Insulin-like Insulinase-resistant Material, Distinguishable from Normal Insulin, in Juvenile Diabetes

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SUMMARY

This study compares some properties of the immunoreactive insulin-like material extracted from the urine of children with overt diabetes with that from normal children. Insulin-like species were fractionated by gel filtration and by isoelectric focusing and were tested for sensitivity to an insulin-specific degradative enzyme. Insulin concentration was measured by radioimmunoassay. The major insulin-like component from the urine of ten normal children and fifteen untreated juvenile diabetics and from the urine of four and the serum of one latent diabetics behaved (on gel filtration) as normal insulin, was sensitive to insulinase, and (in all cases studied) had an identical isoelectric point (resolution 0.1 pH units).

A proportion of the immunoreactivity extracted from urine (0-4 per cent from normal children, 5-30 per cent from twelve of the thirteen nonobese untreated diabetic children) eluted from the gel filtration column before insulin. This material from diabetic urine was of two size classes, "proinsulin-like" and "mid-insulin," both resistant to degradation by insulinase. Insulinase-resistant immunoreactivity from one patient was analyzed by isoelectric focusing. Urine samples from two obese children with overt diabetes and four children with latent diabetes contained normal proportions (less than 4 per cent) of immunoreactive species larger than insulin. The possible nature and significance of the present insulinase-resistant species are briefly considered. DIABETES *24* :609-17, July, 1975.

In 1965, Elliott, O'Brien, and Roy[1] reported that insulin immunoreactivity extracted from the serum of untreated juvenile diabetics was less degraded by insulinase than was a similar extract from normal serum. They postulated that there might be a genetically determined structural difference in the insulin of diabetic children that could contribute importantly to the underlying biochemical disorders of the disease. After the discovery of the existence of proinsulin[2] and the detection of proinsulin-like species in human serum,[3] it was considered likely that the abnormal insulin-like material detected by Elliott might be proinsulin.[3] Indeed, more careful gel filtration of the insulin-like immunoreactivity (ILI)* to partially resolve insulin and proinsulin yielded a proinsulin-like species from pancreatic extracts of maturity-onset diabetics that was resistant to degradation by insulinase.[5] However, the partial resistance to degradation of the "insulin" peak was more than could be explained by slight trailing from the "proinsulin" peak. The possibility that the insulin produced by some diabetics reacted differently with insulinase than did normal insulin had not been excluded.

The investigation of the nature of the insulin produced by untreated juvenile diabetics has been hampered by the very low concentration of insulin-like immunoreactivity usually present in their serum, by the limited serum available, and by the unavailability of methods for simple and reliable isolation of the insulin immunoreactive material in relatively pure form.

For the present study, urine was chosen as the source of insulin because of the larger volumes available, usually containing a higher concentration of insulin than serum. ILI was extracted by an improved

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*Abbreviations used in this paper: BSA, bovine serum albumin; ILI, insulin-like immunoreactivity; IR, immunoreactivity; NEM, N-ethylmaleimide; pI, isoelectric point; PLCU, proinsulin-like component from urine; RIA, radioimmunoassay; W/V, weight for volume. Convention for porcine proinsulin- and insulin-like species, according to Chance:[4] split proinsulin, $leucine_{54}$-$alanine_{55}$ bond split in C-peptide; desdipeptide proinsulin, $lysine_{62}$-$arginine_{63}$ absent in C-peptide; desnonapeptide proinsulin, nonapeptide B_{55-63} absent in C-peptide; monoarginine (diarginine)-B30 insulin, arginine (arginine-arginine) attached to the C-terminal alanine of the B chain; by analogy, monoarginine A_1-(bovine) insulin refers to insulin with arginine attached to the N-terminal glycine of the A chain.

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procedure[6] that permits high and reproducible yields of pure hormone. The insulin subsequently resolved from the extract by gel filtration was analyzed for insulinase sensitivity and by isoelectric focusing in order to test the hypothesis that juvenile diabetics may produce altered insulin molecules.

METHODS

Collection of Urine Samples

Urine samples (whenever possible of twenty-four hours' duration) were obtained from children admitted to hospital with overt untreated diabetes mellitus. All children subsequently required insulin permanently to control their severe diabetes. Twenty-four-hour samples were obtained from eleven normal healthy children with no family history of diabetes and from four children with latent diabetes (abnormal oral glucose tolerance). All samples were kept refrigerated (4° C.) or frozen until the collection was completed and were then stored frozen (−10° C.). An aliquot of each completed collection was analyzed for sodium, potassium, urea, and creatinine by standard AutoAnalyzer procedures. Total urinary protein was measured by an automated method based on that described by Brugerie and James.[7]

Extraction of ILI

ILI was extracted by adsorption to anti-insulin globulins coupled to insoluble agarose beads (Sepharose 4B from Pharmacia Fine Chemicals, Sweden). The method, which is described in detail elsewhere,[6] quantitatively extracts insulin and proinsulin in any proportions, up to the amounts found in urine. The bulk of the ILI was recovered in two 2-ml. portions of 1 M acetic-acid/0.3 per cent bovine serum albumin (BSA).

