Insulin-like Activity in Children as Measured by Epididymal Fat Pad Assay

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SUMMARY

Age-matched series of control and "latent diabetic" children were given intravenous glucose tolerance tests and sera obtained for assay of insulin-like activity (ILA). The rat epididymal fat pad method was used, measuring incorporation of C-14 of glucose-1-C-14 into both CO_2 and glycogen. Glycogen yielded the lower results and the greater statistical precision.

ILA was divided into "suppressible" and "nonsuppressible" fractions on the basis of inactivation by a guinea pig anti-insulin serum. Extraction with acid-ethanol did not increase the "suppressible" fraction, and added very little useful information to the study.

The chief difference between the two series was the smaller response of "suppressible" ILA after glucose in the "latent diabetics" than in the controls. This difference was most marked ($p < .001$) at fifteen minutes when the control response was maximal. By contrast, "nonsuppressible" ILA was not altered by a glucose load, but the baseline level was higher in the controls ($p < .05$).

In the fasting state, "suppressible" ILA was higher in the "latent diabetics" than in the controls when CO_2 was measured, but this difference was not seen with glycogen.

In each series, the fifteen-minute ILA response depended on the k value for rate of glucose disappearance as determined by simultaneous capillary blood glucose levels. But even when matched for similar k values, this response was greater in the controls than in the "latent diabetics." In the controls with k values > 2.3, "suppressible" ILA attained fifteen minutes after glucose showed an upward trend with increasing age. DIABETES *16*:695-703, October, 1967.

The purpose of this study was to determine normal serum levels of insulin-like activity (ILA) in children, as measured by rat epididymal fat pad assay, and to compare these in an age-matched series with the levels of ILA in children suspected of having latent diabetes.

Intravenous glucose tolerance tests (GTT) were performed, and sera obtained at timed intervals for assay. In a manner similar to that used by Samaan et al.[1] and by Froesch et al.,[2] each was divided into "suppressible" and "nonsuppressible" fractions of ILA on the basis of inactivation by a guinea pig anti-insulin serum (GPAS). In addition, each was subjected to acid-ethanol extraction to determine "extractable" ILA.

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EXPERIMENTAL SUBJECTS

Thirteen control and thirteen latent diabetic children constituted the study. As listed in table 1, they were matched as closely as possible for age, sex, weight, and sexual maturation. The term "latent diabetics," in contrast to its usual usage, refers here to subjects who fulfilled the following criteria: (1) a past history of glucosuria and/or grossly abnormal GTT (k value $<$ .7), (2) a strong family history of diabetes (a sibling, a parent, or at least three more distant relatives), and (3) did not yet require insulin therapy. Control subjects were hospitalized children who were awaiting elective procedures or had undergone plastic or orthopedic surgery five or more days previous to being studied. They fulfilled the following criteria: (1) no signs, symptoms or family history suggestive of diabetes, (2) normal growth and development, and (3) absence of any illness or the use of any medication known to alter carbohydrate metabolism. All the children in both series had been afebrile, active, and eating regularly for at least three days prior to the GTT.

METHODS

Glucose tolerance tests

One gram per kilogram of glucose, to a maximum of fifty grams was given by intravenous infusion as a 20 per cent solution over a five-minute period. Peripheral venous blood was obtained for assay just prior to the infusion, and fifteen and thirty-five minutes after the midpoint of the infusion. In addition, capillary blood was obtained at ten- to fifteen-minute intervals for glucose concentration which was measured in Somogyi filtrates[3] by the glucose oxidase method (Glucostat,

TABLE 1

Subjects by pairs (pairs were matched according to age, sex, weight, and sexual maturity. Data concerning individual glucose tolerance and diabetic history are included.)

