

# INSULIN-I<sup>131</sup> METABOLISM IN HUMAN SUBJECTS: DEMONSTRATION OF INSULIN BINDING GLOBULIN IN THE CIRCULATION OF INSULIN TREATED SUBJECTS<sup>1</sup>

BY SOLOMON A. BERSON, ROSALYN S. YALOW, ARTHUR BAUMAN,<sup>2</sup> MARCUS A. ROTHSCHILD,<sup>3</sup> AND KATHARINA NEWERLY

(From the Radioisotope Service, Veterans Administration Hospital, Bronx, N. Y.)

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In recent tracer studies utilizing I<sup>131</sup> labeled insulin, *in vivo* and *in vitro* metabolic degradation of the labeled insulin has generally been presumed to parallel the disappearance of protein precipitable radioactivity and the appearance of non-precipitable radioactivity (1, 2). The present investigations were prompted by the paucity of data on the metabolic fate of insulin in human subjects and the need for a more specific identification of insulin-I<sup>131</sup> in blood and tissues than has heretofore been employed. The methods developed during this study have permitted quantitative evaluation of the rate of insulin-I<sup>131</sup> metabolism and have led to the discovery of an insulin-transporting globulin in the blood of insulin treated subjects.

## METHODS

Subjects were patients and healthy volunteer laboratory personnel of the Veterans Administration Hospital, Bronx, N. Y.

Regular crystalline insulin<sup>4</sup> was iodinated by the method of Pressman and Eisen (3) (Method A) or by Newerly's modification of their method as previously described (4) (Method B). Insulin-I<sup>131</sup> supplied by Abbott Laboratories was also employed in some experiments. The insulin-I<sup>131</sup> solutions prepared in our laboratory were dialyzed against distilled water with frequent changes for 24 to 72 hours, acidified to pH 3.0 with HCl, maintained sterile by the addition of phenol and cultured before use. These lots of insulin-I<sup>131</sup> contained an average of 0.1 to 1.0 iodine atoms per molecule, 12,000 molecular weight, insulin. As deduced from the blood sugar responses in fasting rabbits following intravenous administration of labeled and unlabeled insulin, there was no obvious loss in hypoglycemic potency as a result of the

manipulations involved in iodination even with preparations containing as many as 2 iodine atoms per molecule insulin of 23,000 M.W., which has been suggested as the functional unit of hormonal activity (5). For most of the *in vivo* experiments presented here, the labeled insulin contained an average of 0.1 to 0.5 iodine atoms per molecule 23,000 M.W. insulin.

The specific activity of the labeled insulin at the time of use ranged from 1.0  $\mu\text{c}$  to 7.5  $\mu\text{c}$  I<sup>131</sup> per microgram insulin. Doses of 15 to 200  $\mu\text{c}$  containing 0.1 to 7.0 units of insulin were administered intravenously to human subjects and urine collections and heparinized blood samples were taken at intervals thereafter. On the first day of the studies, insulin was withheld from patients receiving daily insulin therapy except in the case of schizophrenic subjects undergoing insulin shock treatment. Assays of radioactivity were carried out on urine, plasma and packed erythrocytes and on the filtrates and washed precipitates of plasma treated with cold 10 per cent trichloroacetic acid (TCA) and with 23 per cent sodium sulfate and ether according to the method of Kingsley (6). In many cases radioactivity in the filtrates of the sodium sulfate fractionations was also precipitated with cold 10 per cent TCA after the addition of carrier non-radioactive plasma. By these methods plasma radioactivity was identified as: (a) I<sup>131</sup> precipitable with TCA, (b) I<sup>131</sup> not precipitable with TCA, (c) precipitable I<sup>131</sup> salted out with Na<sub>2</sub>SO<sub>4</sub> and ether, and (d) precipitable I<sup>131</sup> not salted out with Na<sub>2</sub>SO<sub>4</sub> and ether. These same procedures were also carried out on control plasma samples to which insulin-I<sup>131</sup> was added *in vitro*.

Paper electrophoresis was carried out in barbital (pH 8.6) or phosphate (pH 7.3) buffers,  $\mu = 0.025$  to 0.10. Strips of Whatman No. 3 MM filter paper, 37 by 550 mm., were stretched horizontally across vertical plastic supports, the ends of the paper dipping down 10 cm. into the buffer vessels. A constant voltage supply was set at levels of 150 to 250 volts. At the end of "runs" lasting 6 to 24 hours, the currents registered were 1 to 2 ma per strip in buffers of 0.025 and 0.05 ionic strength and about 3 ma per strip in buffers of 0.1 ionic strength. After electrophoresis, the strips were stretched horizontally across aluminum pans without contact except at the ends and dry heated in an oven for 45 to 60 minutes at 115° C. After staining with naphthalene black<sup>5</sup> and destaining

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<sup>2</sup> National Heart Institute Research Fellow.

<sup>3</sup> Dazian Foundation Fellow.

<sup>4</sup> We are indebted to Doctors O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for a generous supply of crystalline regular insulin lot No. 535664 which is stated to contain 27 units per mg. This lot served as the source of all the I<sup>131</sup> labeled insulin prepared in our laboratory.

<sup>5</sup> We are indebted to the Imperial Chemical Industries, Ltd., Manchester, England for a generous supply of this dye.

with 10 per cent acetic acid in methyl alcohol, the plasma protein patterns were traced and the strips divided into 0.5 cm. or 1.0 cm. segments for assay of radioactivity. In some cases the paper strips were counted before and after staining in an automatic strip counting flow gas counter. The counting rates were recorded on moving paper by an Esterline-Angus recorder synchronized with the strip counter. Further details of the paper electrophoresis experiments are given under Results.

Starch block electrophoresis was performed according to the method of Kunkel and Slater (7) in barbital buffer; pH 8.6,  $\mu = 0.05$ , constant voltage 250 V. After electrophoresis, the blocks were allowed to dry out partly and were divided into 0.5 cm., 1.0 cm. or 2.0 cm. segments for assay of radioactivity.

Ultracentrifugation experiments<sup>6</sup> were carried out in a Spinco preparatory ultracentrifuge, model L. Duplicate specimens of plasma or serum (each 12 ml.) were centrifuged at 40,000 RPM for 7 to 14 hours. The tubes were then removed gently and successive 1- $\frac{1}{4}$  ml. portions were aspirated from top to bottom for assays of radioactivity and determinations of albumin and globulin concentrations. Nitrogen determinations were performed by the Kjeldahl procedure after fractionation of the serum proteins according to the method of Kingsley (6).

Radioactivities in blood and urine samples and in sectioned paper electrophoretograms were assayed in a 5-ml. capacity well-type scintillation counter with a sensitivity of  $1.00 \times 10^6$  counts per minute per  $\mu\text{c I}^{131}$  above a background of approximately 200 counts per minute. The automatic paper strip counter had a sensitivity of  $0.60 \times 10^6$  counts per minute per  $\mu\text{c I}^{131}$  above a background of 20 counts per minute. Starch block sections were assayed in a 10-ml. capacity well-type scintillation counter with a sensitivity of  $0.85 \times 10^6$  counts per minute per  $\mu\text{c I}^{131}$  above a background of 350 counts per minute.

*In vivo* counting rates over liver, thigh and kidneys were determined with a scintillation counter or a bismuth-wall Geiger counter connected to a Streeter-Amet printing register which recorded cumulative counts at  $\frac{1}{2}$  minute or 1-minute intervals.

To compensate for differences in the volume of distribution of insulin-I<sup>131</sup> in different sized human subjects, blood concentrations were generally plotted as body wt<sub>kg</sub>  $\times$  fraction of dose administered/liter plasma.

## RESULTS

Because of differences in the behavior of insulin-I<sup>131</sup> in insulin treated and non-insulin treated subjects, it is convenient to distinguish between "control" subjects and insulin treated subjects. As used here, "control" subjects refer either to normal healthy volunteers or hospitalized patients, dia-

betic or non-diabetic, who had never received injections of insulin prior to this study.

### I. *In Vivo* Experiments

#### A. Typical radioactivity concentration—time curves in plasma, RBC and urine following intravenous administration of insulin-I<sup>131</sup>

1. *Control subjects*: Following intravenous injection of insulin-I<sup>131</sup> in control subjects the concentration of radioactivity falls rapidly in the plasma and rises in the red blood cells and urine (Figure 1a). Even in the earliest collections, no more than 3 per cent of the urinary radioactivity is TCA precipitable with added carrier protein as contrasted with insulin-I<sup>131</sup> which, when added with carrier protein to urine *in vitro*, is almost entirely (95 to 100 per cent) TCA precipitable. Plasma radioactivity 2 to 4 minutes after injection is largely precipitable with TCA but very shortly thereafter the non-precipitable radioactivity becomes significant and progressively increases for about an hour. Although the concentrations of TCA precipitable radioactivity fall rapidly to quite low levels, the further disappearance of precipitable radioactivity after about 3 hours proceeds at a much slower rate with a half time ranging from 6 to 24 hours. Non-precipitable radioactivity reaches a peak at the end of about one hour, remains at a plateau for an hour or two, and then declines as a result of urinary excretion and an increasing volume of distribution. The curve of erythrocyte radioactivity parallels closely that of the non-precipitable plasma radioactivity at a concentration of about 55 to 60 per cent that of the latter. Since iodide<sup>131</sup> also distributes between RBC and plasma in this concentration ratio and since we have observed that most of the non-precipitable I<sup>131</sup> migrates with the same mobility as iodide<sup>131</sup> on paper electrophoresis, it is quite probable that plasma non-precipitable radioactivity is largely composed of iodide<sup>131</sup> which represents the metabolic end product of the radioactive moiety of the labeled hormone. Furthermore, the renal clearance of non-precipitable plasma radioactivity averages about 30 ml. per minute which is in good agreement with the average normal rate of iodide clearance (8). Rough calculations based on the concentrations of non-precipitable plasma radioactivity and of the space of distribution of iodide

<sup>6</sup> We are indebted to Mr. Ted Marcus in the laboratory of Dr. Ludwik Gross for aid in these experiments.