Radioimmunoassay (RIA) of ILI was performed essentially as described by Albano and Ekins.[8] The assay buffer and reagent diluent was 0.05 M sodium phosphate pH 7.4 containing 0.3 per cent W/V BSA. Human insulin standard (MR71) and guinea pig antibovine insulin serum (MR41, batches K1769 and K3481) were purchased from Wellcome Reagents Ltd., Beckenham, England. Working insulin standards and diluted antiserum were stored frozen (−10° C.) for up to three months with no measurable loss of potency. ^{125}I-porcine insulin (specific activity 180 μCi./μg.), prepared by a modification of the method of Hunter and Greenwood,[9] was kindly provided by the Department of Endocrinology, Auckland University. Insulin-free human plasma[8] was added to each tube not containing a test serum or plasma sample. "Free" and antibody-bound hormone were separated by addition of albumin-coated charcoal. The tubes were centrifuged, the supernatant removed by suction and discarded, and the ^{125}I associated with the charcoal pellet measured by use of a solid crystal well-type gamma-radiation counter. ILI determinations were carried out in duplicate. Porcine proinsulin had about 20 per cent of the IR of standard human insulin on a weight-for-weight basis. Within-assay variation (per cent SD from mean) was 2 per cent, between-assay variation 15 per cent.

Gel Filtration of ILI

Extracts (3-5 ml.) were fractionated at room temperature on a 2.5 × 38-cm. column of Bio-Gel P-30, 100-200 mesh. Acetic acid (2M) containing 0.01 per cent BSA was used as eluent, flow rate 40 ml. per hour, and 120-drop fractions (4 ml.) were collected with a fraction collector equipped with a drop counter. The elution position of the BSA in each sample (void volume) was measured by ultraviolet adsorption at 280 nm.

Fractions were collected in siliconized (Siliclad, Clay-Adams, New Jersey) glass tubes, freeze-dried, and then reconstituted with a suitable volume of buffer. Resuspension volumes and the volume of the aliquots removed for RIA were varied so that fractions before and after the insulin peak were assayed at a two- to fourfold higher concentration than the insulin fractions. This ensured more reliable estimates of the percentage of the ILI eluting in positions other than the insulin region.

Isoelectric Focusing

An Isco (Instrument Specialties Co., Lincoln, Nebraska) Model 210 Density Gradient Electrophoresis Column was used, coupled to an Isco Model 430 Programmed Electrophoresis Pump and a 0-1,500 V, 0-200 mA constant voltage power source. The electrode solutions and ampholyte (Ampholine, pH range 3-10, LKB Produckter, Sweden) concentrations were as suggested by Grant and Leaback.[10] The column was cooled to a steady temperature of about 10° C. by circulating water. The sample for analysis was dissolved in fraction 3 of the 9-ml. manually prepared density gradient. Once the gradient was in position, a voltage of 250 V was applied. This was increased stepwise over the first two hours to 700 V, the power consumption being kept below 0.5 W to minimize heating. The voltage was maintained at about 700 V for twenty to twenty-two hours.

At the completion of the run, the electric field was disconnected and the gradient was pumped from the

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top of the column through a narrow outlet spout. Two- to three-drop fractions were collected in siliconized glass tubes. An aliquot of each fraction was diluted into buffer for RIA. The pH of the remainder of the fraction was measured at 37° C. with a Radiometer capillary glass microelectrode on a model PHM 22 pH meter.

Determination of Rate of Degradation by Insulinase

(a) *Preparation of insulinase.* The method described by Elliott[11] was used. The skeletal muscle from the front and back legs of four albino rats was removed and homogenized in ice-cold 0.1 M phosphate buffer at pH 7.4 (about 250 ml.). The homogenate was centrifuged for twenty minutes at 18,800 *g* (Sorvall RC2B, SS-34 rotor), and the supernatant added with stirring to 10 vol. ice-cold acetone. The precipitate was collected by filtration under vacuum and then air-dried for about an hour at room temperature. The brown solid was resuspended in about 50 ml. phosphate buffer. After centrifugation (18,800 *g*) the supernatant was adjusted to 30 per cent saturation in ammonium sulfate. The slight precipitate was removed by centrifugation (18,800 *g*), and the supernatant adjusted to 60 per cent saturation in ammonium sulfate. The precipitate was collected by centrifugation, drained, and resuspended in 10 ml. phosphate buffer. Aliquots of 0.25 ml. and 0.5 ml. in small capped polystyrene tubes were stored frozen.