Latent diabetics							Controls				
Past history	Family history	Age (yrs.)	Sex	Wgt. (kg.)	Blood glucose at 10 min. (mg. per 100 ml.)	k value	Age (yrs.)	Sex	Wgt. (kg.)	Blood glucose at 10 min. (mg. per 100 ml.)	k value
Glucosuria when ill	Three paternal relatives	4	M	17	320	1.9	3	M	15	404	1.7
Glucosuria when ill	Identical twin	3	F	18	265	1.7	5	F	16	278	3.5
Glucosuria when ill	One paternal and two maternal relatives, including grandmother	5	M	20	390	1.8	5	F	20	404	2.2
Glucosuria when ill	Sibling	5	F	21	315	3.9	5	F	23	420	2.5
Glucosuria when ill	Father, maternal grandmother. (Mother hyperthyroid)	6	M	15	286	2.8	6	M	22	380	2.9
k value .7 when ill	Mother	7	F	22	338	2.0	8	F	26	435	2.7
Glucosuria when ill	Five maternal relatives	10	M	24	368	1.6	9	M	26	517	1.9
Glucosuria & k value .6 when ill	Mother	10	F	35	240	2.4	11	F	36	360	4.9
Glucosuria when well	Sibling (another sibling is 16 yr. M below)	11	F	45	365	3.7	12	F	55	282	1.8
Glucosuria when well	Sibling	13	F	42	420	3.7	12	F	36	420	4.3
Glucosuria and abnormal oral GTT when ill	Mother	13	F (menses)	43	370	1.7	11	F (menses)	48	510	3.5
Formerly proved "renal glucosuria"	Sibling	14	F (menses)	56	344	2.8	13	F (menses)	55	470	1.8
Formerly proved "renal glucosuria"	Sibling (another sibling is 11 yr. F above)	16	M	68	275	2.8	14	M (20 per cent above ideal weight)	75	320	2.0

Worthington Biochemical Corporation, Freehold, New Jersey). The "k value" (rate of glucose disappearance from the blood) for each subject was calculated from the absolute blood glucose levels during the first hour.[4]

Preparation of guinea pig anti-insulin serum

GPAS was prepared by the injection of pork insulin into guinea pigs by the method of Moloney and Coval.[5] The same lot was used for all the assays, its potency being such that one milliliter was capable of neutralizing 0.12 U. of crystalline pork insulin. Thus the amount used in the assay could neutralize 36 mU. per milliliter of original serum which was ten to twenty times the maximum amount of ILA present in any of the samples. In concentrations used in the assay, the GPAS contained no ILA.

Preparation of blood samples

The blood samples were allowed to clot at room temperature for a maximum of forty minutes and then centrifuged at 3,000 rpm at 4° C. Each serum was then divided into two aliquots which were processed imme-

diately, as follows:

Aliquot 1—*Dialysis alone.* Two milliliters of serum were dialyzed* against three changes of 50 × serum volume of Krebs bicarbonate buffer for a total of forty-five hours at 4° C.

Aliquot 2—*Extraction and dialysis.* One milliliter of serum was extracted with 3 ml. acid-ethanol† for five hours at 4° C. This was centrifuged and the precipitate re-extracted for sixteen hours with 0.5 ml. H_2O and 1.5 ml. acid-ethanol, and recentrifuged. The supernatants from the two extractions were combined and dialyzed against 20 per cent polyvinylpyrrolidone for twelve hours, then against two changes of buffer for a total of twelve hours at 4° C.

Just prior to the assay, the 2-ml. aliquots subjected .o dialysis alone were made up with buffer to 6.8 ml. and divided into two 3.4 ml. portions, to one of which was added 0.6 ml. GPAS at 1:20 dilution with buffer (for "nonsuppressible" ILA), and to the other, an equal volume of buffer alone (for "total" ILA). The 1-ml. samples subjected to extraction were made up to 3.4 ml. with buffer; to one of the fifteen-minute samples was added 0.6 ml. GPAS, and to all the other samples an equal volume of buffer alone (for "extractable" ILA).

Assay for insulin-like activity

The rat epididymal fat pad assay technic was used, and the incorporation of C-14 of glucose-1-C-14 into CO_2 and glycogen was used as the measure of ILA. These are referred to subsequently as CO_2 production and glycogen "synthesis." The animals and procedures used have been described elsewhere.[6] They were modified for the present experiments only by the use of the random block design shown in figure 1 and described below:

The assay for each subject consisted of two blocks of three rows each, a single row consisting of nine vials: three for insulin standards‡ of 50 and 100 and 200 μU. per milliliter, and six for the unknowns. The unknowns in the first block were the three timed sera with and without GPAS; in the second block were duplicates of the fifteen-minute serum with and without GPAS, extracts of all three timed sera, of which only the fifteen-minute extract was run both with and without GPAS. All of these unknowns were run at 1:4 dilution of the original serum.

