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BINDING TO HUMAN INSULIN-BINDING ANTIBODIES**

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# PREPARATION AND PURIFICATION OF HUMAN INSULIN- $I^{131}$ ; BINDING TO HUMAN INSULIN-BINDING ANTIBODIES

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Indirect evidence has been adduced that insulin-binding antibodies in man, formed in response to treatment with commercial mixtures of beef-pork insulin, react also with human insulin (1). Thus, human insulin inhibits competitively the binding of  $I^{131}$ -labeled beef, pork, sheep or horse insulin to antibeef-pork insulin antibodies. However, the observation that much higher concentrations of human insulin than of animal insulins may be required to effect a comparable reduction in the binding of animal insulin- $I^{131}$  suggests that the reaction with human insulin is, in such cases, considerably weaker than with animal insulins (1). The failure to detect reaction with human insulin in some studies (2) may be interpreted (1) on this basis. It also appears likely that these quantitative relationships may explain why certain diabetic subjects can exhibit a moderate degree of resistance to animal insulins and yet maintain fairly good control of blood sugar through stimulation of their own endogenous insulin secretion in the absence of exogenously administered insulin (3).

In order to study the reaction of human insulin with antibody in a direct manner, the use of  $I^{131}$ -labeled human insulin is mandatory. Since pure crystalline human insulin is not generally available,<sup>1</sup> a crude preparation must serve as starting material. The purposes of this communication are to describe the preparation and purification of human insulin- $I^{131}$ , to demonstrate its reaction with human antibodies against beef-pork insulin and to consider the clinical implications of such reaction.

## METHODS

All serums were taken at least 24 hours after the last dose of insulin and were allowed to remain at 4° C for at least 2 months to permit destruction by plasma (3) of any residual insulin. Two patients (H.L. and M.N.) had

<sup>1</sup> To our knowledge, human insulin has not yet been obtained in crystalline form.

not received insulin for about 1 year and 4 years, respectively, prior to withdrawal of the serum tested.

The starting material employed for iodination was a powdery preparation of human insulin generously supplied by Dr. A. M. Fisher, Connaught Laboratories, Toronto, which assayed at 6.8 U per mg in March 1956 (4), but was thought possibly to have decreased slightly in hormonal potency by 1959. Since several crystalline animal insulins obtained from Dr. Fisher were estimated at about 22 U per mg, we had tentatively assigned a value of 6.0 U per mg to the human insulin based on a reference standard of 22 U per mg for pure insulin (5). This material was tested by immunoassay in 1959 against a preparation of human insulin, obtained from Dr. F. Tietze (through the courtesy of Dr. J. Field, National Institutes of Health), which had recently been assayed in the Lilly Research Laboratories. The assigned value of 6.0 U per mg for the Fisher insulin referred to a standard of 22 U per mg for pure insulin agreed excellently with the recently standardized Tietze insulin and was therefore accepted as essentially correct and indicating an insulin content of 6.0 U per mg/22 U per mg = 27.3 per cent insulin by weight (5). More recently we have assayed the Fisher insulin against a preparation of pure human insulin<sup>2</sup> supplied through Dr. Ellis Samols, London, by courtesy of Prof. F. G. Young, Cambridge. The assigned value of 27.3 per cent insulin by weight for the Fisher preparation was found to agree excellently with the Cambridge pure insulin, in confirmation of the results recently obtained by Dr. Samols (6) who found the Fisher crude powder to be "consistently equivalent to 25 to 30 per cent Cambridge insulin by weight." The Fisher powder was dissolved in distilled water brought to pH 2.3 with HCl to a final concentration of 200  $\mu$ g per ml; 2 to 4 vol of 0.2 M borate buffer, pH 8.0, was added and 50  $\mu$ g of the material was iodinated with  $I^{131}$  by methods described previously (5). Carrier- $I^{127}$  was added in amounts not exceeding a ratio of 0.2 iodine atom per insulin monomer (mol wt 6,000) in the final labeled mixture. After dialysis against distilled water at 4° C to remove unbound iodine, human serum albumin was added to a concentration of 2 per cent and the solutions kept frozen until used.

All preparations and fractions obtained during the purification procedure were analyzed by paper chromatography<sup>3</sup> and electrophoresis. It has previously been

<sup>2</sup> Purified on cellulose ion-exchange columns by the method of Dr. J. F. Smith, Cambridge, England (6).