Although more extensive methods of purification of insulin-degrading enzymes have been described, e.g. by Brush,[12] the present procedure was used so that the results obtained would be directly comparable to those of Elliott and his associates.[1,11] Their work had previously established the substrate specificity of this relatively crude preparation. The results described in this paper were all obtained using the same batch of enzyme, which caused negligible degradation of porcine proinsulin relative to standard insulin. No significant loss of enzymic activity was detected after five months.

(b) *Measurement of enzyme activity.* The rate of the reaction catalyzed by the insulinase preparation was measured by incubating various dilutions with standard insulin in buffer as described below for test samples.

That amount of enzyme which would degrade a standard insulin solution by about 50 per cent over sixty minutes at 37° C. was used for analyzing each test sample. The reaction appeared to be first-order.

(c) *Analysis of insulin-like species.* Samples of known immunoreactive concentration, in 1-2 ml. 0.1 M sodium phosphate pH 7.4 containing 2 per cent BSA (insulinase buffer), were incubated with a fixed amount of insulinase at 37° C. for sixty minutes. Aliquots were withdrawn at various times (usually 0, 30, 60 min.), and the enzyme in them inactivated by dilution into an equal volume of ice-cold 0.002 M N-ethylmaleimide (NEM) in assay buffer. Two pairs of porcine proinsulin and human insulin standards were included with each batch. The concentration of immunoreactivity in the test samples was adjusted to the concentration of one of the standard pairs; this allowed measurement of immunoreactivity with the same degree of precision for both standard and test samples. No evidence was found for variation in the fractional rate of degradation with ILI concentration over the range of concentrations studied: 10-160 μU./ml.

Since the samples for RIA contained insulinase buffer and some insulinase inactivated by NEM, equivalent concentrations of these species were also added to the standard samples. Additional NEM was added to all assay tubes to give a final concentration of 0.001 M. Apart from the inclusion of these additional components in the incubation mixture, the standard procedure for RIA was followed.

Standard Samples

Porcine proinsulin and proinsulin-insulin intermediates were a generous gift from Eli Lilly and Co., Indianapolis. Monoarginine-Al-bovine insulin[13] was kindly provided by Dr. D. Brandenburg, Deutsches Wollforschungsinstitut, 51 Aachen, Veltmanplatz, Federal Republic of Germany. On gel filtration, desdipeptide and desnonapeptide proinsulin (peak tubes 25 and 26 respectively) behaved like proinsulin (figure 1). Split proinsulin (peak tube 24) eluted just before proinsulin. Mono- and diarginine-B30-insulins (peak tubes 31-32) eluted between proinsulin and insulin.

Human insulin (the standard used for RIA) had an isoelectric point (pI) of 5.2, with a small peak of presumed deamidated insulin at pH 4.7.[14] Monoarginine-B30-insulin had a pI of 5.9, diarginine-B30-insulin of 6.7. Monoarginine-Al-insulin focused in two main bands at pH 6.3 and pH 5.7, the latter probably corresponding to the deamidated species. Desdipeptide proinsulin and split proinsulin were fully resistant to degradation by insulinase. Desnonapeptide proinsulin was partially resistant. Mono- and diarginine-B30-insulins and monoarginine-Al-insulin were fully sensitive.

RESULTS

Total Urinary ILI

The twenty-four-hour samples from eleven normal

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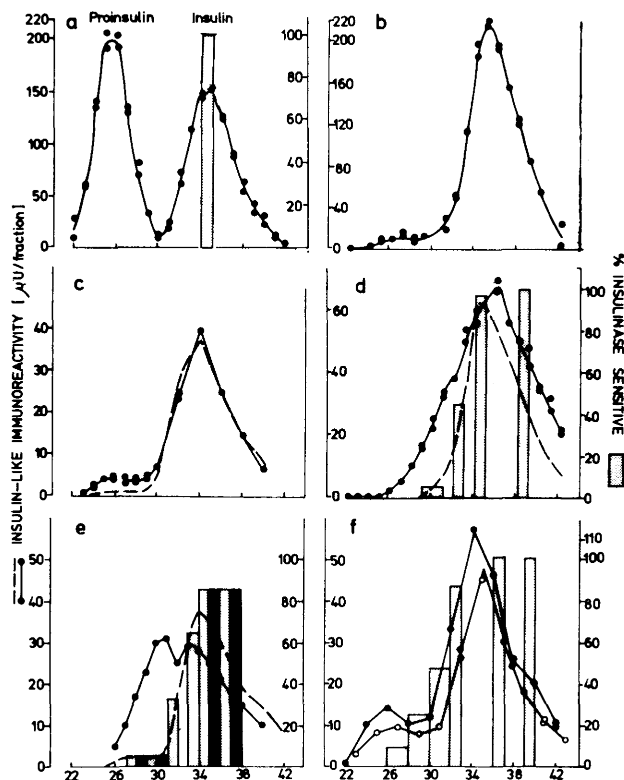

