Subcutaneous Insulin Absorption Explained by Insulin's Physicochemical Properties

Evidence From Absorption Studies of Soluble Human Insulin and Insulin Analogues in Humans

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Objective: To study the influence of molecular aggregation on rates of subcutaneous insulin absorption and to attempt to elucidate the mechanism of absorption of conventional soluble human insulin in humans. Research Design and Methods: Seven healthy male volunteers aged 22–43 yr and not receiving any drugs comprised the study. This study consisted of a single-blind randomized comparison of equimolar dosages of ^{125}I-labeled forms of soluble hexameric 2 Zn^{2+} human insulin and human insulin analogues with differing association states at pharmaceutical concentrations (Asp^{B10}, dimeric; Asp^{B28}, mixture of monomers and dimers; Asp^{B9}, Glu^{B27}, monomeric). After an overnight fast and a basal period of 1 h, 0.6 nmol/kg of either ^{125}I-labeled human soluble insulin (Actrapid HM U-100) or ^{125}I-labeled analogue was injected subcutaneously on 4 separate days 1 wk apart. Absorption was assessed by measurement of residual radioactivity at the injection site by external γ-counting. Results: The mean ± SE initial fractional disappearance rates for the four preparations were 20.7 ± 1.9 (hexameric soluble human insulin), 44.4 ± 2.5 (dimeric analogue Asp^{B10}), 50.6 ± 3.9 (analogue Asp^{B28}), and 67.4 ± 7.4%/h (monomeric analogue Asp^{B9}, Glu^{B27}). Absorption of the dimeric analogue was significantly faster than that of hexameric human insulin ($P < 0.001$); absorption of monomeric insulin analogue Asp^{B9}, Glu^{B27} was significantly faster than that of dimeric analogue Asp^{B10} ($P < 0.01$). There was an inverse linear correlation between association state and the initial fractional disappearance rates ($r = -0.98$, $P < 0.02$). Analysis of the disappearance data on a log linear scale showed that only the monomeric analogue had a monoexponential course throughout. Two phases in the rates of absorption were identified for the dimer and three for hexameric human insulin. The fractional disappearance rates (%/h) calculated by log linear regression analysis were monomer 73.3 ± 6.8; dimer 44.4 ± 2.5 from 0 to 2 h and 68.9 ± 3.5 from 2.5 h onward; and hexameric insulin 20.7 ± 1.9 from 0 to 2 h, 45.6 ± 5.0 from 2.5 to 5 h, and 70.6 ± 6.3 from 5 h onward. Conclusions: Association state is a major determinant of rates of absorption of insulin and insulin analogues. The lag phase and the subsequent increasing rate of subcutaneous soluble insulin absorption can be explained by the associated state of native insulin in pharmaceutical formulation and its progressive dissociation into smaller units during the absorption process. *Diabetes Care* 14:942–48, 1991

Insulin in solution exists as an equilibrium mixture of monomers, dimers, tetramers, and zinc-containing hexamers (1). At pharmaceutical concentrations in neutral solution, hexamers predominate; dimer formation requires removal of zinc and dilution, and monomers are only seen in very diluted solutions (2,3; Fig. 1). This self-association of insulin, which in the β-cell serves to facilitate proinsulin transport and conversion and intracellular storage (4), may limit the rate of absorption of subcutaneously administered insulins (5). However, the detailed mechanisms of subcutaneous absorption are poorly understood.

Subcutaneously administered soluble insulin is absorbed into the blood stream (6); local blood flow is a major factor controlling absorption rate (6–8), and many variables known to affect absorption rate, includ-

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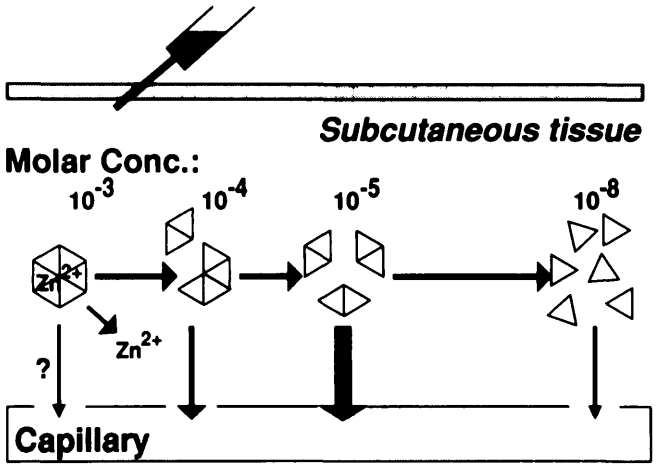