<sup>3</sup> During this procedure, the strips are also subjected to electrophoresis, but the principal separation of pro-

TABLE I  
Purification of I<sup>131</sup>-labeled human insulin  
on cellulose column

	Radioactivity	
	%	
Original effluent + 1st veronal wash	71.0	
2nd veronal wash	4.4	
3rd veronal wash	1.3	
4th veronal wash	.7	
1st plasma eluate (2 ml plasma)	9.7	
2nd plasma eluate (1 ml plasma)	3.7	
Remaining on cellulose column	9.3	

demonstrated that undamaged crystalline animal I<sup>131</sup>-insulins in plasma adsorb to Whatman 3 MM paper at the site of application (7, 8), whereas damaged fractions (8) and antibody-bound insulin (8) migrate with the serum proteins. It can therefore be accepted that any I<sup>131</sup>-labeled material which migrates with the serum proteins of nonimmune plasma is definitely not undamaged insulin-I<sup>131</sup>, whereas any labeled fraction that is adsorbed to paper under these conditions *might be* insulin-I<sup>131</sup>. The final criterion required to establish that I<sup>131</sup>-labeled material adsorbed to paper in the presence of control plasma is, in fact, insulin-I<sup>131</sup> is the demonstration of its binding to antibody in antisera from animals (or man) immunized with animal insulins of high purity. Only that fraction of adsorbed radioactivity which is demonstrably reactive with insulin-binding antibodies can be considered unequivocally to represent insulin-I<sup>131</sup>.

The cellulose adsorption characteristics of insulin were exploited in the purification procedure. Powdered cellulose (Whatman) columns approximately 3.5 cm(ht) × 0.5 cm (diam) were packed tightly and moistened with veronal buffer (ionic strength 0.1, pH 8.6). Aliquots of I<sup>131</sup>-labeled crude human insulin were added to 100 μl control human plasma and passed through the column. The original effluent and several washes with veronal buffer were monitored for radioactivity; when radioactivity in the wash had been reduced to a negligible level, the residual adsorbed I<sup>131</sup>-labeled material was eluted with several aliquots of whole control plasma, previously treated with iodoacetamide as described below.

The principle of separation of insulin-I<sup>131</sup> from other I<sup>131</sup>-labeled compounds in the mixture depends on the capacity of the cellulose to adsorb small amounts of insulin preferentially in the presence of limited amounts of plasma and the ability of high concentrations of plasma to elute the adsorbed insulin-I<sup>131</sup>.<sup>4</sup> Since the insulin-adsorbing capacity of the cellulose is limited, use of large amounts of insulin results in a considerable fraction be-

teins from the site of application is a result of hydrodynamic flow chromatography rather than electrophoresis (8).

<sup>4</sup>This phenomenon has a counterpart in paper strip chromatography or electrophoresis. If the paper strip is first *soaked* in plasma, insulin-I<sup>131</sup> will be adsorbed only slightly or not at all.

ing lost with the original effluent. If the total amount passed through the column does not exceed 1 to 2 μg, approximately 20 to 50 per cent or more is adsorbed by the column, whereas contaminants and damaged fractions which bind to or migrate with plasma proteins on paper strips pass through the column with the original effluent and veronal washes. One ml plasma will generally elute about 20 to 30 per cent of the adsorbed radioactivity. However, plasma itself, especially at 37° C and after extended periods of incubation even at 4° C, can damage insulin (8). Since the chromatographic and electrophoretic characteristics of such damaged insulin resemble, in many respects, insulin altered by cysteine or irradiation (9), both of which are known to effect reductive splitting of disulfide bonds (10, 11), it seemed possible that free sulfhydryl groups in plasma (sulfhydryl groups of serum proteins, free cysteine, reduced glutathione, and so forth) might be responsible in part for "plasma damage" to insulin-I<sup>131</sup>. This hypothesis was tested by treating control plasma with 0.5 per cent iodoacetate or iodoacetamide (30 minutes at 37° C) prior to incubation with insulin-I<sup>131</sup>. This treatment was effective to a large extent in protecting crystalline beef insulin-I<sup>131</sup> against damage by plasma.<sup>5</sup> Accordingly, plasma used for elution of insulin-I<sup>131</sup> was first treated with iodoacetamide as indicated.