FIG. 1. Gel filtration of urinary ILI extracts from diabetic children (●——●) and the insulinase sensitivity of the ILI in selected fractions. The gel-filtration profiles of some normal extracts (---) are included for comparison. The percentage of ILI eluting before fraction 31 is taken as an approximation of the contribution of larger ILI species to the sample. (a) Standard porcine proinsulin and human insulin added to a normal urine sample of low endogenous insulin and extracted by the usual method. (Standards dissolved directly in acetic acid eluted similarly.) The sensitivity of the standards to insulinase was not affected by extraction and gel filtration. The peak of BSA elution was at fraction 15; tryptophan at fraction 55. (b) K.M. (similarly J.J., N.R. and B.C.):<5 per cent "larger" ILI. (c) W.G. (similarly A.A.): 5-10 per cent "larger" ILI evenly distributed over the Components II and III regions. Normal: A.B. (d) P.B. II: 8 per cent "larger" ILI, predominantly Component II. Similarly M.M. (5 per cent), E.F. (8 per cent), P.B.I (8 per cent), G.W. (16 per cent). Normal: V.M. (e) S.B.: Approximately 50 per cent Component II. Some fractions were pooled for measurement of insulinase sensitivity: 27 + 29, 28 + 30, 34 + 36, 35 + 37. Normal: J.F.H. (f)C.W., two separate aliquots: 20 per cent "larger" ILI. Similarly K.J. (13 per cent), D.N. (25 per cent), D.B. (30 per cent). Note: The human insulin standard used in this study contained 6 per cent larger ILI species and hence was less sensitive to insulinase than was insulin further purified by gel filtration. The degree of insulinase sensitivity of the ILI in selected fractions of the urinary samples has therefore been calculated relative to that of the insulin component in each sample, instead of the human insulin standard.

children (table 1) contained 2-20 μU. ILI/ml. (5-20 μU. ILI/mg. creatinine, mean 12.1, S.D. 5.4). The mean twenty-four-hour excretion of ILI per kilogram body weight was 252 μU./kg. (S.D. 108); this value was not significantly different from that reported by McArthur and Stimmler[15] (202 ± 59 μU. ILI/kg.). The urine samples from four children with latent diabetes contained a normal concentration of ILI and normal relative proportions of ILI and creatinine.

Urine samples from four of the children with overt untreated diabetes contained no assayable or extractable ILI. In samples from the other fifteen children, the concentration varied from less than 1 to 65 μU. ILI/ml. For about half of these, the ratio of ILI to creatinine was within 2 S.D. of the mean for the normal children. For the others, the ratio was greater than 3 S.D. above the normal mean. The thirteen nonobese diabetics had characteristically low (<5 μU./ml.) concentrations of ILI in their serum; all fifteen had values for creatinine excretion within the normal range. Thus, high values of urinary insulin:creatinine would seem to indicate altered renal handling of insulin in about half of these patients.

Analysis of Major IR Components from Urine

Normals. When the extracted ILI was analyzed by gel filtration, most of the immunoreactivity (≥96.8 per cent) eluted at the position of standard human insulin. This major species, Component I, was fully sensitive to degradation by insulinase. When Component I from one extract (D.H.) was further analyzed by IEF, 87 per cent of the recovered IR focused at the position of human insulin standard (pH 5.2); 13 per cent focused at pH 4.7, the position expected for monodesamidoinsulin[14] (see figure 2a). It was therefore concluded that Component I was insulin.

Children with latent diabetes. In general, a normal proportion of the ILI eluted before insulin from the gel filtration column. A proportion of "larger" ILI outside the normal range (5 per cent) was measured, however, in a twenty-four-hour sample from one child on the day prior to commencement of insulin therapy; she had previously shown a normal ILI profile. The other children have not received any insulin to date.

Children with overt untreated diabetes. When the extracted ILI was analyzed by gel filtration, more than 70 per cent eluted at the position of standard human insulin. This Component I was in all cases fully sensitive to degradation by insulinase.

