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The Excretion of Proinsulin and Insulin in Urine

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Summary. Proinsulin-like components (PLC) and insulin have been measured in 24 hr urine samples from 8 healthy subjects. The mean excretion of PLC was 45.8 ng and that of insulin 314 ng; the PLC: insulin ratio was 0.14. Urinary PLC was increased 3.5 fold in a patient with a pancreatic islet cell tumor and the PLC: insulin ratio was 0.35. The urinary PLC: insulin ratio is lower than that of serum, presumably because of the relatively lower urinary clearance of the larger molecular weight PLC.

Key words: Proinsulin, insulin, urine, kidney, insulinoma.

It is well established that proinsulin, the single chain precursor of insulin, is secreted by pancreatic beta cells and circulates in the blood [1, 2]. Its levels in the fasting state and after the oral administration of glucose have been measured in healthy subjects and patients with metabolic and endocrine disorders [3–5]. In the former group, proinsulin comprises 5–20% of the total immunoreactive insulin concentration, being relatively higher in the fasting state and 3 to 5 hrs after glucose [3–5].

Although most studies of insulin metabolism have been directed towards its measurement in blood, a number of recent reports have been concerned with its excretion in urine [6–9]. As the urinary clearance of insulin is relatively constant over a wide range of serum concentrations [10], its level in urine has proved to be a useful indicator of average serum values during extended periods of time. In this study we have measured the levels of both insulin and proinsulin in 24 hr samples of urine in order to compare their relative excretory patterns during this period.

Materials and Methods

Subjects

Eight healthy males aged 23–28 years were studied. All were within 10% of their ideal body weight. In addition, a urine sample was obtained preoperatively from a 44 year old woman with a pancreatic islet cell tumor.

Methods

Urine Extraction. Twenty-four hour samples of urine (1250 to 2420 ml) were collected in siliconized plastic bottles, containing 100 mg bovine serum albumin, and kept at 4° C. Urine proteins were precipitated by the addition of 10% trichloracetic acid. After standing at 4° C for 4 hrs, the solution was centrifuged in the same 250 ml container for 15 min at 1500 g, the supernatant fluid being discarded. The precipitate was suspended in 12 ml water and 30 ml acid-ethanol (375 ml 95% ethanol; 7.5 ml concentrated hydrochloric acid) was added [11]. After standing at 4° C for 4 to 6 hrs the precipitate was removed by centrifugation and the supernatant fluid was adjusted to pH 8.5 with concentrated ammonium hydroxide. The precipitate was again removed and the supernatant adjusted to pH 5.3 with hydrochloric acid, using methyl red as the indicator. After adding 0.025 ml 2N ammonium acetate (pH 5.3) for each milliliter of extract, the proteins were precipitated at 4° C for 16 hrs, with 1.5 ml absolute alcohol and 2.5 ml diethyl ether per ml extract. The precipitate was then centrifuged, dried in vacuo and dissolved in 1.0 ml 3M acetic acid. All glassware used during the extraction was coated with an aqueous solution of 1% silicone (Siliclad, Clay-Adams, New York).

Gel Filtration. Proinsulin and insulin were separated on 1 × 50 cm columns of Bio-Gel P-30 (Bio-Rad Labs, Richmond, Calif.), 100–200 mesh, equilibrated in 3M acetic acid and calibrated with a mixture of ^{125}I-insulin and ^{131}I-proinsulin. The fraction size was 1.2–1.3 ml and the void volume 12–13 ml. The fractions were collected in tubes which had been coated with albumin. After the acetic acid was evaporated to dryness in vacuo, 1.0 ml of the immunoassay buffer was added to each tube. Because this method does not separate proinsulin from the closely related two chain biosynthetic intermediates [12], the

material in the early eluting peak has been called proinsulin-like-components (PLC). Recoveries of proinsulin and insulin after gel filtration were comparable and ranged between 98 and 102%.

