.2 × 10⁻⁵ M with 1 mM glutathione.

Degradation products of ¹²⁵I-insulin incubated with the 100,000 g supernate of adipose tissue extract were separated on a Sephadex G-75 column calibrated with labeled B chain* of insulin, utilizing 50% acetic acid to equilibrate and elute the column.⁶

Protein content of adipose tissue extracts was determined by the method of Lowry et al.⁷ using human serum albumin as the standard.

Plasma concentrations of free insulin were measured utilizing an antiserum bound to Sephadex (Pharmacia), obviating interference in the radioimmunoassay of antibodies to exogenous insulin.

RESULTS

The patient's serum contained degrading activity in the non-ketotic state, but the levels did not attain significance. Significant levels were present in the ketotic state, however (Table 1). The degrading activity in ketotic sera was abolished by heat and reduced by incubation with sera from a normal subject or an insulin-dependent diabetic subject in good control. It was unaffected by 15 mM n-ethylmaleimide, 15 mM tryptophan, or 5 μg B chain of insulin.

The insulin-degrading activity of the soluble fraction (100,000 g) of the first adipose tissue biopsy, taken during marked resistance, was 1.93%/mg protein/min—the normal value is 0.38 ± 0.11%/mg protein/min (n = 7); glucagon-degrading activity was 2.27%/mg protein/min—the normal

value is 0.40 ± 0.16%/mg protein/min (Table 2). The soluble fraction degraded B chain to the same degree as insulin but did not degrade proinsulin, human growth hormone, or myoglobin (Table 3).

Insulin-degrading activity of the soluble fraction of adipose tissue extract from the first biopsy was abolished by heat (50 °C for 30 min) or by incubating with the patient's nonketotic serum or with nondiabetic serum. It was reduced by the patient's ketotic serum to 40% and by Trasylol to 22% of the original activity (Table 3).

GIT activity was 33% below normal in the adipose tissue from the first biopsy (10.2 U/mg protein vs. the control value of 15.1 U/mg protein).

Separation of radioactive fragments after incubation of ¹²⁵I-insulin with either whole homogenate of the first biopsy or its soluble fraction showed that 64 to 83% of the TCA-soluble radioactivity chromatographed with monoiodotyrosine for both patient and control adipose tissue samples.

The insulin- and glucagon-degrading activities of the soluble fraction of the second adipose tissue biopsy, taken at the onset of remission, were normal (0.31% and 0.45%/mg protein/min, respectively). The activities eluted as a single peak on G-200 Sephadex in a manner identical to those in the soluble fraction of control tissue.⁸ The insulin-degrading activity of the peak was 3.42%/mg protein/min but only 0.15% for the original homogenate of the patient's tissue; for the control it was 2.17%/mg protein/min, which compared with homogenate activity of 0.13%. The enzymes that eluted from the patient's and the control's soluble fraction were inhibited by sulfhydryl blockers and by ACTH, were stimulated slightly by glutathione and EDTA, and were unaffected by the trypsin inhibitor tosylamide-2-phenylethyl-chloromethyl ketone (TPCK), which are properties consistent with those described for insulin-glucagon protease in rat skeletal muscle.⁹ No GIT activity was detectable in the soluble fraction.

DISCUSSION

Insulin resistance in the patient was not accounted for on the basis of excessive, circulating antibodies or insulin

TABLE 3
Protein-degrading activity of patient's adipose tissue (first biopsy; soluble fraction)

	Degradation† compared with patient's soluble fraction (%)
100,000 g-soluble fraction incubated with ¹²⁵ I-insulin plus	
Buffer* (1:1)	100
Patient's nonketotic serum (1:1)	0
Patient's ketotic serum (1:1)	40
Nondiabetic serum (1:1)	0
Trasylol (7500 U/ml) (1:1)	22
100,000 g-soluble fraction incubated with ¹²⁵ I-proteins	
Proinsulin	0
B Chain	91
Human growth hormone	0
Myoglobin	0

* Taken as maximal, or 100%, degradation.

† Trichloroacetic acid-soluble fragments.

* ¹²⁵I-labeled A chain was completely precipitated in 5% trichloroacetic acid, suggesting that polymerization had occurred, which rendered it unacceptable for study.

receptor antibodies. Yet 5000 U of subcutaneous insulin produced little change in her plasma insulin or glucose concentrations. She responded well, however, to intravenous insulin, suggesting that the subcutaneous insulin was being destroyed by the tissue before it reached the bloodstream.

Analysis of adipose tissue during the period of insulin resistance revealed a marked increase in insulin-degrading activity that was sixfold greater than normal. The patient's ketotic sera also contained varying levels of degrading activity, whereas none was detectable in the normal sera. A second biopsy, taken after several months of continuous intravenous insulin treatment, fortuitously coincided with her remission. Analysis of her adipose tissue at this time revealed normal levels of insulin-degrading activity, thus strongly suggesting a causal relationship between her resistance to insulin and the increased degrading activity.