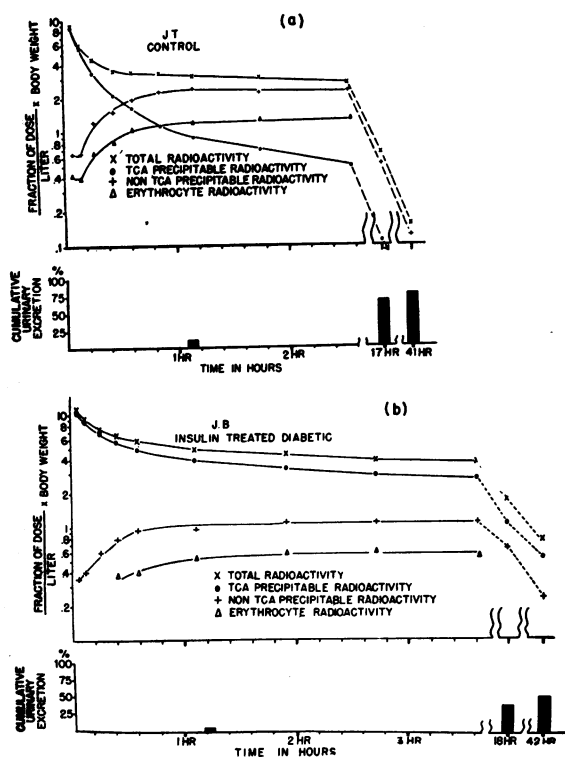


FIG. 1. TYPICAL CURVES OF RADIOACTIVITY IN PLASMA FRACTIONS, ERYTHROCYTES AND URINE AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $I^{131}$ , a) TO SUBJECTS NOT PREVIOUSLY TREATED WITH INSULIN ("CONTROL" SUBJECTS) AND b) TO SUBJECTS TREATED WITH INSULIN FOR MONTHS TO YEARS—BODY WEIGHT IN KILOGRAMS

indicate that from  $\frac{2}{3}$  to  $\frac{3}{4}$  of the injected insulin- $I^{131}$  is degraded within the first hour.<sup>7</sup>

2. *Insulin treated subjects:* Following insulin- $I^{131}$  administration to patients who have received injections of exogenous insulin for months to years, the general characteristics of the blood radioactivity curves are somewhat similar to those

<sup>7</sup> *E.g.*, In subject J. T. (weight 66 kg.), the concentration of non-precipitable  $I^{131}$  in the plasma at 51 minutes was 3.5 per cent per liter of the total radioactivity administered (Figure 1a). The approximate space of distribution of non-precipitable  $I^{131}$  at this time was probably about 17 liters since the space of distribution of iodide<sup>131</sup>, 30 minutes after intravenous injection, is about 26 per cent of body weight and does not change very much between about 10 minutes and an hour or so (8). To the non-precipitable  $I^{131}$  still in the body (17 L.  $\times$  3.5 per cent per L. = 59.5 per cent) must be added the 7 per cent excreted in the urine by this time to make a total of 66.5 per cent of the insulin- $I^{131}$  degraded in 51 minutes.

described above. However, the rates of decline of TCA precipitable radioactivity in plasma and of increase in RBC radioactivity and non-precipitable plasma radioactivity are strikingly slower than in control subjects (Figure 1b). Similarly, the early urinary excretions of radioactivity are significantly lower than in control subjects.

The early time courses of TCA precipitable plasma radioactivity are summarized for all subjects in Figure 2. The curves demonstrate a persistence of relatively high concentrations of precipitable radioactivity in the plasma of subjects treated with insulin for more than a few weeks. This group includes 14 patients with diabetes mellitus who had received insulin for 3 months to 17 years, 1 patient (S. P.) with acromegaly and diabetes who had received insulin for 2 years, and 1 schizophrenic patient who had received insulin for 6 weeks. In contrast, a much more rapid disappearance of precipitable radioactivity was observed in 22 non-diabetic control subjects who had never received insulin, 2 diabetic subjects (J. K., J. T.) who had never received insulin, 2 diabetic subjects (M. N., B. B.) who had received insulin for only 2½ and 3½ weeks, respectively, 1 subject (C. B.) with cortisone induced diabetes who had never received insulin and 1 schizophrenic subject (J. Ves.) who had received insulin for 3½ weeks. Subject M. N. showed a rapid disappearance of TCA precipitable radioactivity in the study performed 2½ weeks after insulin therapy was initiated (M. N.<sub>1</sub>) and a slow disappearance in the study performed after 4½ months of insulin therapy (M. N.<sub>2</sub>). Thus, the rate of disappearance of precipitable radioactivity for plasma was correlated only with the history of previous insulin administration and not with the presence or absence of diabetes.

#### B. Salting out characteristics of TCA precipitable radioactivity in plasma as a function of time following intravenous administration of insulin- $I^{131}$

When insulin- $I^{131}$  was added *in vitro* to control plasma samples, 90 to 102 per cent of the radioactivity was found in the globulin plaque after the salting out procedure and almost as high a recovery was obtained in venous plasma samples taken 2 to 3 minutes after injection of insulin- $I^{131}$

to control subjects. However, in subsequent samples taken from control subjects the fraction of precipitable radioactivity which was salted out by 23 per cent  $\text{Na}_2\text{SO}_4$  gradually decreased to about 50 per cent by 2 to 3 hours following administration of insulin- $I^{131}$  and persisted at that level without further decrease for another 24 to 30 hours (Figure 3). In contrast with the findings in control subjects, the salting out procedures performed on the plasma of insulin treated subjects almost always revealed a relatively high fraction of the TCA precipitable radioactivity in the globulin plaque even many hours after the administration of the labeled insulin (Figure 3).

### C. Analysis of insulin- $I^{131}$ preparations by zone electrophoresis

Since the following observations obtained in barbital or phosphate buffers throughout a range in ionic strength of 0.025 to 0.10, no distinction will be made in the buffer conditions of the various experiments. When tracer amounts of insulin- $I^{131}$  alone or in 10 to 50 microliters of plasma from control subjects are run on Whatman 3 MM paper, the radioactivity is almost quantitatively bound to the paper near the site of application (Figure 4a, b). However, if the paper is soaked in a solution of insulin (3 mg. per ml.) prior to

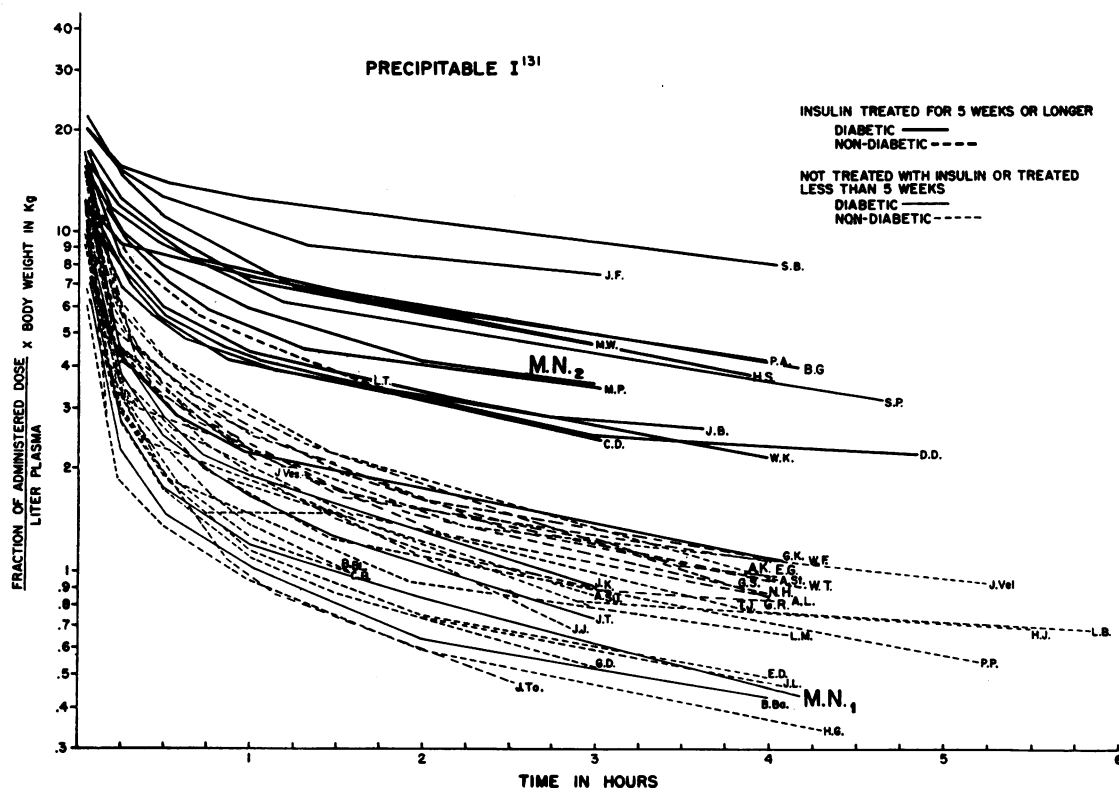


FIG. 2. TRICHLORACETIC ACID PRECIPITABLE RADIOACTIVITY IN PLASMA AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $I^{131}$

Two studies were performed on subject D. D. In the first of these insulin was withheld for 24 hours prior to intravenous administration of 0.1 unit insulin- $I^{131}$ . In the second study, carried out on the following day, 45 units protamine zinc insulin were given subcutaneously 1 hour prior to the intravenous administration of 7.0 units insulin- $I^{131}$ . The curves were virtually identical and are therefore represented as a single curve. Subject J. Ves. (schizophrenia) and subject L. T. (schizophrenia) received 640 units and 120 units regular insulin, respectively, by subcutaneous injection 45 minutes prior to the intravenous administration of insulin- $I^{131}$ . All other subjects received no stable insulin for at least 24 hours prior to these studies. M. N.<sub>1</sub>—after treatment with insulin for 2½ weeks; M. N.<sub>2</sub>—after treatment with insulin for 4½ months. See text for further identification of subjects.

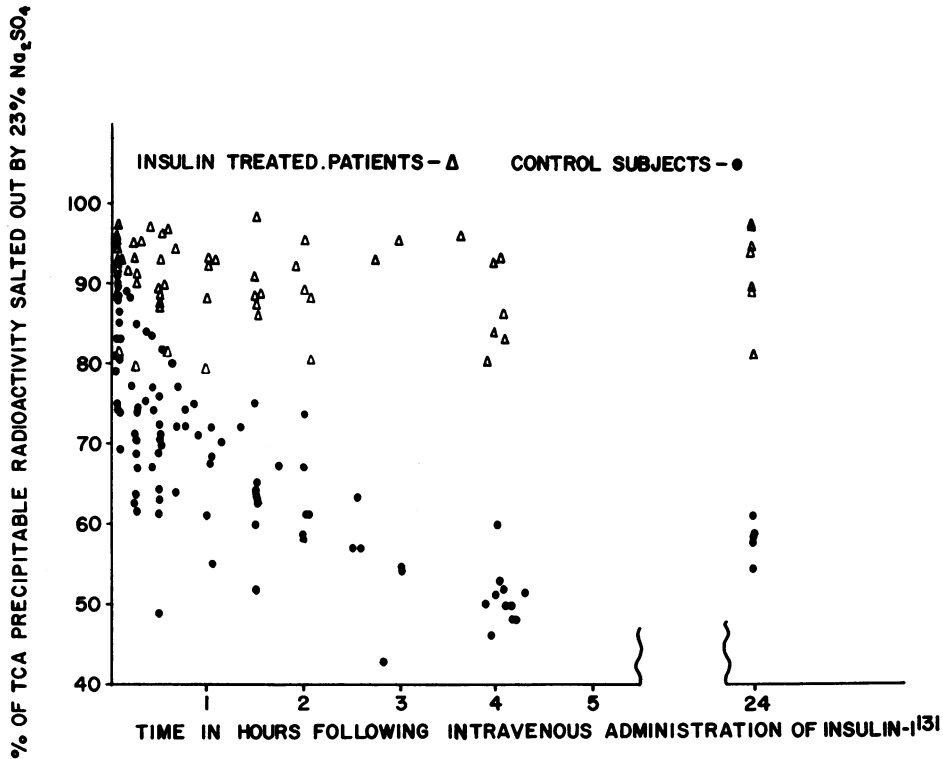


FIG. 3. PER CENT OF TRICHLOROACETIC ACID PRECIPITABLE RADIOACTIVITY WHICH WAS SALTED OUT BY 23 PER CENT  $\text{Na}_2\text{SO}_4$  AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $\text{I}^{131}$

electrophoresis in order to saturate the binding sites, the radioactivity migrates with a mobility somewhat slower than serum albumin whether or not plasma is present (Figure 4c, d). Under

the latter conditions the peak of the  $\text{I}^{131}$  labeled crystalline insulin prepared in our laboratory generally appeared in the  $\alpha_1$  region while in at least one lot prepared by the commercial supplier the