Immunoassay. This was a modification of the double antibody method of Morgan and Lazarow [13]. The buffer consisted of 0.1 M Tris (hydroxymethyl aminoethane/hydrochloric acid), 0.05 M sodium chloride and 0.25% bovine serum albumin, pH 7.7. Human insulin and proinsulin [14] standards, porcine insulin antiserum (final dilution 1:320,000) and ^{125}I-porcine insulin (100 mc per mg) were used in the assay. Human proinsulin reacts with this particular antiserum approximately four times less well than insulin, on a weight basis. However, the curves of the two standards were almost parallel.

Trypsin Conversion. After gel filtration, samples from the proinsulin peak of 7 subjects were assayed before and after incubation with trypsin. TCPK trypsin (0.5 μg in 0.1 ml) was added to 0.2 ml buffer containing 0.64 to 1.64 ng proinsulin and incubated at 37° C for 10 min. Then 0.2 ml Trasylol (5000 Kallikrein inactivator units per ml; FBA Pharmaceuticals, New York) and 0.6 ml buffer were added to stop the reaction, and the samples were reassayed. In control experiments, labelled proinsulin was added to urine and allowed to stand at 4° C for 24 hrs. No conversion of the proinsulin to insulin occurred as determined by gel filtration.

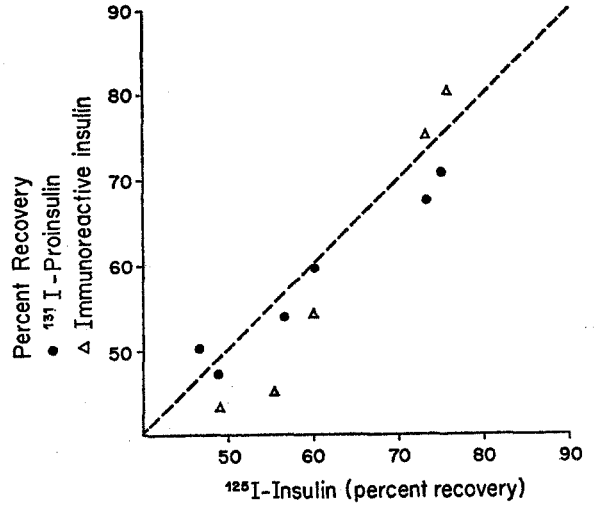

Fig. 1. Recovery of ^{131}I-proinsulin and ^{125}I-insulin after trichloracetic acid precipitation and acid ethanol extraction (●) ($r = 0.9$). In separate experiments the recovery of ^{125}I-insulin and total immunorective insulin was compared (△) ($r = 0.9$)

Results

In order to ensure that the recoveries of insulin and proinsulin were similar, ^{125}I-insulin and ^{131}I-proinsulin were added to 6 urines before extraction. No systematic differences in their recoveries in the alcohol ether precipitate were noted (Fig. 1). In addition, the recoveries of ^{125}I-insulin were compared to the values obtained by direct immunoassay of the urines before and after extraction, and a close correlation was again observed (Fig. 1). In these experiments the immunoreactive insulin concentration was measured using a tracer labelled with ^{131}I.

Immunoassay of the fractions after gel filtration showed two peaks of immunological activity, the early peak corresponding to the elution position of proinsulin and the later to that of insulin (Fig. 2). The immunologic relationship of urine PLC to the standard of human pancreatic proinsulin was determined in two sets of experiments. Firstly, assay of the material from the early peak in several dilutions resulted in values

