Immunologic Assay of Insulin in Plasma of Children

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SUMMARY

A brief description of our experience with the immunoassay of insulin by the technic of Hales and Randle is given. Insulin values determined in twelve normal children and in seven cases of untreated diabetes mellitus in the fasting state and following a glucose load are presented. A newly diagnosed untreated child with diabetes mellitus had a low fasting insulin and gave very little response to test meal of glucose. Implications of these findings are discussed briefly.

The results of attempts to measure insulin in human blood have given confusing and often conflicting data.[1] Until recently the principal methods have been in the nature of bioassays. They have measured the response in a tissue (usually muscle or adipose) to the net effect of all the factors in plasma which enhance or antagonize the biological action of insulin. Because of the multiple factors present in plasma the quantitation of the results of these assays as a measure of insulin per se has been difficult.[2] Therefore, many consider that these assays measure something vague, usually referred to as insulin-like activity. The more precise and specific immunological methods have been developed to overcome the defects inherent in the biological methods. Yalow and Berson pioneered in this field and their immunoassay for plasma insulin was a major advance.[3]

In the investigations to be described, the Hales and Randle double antibody assay for insulin was employed.[4,5] A brief description of the assay and the results obtained in normal children and children with untreated diabetes mellitus will be given.

MATERIALS AND METHODS

Anti-human insulin serum was used in these experiments as it was found to give a more sensitive assay.[4] The human insulin was prepared from pancreases obtained at autopsy by the method of Smith.[6] It was injected into guinea pigs with complete Freund's adjuvant according to the method of Robinson and Wright.[7] (The anti-human insulin sera were supplied through the kindness of Dr. N. Hales, Cambridge University, England.) The insulin I-131 was of porcine origin and was obtained from the Abbott Pharmaceutical Company, Oak Ridge, Tennessee. The specific activity was always greater than 100 millicuries per milligram. Antibody to globulin of guinea pigs was prepared in rabbits by injecting subcutaneously 10 mg. of guinea pig gamma globulin in complete Freund's adjuvant at weekly intervals for seven to eight weeks. Antisera were obtained from the animals by cardiac puncture under light ether anesthesia, taking care to avoid hemolysis since it interferes with the assay. Antisera were stored at —20°C. in 0.04 M Tris buffer *p*H 7.4 in volumes sufficient for one day's assay. Merthiolate to a final concentration of 1:10,000 and bovine plasma albumin* to a concentration of 1 mg. per milliliter were added to all buffers. Phosphate buffer (*p*H 7.4 and 0.04 M) was used for the dilution of insulin I-131 and further dilution of the antisera. Crystalline bovine insulin generously provided by Dr. P. J. Moloney of the Connaught Laboratories, Toronto, was used as the standard insulin preparation. It was assayed at 22.5 units per milligram. All incubations were carried out in 5 x 1 cm. tubes at 4°C. Measurements were made with Hamilton Microliter syringes.† Filtration was performed in Pyrex Hydrosol Micro Analysis Filter Holders,‡ through two centimeter Oxoid membrane filters§ both obtained through the British Drug Houses, Queensway, Toronto. The precipitates on the membranes after filtration were washed with normal human plasma obtained from outdated blood. The plasma was passed through Hyflo Super Cel prior to use. Radioactivity was estimated by placing the membrane filters in 3 ml. of 1N HCl and counted in a well type scintillation counter using a channel selector. At least 1,000 disintegrations were counted for each sample.

Levels of blood glucose were determined by means of the AutoAnalyzer.|| Glucose tolerance tests were per-

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† Hamilton Corporation, Whittier, Calif.

‡ Millipore Filter Corporation, Bedford, Mass.

§ Oxo Limited, London, England

|| Technicon Instrument Corporation, Chauncey, New York

formed after an overnight fast of at least ten hours. One gram of glucose per pound of body weight up to 50 gm. was administered orally. Blood samples for insulin and glucose were obtained by venipuncture zero, thirty, sixty, ninety and 150 minutes. Coagulation was prevented by the use of heparin solution* or Sequester-Sol.†

PROCEDURE

The principle underlying the assay has been amply reviewed and will not be described in detail.[4] Insulin, insulin I-131 and anti-insulin serum when reacted together form a soluble complex. This complex can be isolated by chromatoelectrophoresis or by the use of a second antibody. The second antibody is an anti-guinea pig gamma globulin prepared in rabbits. The precipitate thus obtained can be isolated by filtration (Hales and Randle[4]) or centrifugation (Morgan and Lazarow[8]) and the radioactivity of the precipitate measured (figure 1).