FIG. 1. Putative events in subcutis after subcutaneous injection of soluble (regular) human insulin. Concentration of hexameric Zn^{2+} insulin, predominant association state of soluble insulin in U-40 or U-100 strength (U-100 ~0.6 mM), is lowered by diffusion in interstitial space. During this process, zinc-insulin-hexamer complex disintegrates into smaller units. For dissociation into mainly dimeric insulin, only 50- to 100-fold dilution is needed, whereas dominant population of monomeric insulin would require further 1000-fold dilution. Passage of more associated forms through capillary membrane is believed to be restricted due to steric hindrance. From Brange et al. (3). © by the American Diabetes Association.

ing site (9–11) and depth (9,12) of injection, exercise (13,14), smoking (15), and temperature (10,16,17), do so by their influence on blood flow. Other major factors include the rate of diffusion to the capillary and the rate of restriction for transport across the capillary membrane (total area and permeability); these factors are mainly influenced by the size of the transported molecule (18).

In the first 2–4 h after subcutaneous injection, the rate of soluble insulin absorption rises only slowly to its maximum (6,19,20). During this lag phase of slower absorption, there is an inverse relationship between absorption rate and concentration and volume of the insulin preparation (6). The early delay in absorption has been attributed to the breakdown of hexameric units into dimers and monomers (5). Alternatively, Hildebrandt et al. (18) suggest that insulin is mainly absorbed in the hexameric form and that, although subcutaneous blood flow is the predominant influence on absorption rate at low blood flow rates, diffusion becomes rate limiting in the high blood flow range.

Subcutaneous absorption is reliably studied by the disappearance of radiolabeled insulin from the injection site (6,21). Several mathematical models (5,21–28) have been developed to quantitatively study subcutaneous absorption, but most are descriptive or predictive rather than explanatory, and all depend on assumptions about the mechanisms of absorption (29).

Studies with insulin analogues with reduced association states are helping to elucidate the absorption process. We studied the absorption in nondiabetic humans of three ^{125}I-labeled insulin analogues and ^{125}I-labeled human insulin with differing association states and report herein on the analysis of the disappearance data to examine the mechanisms of absorption of soluble insulins.

RESEARCH DESIGN AND METHODS

This study was approved by the local ethical committee and was performed in accordance with the Declaration of Helsinki. All subjects gave informed written consent.

Seven healthy male volunteers aged between 22 and 43 yr and with a body mass index between 20.7 and 24.8 kg/m^2 were studied on five occasions over 4 mo, receiving in random order for the first study 0.6 nmol/kg of ^{125}I-labeled soluble human insulin (Actrapid HM U-100, sp act 189 MBq/L, Novo, Copenhagen) and the same dose of ^{125}I-labeled analogue Asp^{B10} (sp act 183 MBq/L). In the second study, subjects received in random order 0.6 nmol/kg ^{125}I-labeled soluble human insulin (Actrapid HM U-100, sp act 180 MBq/L) and the same dosages of ^{125}I-labeled analogues Asp^{B9}, Glu^{B27} (sp act 139 MBq/L) and Asp^{B28} (sp act 149 MBq/L). The production and purification procedures of the analogues are identical to those used in the production of human insulin by genetic engineering (30). All preparations were formulated to the same concentration (0.6 mM), and all were mono-^{125}I-labeled at the Tyr^{A14} position (31).

After an overnight fast and a basal period of 1 h, the ^{125}I-labeled preparations were injected subcutaneously into the anterior abdominal wall midway between the umbilicus and the anterior superior iliac spine with a 0.5-ml disposable syringe (Lo-Dose, Becton Dickinson, New York). All injections were given by the same physician, and a lifted skin-fold technique was used to avoid intramuscular injection.