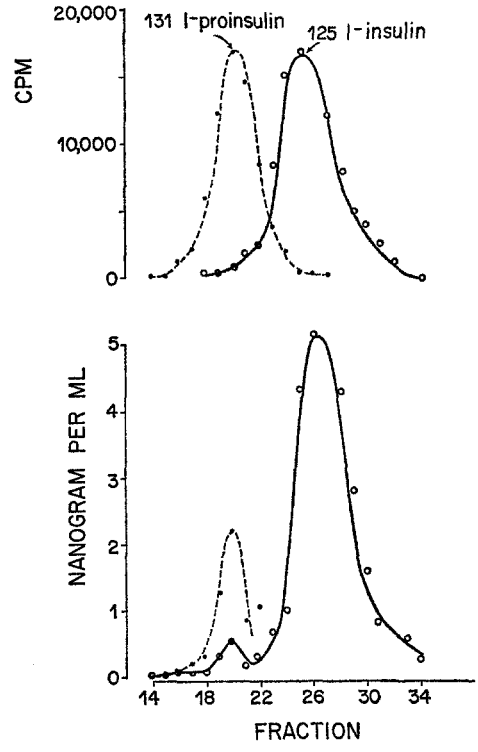

Fig. 2. Gel filtration of an acid-ethanol extract of urine on 1 × 50 cm Bio-Gel column equilibrated in 3M acetic acid. After drying in vacuo, the fractions were assayed and the values read against the human insulin standard (fractions 23 to 34; ○——○) or the human proinsulin standard (fractions 17 to 21; ●——●) (bottom). The column was calibrated with ^{131}I-proinsulin and ^{125}I-insulin (top)

which lay parallel to the human proinsulin standard curve. Secondly, because PLC is underestimated in terms of the insulin standard in our assay system (Fig. 3), its conversion to desthreonine insulin by trypsin

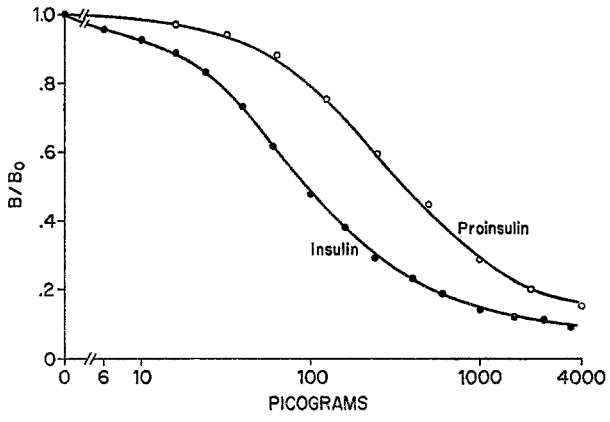

Fig. 3. Standard curves of human proinsulin and insulin. The insulin antibody dilution was 1:320,000 and the total radioactivity (^{125}I-insulin) in each tube was 36,000 CPM (0.4 ng insulin). The ordinate represents the counts in the precipitate at each concentration of the peptide (B) divided by the precipitate counts in the zero dose tubes (B_0)

should theoretically result in an increased insulin value when read against this standard (Fig. 3). In fact, an increase in immunological activity was observed in all 7 samples after exposure to trypsin for a short time (mean control value 1.1 ng and mean post-trypsin value 1.9 ng). These results support the conclusion that the early eluting material in urine is proinsulin or the closely related intermediate or partially cleaved forms [13], and we have thus determined its concentration from the human proinsulin standard.

Insulin and PLC levels in 24 hr urine samples are shown in Table 1. The values have been corrected for the percentage recovery (46 ± 7), which was calculated by comparing the total immunological activity in all the column fractions (in terms of the insulin standard) with that obtained by direct assay of the unextracted urine. In order to correct for variations in body size, the values have also been expressed as ng/kg/hr and ng/mg creatinine. The ratio of PLC to insulin was determined from the absolute values of each protein in their respective peaks following gel filtration.

Discussion

Previous studies have shown that insulin is stable in urine provided that it is kept at 4° C and maintained at a pH between 6 and 8 [6, 7, 9, 10]. Under these conditions exogenous insulin added to urine is quantitatively recovered and conversion of proinsulin to insulin does not occur [2]. Although we have concentrated urines by dialysis and flash evaporation, the resulting samples were often unsuitable for gel filtration and recoveries were low. Albumin was added to urine to prevent absorption of insulin and PLC to the container and to enhance their precipitability with trichloracetic acid.