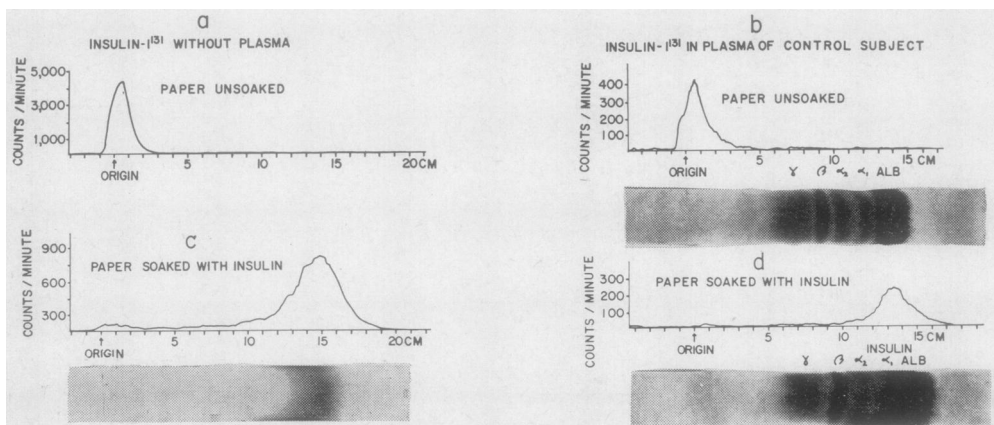


FIG. 4. PAPER RADIOELECTROPHORETOGRAMS: a, c) INSULIN- $\text{I}^{131}$  ALONE, b, d) INSULIN- $\text{I}^{131}$  IN PLASMA OF A CONTROL SUBJECT

a and b—papers not soaked in insulin prior to electrophoresis; c and d—papers soaked in insulin at origin prior to electrophoresis. Assays of radioactivity by automatic strip counter. The radioactivities which were recorded from the moving paper strip on a curvilinear ordinate scale have been projected on a rectilinear scale.

peak occurred between the  $\beta$  and  $\alpha_2$  proteins. These differences are probably related to variations in the electrophoretic mobility of different native crystalline insulin preparations previously reported by Hall (9) rather than to differences in the methods of iodination. Separation of insulin- $I^{131}$  from the plasma proteins is best demonstrated when the samples containing plasma and insulin- $I^{131}$  are applied along a line close to the cathodal vessel on papers not soaked with insulin and hydrodynamic flow<sup>8</sup> is permitted to move the whole plasma an inch or two towards the anode prior to or during electrophoresis. There is then a protein free zone between the insulin- $I^{131}$  radioactivity bound to the paper at the origin and the slowest migrating  $\gamma$  globulin proteins (Figure 4b).

With all lots of radioiodinated insulin, however, some of the radioactivity migrated with the serum protein fractions. In the most satisfactory preparations only about 2 to 5 per cent of the total activity migrated with the serum proteins, the remainder being adsorbed at the origin (Figure 4b). In some lots, however, as much as 25 per cent of the total radioactivity migrated with the serum proteins which, for reasons indicated below, rendered these preparations much less satisfactory for the study of insulin- $I^{131}$  metabolism.

The chemical nature of the radioactivity accompanying the serum proteins is not clear. Control studies with iodide $^{131}$  added *in vitro* to plasma indicated that less than  $\frac{1}{3}$  of 1 per cent of iodide $^{131}$  remains on the paper under the conditions of the experiments reported here, so that the radioactivity migrating with the serum proteins is definitely not due to iodide $^{131}$ , which would be unlikely in any event, since all preparations were dialyzed thoroughly. The possibility that contaminating proteins in the original crystalline insulin are responsible is rendered unlikely by the following experimental observations which indicate that the radioactive material migrating with the serum proteins is somehow produced in the iodination

<sup>8</sup> This is effected by leaving open the top of the vapor-saturated enclosure employed for electrophoresis. Under these conditions evaporation of water from the paper strip produces a concentration of buffer salts on the paper thus causing fluid to be drawn up osmotically from the buffer vessels. This produces a rapid movement of the plasma towards the center of the paper strip. If the plasma is applied near the cathodal vessel, hydrodynamic flow moves the plasma in the direction of the anode.

procedures or by other unknown factors. A small quantity of crystalline insulin was divided into two portions. To one portion was added the neutralized iodination mixture used in Method A (3) which contains  $\text{NaNO}_2$ ,  $\text{KI}^{127}$  and  $^{131}$ ,  $\text{NaCl}$  and elemental iodine $^{127}$  and  $^{131}$  at a pH of 8.0. From a similar mixture at a pH of about 1.0, elemental iodine was extracted with chloroform according to Method B(4) and added to the other portion of crystalline insulin. At the end of one hour, half of each portion was removed and dialyzed immediately against water for 48 hours. The remainder of each portion was permitted to remain with the iodinating solutions for 48 hours, after which dialysis was carried out in the same manner. All procedures were performed at about 4° C. One ml. of the same plasma was added to a minute amount of each of the four preparations and the mixtures were simultaneously subjected to electrophoresis on paper. In the insulin- $I^{131}$  solutions prepared by iodination for one hour only a small fraction of the total activity migrated with the serum proteins (Figures 5a, b), while the solutions prepared by iodination for 48 hours contained significantly larger fractions of migrating components (Figures 5c, d).<sup>9</sup> These findings were reproducible. Differences in the amounts of iodine bound to the insulin did not appear to be a significant factor. The alterations of insulin- $I^{131}$  which caused a variable fraction to migrate with the serum proteins were not related to the methods of iodination employed since such migrating components were observed in lots prepared by both of the methods used in our laboratory as well as in those produced by the commercial supplier.

#### D. Electrophoretic behavior of radioactivity in plasma following intravenous administration of insulin- $I^{131}$

1. Paper electrophoresis: (a) Animal experiments. Since the concentrations of TCA precipitable radioactivity in the plasma of control subjects after about an hour are generally too low to yield quantitatively precise data by paper electrophoresis, two experiments in rabbits receiving

<sup>9</sup> Occasionally, even lots prepared with short iodination periods contained a relatively large fraction of migrating components.

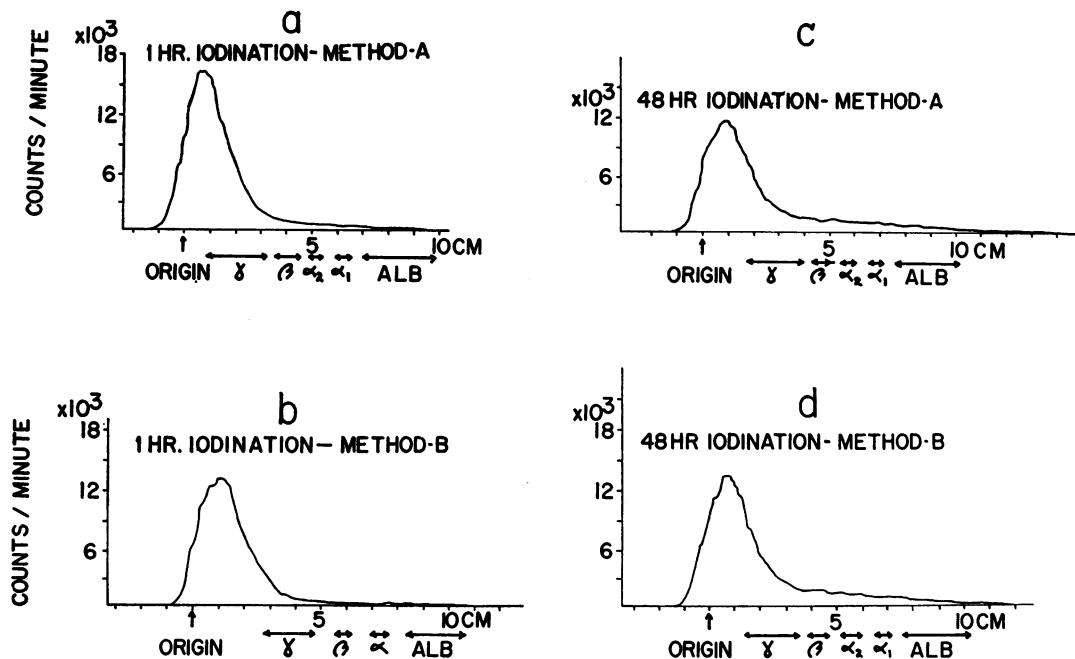


FIG. 5. PAPER RADIOELECTROPHORETOGRAMS OF VARIOUS INSULIN- $I^{131}$  PREPARATIONS IN PLASMA

a) Method A 1-hour iodination

b) Method B 1-hour iodination

c) Method A 48-hour iodination

d) Method B 48-hour iodination

In this and all subsequent figures the paper strips were untreated prior to electrophoresis unless otherwise indicated.

different lots of insulin- $I^{131}$  are shown in Figure 6 for the purpose of illustrating the behavior of the electrophoretic fractions of insulin- $I^{131}$ . Because of the relatively larger amounts of radioactivity administered, high concentrations of TCA precipitable radioactivity were still present at the end of 3 or 4 hours. In rabbit experiment No. 55 insulin- $I^{131}$  lot No. 50 was employed. The radioelectrophoretogram obtained when the labeled insulin was added *in vitro* to a control plasma sample revealed that only about 4 per cent of the total radioactivity migrated with the serum proteins (Figure 6a). Radioelectrophoretograms of plasma samples taken 2 minutes to 3 hours after intravenous administration of this lot of insulin- $I^{131}$  revealed a rapid decrease in the concentration of the unaltered insulin- $I^{131}$  bound to the paper at the origin and a much slower fall in the radioactivity migrating with the serum proteins. Therefore, in later samples, the unaltered insulin- $I^{131}$  gradually became a smaller and smaller fraction of the total radioactivity. The accompanying graph shows that the total number of counts remaining on the paper strips after heat coagulation, staining and

destaining was generally in good agreement with the total TCA precipitable radioactivity of the samples. The total counts in the segments corresponding to the unaltered insulin- $I^{131}$ , however, decreased at a much more rapid rate than the originally small moiety of altered insulin migrating with the serum proteins. At the end of one hour, unaltered insulin- $I^{131}$  still comprised over 50 per cent of the total TCA precipitable radioactivity whereas at the 2½-hour point only 15 per cent of the TCA precipitable radioactivity was unaltered insulin- $I^{131}$ .