The disappearance of the injected preparations from subcutaneous tissues was assessed from the amount of residual radioactivity at the injection site. External emission of γ-rays was measured with a 50 × 57-mm thallium-activated sodium iodide scintillation detector with a cylindrical lead collimator fixed 50 mm above the skin surface. Residual radioactivity at the injection site was measured continuously for the first 2 h after injection of ^{125}I-labeled preparations and thereafter over 5-min periods every 0.5 h up to 3 h and hourly up to 8 h. All counts were corrected for background and the residual activity at a given time expressed as a percentage of initial counts.

During each study period, subjects remained fasted and supine, and smoking was not permitted. Room temperature was maintained constant at 22°C.

Values for the association states of the four preparations, measured at pharmaceutical concentration, were used as previously reported (hexameric 2 Zn^{2+} human insulin, 6; analogue Asp^{B10}, 2.2; analogue Asp^{B28}, 1.3;

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TABLE 1
Association states and absorption rates

Insulin	Association state	Initial absorption rate (%/h)	Absorption (min)		
			t_{10}	t_{25}	t_{50}
Hexameric 2 Zn^{2+} human	6	20.7 ± 1.9	35.4 ± 2.8	92.6 ± 11.9	182.9 ± 14
Asp^{B10}	2.2	44.4 ± 2.5	14.0 ± 1.6	36.6 ± 3.3	94.1 ± 6.2
Asp^{B28}	1.3	50.6 ± 3.9	14.7 ± 2.2	36.9 ± 3.1	83.4 ± 7.5
Asp^{B9},Glu^{B27}	1.1	67.4 ± 7.4	10.3 ± 2.3	27.4 ± 4.5	62.6 ± 8.7

Association states, mean ± SE initial fractional absorption rates, and time (t) to 10, 25, and 50% absorption for soluble hexameric 2 Zn^{2+} human insulin and 3 insulin analogues.

and analogue Asp, 1.1) (30; Table 1). These values represent the ratio between the osmotic pressure of the analogue and six times that of 2 Zn^{2+} human insulin (hexameric at this concentration with calculated mean relative M_r 37,500).

Statistical analysis. Results are expressed as means ± SE, unless otherwise stated. Comparison between multiple group means was by repeated-measures analysis of variance. Individual pairs of treatment points of interest were analyzed by Student's paired t tests. Tukey's honestly significant difference test for multiple comparisons was used to test between the different preparations. $P < 0.05$ was significant. Correlation between two variables was by Pearson's method. Linear regression, by the method of least squares, was used to analyze the log-transformed disappearance curves. The absorption rate constants (or fractional disappearance rates) thus obtained represent the value K ($\times 100$ to give %/h) in the equation $R_t = R_0 \cdot e^{-Kt}$, where R_t is the amount of radioactivity at the injection site at time t hours after injection.

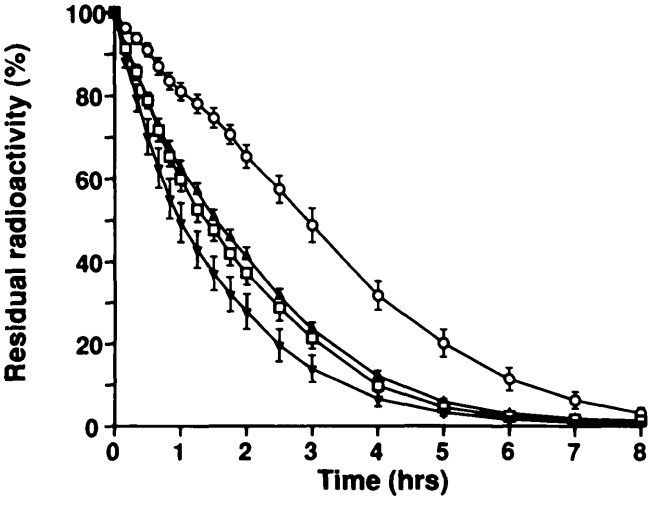

FIG. 2. Mean ± SE residual radioactivity at injection site after injection of 0.6 nmol/kg of ^{125}I-labeled preparations of soluble hexameric 2 Zn^{2+} human insulin (○), dimeric insulin analogue Asp^{B10} (▲), analogue Asp^{B28} (□), and monomeric analogue Asp^{B9},Glu^{B27} (▼) to healthy men (n = 7).