The details of the procedure and the experimental rationale behind the steps have been published elsewhere.[4] Briefly, the antihuman insulin serum in appropriate dilution (previously determined) is precipitated with the anti-guinea pig gamma globulin (in appropriate dilution previously determined) for sixteen to twenty hours at 4°C. Standard insulin solutions (0-640 microunits per milliliter) or unknown (plasma) samples are then added and allowed to react for six to seven hours at 4°C. Finally the insulin I-131 (about 200 $\mu\mu$ or 4 μ units) is added and a further incubation of sixteen to twenty hours at 4°C. is performed (figure 2). The reaction mixtures are then filtered and washed and the radioactivity estimated. Each sample is assayed in duplicate or triplicate on at least two occasions. Results are expressed in microunits of insulin per milliliter of plasma. Hales and Randle have shown that the standard curves employing bovine and human insulin are very similar. Therefore the bovine insulin may be used as a standard for assaying insulin in human plasma. All plasma samples were assayed undiluted unless high insulin values were found and the appropriate dilutions were made.

RESULTS

Standard curves can be plotted in several ways (figure 3, 4). As can be seen, the greatest displacement of radioactivity has occurred at approximately 100 microunits. Therefore, small changes in concentration of insulin produced large changes in antibody bound radio-

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†Esbe Laboratory, Toronto, Ont.

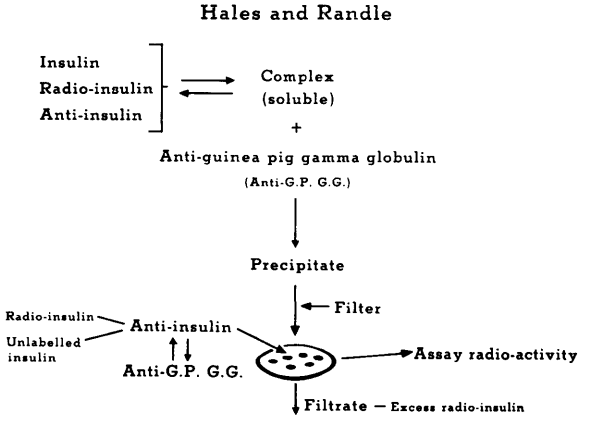

FIG. 1. Diagrammatic representations of the Hales and Randle double antibody assay for insulin. Radio-insulin = Insulin I-131.

Procedure for Immuno-Assay

1. Anti-guinea pig gamma globulin plus anti-insulin	16-20 hrs.
2. Addition of standards and unknown	6-7 hrs.
3. Addition of radio-insulin	16-20 hrs.
4. Filtration	
5. Wash filtrate	
6. Assay radio activity of washed precipitate	

FIG. 2. Steps in procedure of immunoassay for insulin (see text for details). Radio-insulin = Insulin I-131.

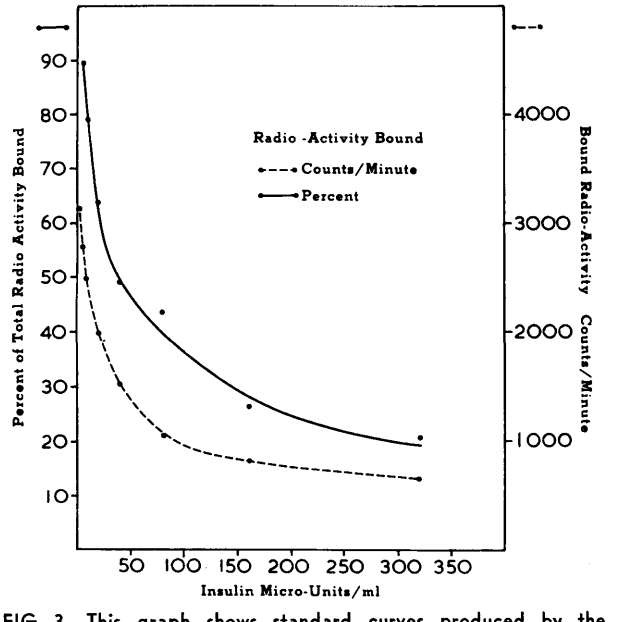

FIG. 3. This graph shows standard curves produced by the addition of known amounts of unlabeled insulin. Per cent total radioactivity added which is bound to antibody is plotted on the left ordinate. Total radioactivity bound is plotted on the right ordinate.