In experiment No. 59 in which insulin- $I^{131}$  lot No. A611-1674 was employed, the discrepancy between insulin- $I^{131}$  and TCA precipitable radioactivity was much more exaggerated. Approximately 25 per cent of the original radioactivity represented altered products as indicated in the control electrophoretogram (Figure 6b). As early as fifteen minutes after intravenous injection only about 35 per cent of the TCA precipitable radioactivity which remained in the plasma was unaltered insulin- $I^{131}$  and at the end of one hour less than 15 per cent was insulin- $I^{131}$ . This

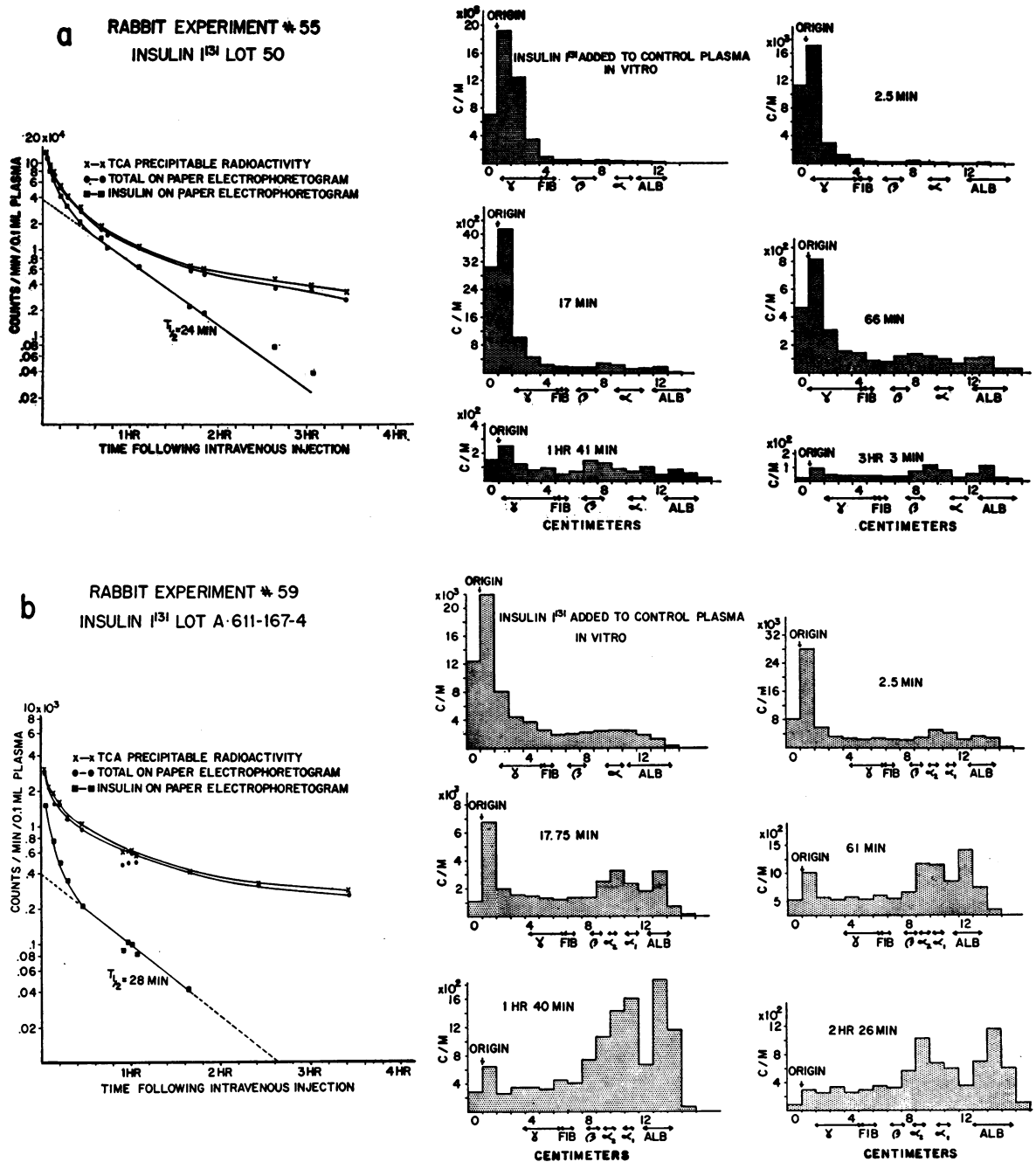


FIG. 6. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA AND CURVES OF TRICHLORACETIC ACID PRECIPITABLE PLASMA RADIOACTIVITY, TOTAL RADIOACTIVITY ON PAPER STRIP AND RADIOACTIVITY ADSORBED TO PAPER AT ORIGIN (INSULIN- $I^{131}$ ) AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF TWO DIFFERENT LOTS OF INSULIN- $I^{131}$  TO RABBITS

- a) Preparation of insulin- $I^{131}$  which contained a small fraction of radioactivity migrating with the plasma proteins.
- b) Preparation of insulin- $I^{131}$  which contained a relatively large fraction of radioactivity migrating with plasma proteins.



lot of insulin- $I^{131}$  was also responsible for two of the highest curves of TCA precipitable plasma radioactivity obtained in control human subjects (Figure 2, W. F., G. S.).

The approximate half times for disappearance of unaltered insulin- $I^{131}$ , after the initial distribution phase, were about 25 minutes in both curves.

Electrophoretic patterns similar to those described above have been observed in many other experiments in rabbits and dogs (10), as well as in man, and it may be noted that cross circulation experiments in animals (10) have confirmed that the radioactivity migrating with the serum proteins has a much smaller space of distribution (about  $2\frac{1}{2}$  times plasma volume) and a very much slower degradation rate ( $T_{1/2}$  equals approximately 18 hours) than that of unaltered insulin- $I^{131}$ .

(b) *Control human subjects.* Because of the much lower concentrations of radioactivity obtained in human subjects, plasma samples after the first hour or so are usually not suitable for such electrophoretic analysis. However, in a group of control subjects, 3 to 4 paper strips were run for each plasma sample and the cut sections of each were pooled to yield higher counting rates. This permitted assays of insulin- $I^{131}$  concentrations for periods up to 2 to 3 hours. In several of these cases, a short run of about an hour employing hydrodynamic flow alone without electrophoresis permitted the plasma to be moved through the paper by liquid flow (*i.e.*, simple chromatographic development) and effected a satisfactory separation between insulin- $I^{131}$  adsorbed at the origin and the components migrating with the serum proteins (Figure 7a). The concentrations of un-

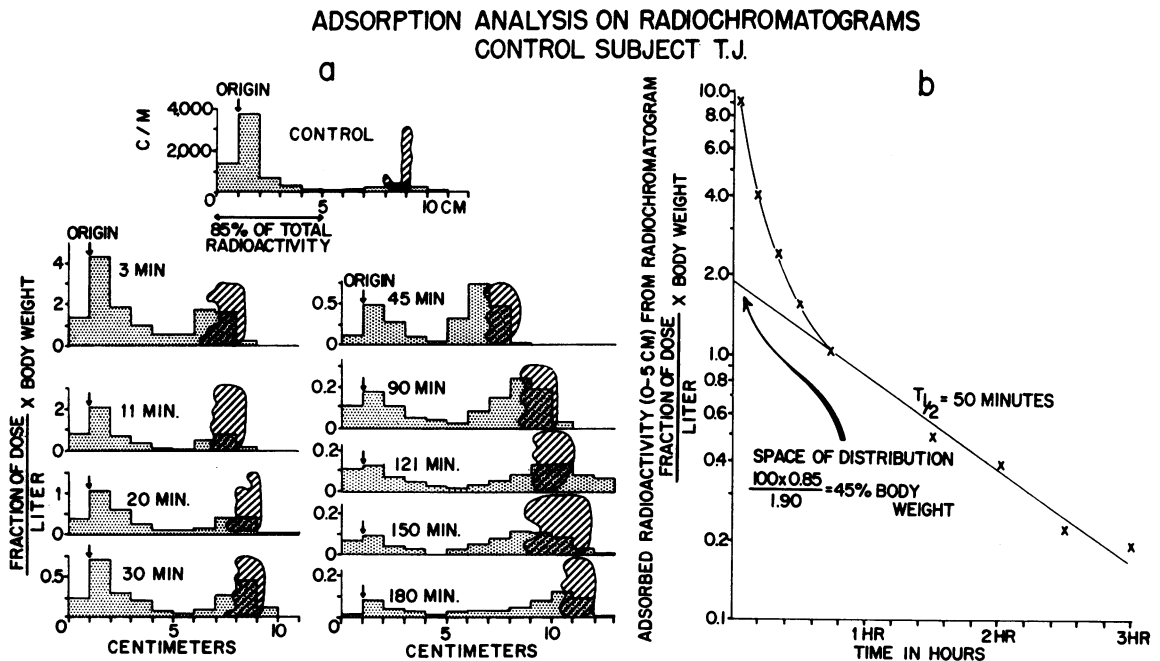


FIG. 7. a) PAPER RADIOCHROMATOGRAMS OF PLASMA AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $I^{131}$  TO CONTROL HUMAN SUBJECT T. J.

Four strips were run for each of the later plasma samples and the cut segments pooled for assay. Bromphenol blue was added to the plasma prior to the runs. The diagonally hatched zones represent the areas of albumin-bound stain.

b) PLASMA CONCENTRATION OF RADIOACTIVITY ADSORBED AT ORIGIN (INSULIN- $I^{131}$ ) IN PAPER CHROMATOGRAMS AS A FUNCTION OF TIME IN CONTROL HUMAN SUBJECT T. J.

The early portion of the curve represents distribution and degradation of insulin- $I^{131}$ . The latter exponential segment is presumed to represent degradation primarily or entirely. The zero time extrapolation of the exponential segment gives only an approximate measure of the volume of distribution of the insulin- $I^{131}$ , since it cannot be assumed that the rate of degradation is constant throughout the entire period.

altered insulin-I<sup>131</sup> as determined from the radioelectrophoretograms or radiochromatograms (Figure 7a), were then plotted as a function of time (Figure 7b). Distribution into an apparent volume averaging about 37 per cent of body weight (Table I) appeared to take place within about 30 to 40 minutes (Figure 7b) following which the plasma concentration fell with an average half time of about 35 minutes (Table I). With some lots of insulin-I<sup>131</sup>, particularly those containing a relatively large amount of radioactivity migrating with the serum proteins, a decrease in the rate of disappearance from plasma of radioactivity adsorbed at the origin was observed after the concentration had fallen to about 0.2 per cent to 0.4 per cent of the administered dose per liter plasma. It is believed that this small moiety of slowly disappearing radioactivity also represented altered insulin. The length of time required for complete distribution of the labeled insulin in man cannot be precisely determined because of uncertainty regarding the entire course of disappearance from the plasma. However, the exponential decay rates of the electrophoretically determined insulin-I<sup>131</sup> concentrations are consistent with the rates of metabolic degradation of insulin-I<sup>131</sup> estimated from the rate of appearance of non-precipitable I<sup>131</sup> as given above.