For each row of nine vials, three rats were killed, their epididymal fat pads divided into nine segments, and one segment from each placed into each of the nine vials. Thus each unknown was run in triplicate, each triplicate vial containing three pieces of fat, 1 ml. of assay medium, and 0.12 μc. of glucose-1-C-14.* Each value reported, therefore, represents the average metabolic activity of fat from nine different rats, and the average of two countings of radioactivity of each planchet.

In most assays, the first block was internally controlled for dilutional effect by repeating the thirty-five-minute sample at 1:8 dilution as a tenth vial in each row.

Calculations and statistical analysis

The values for "total" and "nonsuppressible" ILA for each sample were direct results of the assay read against the subject's individual standard curve. "Suppressible" ILA was calculated by subtracting "nonsuppressible" from "total." All were multiplied by the dilution (4 ×) and expressed in μU. per milliliter original serum.

Statistical analysis was performed by a two-way crossed and nested analysis of variance, subtracting patient interaction as a significant variable ($p < .001$). Part of this interaction was due to inability to match the two series perfectly, and part to a difference in clinical state from one member of a series to another. The raw data, in cts./min./100 mg. fat/2-hr. incubation period, minus background, were used in the statistical analysis since there was no significant difference between the mean standard curves for the two series.

Student's *t* was used to determine the significance of the dilutional effect since the vials at 1:8 dilution were not included in the analysis of variance.

RESULTS

Results related to accuracy of assay

The *index of precision* (λ) for the standard curves for the first blocks of the twenty-six assays was .375 for CO_2 production and .298 for glycogen "synthesis"; for the second blocks, .338 and .287. The composite standard curves showed no significant curvature within the range of concentrations used, by either of the measures of ILA.

In figure 2 the *effects of doubling the dilution* of the thirty-five-minute sera are shown (1:4 to 1:8), and

*Dialysis tubing was 18/32 Visking Cellophane.

†Acid-ethanol for serum extraction was made by adding 5 ml. concentrated HCl to 250 ml. absolute ethanol.

‡Glucagon-free pork insulin, lot No. 499667 of the Eli Lilly Co.

*New England Nuclear Corporation, Boston, Mass. (Specific activity 2.42 millicuries/millimole.)