RESULTS

Figure 2 shows the mean disappearance curves for the four preparations tested. Because there was no statistically significant difference between the mean disappearance curves for the 2 study days involving hexameric human insulin at any measured time point between 0 and 480 min after injection (e.g., time to 50% absorption was 184 ± 11 min on the 1st occasion and 182 ± 20 min on the 2nd), the mean data derived from each subject's two Actrapid curves have been used. Figure 3 shows the log-transformed mean disappearance curves (data for analogue Asp^{B28} have been omitted for clarity). Linear regression analysis of the log-transformed data shows that disappearance from the injection site was linear for all four insulins during the initial period between 0 and 2 h postinjection. The mean initial absorption rate constants for the four

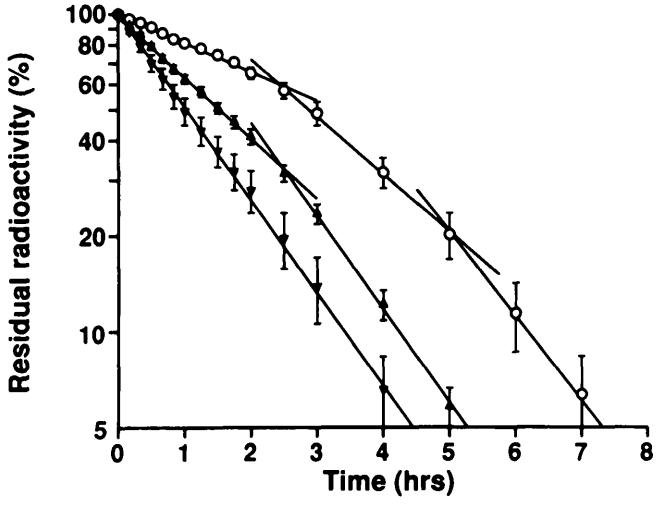

FIG. 3. Replot of mean ± SE disappearance curves (Fig. 2) of hexameric human insulin (○), dimeric insulin analogue Asp^{B10} (▲), and monomeric insulin analogue Asp^{B9},Glu^{B27} (▼) on logarithmic scale. Straight-line segments were calculated by linear regression analysis with data from relevant time intervals (see Table 2 for relative rates of absorption calculated by similar analysis on each subject's individual curves).

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preparations were 20.7 ± 1.9 %/h (hexameric soluble human insulin), 44.4 ± 2.5 %/h (dimeric analogue Asp^{B10}), 50.6 ± 3.9 %/h (analogue Asp^{B28}), and 67.4 ± 7.4 %/h (monomeric analogue Asp^{B9}, Glu^{B27}; Table 1). Absorption of the dimeric analogue was significantly faster than that of hexameric 2 Zn^{2+} human insulin ($P < 0.001$); absorption of monomeric insulin analogue Asp^{B9}, Glu^{B27} was significantly faster than that of dimeric analogue Asp^{B10} ($P < 0.01$). Absorption of analogue Asp^{B28} (mixture of dimeric and monomeric units) was intermediate between that of Asp^{B10} and Asp^{B9}, Glu^{B27} but was not significantly different compared with either analogue.

From each subject's individual curves, times to 10, 25, and 50% absorption (corresponding to times to 90, 75, and 50% residual activity, respectively) were calculated by linear interpolation between two measured time points on the log-transformed disappearance curves (Table 1). Analysis of these data shows essentially the same differences between the preparations as for the initial fractional absorption rates.

There were significant linear correlations between the measured association states of the preparations at pharmaceutical concentration and their initial absorption rate constants ($r = -0.98$, $P = 0.02$; Fig. 4) and between association state and time to 10, 25, and 50% absorption in minutes ($r = 0.98–0.99$, $P < 0.01–0.02$; Fig. 5).