(c) *Insulin treated subjects.* The radioelectrophoretograms of plasma samples obtained following intravenous administration of insulin-I<sup>131</sup> to insulin treated subjects differed characteristically from those just described. Plasma samples taken

TABLE I  
Space of distribution and rate of degradation of electrophoretically determined insulin-I<sup>131</sup> in control subjects

Subject	Space of distribution % body weight	Rate of degradation	
		T <sub>1/2</sub> (min.)	%/min.
A. Su.	38	35	1.97
H. G.	55	42	1.65
J. K.	23	25	2.77
M. N.	39	31	2.23
J. L.	35	30	2.31
B. Ba.	36	38	1.82
N. H.	42	40	1.73
W. T.	48	54	1.28
E. G.	33	43	1.61
W. F.	21	29	2.39
G. S.	23	22	3.16
T. J.	45	50	1.38
Mean	37		2.02

## INSULIN TREATED DIABETIC SUBJECTS

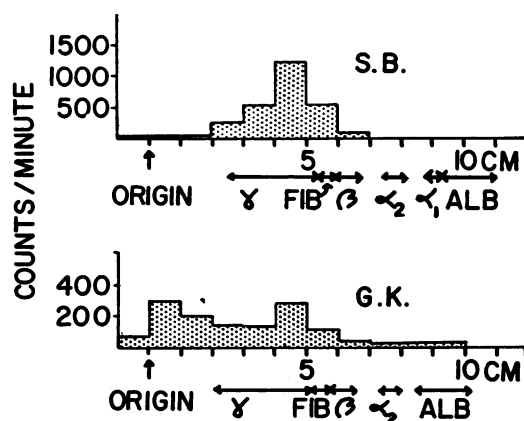


FIG. 8. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA OBTAINED FROM INSULIN-TREATED SUBJECTS 3 MINUTES AFTER INTRAVENOUS ADMINISTRATION OF INSULIN-I<sup>131</sup>

Subject S. B. Almost all the radioactivity migrated in the region of the front running  $\gamma$  globulin.

Subject G. K. The radioactivity is divided into two peaks, one in the front running  $\gamma$  globulin region, the other near the origin.

as early as 2 to 3 minutes following injection revealed a significant fraction of the radioactivity migrating with the front running  $\gamma$  globulin (" $\gamma_1$ " globulins or " $\beta_2$ " globulins) components of the serum.<sup>10</sup> In some subjects virtually all the radioactivity migrated in this region (Figure 8, S. B.) in all samples taken over several hours while in others there were two distinct peaks, one at the origin representing "free" insulin-I<sup>131</sup>, the other in the region of the faster migrating  $\gamma$  globulins (Figure 8, G. K.). In some of the latter cases, the patterns obtained in samples taken from the same subject at various intervals after injection were quite similar except for the absolute heights of the peaks, suggesting reestablishment of an equilibrium ratio between the free insulin-I<sup>131</sup> and the radioactivity migrating with the  $\gamma$  globulins. In other cases, samples taken over the first hour

<sup>10</sup> Preliminary studies (10) with protein fractions separated with cold ethanol according to the method of Lever and associates (11) suggest that the insulin-I<sup>131</sup> is complexed to the slowest migrating component in Fraction I and III which contains  $\beta_2$  and fibrinogen,  $\beta_1$ ,  $\alpha_1$  and  $\alpha_2$ , rather than to a component of Fraction II which is almost pure  $\gamma$  globulin. Until this is definitely established, however, the site of migration is simply designated as the "front running  $\gamma$  globulin zone."

or so showed two peaks, but samples taken subsequently, when the plasma concentration had decreased significantly, showed only a single peak in the  $\gamma$  globulin region (Figure 9a). This suggested that the earlier concentrations were too high to permit complete binding to the insulin

complexing protein. In confirmation of this suggestion the *in vitro* addition of successively larger amounts of labeled insulin to plasma produced a gradual decrease in the fraction bound to the  $\gamma$  globulin even though the absolute amount bound increased (Figure 9b).

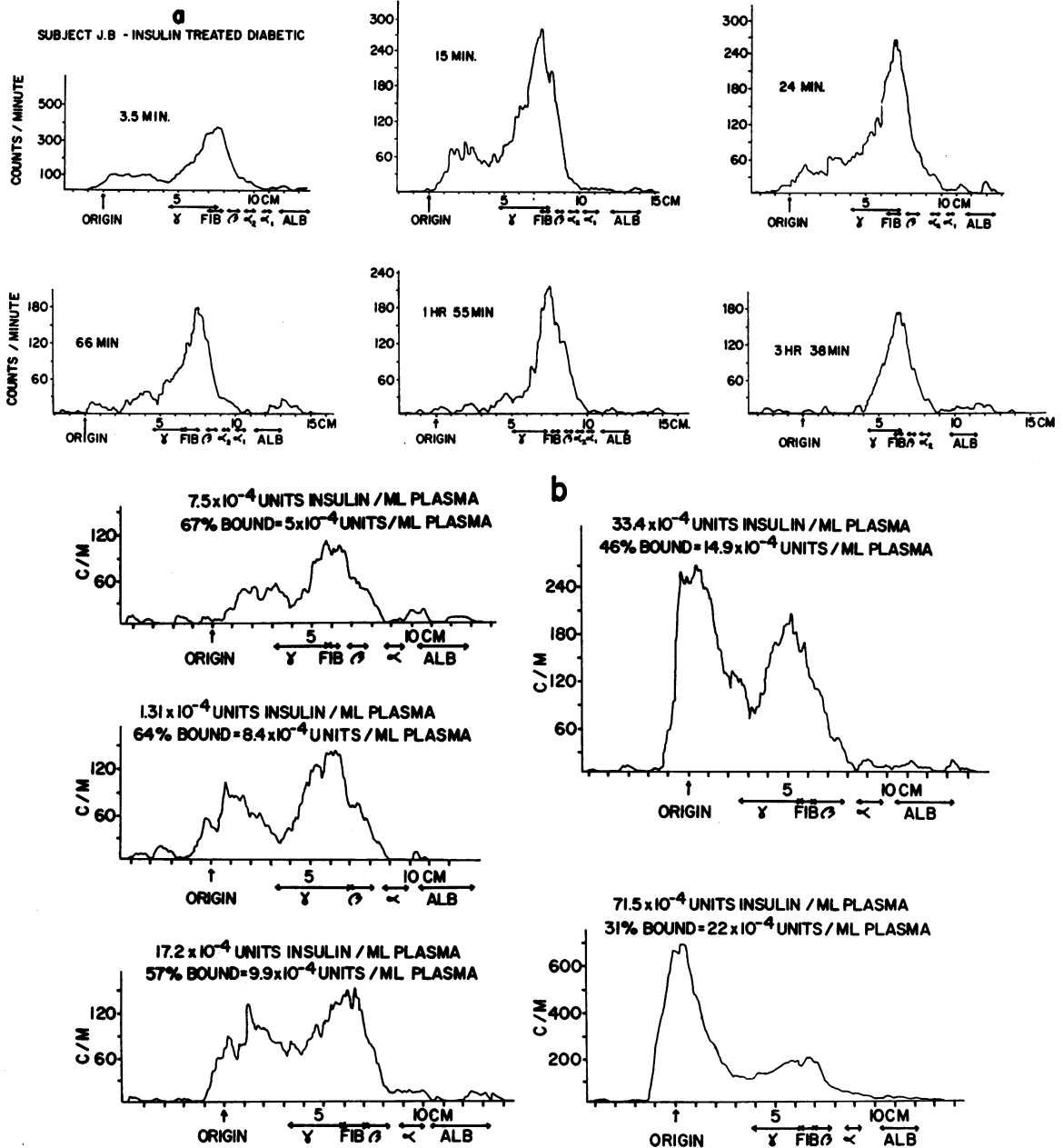


FIG. 9. a) PAPER RADIOELECTROPHORETOGRAMS OF PLASMA SAMPLES TAKEN AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-<sup>125</sup>I TO SUBJECT J. B., A DIABETIC PATIENT TREATED WITH INSULIN FOR 15 YEARS

b) TWO-HOUR PLASMA SAMPLE FROM J. B., TO WHICH VARYING AMOUNTS OF INSULIN-<sup>125</sup>I WERE ADDED *in Vitro*

Frequently, when the same sample was run under somewhat different conditions, variations in the relative heights of the two peaks was observed. The results of a large number of experiments of this sort indicated quite definitely that dissociation of the insulin- $\gamma$  globulin complex was taking place during electrophoresis, and that the paper was competing with the  $\gamma$  globulin for the free insulin- $I^{131}$ . Thus, in cases where dissociation of the insulin- $\gamma$  globulin complex appeared to be relatively rapid, the tendency towards dissociation could be enhanced by permitting the plasma to remain at the origin for a few hours prior to slow electrophoretic migration in high ionic strength buffers. A larger peak at the origin and a considerable degree of trailing towards the front running  $\gamma$  globulin peak were then observed and consequently a smaller peak was obtained in the front running  $\gamma$  globulin region. Conversely, dissociation could be inhibited by producing a rapid hydrodynamic movement of the whole plasma towards the anode prior to electrophoresis, with the consequent maintenance of a high peak in the front running  $\gamma$  globulin zone and a lesser degree of trailing between this region and the free insulin- $I^{131}$  bound at the origin. Because of the competition for insulin- $I^{131}$  between the paper and the  $\gamma$  globulin, the kinetics of association and dissociation of the insulin- $\gamma$  globulin complex could not be studied satisfactorily by this means.

When plasma samples from these subjects were run on paper strips presoaked in nonradioactive insulin solutions, two peaks were again observed, with the "free" insulin- $I^{131}$  no longer bound to the paper at the origin but running just behind the albumin (Figure 10). Generally, the ratio,  $\frac{\gamma \text{ globulin bound insulin-}I^{131}}{\text{"free" insulin-}I^{131}}$  was decreased by this

procedure, apparently as a result of exchange between the nonradioactive insulin used in soaking the paper and radioactive insulin in the  $\gamma$  globulin complex and a consequent lowering of the ratio of total  $\gamma$  globulin bound insulin to total free insulin. Even when the insulin presoaked papers were heat coagulated, stained and destained, this exchange could not be entirely prevented. Such displacement of radioactive insulin by nonradioactive insulin could readily be demonstrated even in samples where all the radioactive insulin was

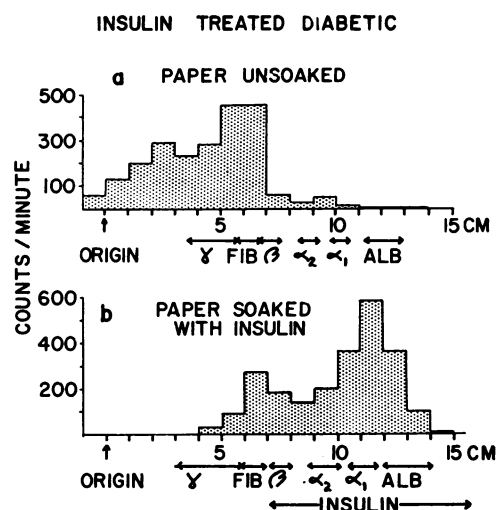


FIG. 10. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA FROM SUBJECT B. G., A DIABETIC PATIENT TREATED WITH INSULIN FOR 10 YEARS

- Paper not soaked in insulin prior to electrophoresis.
- Paper soaked in insulin prior to electrophoresis.

originally  $\gamma$  globulin bound prior to the addition of nonradioactive insulin.