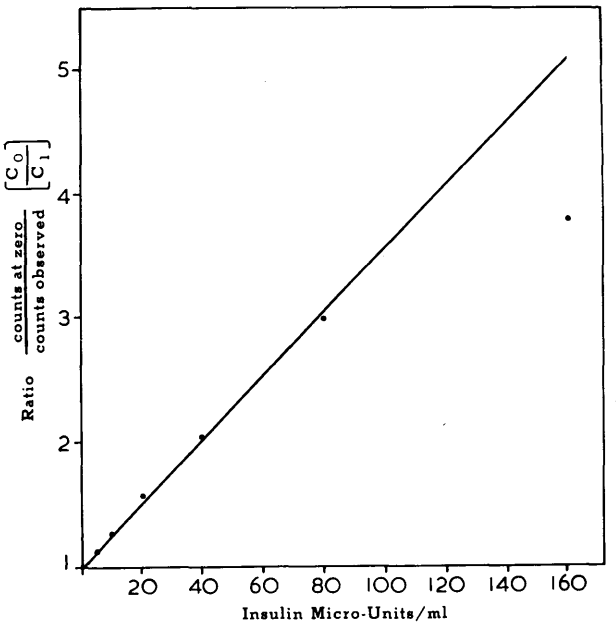

FIG. 4. Standard curve: the ratio of the counts bound at zero (Co) over the counts bound at various insulin concentrations (C_1) is plotted on the abscissa.

activity, giving a very sensitive assay.

Samples with high insulin content were diluted so that the value would fall in the range of 0-160 micro-units.

Figure 5 compares antisera from two guinea pigs and two different insulin I-131 preparations. As can be seen, the Hales preparation of insulin I-131 with antiserum No. 2 gave the steepest slope. The Abbott insulin I-131 gave a satisfactory slope with antiserum No. 2 but not with antiserum No. 4. Therefore it is important to determine the potency of the antiserum from each guinea pig separately. Standard curves were obtained with each assay. The results were pooled and a mean standard curve of ten assays was used to determine plasma insulin values.

The fasting blood glucose and fasting insulin values are shown in figure 6 for both normal and untreated diabetic children. In twelve normal children the range was 0-101 microunits per milliliter with a mean of 25. Levels of fasting glucose were all within the normal range. In the diabetics, all except one showed elevated fasting levels of blood glucose and low fasting levels of insulin. The one child with a normal or low fasting level of glucose revealed an elevated value for fasting insulin. Table I presents the results of glucose tolerance tests on a group of normal children, with their corresponding levels of plasma insulins.