By linear regression analysis of the log-transformed data (Fig. 3), three phases with increasing absorption rates could be identified for the hexamer and two for the dimer. No discernible lag phase could be attributed to the monomeric analogue, absorption being monoexponential from injection onward. The mean ± SE rates of subcutaneous absorption (calculated as the slope of the regression lines for the logarithms of the residual radioactivity at the injection site vs. time with all measurements in the indicated time interval) from each subject's individual curves are shown in Table 2. For the hexamer, the fractional absorption rate was significantly faster during the intermediate phase (2.5–5 h) than during the initial phase (0–2 h, $P < 0.001$) and significantly faster during the final phase (5–8 h) compared with the intermediate phase ($P < 0.01$). For the dimer, the fractional absorption rate was significantly faster during its second phase (2.5 h until time to 5% residual counts) than in its initial phase (0–2 h, $P < 0.001$). The fractional absorption rate of the hexamer during the intermediate phase (2.5–5 h postinjection) was similar to that of the dimer during its initial course (0–2 h). The fractional absorption rate of the hexamer during the last ~20% of its absorption course and that of the

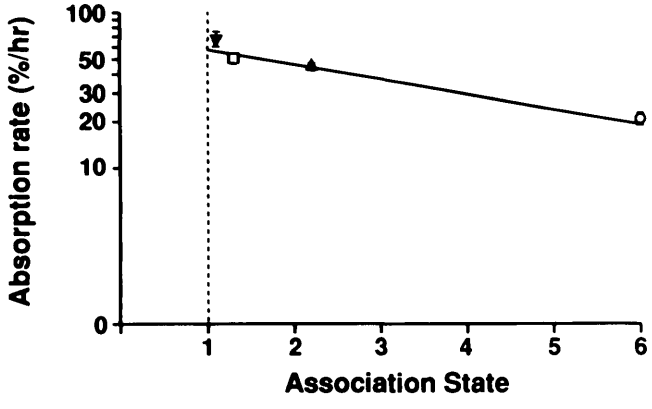

FIG. 4. Correlation between association state of hexameric 2 Zn^{2+} human insulin and insulin analogues Asp^{B10}, Asp^{B28} and Asp^{B9},Glu^{B27} and mean ± SE initial fractional absorption rates ($r = -0.98$, $P = 0.02$). ○, Soluble hexameric 2 Zn^{2+} human insulin; ▲, dimeric insulin analogue; ▼, monomeric analogue Asp^{B9},Glu^{B27}; □, analogue Asp^{B28}.

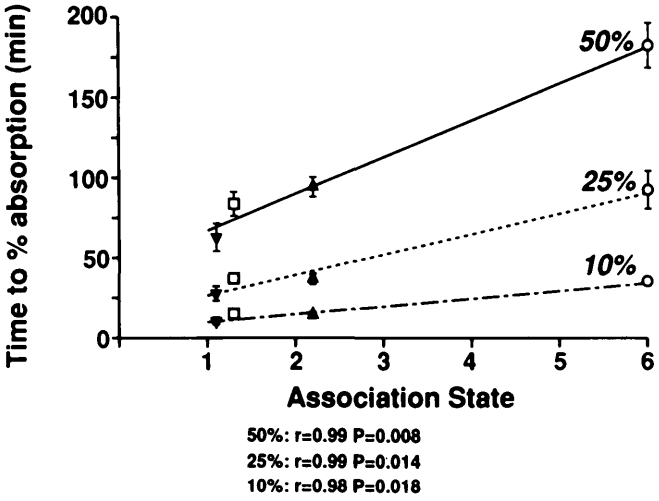

FIG. 5. Correlation of measured association states of hexameric 2 Zn^{2+} human insulin and insulin analogues Asp^{B10}, Asp^{B28} and Asp^{B9}, Glu^{B27} with times to 10, 25, and 50% absorption (corresponding to times to 90, 75, and 50% residual radioactivity, respectively); error bars are means ± SE. ○, Soluble hexameric 2 Zn^{2+} human insulin; ▲, dimeric insulin analogue; ▼, monomeric analogue Asp^{B9},Glu^{B27}; □, analogue Asp^{B28}.

TABLE 2
Rates of subcutaneous absorption (%/h)

Rate (h)	Human insulin	Insulin analogues	
		Asp^{B10}	Asp^{B9},Glu^{B27}
Initial			
0–2	20.7 ± 1.9	44.4 ± 2.5	73.3 ± 6.8*
Intermediate			
2.5–5	45.6 ± 5.0	68.9 ± 3.5†	
Final			
5–8	70.6 ± 6.3		

Mean ± SE fractional absorption rates were calculated (from each subject's individual curves) as slope of regression lines for logarithms of residual radioactivity at injection site vs. time with all measurements in indicated time intervals (Fig. 3).
*Calculated from 0 h until time to 5% residual radioactivity.
†Calculated from 2.5 h until time to 5% residual radioactivity.