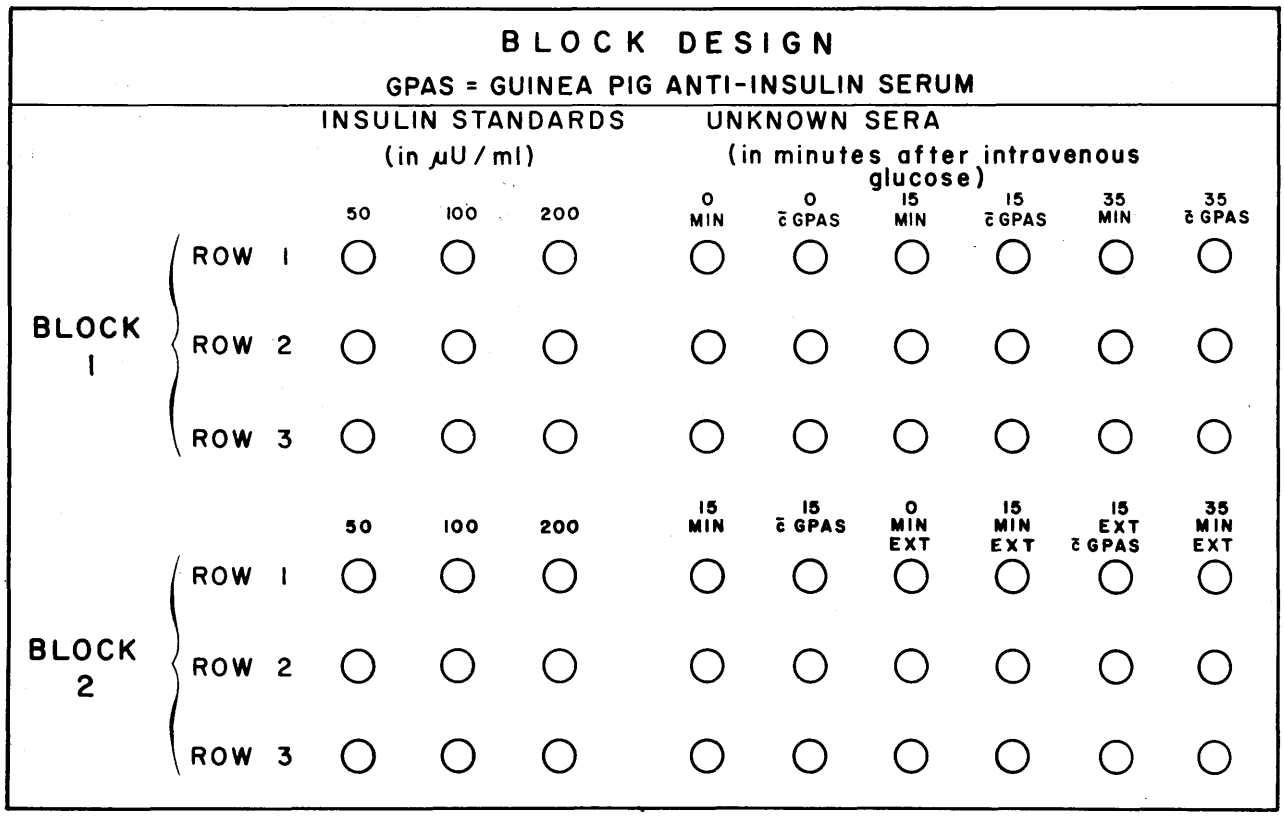

FIG. 1. Random block design used for the assay of each individual subject. All vials were run simultaneously. For each row, three rats were killed and a piece of fat from each was placed in each of the nine vials in that row.
Block 1: for "total" (without GPAS) and "nonsuppressible" (with GPAS) ILA.
Block 2: for "extractable" ILA and for comparing suppressibility by GPAS of "total" and "extractable" ILA.
All sera were run at 1:4 dilution.

the slope of the curve compared to that of the standard curve. The dilutional effect appeared less marked for serum than for the standards but the difference between the regression coefficients did not reach the level of significance. This was true for both CO_2 and glycogen, and for both series of subjects.

Results related to matched series

In figure 3 are given the *mean timed responses to intravenous glucose* of "suppressible," "nonsuppressible," and "extractable" ILA in the *control vs. the latent diabetic series.* The negative numbers for "suppressible" ILA at fasting time indicate that GPAS raised the ILA of the samples rather than lowering it. This was frequently true of fasting sera, and occasionally of thirty-five-minute sera. This elevation, however, was not statistically significant for either series of thirteen subjects. (See also table 2.)

The response to glucose in the control series was maximal at fifteen minutes in "suppressible" and "extractable" ILA. "Nonsuppressible" ILA did not change significantly in either series after glucose infusion.

The means for the control series were significantly greater than those for the latent diabetic series for each of the following fractions of ILA: (1) "suppressible" ILA at fifteen minutes (CO_2: $p < .01$, Glyc.: $p < .001$), and at thirty-five minutes (CO_2 only: $p < .03$); (2) "nonsuppressible" ILA at fifteen and thirty-five minutes (Glyc. only: $p < .05$); and (3) "extractable" ILA at fifteen minutes (CO_2: $p < .01$, Glyc.: $p < .001$), and at thirty-five minutes (Glyc. only: $p < .001$).

Results obtained from glycogen "synthesis" and from CO_2 production were compared. Glycogen gave the more significant separation between the two series at fifteen minutes in "suppressible," "nonsuppressible," and "extractable" ILA (see p values in the preceding paragraph). In only two instances were the means using CO_2 production significantly different for the two series when means using glycogen "synthesis" were not. First, the "suppressible" ILA in the fasting state was higher in the latent diabetic than in the control series (CO_2 only:

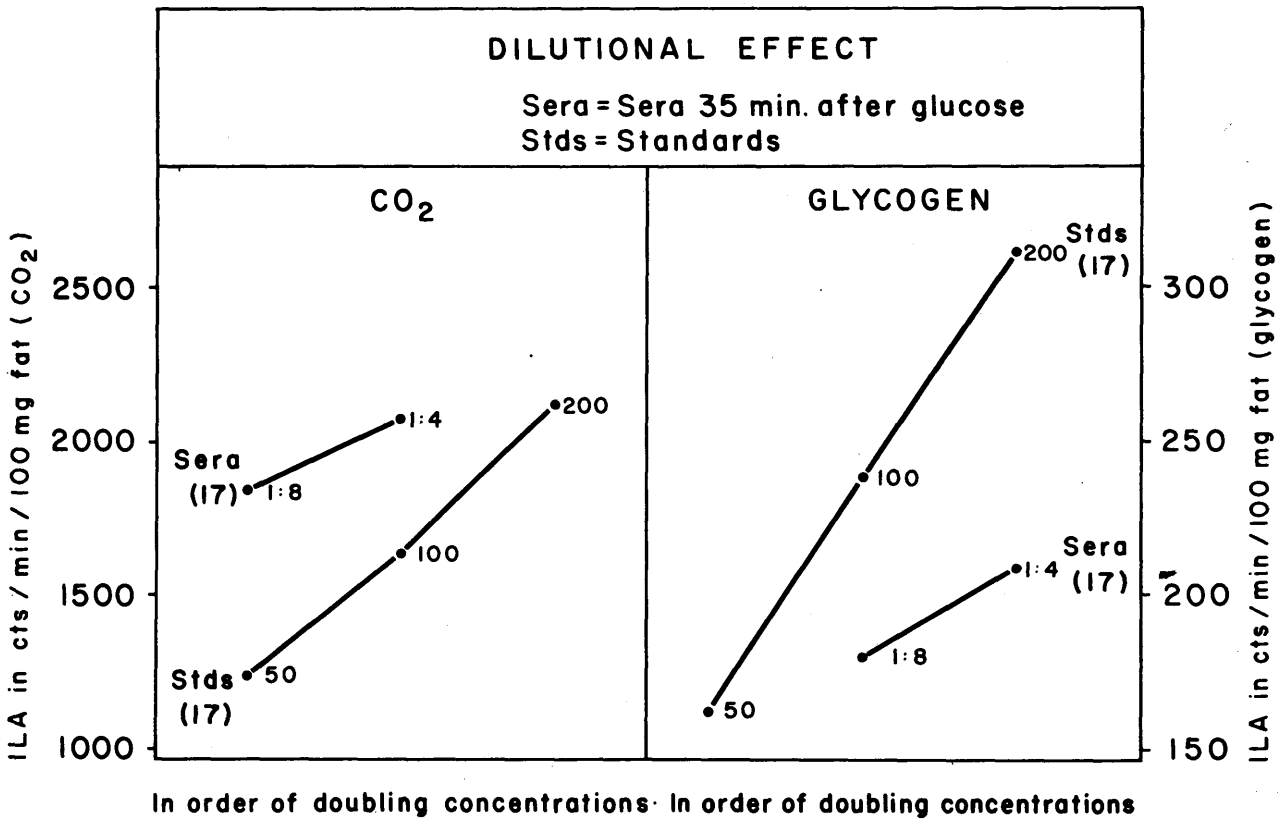

FIG. 2. Dilutional effect. Compares the effect of diluting the thirty-five-minute sera to one-half strength with that of diluting the insulin standards to one-half strength. Neither by CO_2 nor by glycogen were the regression coefficients for the sera significantly different from those of the standard curves. Results are expressed in counts/min./100 mg. fat.

TABLE 2

Mean timed responses to intravenous glucose in subgroups of subjects divided according to k value

		Number of subjects	CO_2 μU./ml. original serum at: 0 min.	15 min.	35 min.	Glycogen μU./ml. original serum at: 0 min.	15 min.	35 min.
"Suppressible" ILA								
$k > 2.3$	Controls	(7)	—76	700	—36	—12	448	68
	Latent diabetics	(7)	632	52	28	—12	128	60
$k < 2.3$	Controls	(6)	—800	1,656	3,004	—56	224	184
	Latent diabetics	(6)	—4	76	—12	—32	8	24
"Nonsuppressible" ILA								
$k > 2.3$	Controls	(7)	668	684	756	308	328	344
	Latent diabetics	(7)	804	676	684	288	252	264
$k < 2.3$	Controls	(6)	2,000	1,700	1,228	344	304	312
	Latent diabetics	(6)	548	444	588	248	240	224

Controls and latent diabetics were both divided into two subgroups with k values above and below 2.3. Both "suppressible" and "nonsuppressible" ILA by glycogen, at fifteen minutes after glucose, were higher for controls than for latent diabetics in the same k range, and higher for controls (or latent diabetics) with higher k values than for those in the same series with lower k values. ILA by CO_2 was disproportionately higher in the controls with lower k values, both in the "suppressible" and "nonsuppressible" fractions. Results are expressed in μU. per milliliter of original serum.