Table 2 shows the results of glucose tolerance tests

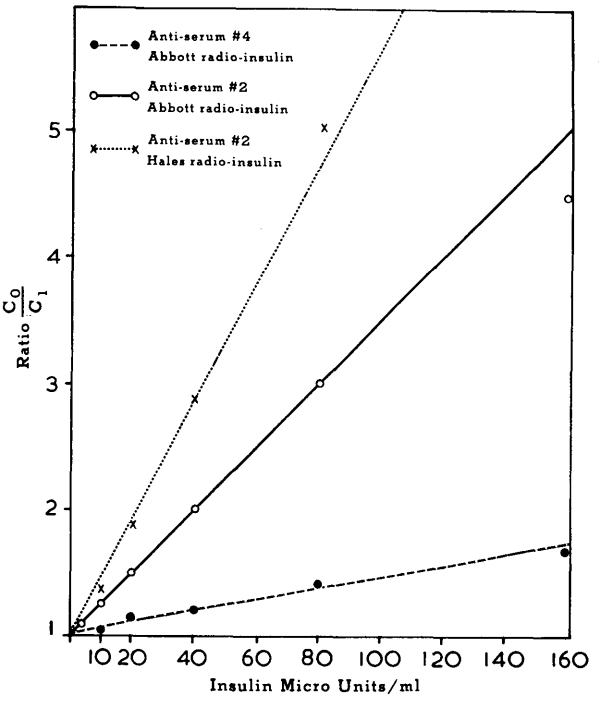

FIG. 5. A comparison of two insulin antisera and two insulin I-131 presentations is depicted. The dilution of each antiserum was the same. Radio-insulin = insulin I-131.

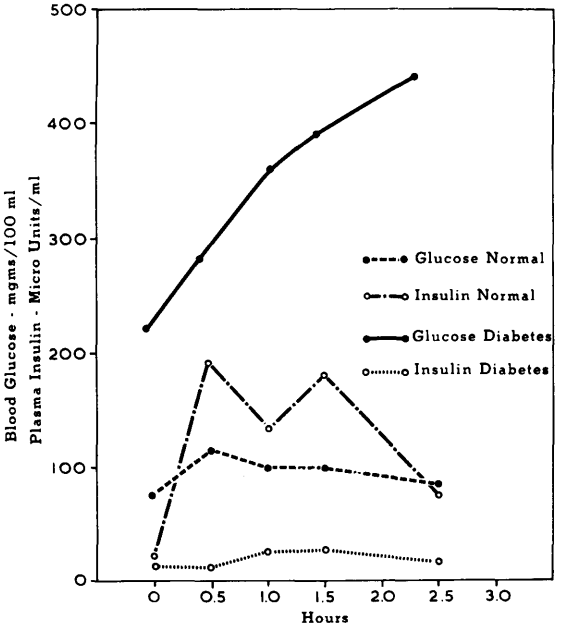

FIG. 6. Blood glucose and plasma insulin values of normal and diabetic children after a standard glucose tolerance test.

TABLE 1

Values for blood glucose and insulin in a group of normal children at various times after administration of glucose in a glucose tolerance test

		Normal									
		Blood glucose mg. per 100 ml.					Plasma insulin Microunits per ml.				
Case	Age (yrs.)	0	0.5	1.0	1.5	2.5	0	0.5	1.0	1.5	2.5
		(hours)					(hours)				
I.Z.	14	84	127	126	117	87	101	298	250	500	290
D.C.	5	83	143	119	105	90	24	295	160	230	109
B.H.	7	64	132	97	105	90	4	125	40	70	2
G.G.	6	75	83	83	109	80	ND*	250	95	160	6
T.M.	1½	69	80	88	93	69	ND*	60	180	40	ND*
D.P.	3	82	153	157	141	105	23	135	105	105	75
Mean		76	119	112	112	87	25	194	138	184	80
					S.D. ±		11.2	27.8	21.8	154	103
					S.E. ±		4.5	11.3	8.8	63	41.5

*ND — None Detectable.

in a group of seven diabetic children. Abnormal curves were found in all. Measurements of plasma insulin, which were all low, showed very little, if any, response to glucose. Tables 1 and 2 are depicted graphically in figure 6. The one case of the child with a normal fasting blood glucose differed from the others. The fasting insulin was elevated, with a rapid fall and then a rise in response to glucose (figure 7).

DISCUSSION

The development of immunological methods for insulin assay has led to some clarification in the field of blood insulin. In contrast to the biological methods the

TABLE 2

Values for blood glucose and insulin in a group of diabetic children

		Diabetes mellitus									
		Blood glucose mg. per 100 ml.					Plasma insulin Microunits per ml.				
Case	Age (yrs.)	0	0.5	1.0	1.5	2.5	0	0.5	1.0	1.5	2.5
		(hours)					(hours)				
P.B.	7	175	194	231	266	392	21	14	11	18	7
W.P.	10	216	312	404	426	433	ND*	ND*	52	ND*	2
T.M.	14	212	273	288	277	328	25	26	27	24	10
T.M.	14	212	261	338	330	341	ND*	14	ND*	35	5
J.R.	9	254	318	470	550	600	3	14	4	3	7
S.W.	10	164	241	308	320	369	15	5	13	24	21
D.R.	5	252	324	352	394	429	8	ND*	ND*	13	4
Mean		212	277	342	372	429	12	10	18	19	9
					S.D. ±		7.55	9.15	12.5	7.05	4.12
					S.E. ±		2.7	3.4	4.7	2.66	1.55
T.R.	3½	51	258	405	444	319	196	50		21	57

*ND — None Detectable.