Insulin is degraded primarily in the liver, but nearly all peripheral tissues contain insulin-degrading enzymes. In 1966, Rudman et al.¹⁰ demonstrated the presence of an enzyme system in adipose tissue of the rat, hamster, guinea pig, and rabbit that cleaved bovine insulin into TCA-soluble fragments. Kitabchi and Stentz¹¹ demonstrated an insulin-degrading activity in the 100,000 g supernate of fat tissue homogenates in rats but the levels were low in comparison with all other tissues examined (liver, pancreas, kidney, spleen, testis, ovary, lung, heart, muscle, and brain). Varandani¹² demonstrated the presence of GIT in rat adipose tissue, as well as kidney, heart, and skeletal muscle. The enzyme catalyzes inactivation of insulin by cleaving its disulfide bonds. Varandani postulates that this is the first step in insulin degradation, which is then followed by proteolytic degradation of the A and B chains. Other workers have not reported any studies of insulin-degrading enzyme systems in human adipose tissue.

Duckworth et al.⁹ isolated an insulin-specific protease (ISP) from rat muscle that rapidly degrades insulin without requiring initial disulfide cleavage. Glucagon but not proinsulin, A or B chains, is also proteolytically degraded by ISP. N-ethylmaleimide is a protein inhibitor of the enzyme.

In the present study insulin-degrading activity from the patient's second biopsy and from the control biopsy were eluted as a single peak on Sephadex G200. Both peaks had properties consistent with those of ISP of rat muscle.

The exact nature of the abnormal degrading activity from the first biopsy is not clear. Unfortunately, sufficient material for isolation and characterization of enzymes was not obtained in the first biopsy, and remission had occurred when the second was obtained. However, the facts that both degrading activity in the patient's serum was not inhibited by n-ethylmaleimide and the first adipose tissue biopsy degraded B chain suggest that the abnormal degrading activity was not a result of increased amounts of ISP.¹³ GIT activity in the patient's first adipose tissue biopsy was significantly decreased below normal and therefore cannot be implicated. Thus, the increased degrading activity would seem to be due to an as yet unidentified enzyme.

The abnormal degrading activity in the patient's adipose tissue and ketotic sera was suppressed by a factor(s) in normal sera or in the patient's nonketotic sera. Burghen et al.¹⁴ reported finding potent competitive inhibitors to rat liver ISP in extracts of human plasma. Subsequently, in-

hibitory activity to rat liver ISP was detected in an alpha₂ globulin preparation (Cohn fraction IV₁) of human serum.¹⁵ The activity separated into four heat-stable components, with molecular weights of 4000 to 7000, that acted by competitive inhibition.

Therapeutic use of a proteolytic inhibitor, Trasylol, was considered when it was found to suppress 80% of the degrading activity of soluble fraction of adipose tissue extract. This was abandoned when the Alza LIS unit became available. However, a recent study of chicks indicates that Trasylol and E-aminocaproic acid protect peptide hormones from local degradation when injected subcutaneously.¹⁶

The appearance of the patient's insulin resistance subsequent to a severe and extensive infection raises the speculation that enzymes of bacterial origin set in motion a destructive process of insulin or enzyme inhibitors and that two recurrences of infection in the same area served to perpetuate it. It is also possible that a continuous, low grade infection was present until the remission of insulin resistance, though it was not clinically evident.

Recently, bioavailability of subcutaneous regular insulin was studied in normal subjects.¹⁷ Nelson et al. found that only 50% of the injected insulin reached the bloodstream; the percentage was even less for NPH insulin. The authors suggested that injected insulin is enzymatically destroyed at the injection site, or concentrations are artificially low as a consequence of nonlinear absorption and elimination kinetics, or both.

Henry et al.⁴ suggested, from their studies of the disappearance of radiolabeled insulin from injection sites in their patients, that gross delay of absorption from repeatedly injected sites is a factor in producing a state of apparent insulin resistance. Unfortunately, such studies do not reveal the physiologic activity of whatever is attached to the absorbed label. That increased destruction of insulin at the site of injection is the primary factor is, actually, suggested by their data. Three to four hours after subcutaneous injection of the labeled insulin, they found that 30% had disappeared. Thus, 750 U of the patient's daily insulin requirement of 2500 U subcutaneously would be absorbed in three or four hours. If physiologically effective, this would certainly lower blood sugar effectively.

Four other young adult females and one young adult male have come to our attention in the past two years who are resistant to subcutaneous but not to intravenous insulin. The incidence, plus the surprising observation that 50% or more of subcutaneously injected insulin is destroyed in situ in normal subjects, makes the fate of insulin in adipose tissue under normal and pathologic circumstances a compelling area to study further.

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