## RESULTS

A typical purification experiment is summarized in Table I. Scans of radioactivity on paper chromatoelectrophoretograms and electrophoretograms of the unpurified solution, the original effluent and plasma eluates 1 and 2 are shown in Figure 1. In all preparations (13 separate iodination procedures) the fraction of radioactivity in the unpurified solutions that migrated with the serum proteins on chromatography was about 35 ± 5 per cent of the total protein-bound I<sup>131</sup>. On electrophoresis (Figure 1), the migrating radioactivity is seen to be randomly distributed throughout the zone of migration of serum proteins, but there is, in addition, a small fraction extending out beyond serum albumin.

The original effluents contained, as expected, a greater proportion of migrating activity as well

<sup>5</sup>Prolonged incubation of plasma containing 0.05 to 0.5 per cent iodoacetate with insulin-I<sup>131</sup> for periods longer than 6 to 8 hours at 37° C results in loss of ability of insulin-I<sup>131</sup> to adsorb to cellulose, possibly a result of slow interaction of iodoacetate with amino groups as is suggested by an increase in electrophoretic mobility of all the serum proteins. (Incubation at 4° C for as long as 9 days does not generally lead to this change.) However, the ability of the altered insulin-I<sup>131</sup> to bind to antibody is not detectably impaired.

as virtually all the iodide $^{131}$  not removed by dialysis. Adsorbed radioactivity eluted with plasma contained 2 to 6 per cent migrating components. Evidence that the fraction remaining at the origin (in control plasma) is largely, if not entirely, insulin- $I^{131}$  is provided by experiments with specific insulin antisera. When tracer quantities of the purified  $I^{131}$ -labeled human insulin were incubated with guinea pig anti-beef insulin or anti-pork insulin serums or with human anti-beef-pork insulin serums containing high concentrations of insulin-binding antibodies, at least 80 to 90 per cent of the adsorbed radioactivity was bound to antibody (Figure 2). This is true also of the adsorbed radioactivity in the unpurified preparations. It is possible that even higher fractions were bound to antibody in the test tubes and that dissociation of the insulin-antibody complexes during migration resulted in the observed trailing of 10 to 20 per cent. In support of this suggestion is the observation that the percentage remaining at the origin and trailing up to the antibody region

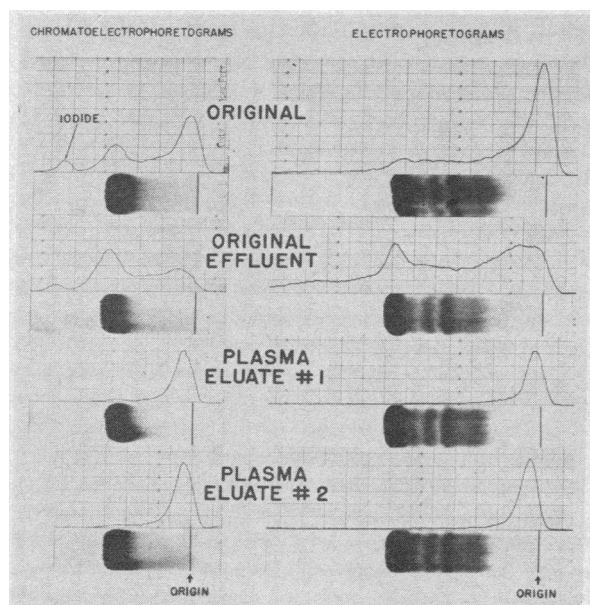


FIG. 1. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS AND CONVENTIONAL ELECTROPHORESIS OF CRUDE HUMAN INSULIN- $I^{131}$  ("ORIGINAL") AND FRACTIONS OBTAINED DURING PURIFICATION ON A CELLULOSE COLUMN. All fractions were added to control (nonimmune) plasma prior to application on the paper strips. Chromatoelectrophoresis requires about 30 to 60 minutes, resolves the iodide peak, and lumps most non-insulin- $I^{131}$  components into a single peak moving away from the origin with the serum proteins.