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dimer during the last ~30% of its absorption course were similar to that of the monomeric preparation (Fig. 3, Table 2).

CONCLUSIONS

The demonstration in this study of a strong relationship between the association state of insulin or analogue and its rate of absorption from the subcutaneous injection site confirms for the first time that the size of the insulin molecular aggregate is a major determinant of absorption rate in humans and is in agreement with some animal studies (32). Factors explaining this relationship presumably include the more rapid diffusion of smaller insulin units (33) and the lesser restriction of smaller molecules at the capillary membrane (34).

Analysis of the disappearance curves on a log-linear scale reveals that only the monomer has a monoexponential course from the time of injection. The dimer has an initial slower phase followed by a phase in which the absorption rate is similar to that of the monomer. Soluble human insulin shows three phases: *1*) an early slow phase, *2*) a middle phase in which the absorption rate is similar to that of the initial phase of dimer absorption, and *3*) a late phase in which the absorption rate approaches that of the monomer. Together, with the relationship already demonstrated between association state and absorption rate, these findings strongly suggest that, in the late phases of absorption of soluble human insulin and the dimeric insulin analogue, both preparations are mainly in monomeric form, in the middle phase of its absorption course, human insulin is in largely dimeric form, and in the initial phase, it is in a more associated form.

Thus, these data support the hypothesis that, after subcutaneous injection, during the lag phase of absorption, hexameric insulin dissociates into dimers and monomers as a result of diffusion and dilution in the subcutaneous tissue (22). The differing absorption rates of hexamers, dimers, and monomers probably provides the biophysical explanation for the need to include several subcutaneous compartments or "pools" in the mathematical models of absorption kinetics of soluble insulin (22,23,26). Only the last ~20% of the soluble human insulin preparation and the last 30% of the dimer are absorbed at the rate of the monomer, presumably reflecting the high degree of dilution necessary for dissociation into monomeric units (Fig. 1). From this study, it is not clear whether in the early stages soluble human insulin is absorbed as both hexamers and newly formed dimers, or as dimers alone. However, contrary to previous opinion (5), it has been shown that the hexameric unit can be absorbed directly into the blood stream: a monomeric analogue is absorbed at 3.3 times the rate of the nondissociating cobalt hexamer of insulin in the pig (3,35). The similar ratio between the initial fractional absorption rates of the monomeric analogue and soluble insulin (Table 1) is also compatible with soluble insulin being initially partly absorbed in hexameric form in humans.

The known inverse relationship between both volume and concentration and the absorption rate of soluble insulin can now be explained by their influence on association state. The more rapid absorption of human compared with pork insulin (19) may also be understood in terms of the ability of hexameric insulin to dissociate: dilution of insulin in size-exclusion chromatography experiments indicates that human insulin dissociates more readily than pork insulin (3,32). Other factors (e.g., temperature) that affect blood flow and have an independent effect on association state (J.F. Hansen, unpublished observations) increase absorption rate by more than one mechanism. Moreover, increased blood flow itself will, by increasing absorption rate, presumably increase the rate of dilution and hence dissociation of the subcutaneous insulin depot, illustrating the complex interactions between factors affecting absorption. Thus, many of the factors influencing the day-to-day variation in insulin absorption would be expected to affect the rate at which hexameric insulin dissociates after subcutaneous injection.

In conclusion, the increasing relative rate of absorption of soluble human insulin after subcutaneous injection is explained by its dissociation into dimeric and monomeric units. We have already demonstrated the more rapid subcutaneous absorption of monomeric and dimeric insulin analogues accompanied by a more rapid onset of hypoglycemia in nondiabetic humans (36; S.K. et al. this issue, p. 942) and their biological efficacy after subcutaneous injection in diabetic subjects (37,38). It is probable that as well as abolishing the lag phase and increasing the rate of insulin absorption, monomeric insulin analogues reduce the intraindividual variation in insulin absorption, which currently exceeds 25% (39).

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Preliminary analysis of part of these data has been published previously.

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