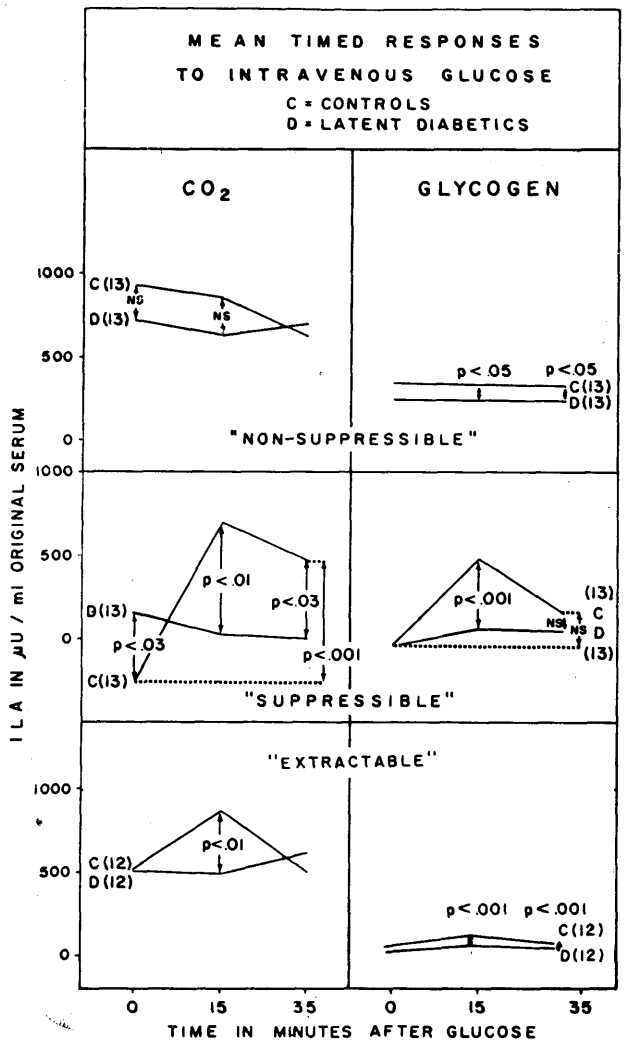

FIG. 3. Mean timed responses to intravenous glucose of "suppressible," "nonsuppressible," and "extractable" ILA in the control vs. the latent diabetic series. Results by CO_2 and by glycogen are treated separately; each is expressed in counts/min./100 mg. fat.

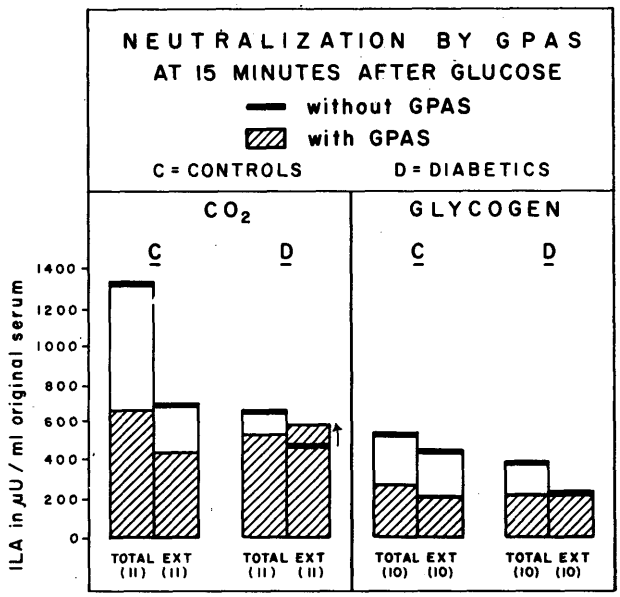

FIG. 4. Neutralization by GPAS at fifteen minutes of the "total" vs. the "extractable" ILA, in controls vs. latent diabetics. Results by CO_2 and by glycogen are treated separately. The proportion of ILA suppressible with GPAS did not increase after extraction, and approached zero in the extracts from the latent diabetics. Results are expressed in μU. ILA per milliliter of original serum.

$p < .03$). Second, the "suppressible" ILA at thirty-five minutes remained elevated in the controls (CO_2 only: $p < .001$).

Glycogen "synthesis" yielded lower values for ILA than did CO_2 production. This was particularly true of the "nonsuppressible" and "extractable" fractions. For the "suppressible" fraction, results using both measures of ILA were in the same general range, but glycogen "synthesis" again yielded the lower values in those instances where insulin release was being measured (controls at fifteen and thirty-five minutes).