Table 1. *Mean and standard deviation of total immunoreactive insulin (IRI), insulin and proinsulin as determined in column fractions after gel filtration, and the proinsulin: insulin ratio (× 100) in urine samples from 8 healthy subjects. The results in a patient with an islet cell tumor are also shown*

		Insulin			Proinsulin			
	Total "I.R.I." (ng/24 hrs)	Total (ng/24 hrs)	ng/kg/hr	ng/mg creat.	Total (ng/24 hrs)	ng/kg/hr	ng/mg creat.	Proinsulin / Insulin (%)
	242	231	0.163	0.179	41.9	0.030	0.032	18.1
	135	133	0.078	0.085	8.9	0.005	0.006	6.7
	223	220	0.103	0.112	10.8	0.005	0.006	4.9
	360	346	0.192	0.172	49.4	0.028	0.024	14.3
	259	252	0.154	0.366	44.8	0.028	0.065	17.8
	510	494	0.348	0.343	106.8	0.075	0.074	21.5
	548	528	0.158	0.416	71.4	0.038	0.056	13.5
	318	306	0.175	0.243	39.1	0.022	0.031	12.7
Mean	324	314	0.172	0.239	45.8	0.029	0.037	13.7
S. D.	143	137	0.081	0.123	31.6	0.012	0.017	5.7
Insulinoma	504	468	0.279	0.298	164.2	0.119	0.104	35.1

Although the double antibody immunoassay has been used to measure total immunoreactive "insulin" directly in unextracted urine samples, the cross reactivity of PLC and insulin with insulin antisera neces-

sitates a preliminary separation procedure. The present results demonstrate that urinary insulin and PLC can be concentrated by trichloracetic acid precipitation and acid ethanol extraction and that similar recoveries of each are achieved. Bio-Gel P-30 columns were used for gel filtration because the resolution achieved was, in general, superior to that obtained previously with Sephadex G-50.

Details of the purification and characterization of human pancreatic proinsulin used as the immunoassay standard have been described [14]. The early eluting material from urine showed immunological identity with this standard when measured in several dilutions. Furthermore, incubation with trypsin resulted in the expected increase in insulin values when read against the insulin standard. These findings are similar in every respect to the results obtained with serum [3–5] and are compatible with the conclusion that urinary PLC is derived from the circulation.

Urinary insulin has been measured in children [15], adults [6, 16] and infants [9, 16], and in patients with renal failure and diabetes mellitus [7]. Because the insulin clearance does not vary with the serum insulin level, urinary insulin represents an integrated measurement of the serum concentration over a period of time [10]. Experiments in dogs [17] and rats [18] have indicated that the clearance of proinsulin is similarly constant over a wide range of serum levels and that urinary proinsulin represents a constant fraction of the filtered load. The twenty-four hour excretion of these hormones is thus a useful indicator of the prevailing mean daily serum level.

The values for total urine insulin, as well as its correction for body size and creatinine excretion, are similar to previously reported levels. The 24-hour excretion of PLC in healthy subjects was 45.8 ng, and the ratio of proinsulin to insulin was 0.137. In contrast, mean serum ratios are higher both in the fasting state, which accounted for almost half the 24-hour collection period, and at 60 and 180 min after oral administration of glucose, which may be representative of the absorptive state. For example, the mean serum ratio of proinsulin to insulin in 66 fasting healthy subjects, measured by similar methods in our laboratory, is 0.62. This difference is probably accounted for by the lower urinary clearance of proinsulin compared to insulin, the values of the former being at least 2 to 3 times lower than that of insulin in dogs [17] and rats [18]. Based on the lower urinary clearance (0.043 ml/min) of β_2-microglobulin (MW 11,600) [19] compared to insulin (0.42 ml/min; MW 5,900), it is not surprising that proinsulin with a molecular weight of approximately 9,000 is also cleared more slowly than insulin.

Urinary PLC and the PLC: insulin ratio were markedly elevated in the patient with an islet cell tumor, being 3–4 times higher than the mean values in healthy subjects. The basal IRI varied between 114 and 372 μU/ml and PLC comprised 60–80 per cent of the fasting serum "insulin" immunoreactivity in this patient [20]. Others have also reported high serum concentrations of proinsulin in patients with insulinomas [21–24], and this finding has proved to be a useful adjunct in the diagnosis of this condition. Further studies are now needed to determine the urinary levels of PLC in diabetic patients and in other disorders of carbohydrate metabolism.

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