There was a very close correlation between the extent of  $\gamma$  globulin binding of insulin and the rate of fall in the precipitable radioactivity of the plasma. Where almost all the radioactivity was present in the  $\gamma$  globulin peak, the plasma concentrations decreased at a very slow rate. Conversely, when the  $\gamma$  globulin peak was smaller than the free insulin peak, the precipitable radioactivity in the plasma declined more rapidly. No definite correlation could be established between the extent of insulin binding to  $\gamma$  globulin or the rate of fall of plasma precipitable radioactivity and the insulin requirements of the patients studied. Furthermore, some patients who had taken insulin for 15 years or longer manifested relatively weak binding of insulin to  $\gamma$  globulin while others with much shorter histories of insulin therapy revealed strong binding. In subject M. N. no evidence of  $\gamma$  globulin binding was obtained after 2½ weeks of insulin therapy (Figure 11a). However, on repeat study 4½ months later there was obvious strong binding to  $\gamma$  globulin (Figure 11b). These findings correlated well with the different rates of disappearance of TCA precipitable radioactivity from the plasma (Figure 2, M. N.<sub>1</sub> and M. N.<sub>2</sub>).

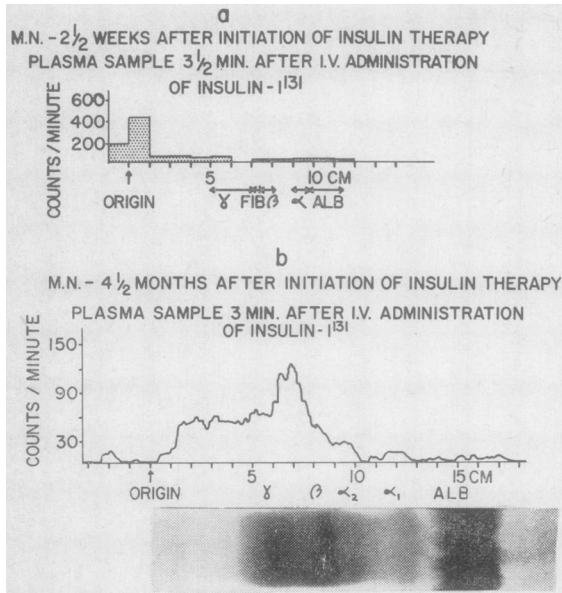


FIG. 11. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $I^{131}$  TO SUBJECT M. N.

- a) After 2½ weeks of insulin therapy.
- b) After 4½ months of insulin therapy.

Binding of the insulin- $I^{131}$  to the  $\gamma$  globulin also occurred upon the addition of insulin- $I^{131}$  *in vitro* to the plasma of these subjects (Figure 9b). Other *in vitro* studies are presented below.

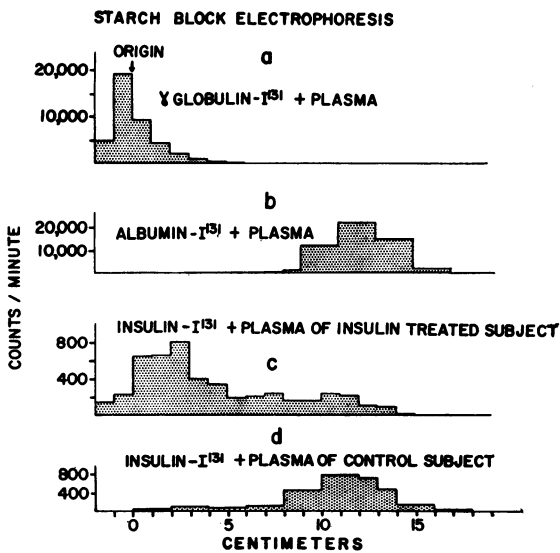


FIG. 12. STARCH BLOCK RADIOELECTROPHORETOGRAMS  
The four specimens were run simultaneously on the same block.

2. *Starch block electrophoresis*: When insulin- $I^{131}$  in the plasma of control subjects is run on the starch block, binding to starch does not occur to any significant extent since there is a single peak of radioactivity just behind that of serum albumin<sup>11</sup> (Figure 12d) and the small extent of trailing which is observed can be explained on the basis of altered components migrating with the serum proteins. In the plasma of insulin treated subjects, however, there is again either a single peak in the fore-running  $\gamma$  globulin zone, or two peaks, one corresponding to the front of the  $\gamma$  globulin region and one in the region of free insulin migration behind the albumin (Figure 12c). At least partial displacement of insulin- $I^{131}$  from the  $\gamma$

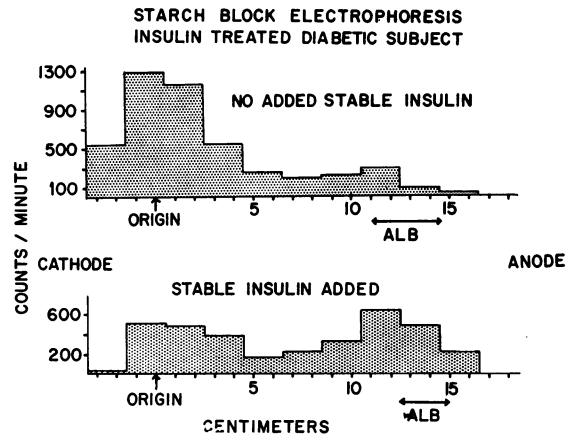


FIG. 13. STARCH BLOCK RADIOELECTROPHORETOGRAMS OF PLASMA FROM SUBJECT J. B. WHO HAD BEEN TREATED WITH INSULIN FOR 15 YEARS

Bromphenol blue was added to the plasma to identify the zone of albumin migration.

Top—without added stable insulin.

Bottom—stable insulin added to plasma 8 hours prior to electrophoresis.

globulin complex to the region of free insulin migration is readily demonstrated by the addition of nonradioactive insulin to the labeled plasma (Figure 13).

*E. External assays of radioactivity over liver, kidneys and thigh*

Simultaneous counting rates over liver and thigh, or over liver and kidneys, were recorded in

<sup>11</sup> When a small amount of insulin- $I^{131}$  in water is run on the starch block the peak occurs in the same position as when the insulin- $I^{131}$  is run in plasma.

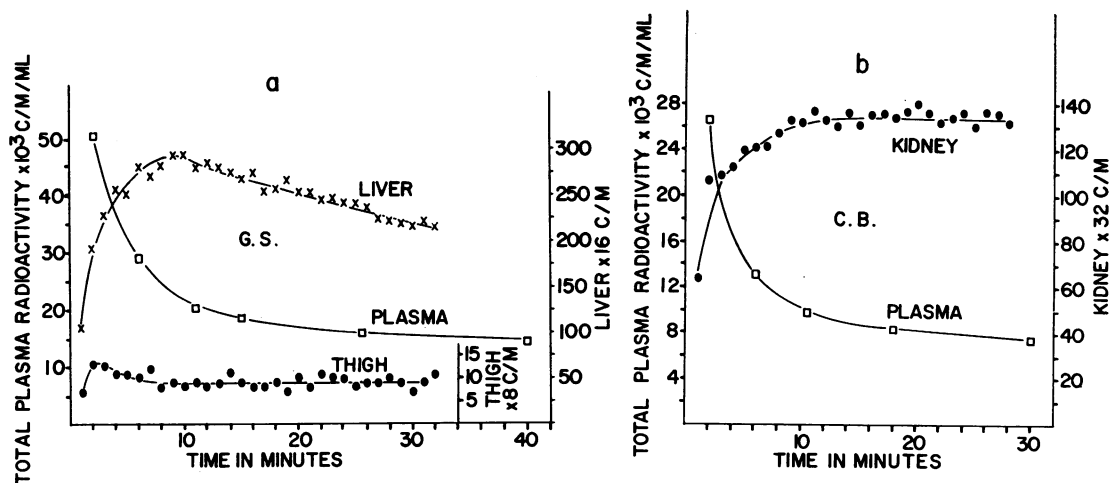


FIG. 14. RADIOACTIVITIES OVER LIVER, THIGH AND KIDNEY COMPARED TO CONCENTRATIONS OF RADIOACTIVITY IN PLASMA AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN- $I^{131}$  TO CONTROL HUMAN SUBJECTS

several patients (Figure 14). The thigh counts reached a peak within about one-half to 2 minutes after injection and then decreased in a manner roughly parallel to the curve of total plasma radioactivity. Over the liver (Figure 14a), however, there was occasionally recorded a continual buildup to a peak for a period of about 10 minutes following which radioactivity decreased at a some-

what faster rate than over the thigh or in the blood. Increasing radioactivity over the kidneys for about 8 to 10 minutes was also frequently observed (Figure 14b). Although the methods of *in vivo* assay are so rough as to permit conclusions only of a qualitative nature, the increase in radioactivity over liver and kidneys at a time when radioactivity in plasma and over the thigh are de-

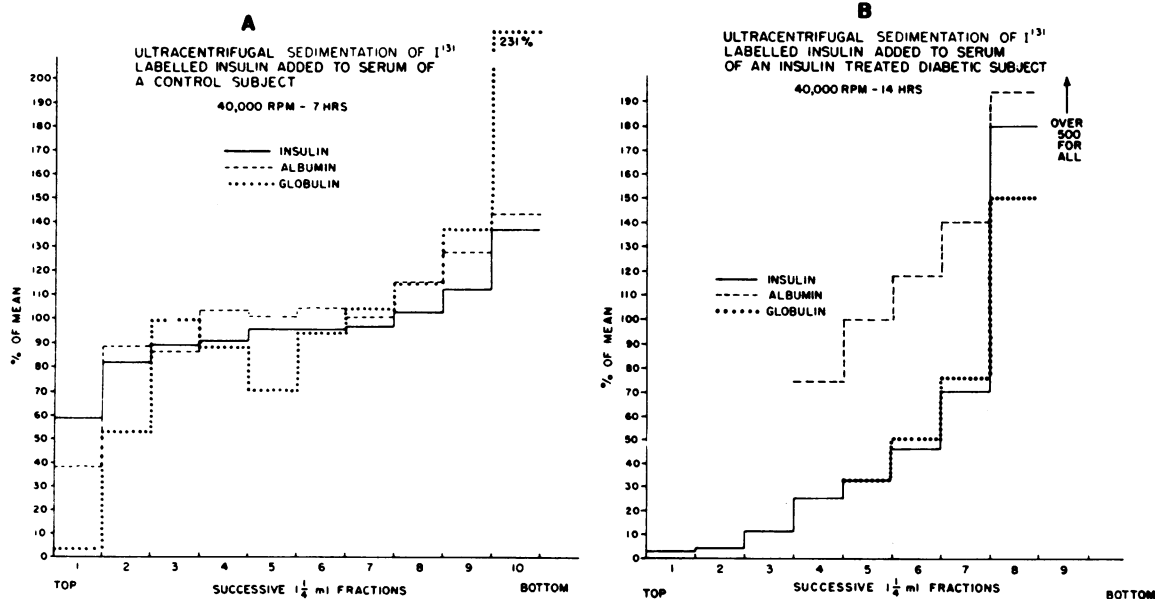


FIG. 15. ANALYSIS AFTER ULTRACENTRIFUGATION OF INSULIN- $I^{131}$  AND SEDIMENTING PROTEIN FRACTIONS  
 a) Insulin- $I^{131}$  in serum of control subject.  
 b) Insulin- $I^{131}$  in serum of D. D., a diabetic patient treated with insulin for 13 years.

creasing would seem to indicate a heavy concentration of insulin- $I^{131}$  in these organs, which probably represent sites of insulin degradation. This is in accord with the *in vitro* studies of Elgee, Williams, and Lee (2).