Component I from two ILI extracts was further analyzed by IEF. Those children with the highest (K.M.) and lowest (D.B.) proportion of Component I in the extract were chosen. When equal IR amounts of Component I from normal and diabetic urine were analyzed together, the IR focused into a single main peak. There was no evidence for broadening of the peak, which could have indicated the presence of two

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TABLE 1

Details of the normal children and their twenty-four-hour urine samples

Name	A.M.	V.M.	D.C.	A.B.	R.W.	J.F.H.	J.M.H.	M.W.	D.H.	R.H.	G.C.
Sex	M	F	F	F	F	F	M	M	M	M	M
Weight (kg.)	38	24	37	23	23	28.5	25.5	17	44	38	30
Age when urine sample collected (yr.)	11	8.5	11.5	5.5	6	11	9	4	14.5	13	8
Volume (ml.)	1,600	700	925	950	1,000	600	700	500	630	1,100	780
Protein (mg./100 ml.)	Nil	Nil	10	5	Nil	5	Nil	Nil	5	5	5
Creatinine (mg./100 ml.)	69	97	70	42	54	82	94	73	168	62	50
(mg./kg. body wt./hr.)	1.21	1.18	0.73	0.72	0.98	0.72	1.07	0.89	1.00	0.74	0.61
ILI (μU./ml.)	3.5	12	14	2	10	8	5.5	12	20	6	10.5
(mU./24 hr.)	5.6	8.4	13.0	1.9	10.0	4.8	3.8	6.0	12.6	6.6	8.2
(μU./kg. body wt.)	147	350	351	83	435	168	149	353	286	174	273
ILI (μU./mg. creatinine)	5.1	12.4	20.0	4.8	18.5	9.2	6.2	16.5	11.9	9.7	18.7
% ILI eluting before insulin from gel-filtration column	1.8	0	2.2	1.7	1.2	2.3	3.2	0	1.4	2.9	*

*Not Determined

species of slightly different isoelectric point (figure 2). The peak of presumed deamidated insulin was fully sensitive to degradation by insulinase.

Analysis of Other IR Components from Urine

Normal and latent diabetics. Up to 3.2 per cent of the ILI in the normal urine extracts eluted before insulin from the gel filtration column (see table 2). The low concentration precluded further analysis of insulinase-sensitivity or isoelectric properties. This proportion of larger ILI confirms the results of Constan, Mako, Rubenstein, and Steiner.[16]

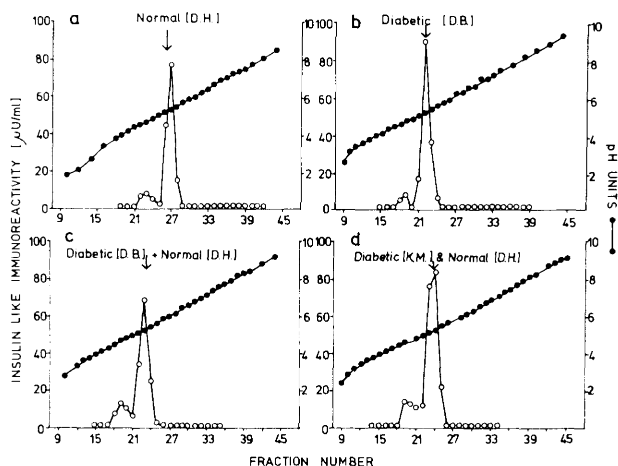

FIG. 2. Isoelectric focusing of Component I (insulin) from urine extracts. No difference in isoelectric behavior of Component I from normal and diabetic children could be shown. Details are given in the text.

Children with Overt Untreated Diabetes

Gel-filtration profiles. Up to 30 per cent of the extracted ILI eluted from the gel-filtration column before insulin. In four samples, the larger ILI comprised 13-30 per cent of the total, most eluting at the position of porcine proinsulin (Component III) and some between proinsulin and insulin (Component II), figure 1f. In five samples most of the noninsulin IR eluted as Component II only (figure 1, d and e). Six samples (two from obese children) contained normal or near normal proportions of the larger ILI species (figure 1, b and c).

The proportion and type of larger immunoreactive species in the urinary ILI extracts did not apparently correlate with the severity or duration of onset symptoms or with family history. Further, in these children where two consecutive samples were extracted (e.g. P.B., sixteen hours, seven hours; K.M., twenty-two hours, two hours), no trend was measured towards increased or decreased "precursor" production with time.

Insulinase sensitivity. Components II and III were both resistant to degradation by insulinase (figure 1). Although Component II was poorly resolved from insulin on gel filtration, its presence, suspected from the shape of the elution profile, could be confirmed by analysis of the leading edge of the insulin peak with insulinase.

Isoelectric properties. Most of ILI extracts from diabetic urine samples contained insufficient insulinase-