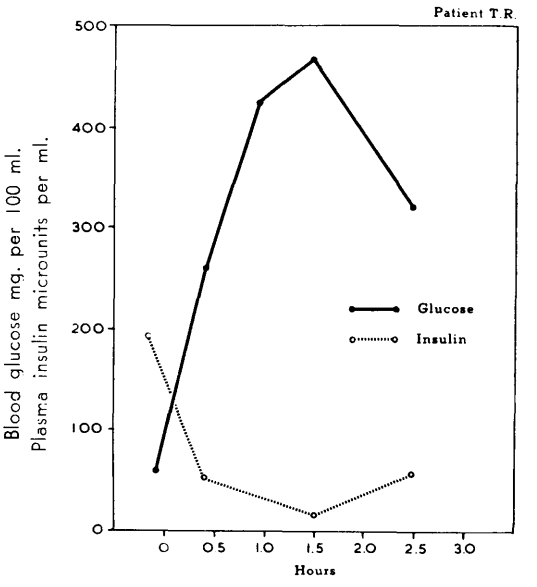

FIG. 7. Blood glucose and insulin values in patient T.R. (see text for details).

data from the immunoassays have been in close agreement with fasting blood insulin and insulin values following glucose stimulation.[4-6, 9-11] Our results complement those of others (Yalow and Berson, Hales and Randle, Samols and Marks) by showing that children have similar values for plasma insulin as do adults. The fasting insulin in infants and children, excluding the neonatal period, and the response to a glucose tolerance test are very similar to those found in adults. The differences are slight and may be due to technical error, although data are too insufficient to be certain of this interpretation at present.

The finding of low plasma insulin levels and little if any response to glucose stimulation in diabetics confirm the observations of Hales and Randle[11] who showed that in a group of adult diabetics, with the most severe abnormalities of carbohydrate tolerance, the level of plasma-insulin did not rise in response to a glucose load to the same degree as did milder diabetic and normal persons. They observed also that in this group the fasting plasma insulin was elevated. In a group of ketotic diabetics, plasma insulin was low. Six of our seven cases had severe abnormalities of carbohydrate metabolism as well as mild ketoses. The one case with a normal or even low fasting glucose and elevated fasting insulin was not ketotic. This child was the only case in which a drop in blood glucose was found within the two and one-half hour period of the test. It was associated with a small rise in

plasma insulin. That the plasma insulin in children with diabetes mellitus is low is not surprising considering their pancreatic insulin content.[12] In this connection it would be of interest to learn if there is any recovery of the pancreas' ability to produce insulin after the diabetes has been brought under control, since in many children there is an initial rise and then fall in the requirement of exogenous insulin as control is achieved.

The finding of an elevated level of plasma insulin in one case is of interest because there is now much evidence[5,13] to suggest that an elevated plasma insulin may be present prior to the onset of overt diabetes. The theory of Randle et al.[13] suggests that there is a block in the intracellular utilization of glucose, leading to an elevated blood glucose and in turn an elevated plasma insulin. The pancreas' ability to compensate by increased production of insulin may be the determining factor in the development of clinical diabetes.

SUMMARIO IN INTERLINGUA

Essayage Immunologic pro Insulina in le Plasma de Juveniles

Es presentate un breve description de nostre experientia con le immuno-essayage pro insulina secundo le technica de Hales e Randle. Es reportate le valores pro insulina determinate in dece-duo juveniles normal e in septe juveniles con non-tractate diabete mellite tanto in stato jejun como etiam post cargation con glucosa. Un novemente diagnosticate e non-tractate juvene con diabete mellite habeva basse valores pro insulina in stato jejun e respondeva pauchissimemente a un repasto experimental de glucosa. Le significations de iste constatationes es discutite brevemente.

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