## II. In Vitro Studies

### A. Ultracentrifugal sedimentation of insulin- $I^{131}$ in the plasma of control and insulin treated subjects

When insulin- $I^{131}$  is added to the plasma of a control subject, the radioactivity sediments less rapidly than serum albumin (Figure 15a) as might be expected from the relatively low molecular weight of insulin. However, when insulin- $I^{131}$  is added to the plasma of an insulin treated subject, the radioactivity sediments with the globulins, indicating a binding of insulin to one of the globulin components (Figure 15b).

### B. Effect of *in vitro* incubation of insulin- $I^{131}$ with plasma

When insulin- $I^{131}$  was incubated with control plasma at 37° C., some non-precipitable radioactivity appeared to the extent of about 30 per cent of the total after 72 hours. A more interesting finding, however, was the gradual disappearance of the free insulin- $I^{131}$  peak on electrophoresis and

a buildup of radioactivity in the  $\alpha_2$  globulin region so that at the end of 72 hours much of the precipitable radioactivity was frequently present in this region (Figure 16). In control experiments no detectable changes were observed in the radioelectrophoretograms of  $I^{131}$  labeled  $\gamma$  globulin or  $I^{131}$  labeled serum albumin after 74 hours' incubation with plasma (Figure 16). Heating of the plasma for one hour at 57° prior to incubation with insulin- $I^{131}$  did not prevent the changes noted above. Incubation of insulin- $I^{131}$  and/or plasma alone for 72 hours did not result in these findings on electrophoresis of the insulin- $I^{131}$  and plasma together. Experiments in dogs showed that the biologic activity of the insulin- $I^{131}$  preparations after 24 hours' incubation was approximately proportional to the free insulin peak remaining. Unlike fresh solutions of insulin- $I^{131}$ , the 72-hour incubated mixtures of insulin- $I^{131}$  and plasma, when injected into dogs, showed a small space of distribution and a slow rate of removal of precipitable radioactivity from the plasma (10).

When insulin- $I^{131}$  was incubated with the plasma of insulin treated subjects who showed strong binding to  $\gamma$  globulin, the amounts of non-precipitable radioactivity which migrated in the  $\alpha_2$  globulin region were much less than in control subjects. Labeled insulin bound to  $\gamma$  globulin appeared to be partially "protected" from degradation in these cases.

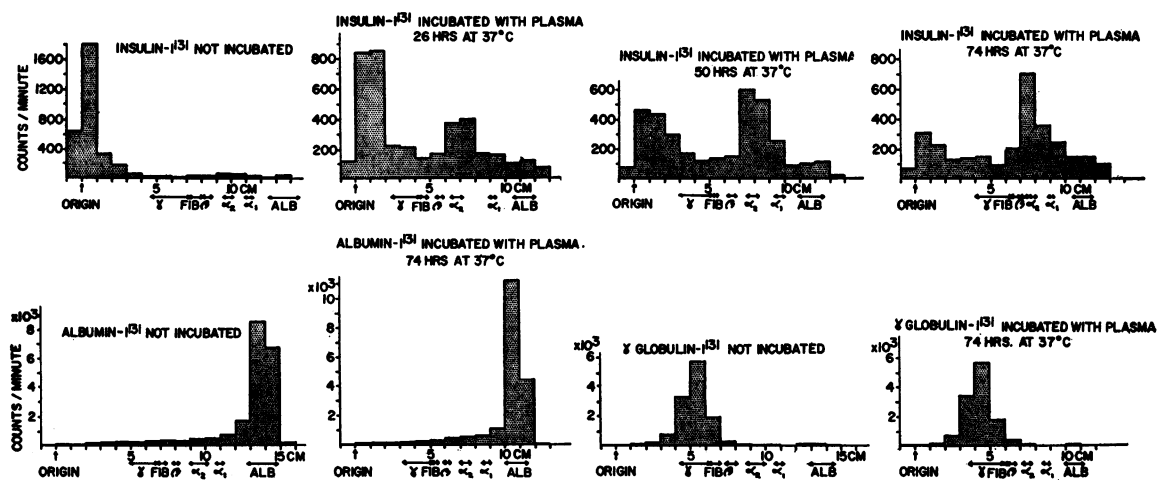


FIG. 16. RADIOELECTROPHORETOGRAMS OF PLASMA INCUBATED WITH INSULIN- $I^{131}$ , ALBUMIN- $I^{131}$  AND  $\gamma$  GLOBULIN- $I^{131}$  FOR VARIOUS PERIODS OF TIME

TABLE II  
*Binding of insulin-I<sup>131</sup> to  $\gamma$  globulin in insulin treated and untreated subjects*

	Diagnosis	Duration of insulin therapy	Number of subjects
Binding present	Diabetes mellitus	7½ weeks to 23 years	25
	Diabetes with acromegaly	14 months	1
	Schizophrenia	6 weeks, 3½ months	2
Binding absent	Diabetes mellitus	0	5
		8 days-12 weeks	6
	Cortisone induced diabetes	0	1
	Schizophrenia	3½ weeks, 8 weeks	2
	Non-diabetic; nonschizophrenic controls	0	33

*C. Electrophoretic analysis of insulin-I<sup>131</sup> added in vitro to plasma of insulin treated and non-treated subjects*

In addition to the patients who received intravenous injections of insulin-I<sup>131</sup> (Figure 2), plasma or serum samples were obtained from a large number of subjects and subjected to paper and starch block electrophoresis after addition of insulin-I<sup>131</sup> *in vitro*. Binding of insulin-I<sup>131</sup> to  $\gamma$  globulin was never observed in the absence of previous insulin therapy but was always present in subjects who had received insulin treatment for 3 months or longer. This latter group included 13 diabetic patients and 1 schizophrenic non-diabetic subject in addition to the schizophrenic subject (W. T.) mentioned above. In another group of diabetic and schizophrenic patients who had received insulin for 12 weeks or less, binding of insulin-I<sup>131</sup> to  $\gamma$  globulin was not observed. In one diabetic patient treated with insulin for 7½ weeks, insulin binding by  $\gamma$  globulin was observed. Table II summarizes the data for the entire group of 75 subjects.

DISCUSSION

It has previously been observed (12) that subtle alterations of proteins produced by isotopic labeling procedures may escape detection in any of a number of physical, chemical or biological testing systems. Thus, even gross inhomogeneities in I<sup>131</sup> labeled human serum albumin frequently remained undiscovered by electrophoretic and ultracentrifugal analysis but showed up as more or less rapidly degraded components following injection into man (12). In the case of a protein or peptide hormone such as insulin, any *drastic*

changes induced by the labeling process are likely to be revealed by a decrease in biologic activity. However, it is well known that insulin can tolerate certain minor changes, such as substitution on some of the tyrosine residues, without sacrifice of biologic potency (13). Furthermore, while the most sensitive of available procedures for testing of insulin potency may be reliable to within about 5 to 10 per cent, the blood sugar response of fasting rabbits utilized here is probably incapable of detecting loss of potency of the order of 20 to 30 per cent or more. Thus, even if each lot of labeled insulin is subjected to exhaustive biologic testing, which is virtually a prohibitive enterprise, biologic alteration of a small fraction of the starting material cannot be excluded.

The problem of whether the separation of the radioactive label from the protein is synonymous with metabolic degradation of the protein molecule has previously been discussed in detail (12). The fact that the rate of separation of I<sup>131</sup> from the labeled insulin can be slowed significantly by the simultaneous administration of very large doses of unlabeled insulin (> 200 U per kg. body weight) to rabbits (10) or by the addition of stable insulin, *in vitro*, to liver preparations which degrade insulin (14) suggests at least that the two processes are related and that the degrading mechanisms do not distinguish between the labeled and native insulin. However, as emphasized above, complete integrity of the labeled insulin cannot be guaranteed and the presence of even a small fraction of altered material is capable of introducing very annoying complexities into the experimental studies. When, in addition, the magnitude of the fraction of altered material varies among different preparations of labeled insulin



derived from the same lot of crystalline insulin, the difficulties are multiplied. In the present study the most specific method for the identification of unaltered insulin- $I^{131}$  in plasma was found to be paper electrophoresis. Even this method, however, is not eminently satisfactory since it simply permits distinction between protein bound radioactivity which migrates with the serum proteins (and is therefore definitely not unaltered insulin) from protein bound radioactivity which is adsorbed at the starting line (and may therefore represent unaltered insulin). Yet, it can at least be accepted that most of the adsorbed material is, in fact, unaltered insulin, since it comprises a major fraction of the insulin- $I^{131}$  preparations which show no definite loss in biologic potency.

On the basis of the foregoing discussion it therefore seems reasonable to regard the fall in plasma concentration of the paper adsorbed radioactivity as reflecting the distribution and metabolism of unaltered insulin- $I^{131}$  and to utilize the data obtained in this manner to attempt an estimation of endogenous insulin concentrations in the plasma of normal subjects. Very roughly, and perhaps arbitrarily, the daily utilization of insulin is taken at 48 units, based in part on the insulin requirements of depancreatized subjects. This reduces to a value of 1 unit of insulin metabolized in 30 minutes. Again roughly, the results of the present study indicate that  $I^{131}$  labeled insulin distributes into an apparent volume of 26 liters in the average sized subject and is degraded at a rate of about 2.0 per cent per minute. In 30 minutes, therefore, approximately 60 per cent of the insulin in body fluids is turned over. Since this amount represents about 1 unit of insulin, the total volume of distribution, 26 liters, contains about  $1/0.60$  units.

The average insulin concentration is then  $\frac{1.7 \text{ units}}{26 \text{ liters}}$  or 0.065 units per liter. Since each unit corresponds to 37 micrograms, this concentration is equivalent to about  $2\frac{1}{2}$  micrograms insulin per liter. These calculations should not be construed as implying a constancy of insulin concentration; on the contrary, it is recognized that there are probably marked fluctuations of insulin secretion in response to food intake or physiologic stresses. The values presented are merely rough estimates for an overall time average. That the calculated average value of 1.7 units for the total insulin

present in body fluids and readily exchangeable with plasma insulin is at least not an inordinately low estimate is confirmed by observations that as little as twice this amount administered intravenously is capable of producing a definite although transient fall in blood sugar (15).