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TABLE 2

Details of untreated diabetic children and their urine samples

Name	P.B.*	P.B.*	S.B.	C.W.	D.B.	W.G.	A.A.	K.J.¶	G.W.	K.M.**
Sex	MI	MII	F	F	M	M	F	F	M	M
Wt. (kg.)	17.4	17.4	13.4	14.1	35.5	41.7	30.7	21.6	14.4	38
Age at onset	5	5	3.25	3.75	13.5	14	9.5	9	2.75	12.5
Duration of collection (hr.)	17	7	24.5	unknown†	4 (approx)	24	10 (approx)	2	23	19.5
Volume (ml.)	2,350	730	475	91	850	1,600	550	443	440	1,100
Protein (mg./100 ml.)	5	5	Nil	50	55	10	10	Nil	Nil	Nil
Creatinine (mg./100 ml.)	12	19	43	23	—	52	—	16	32	35
(mg./kg./hr.)	0.95	1.14	0.62	†	—	0.83	—	1.6	0.43	0.42
ILI (μU./ml.)	0.5-1	6.5	<1	22	37	3	3	<1	7	61
ILI mU. total	2.3	4.7	0.36	2.0	31.4	4.8	1.6	<0.4	3.1	67.1
ILI (μU./mg. creatinine)	8.3	34	<24	96	>37‡	5.8	3-6§	4-8	2.2	175
See elution profile in figure	1d	1d	1e	1f	2	1c	1c	1f	1d	1b

*Consecutive portions (I and II) of a single twenty-four-hour collection.

†A single urine specimen was obtained by catheterization of comatose patient—time of previous voiding unknown.

‡Calculated assuming creatinine concentration < 100 mg./100 ml.

§Calculated assuming creatinine concentration 50-100 mg./100 ml. (Na, K, urea concentrations were normal).

¶350 ml. urine (1-2μU. ILI/ml.) were collected before this fasting sample, and the two were pooled for extraction of the ILI.

TABLE 2 (continued)

Name	K.M.**	M.M.	J.J.	E.F.	D.N.	N.R.††,‡‡	N.R.††,‡‡	B.C.*,‡‡	B.C.††,‡‡
Sex	M	M	M	M	M	M	M	FI	FII
Wt. (kg.)	38	10.8	24.7	40	18	42.5	42.5	50.8	50.8
Age at onset	12.5	1.3	10.5	14	5.7	12.25	12.25	12.7	12.7
Duration of collection (hr.)	1	20.75	18	14.5	1.5	16.5	15	7	17
Volume (ml.)	170	198	2,070	2,390	120	850	400	340	600
Protein (mg./100 ml.)	5	Nil	Nil	Nil	10	5	10	Nil	5
Creatinine (mg./100 ml.)	26	42	32	30	25	59	141	144	124
(mg./kg./hr.)	1.16	0.37	1.44	1.23	1.11	0.71	0.88	1.38	0.86
ILI (μU./ml.)	65	3.5	30	16	28	5	9	6	4.5
ILI mU. total	11.0	0.70	62.1	38	1.2	4.25	3.6	2.04	2.70
ILI (μU./mg. creatinine)	250	8.3	94	53	112	8.5	6.4	4.2	3.6
See elution profile in figure	1b	1d	1b	1d	1f	1b	1b	1b	1b

**A two-hour fasting sample (94 ml., 59 μU./ml.) was collected after the 19.5-hour sample, an oral glucose dose of 1.75 gm./kg. body weight was administered, and then this one-hour sample was collected. A further one-hour sample was collected (160 ml., 19 μU./ml.) prior to the commencement of insulin therapy.

††These two collections were separated by an interval of 4.25 hours.

‡‡Obese.

resistant material to permit analysis of isoelectric properties. Analysis was possible, however, for D.B.

The "proinsulin" fractions from the gel-filtration column (24-27 inclusive) and the trough of ILI between the two peaks of IR (28-32 inclusive) were analyzed separately. Fractions were not individually immunoassayed, as the relatively large resuspension volume and albumin concentration necessitated would render the sample unsuitable for direct isoelectric focusing. Fractions were pooled by analogy with the elution profile of another aliquot of the sample. From the "proinsulin" region proinsulin (pH 5.2), a more acidic band (pH 4.7—presumably deamidated proinsulin and/or desdipeptide proinsulin) and a more basic band (pH 5.9), all insulinase-resistant, were resolved. From the second region, insulin and proinsulin, their more acidic derivatives, and the insulinase-resistant band at pH 5.9 were resolved (figure 3). This result strongly suggests that the species focusing at pH 5.9 in this extract was Component II.

For convenience, Component III will be referred to as PLCU (proinsulin-like component from urine) and Component II as mid-insulin, because of their elution positions on gel filtration.