Figure 4 compares the *GPAS suppressibility* of the "total" and of the "extractable" ILA at fifteen minutes, according to results obtained within the same (second) assay block. The level of "extractable" ILA was 53 to 73 per cent as high as the "total." However, this procedure did not improve the suppressibility of the samples with GPAS. The percentage of activity suppressed from the "total" vs. the "extractable" was, for the controls, 50 per cent vs. 40 per cent by CO_2 production and 52 per cent vs. 40 per cent by glycogen "synthesis." For the latent diabetic series, these values were 20 per cent vs. 0 by CO_2 production and 38 per cent vs. 0 by glycogen "synthesis."

Figure 5 gives the *relationship to age* in the "total" and "suppressible" ILA obtained in the control series fifteen minutes after glucose infusion. An upward trend with age was suggested by the controls with higher k values, but was not found to be significant when all thirteen control subjects were included. In "nonsuppressible" ILA there was no such trend. The control blood glucose levels attained ten minutes after the infusion, and the k values, had an almost straight line relationship throughout the ages studied, and could not have accounted for this trend.

Results related to k values

The control and latent diabetic series were each

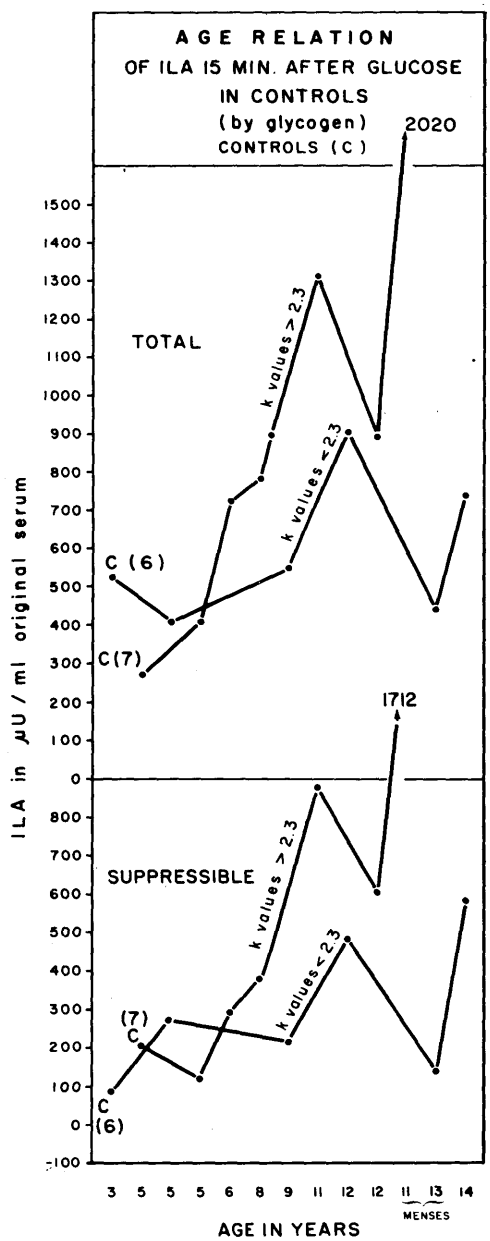

FIG. 5. Age relation of fifteen-minute ILA response in controls. Both the "total" and "suppressible" are plotted, and the controls separated according to their k values. There is an apparent increase with age in the controls with higher k values, but for the entire series of thirteen children, this increase was not significant over the age range studied. Only results by glycogen are given, and are expressed in μU. ILA per milliliter of original serum.

divided into subgroups of seven subjects with k values above, and six subjects with k values below 2.3. The average age of the children in the two control subgroups were comparable, but was younger in the latent diabetic subgroup with lower k values (seven years) than with higher (eleven years).

Table 2 gives the *mean timed responses to glucose infusion* of "suppressible" and "nonsuppressible" ILA in these subgroups *based on k values. "Suppressible"* ILA was greater in the controls than in the latent diabetics of the same k category, except at fasting time. In both the controls and latent diabetics with k values > 2.3, there was a peak at fifteen minutes, but this was higher in the controls. In both the controls and latent diabetics with k values < 2.3, there was a continued rise at thirty-five minutes (or only a slight fall), but again this was more marked in the controls, especially with the higher CO_2 values. Of the latent diabetics, those with lower k values had flatter glycogen responses than did those with higher k values.