Since the estimated total exchangeable insulin is only of the order of one and a half units, it may be questioned whether the doses administered in the present study (0.1 to 7.0 units) can be considered as tracer doses in the sense of being physiologically insignificant. Certainly, the larger doses are actually in the pharmacological range while even the smaller doses, though without discernible effect, cannot be considered negligible. However, it should be noted that in one subject (J. Ves.) who had received 640 units regular insulin subcutaneously 40 minutes prior to intravenous injection of the tagged insulin, the rate of disappearance of the tagged insulin did not differ significantly from that of other control subjects (Figure 2). Since this patient developed progressively severe hypoglycemic symptoms during the course of the study, there is little question that much higher than normal insulin concentrations in plasma were obtained. Furthermore, in rabbit experiments it has been found that there is no significant change in the rate of insulin- $I^{131}$  degradation until accompanying doses of stable insulin administered intravenously reach levels as high as 100 units to 200 units per kg. body weight (10). Above this level the rate of insulin- $I^{131}$  degradation decreases significantly, probably as a result of overloading of the insulin degrading mechanisms. A similar decrease in the rate of degradation of  $I^{131}$  tagged globulin produced by overloading with large doses of stable globin has previously been reported (16). In the case of insulin, therefore, it appears as if the degradation reaction follows first order kinetics over a very wide range of insulin concentrations. Similarly, Hellman and his associates (17) showed that  $C^{14}$  labeled hydrocortisone was conjugated and excreted at the same rate when 0.25 milligrams or 100 milligrams were administered to a patient devoid of endogenous sources of this hormone. These results suggest that at any particular metabolic level the rate constant for inactivation, by degradation or conjugation, of some hormones remains constant over a range of hormone concentrations far exceeding physiologic

limits. In most cases, the rate of inactivation appears to be very rapid (in comparison to the rate of metabolism of serum proteins) with a half time of the order of 30 minutes or so. This is true of insulin, hydrocortisone and several of the anterior pituitary hormones (18, 19). While this pattern of hormone inactivation is undoubtedly responsible for tremendous hormone waste, it allows for temporarily high concentrations in response to physiologic demands or stress situations without the penalty of prolongation of effect after the need has passed.

One notable exception to this scheme is thyroxine which is normally degraded with a half time about 7 days (20) and whose degradation follows second order kinetics (20, 21). The latter behavior is due to the fact that it is the concentration of thyroxine itself which sets the general metabolic level. Thus, with increase in thyroxine concentration the rate constant for metabolism of many substances, including thyroxine as well as other hormones, is increased. Since the level of basal metabolism is controlled by the concentration of thyroid hormone in body fluids it would seem also that constancy of this function, which is more suitable to body economy than constancy of many other endocrine functions is best served by a relatively steady concentration and slow rate of degradation of thyroxine.

The association of persistently high levels of precipitable radioactivity with the presence of this radioactivity in the electrophoretically front running gamma globulin in the plasma of insulin treated subjects indicates an insulin- $\gamma$  globulin complex, since the same electrophoretic patterns are demonstrable upon addition of insulin-I<sup>131</sup> to the serum of these patients *in vitro*. Ultracentrifugal sedimentation of insulin-I<sup>131</sup> with the serum globulins in these cases supports the conclusion that retention of insulin-I<sup>131</sup> in the blood stream results from binding to the globulin and a consequent inability of the insulin molecule to pass rapidly through the capillary wall. Upon dissociation of the insulin-globulin complex, the relatively small insulin molecule either escapes into extravascular compartments or is again bound by a globulin molecule. The affinity of the globulin binding sites for the insulin is then the major factor in determining the rate of loss of insulin-I<sup>131</sup> from the plasma in these subjects. In some cases

this affinity was quite marked and the plasma concentration of insulin-I<sup>131</sup> decreased slowly. In others the rate of fall of insulin-I<sup>131</sup> in plasma was more rapid indicating an apparent lesser degree of affinity of the globulin for the insulin. However, the apparent affinity undoubtedly depends on the concentration of insulin present as is readily demonstrated by *in vitro* experiments with various insulin concentrations. Although insulin was withheld on the day of the studies from all diabetic patients receiving insulin, there may still have been some insulin remaining from the previous day's treatment. In some cases, then, differences in the quantity remaining may have played a role in the relative apparent binding affinities in different subjects. In the one insulin treated patient to whom two markedly different doses were given on successive days the curves of protein precipitable activity were superimposable (see legend to Figure 2) suggesting virtually identical binding and dissociation constants over a relatively wide range of insulin levels; however, if still higher levels of insulin had been achieved, a more rapid rate of disappearance from plasma might have been observed. It seems likely that the relative insensitivity of many insulin treated diabetic subjects to intravenously administered insulin during the glucose-insulin tolerance test (22) is attributable to binding by globulin.

The evidence presented in this paper indicates that an insulin binding globulin is acquired by human subjects in response to the administration of insulin. It would appear that the insulin binding globulin satisfies the requirements for an antibody as defined by Topley and Wilson (23): "An antibody is any substance which makes its appearance in the blood stream or body fluids of an animal, in response to the stimulus provided by the parental introduction of an antigen into the tissues, and reacts specifically with that antigen in some observable way." The reaction in this case is evidenced by the retardation of disappearance of insulin from the blood stream, by the altered migration in zone electrophoresis on paper and starch and by sedimentation with the globulins in the ultracentrifuge. This antibody does not appear to be a precipitating antibody even when concentrated five fold (no evidence of precipitation appears after 48 to 72 hours at 37° or up to 2 to 3 weeks at 4° C.) but merely binds the insulin.

It may be that the concentration of antigen-antibody complex is too low to be in the precipitable range or that the antibody is of the type generally regarded as "monovalent" or "incomplete" (24). Since complexing of insulin to the antibody of the present study does not lead to rapid removal from the circulation, but in fact serves to retain insulin within the circulation, the antibody has been here termed "insulin-transporting antibody." It should be noted that the relative electrophoretic mobility of the uncomplexed antibody is not known from these studies since an alteration in mobility may result following the complexing of insulin.

Whether the insulin-transporting antibody is of any clinical significance remains to be established. Its mere presence is certainly not necessarily related to the degree of insulin tolerance or intolerance presented by patients since all insulin treated patients apparently develop it in time. However, it is possible that fluctuations in its production may be causally related to the lability of insulin requirements in those patients who manifest what has been called "brittle diabetes." It is also quite possible, that in the presence of infections which call for heightened antibody response to bacterial or viral antigens, the increase in insulin requirements so frequently observed may likewise be related to altered production of insulin-transporting antibody. These possibilities represent fields for future investigation.

Whether this antibody in extremely high titer is the same antibody which has occasionally been identified in the rare instances of true insulin resistance is unknown. In the relatively small group which has been studied up to the present no definite relationship between insulin requirements and apparent magnitude of antibody binding of insulin has been established. However, a more quantitative expression of this antibody reaction is required than is available at present. The absence of insulin resistance in subjects who manifest marked binding of insulin- $I^{131}$  to globulin is not unexpected. Since the total binding capacity is not unlimited, quantities of insulin in excess of that required to saturate the globulin binding sites might be expected to be available for metabolism at the same rate as insulin in subjects without antibody. The maximum amount of insulin bound to globulin observed in patients of this study was approximately 60 to 80 units as esti-

mated from the specific activity of the administered insulin- $I^{131}$  and the assumption that the volume of distribution of the globulin antibody is  $2\frac{1}{2}$  times the plasma volume as is the case with serum albumin (12).

Another possible consequence of insulin antibody production is to be considered, although it likewise remains purely speculative at present. This has to do with the possibility of a tissue reaction either to the circulating insulin-antibody complex or from tissue-fixed antibodies of similar nature. Possibly some of the complications of long standing diabetes which were unrecognized in the pre-insulin era, particularly the renal lesion responsible for the Kimmelstiehl-Wilson syndrome, may be causally related to long continued exposure to the circulating insulin-antibody complex.

These and many other problems, such as the longevity of insulin-antibody production after discontinuation of insulin therapy, which can most readily be studied in insulin treated schizophrenics, the degree of cross reaction with insulin of other species, particularly human insulin, and the kinetics of the insulin-antibody reaction, remain to be explored. The present study has not established the clinical significance of the insulin-transporting antibody and it may be that there is none to be established. The ubiquitous presence of such an antibody in insulin treated subjects, however, would appear to be a finding of interest.

#### SUMMARY AND CONCLUSIONS

1. Following intravenous administration of  $I^{131}$  labeled insulin to human subjects the time course of radioactivity in plasma has been studied by chemical and electrophoretic methods.

2. During the trace labeling of regular crystalline insulin with  $I^{131}$  some of the insulin is altered in an unknown fashion so that small but variable fractions of the radioactive material become bound to the serum proteins.

3. The radioactivity bound to serum proteins disappears from plasma much more slowly than unaltered insulin- $I^{131}$ , so that within a short period of time following intravenous administration most of the protein precipitable radioactivity in plasma is not insulin- $I^{131}$ .

4. During paper electrophoresis of plasma containing insulin- $I^{131}$ , the labeled insulin is adsorbed

to the paper at the site of application, thus permitting separation from plasma protein bound radioactivity. The half time of disappearance from plasma of adsorbed radioactivity (insulin-I<sup>131</sup>) after distribution in body fluids is of the order of 40 minutes which is in good agreement with the rate of degradation of insulin-I<sup>131</sup> as estimated from the rate of appearance of non-precipitable radioactivity in the plasma.

5. In subjects treated with insulin for months to years the disappearance of insulin-I<sup>131</sup> from plasma is much slower than in subjects never treated with insulin or treated with insulin for less than two to three months. It has been shown that this persistence of relatively high concentrations of insulin-I<sup>131</sup> in the plasma of insulin-treated subjects is due to binding of insulin-I<sup>131</sup> by an acquired globulin which satisfies the criteria for antibody. The insulin-"insulin transporting antibody" complex migrates in the front running  $\gamma$  globulin region on paper or starch block electrophoresis at pH 7.3 or 8.6. The binding of insulin to circulating globulin in these patients is confirmed by ultracentrifugation studies in which it is observed that insulin-I<sup>131</sup> in the serum of insulin treated subjects sediments with the globulins but in the serum of control subjects sediments at a slower rate than serum albumin.

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#### Addenda

(a) Since this paper was written, a report by Kallee and Seybold (Uber<sup>131</sup>J-signiertes Insulin III, Zeitschrift Fur Naturforschung Bd. 1954, 9b, 307) has come to our attention, in which shifting bands of radioactivity were observed in radioautographs of rat serum at various intervals following administration of I<sup>131</sup> labeled insulin. Many of their unexplained observations appear to be resolved by the results of the present study.

(b) Since the preparation of this paper it has been established by two of us (R.S.Y. and S.A.B.) that the appearance of migrating components is a manifestation of radiation damage to the insulin. Similar changes have been produced by external irradiation with x-rays and radium at dose levels comparable to those delivered by the I<sup>131</sup> present in these preparations.

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