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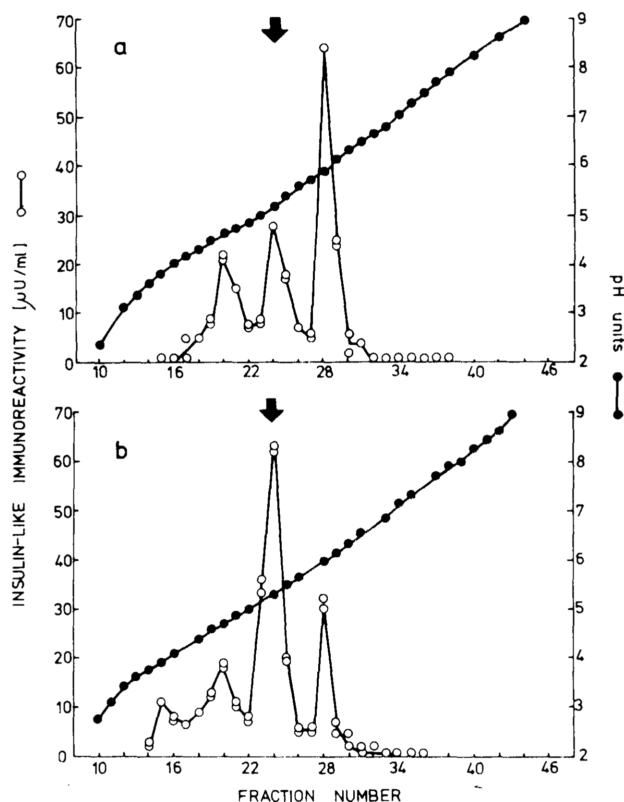

FIG. 3. The insulinase-resistant urinary ILI from a juvenile diabetic (D.B.) was analyzed by IEF in two portions: (a) fractions 24-27 and (b) fractions 28-32, from the gel-filtration column. The arrows indicate the pH (5.2) at which standard human insulin focused. These results suggest that Component II from this extract focused at pH 5.9.

Analysis of Major Immunoreactive Component from Serum

The original reports of insulinase-resistant insulin in untreated juvenile diabetes were based on results with serum extracts. Since most diabetic children have only a trace of ILI in their serum at the time of hospital admission (0-5 μU./ml.), insufficient ILI is usually available for analysis. Serum ILI was not routinely extracted in the present study. However, a sample (12 μU. ILI/ml.) from a girl (eleven years) with latent diabetes that progressed over the following six weeks to overt insulin-requiring diabetes was extracted in order to measure the insulinase-sensitivity of the insulin. A sample of serum from a normal young adult was similarly extracted. The insulin moiety in both samples was fully sensitive to degradation by insulinase (10 per cent of the ILI extracted from the normal sample, and 27 per cent of that from the diabetic sample, eluted before insulin from the gel-filtration column and was insulinase-resistant).

DISCUSSION

The material extracted from urine by adsorption to insolubilized anti-insulin globulins is composed chiefly of insulin: also present, however, are components that differ from insulin in having (a) a larger molecular weight and (b) resistance to the degradative action of insulinase. One of these insulin-like components is likely to be proinsulin, but other species are also present. In this study we have examined the constituents of such urine extracts from children with untreated diabetes mellitus.

Nature of the Insulin Produced by Juvenile Diabetics

The present work has conclusively demonstrated that juvenile diabetics produce insulin that is normal, by the criterion of insulinase-sensitivity, and thus the hypothesis presented earlier[1] cannot be upheld. The insulin from the urine of juvenile diabetics has also been shown in the present work to have the same isoelectric point as normal insulin. (Differences of less than 0.1 pH units would not have been detected.) The possibility remains, however, that the insulin produced by diabetics differs from normal insulin in a manner not detected by the present discriminants.

It is important to note that the consistent and unequivocal results obtained with insulinase were due to the efficient extraction technic used for insulin purification. In contrast, less satisfactory extraction technics, such as aqueous dialysis followed by freeze-drying, or dialysis against carbowax 20M (Union Carbide Co.) then freeze-drying, yielded impure insulin that appeared to be partially and variably insulinase-resistant.[6]

Nature of the Insulinase-resistant ILI in Diabetic Urine

Although the insulin produced by juvenile diabetics is insulinase-sensitive, insulinase resistance is a property of a proinsulin-like component and a previously undescribed IR species partially resolved from insulin by gel filtration. The quantity and variety of these insulinase-resistant components may of course be different in serum and pancreas.

Proinsulin-like Component

Many workers have isolated, from normal and pathological human serum, immunoreactive species eluting at the position of proinsulin (PLC, proinsulin-like component). Occasional reports of a species eluting with PLC but migrating on polyacrylamide gel electrophoresis at the position of desdipeptide proinsulin have also appeared.[16,18] However, very little information has been published concerning the occurrence in urine of insulin precursors: A relatively low concentration of proinsulin-like immunoreactivity was measured in a small number of normal twenty-four-hour samples,[15] and a relatively

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large amount in a sample from a ketotic untreated juvenile diabetic and a patient with an islet-cell tumor.[3] Proinsulin-insulin intermediates have not been reported in urine.