"Nonsuppressible" ILA, when based on glycogen "synthesis," was similar in the two control subgroups and similar in the two latent diabetic subgroups, the former being higher than the latter. However, when based on CO_2 production, the controls with lower k values had much higher levels at all three times measured. "Extractable" ILA, though the data is not given, showed a peak at fifteen minutes in the control group with higher k values by both measures of ILA, but the responses were as flat for the control group with lower k values as for both latent diabetic subgroups.

For comparison, two newly diagnosed, overtly diabetic children (nonketotic, but requiring institution of insulin therapy) were tested by the same methods and showed levels of ILA which were comparable to those in the latent diabetic group with lower k values.

DISCUSSION

Insulin-like activity (ILA) was measured in this study by the rat epididymal fat pad assay. The incorporation of radioactivity of glucose-1-C-14 into both CO_2 and glycogen was measured and their reliability compared. Glycogen "synthesis" provided the better index of precision (λ), the higher peak in "suppressible" ILA response fifteen minutes after glucose, and the more consistent and significant differences between the control and latent diabetic series. The lower values it yielded, particularly for "nonsuppressible" ILA, suggested greater specificity for insulin in addition to being more precise. The chief value of CO_2 production would appear to be in the magnitude of difference between results obtained with it as opposed to those obtained with glycogen "synthesis." This difference possibly indicates an abnor-

mal or bound insulin, or other substance with metabolic activities differing from those of insulin.

Even incorporation into glycogen is not absolutely specific for insulin, as indicated by persistence of part of this activity by fat pad after pancreatectomy.[6,7] However, less activity persisted by glycogen "synthesis" than by CO_2 production in both these studies.

Dilution of serum has been shown to increase its ILA by the rat diaphragm assay, and to a lesser degree by fat pad, using CO_2 production,[8-10] net gas exchange,[11] or incorporation of radioactivity into lipid[12] as the measures of ILA. In this report, the dilutional effect on glycogen "synthesis" was also measured and was found to have regression coefficient similar to that for CO_2 production, but neither was significantly less than that for the corresponding insulin standards. This could be at least in part attributed to there being insufficient serum to include all subjects and a third dilution on each.

The experimental subjects in this study were found to have poor responses of "suppressible" ILA to glucose loading. Similar poor responses have been reported in more strictly chosen groups of prediabetic subjects, both by fat pad,[13,14] and by immunoassay.[15,16] Fasting ILA has been reported to be greater than normal in prediabetes.[13] In the present study this was true of "suppressible" ILA, but only by CO_2 production and not by glycogen "synthesis."

"Nonsuppressible" ILA was lower in the latent diabetic subjects than in the control subjects in this study as in former studies.[17,18] This suggests that the fraction is diabetes-related even though it is not suppressed by specific antibodies, and does not alter in response to a glucose load.[17] The nature of this fraction is unknown, but in the present experiments it stimulated CO_2 production to a greater extent than it did glycogen "synthesis."

The k values reported here cannot be compared with those found in other series of children[19,20] because of the higher glucose dosage used to stimulate maximal insulin release. However, the lower limit of normal suggested in this study (2.3) is consistent with the findings of Loeb.[19]

Six of the control subjects had k values lower than 2.3 and showed the pattern of ILA response seen in adult-onset diabetes with slower immediate rise, but continued or even exaggerated rise at thirty-five minutes. Three of these were adolescents and three were younger children.

Seven of the latent diabetic subjects had normal k values but were retained in the series because they fulfilled the initial criteria for selection. Both children with proven renal glucosuria, and the two others whose glucosuria had been discovered when they were well, were in this group. It cannot be stated whether the rapid disappearance of blood glucose might in part have been due to renal glucosuria or whether glucose was also being utilized or stored more rapidly. Von Euler and Larsson[20] found high k values in three children whom they considered to be prediabetic and suggested that this might be an early manifestation of their disorder.

Over a period of two to three years' follow-up, only one of the experimental subjects in this study developed overt diabetes (the identical twin, k value 1.7). This would suggest that, in children, poor insulin responses with lower mean levels of ILA following glucose might exist for a number of years without development of overt symptoms. The proposed possibility of an age relationship in ILA responsiveness to glucose should be considered when assays are performed on young children, so that a relatively low insulin response will not be interpreted to indicate diabetes.

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