In the present study, the relative contribution of species such as desdipeptide and desnonapeptide proinsulin to the PLCU peak is not known in most cases, since all would co-elute on gel filtration. Further analysis by IEF of the PLCU extracted from the urine of one juvenile diabetic (D.B.), however, revealed presumed proinsulin at pH 5.2 (28 per cent) and a considerable proportion of insulinase-resistant immunoreactivity (20 per cent) at pH 4.7 (the remainder of the IR focused at pH 5.9). Since proinsulin is unlikely to be more readily deamidated than insulin, the IR at pH 4.7 probably contains less than 20 per cent deamidated proinsulin (cf. figure 2), the remainder being related polypeptides such as desdipeptide proinsulin.

Mid-insulin

Because of its elution characteristics on gel filtration, mid-insulin probably corresponds to an insulin moiety with some additional amino acids. By comparison with the behavior of porcine proinsulin-insulin intermediates, the insulinase-resistance of mid-insulin strongly suggests that any additional amino acids are bound to the N-terminus of the α chain rather than the COOH-terminus of the β chain. The insulinase-sensitivity of monoarginine-Al-insulin, however, indicates that mid-insulin must contain more than one additional amino acid. The combined charge effect of the additional amino acids would be approximately equivalent to that of a single basic amino acid, such as arginine.

As the possibility exists that proteases in urine might act on the proinsulin in the diabetic samples and produce an artifactual intermediate, porcine proinsulin was incubated with both normal and diabetic urine samples, for twenty-four hours at room temperature (25° C.) and subsequently gel-filtered. No intermediates were resolved, and the proinsulin: insulin ratio was the same as before incubation.

Evidence that the Appearance in the Urine of Increased Proportions of Larger ILI Was Not Related to Renal Function

Because of the low concentration of ILI in the serum of the juvenile diabetics, it proved impossible to confirm that the nature of the urinary ILI truly reflected the average proportions of the ILI species in the serum. However, the data in table 3 show that the presence of renal abnormality, as indicated by an elevated insulin:creatinine ratio, or by gross proteinuria,

TABLE 3

Evidence that the appearance of insulin "precursors" in the urine was not related to renal function

Patient	Urinary insulin: creatinine (μU./mg.)	Protein (mg./specimen)	Proteinuric? (>200 mg./24 hours)	Nature of Urinary ILI		
				"proins."	"mid-ins."	ins.
	Elevated					
C.W.	96	50 mg./90 ml.*	No?*	+	+?†	+
D.N.	112	12 mg./1.5 hrs.	No	+	+?†	+
D.B.	>37	>440 mg./4 hrs.	Yes	+	+	+
E.F.	53	Nil	No	−	+	+
K.M.	175	Nil	No	−	−	+
J.J.	94	Nil	No	+?§	+?§	+
P.B. II‡	34	117 mg./17 hrs.	No	−	+	+
	Normal					
P.B. I‡	8.3	36 mg./7 hrs.	No	−	+	+
B.C.	4	Nil	No	+?§	?§	+
S.B.	<24	Nil	No	−	+	+
M.M.	8	Nil	No	−	+	+
W.G.	5.8	160 mg./24 hrs.	No	+?§	+?§	+
A.A.	3-6	55 mg./10 hrs.	No	+?§	+	+
N.R.	6-8	82 mg./24 hrs.	No	−	−	+
K.J.	4-8	Nil	No	+	+?§	+
G.W.	2.2	Nil	No	−	+	+

*A single urine sample was obtained by catheterization of comatose patient. Time of previous voiding not known.

†Presence of mid-insulin inferred, because of the reproducibly incomplete separation of the proinsulin-like and insulin-like components on gel filtration (see also figure 1f).

‡I and II represent two consecutive portions of a twenty-four-hour collection. These two extracts contained similar proportions of insulin, mid-insulin, and PLCU.

§The small percentage of "larger" ILI precluded positive identification of "proinsulin" and "mid-insulin."

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did not correlate with the presence or otherwise of the larger ILI species. These observations suggest, but do not confirm, that impaired renal function is not per se the cause of our findings.

Possible Origins of Insulinase-resistant ILI

In view of current uncertainty concerning the normal proinsulin conversion mechanism(s) in the beta cells (compare references 19 and 20), and in the absence of quantitative data on the nature of the trace amounts of larger ILI species extracted from normal urine samples, it is not possible at present to distinguish the effects of genetic alterations in proinsulin or cleavage enzymes from modified control of proinsulin biosynthesis and cleavage to insulin. Although this paper has shown that the earlier evidence for a genetically altered insulin molecule itself in juvenile diabetes (insulinase-resistance) was not valid, the possibility remains that there is indeed a genetic alteration in proinsulin in some cases. As in the rat and some fishes[21] and the mouse,[22,23] two or more genes for proinsulin may occur in man, and one may occasionally have a slightly altered amino acid sequence.

In the absence of metabolic or other in vivo data, the significance of the present insulinase-resistant species is not known. Further analysis of their amino acid composition and of the extent of their occurrence in diabetes and related states may contribute importantly to the understanding of the fundamental defects in diabetes mellitus.

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