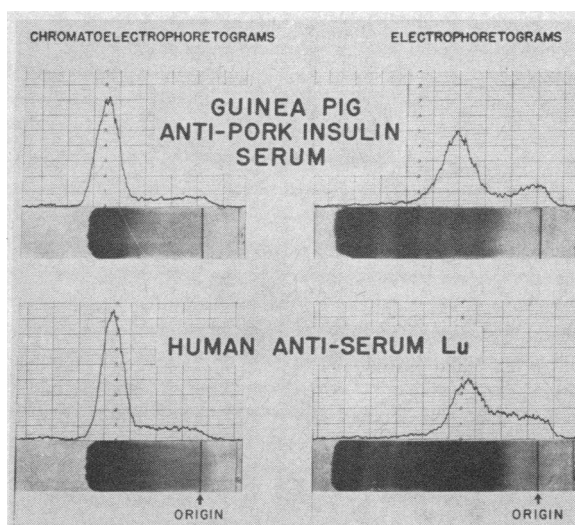


FIG. 2. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS AND CONVENTIONAL ELECTROPHORESIS OF HUMAN INSULIN- $I^{131}$  (PLASMA ELUATE NO. 1 OF FIGURE 1) ADDED TO INSULIN ANTISERUMS. Note that insulin-antibody complex migrates between  $\gamma$ - and  $\beta$ -globulins in both guinea pig and human antisera, whereas only negligible amounts of radioactivity migrate with serum proteins in control (nonimmune) serums (Figure 1).

is greater in the electrophoretograms run over an 18 hour period than in the short-run (30 to 60 minutes) chromatoelectrophoretograms, the longer time required in the former procedure allowing for greater dissociation. On the other hand, in similar experiments with beef insulin- $I^{131}$ , 99 per cent or more of the radioactivity is demonstrably bound to antibody in both chromatoelectrophoretograms and electrophoretograms.

Since generally about 65 per cent of total protein-bound- $I^{131}$  in the unpurified solution is adsorbed by paper in the presence of control serum, these results indicate that approximately 50 to 60 per cent of the protein-bound- $I^{131}$  in the unpurified preparation represents insulin- $I^{131}$ . Since insulin comprises only 27 per cent by weight of the preparation used for iodination, it can be concluded that one-half or more of the non-insulin contaminants is either not able to be iodinated under these conditions, is iodinated less readily than insulin itself, or is dialyzable.

To evaluate quantitative differences between the reaction of human and beef insulin with antibody, antisera from eight insulin-treated diabetic subjects were incubated for 15 minutes at 37° C with mixtures of human insulin- $I^{131}$  and

TABLE II  
*Insulin-binding capacity of serum*

Subject	Insulin dosage at time serum drawn	Maximum beef insulin-binding capacity of serum*	Insulin concentration tested	Insulin bound after 15 min at 37°C†	
				Human	Beef
M.N.	U/day 0	U/L 7	U/L 0.25 1.0	U/L 0.10 0.41	U/L 0.17 0.66
Mo	50	5.5	1.0	0.71	0.70
Low	65	7	0.25 1.0	0.14 0.52	0.19 0.74
H.L.	0	22	3.0	0.56	1.6
Gal	90-110	28	0.25 1.0	0.21 0.81	0.23 0.88
Lo	Unknown, but known to be resistant	97	0.25 1.0	0.20 0.72	0.24 0.93
Gru	800	210	0.25 1.0	0.18 0.74	0.23 0.89
Ge	700	250	0.25 1.0 3.0	0.19 0.71 1.7	0.23 0.94 2.6

\* Determined with diluted antiserum and higher insulin concentrations at equilibrium (after prolonged incubation).

† Corrected for damaged insulin- $I^{131}$  and assuming that only 85% of adsorbed human insulin- $I^{131}$  was available for binding to antibody.

unlabeled Fisher human insulin or beef insulin- $I^{131}$  and unlabeled beef insulin at total insulin concentrations of 0.25, 1.0, or 3.0 U per L, and then applied to paper strips in a cold room (4° C) for paper chromatoelectrophoresis. Of the eight antisera studied (Table II), three (Lo, Gru, Ge) were from patients who were quite definitely insulin-resistant and had insulin-binding capacities ranging from 97 to 250 U per L; one was from a patient (Gal) who could probably be termed mildly resistant and had a binding capacity of 28 U per L, and one was from a subject (H.L.) reported previously (3) who had an insulin-binding capacity of 22 U per L. The lowest of the concentrations used (0.25 U per L) is in the range of values of endogenous insulin found in normal subjects and in patients with early maturity-onset diabetes, 0.5 to 2 hours after oral administration of 100 g glucose (5); the second concentration used (1 U per L) is at the upper limits of the range of values observed after heavier glucose loading. The highest concentration employed (3 U per L) is considerably beyond the range of endogenous insulin concentrations that we have observed in man (5), but is certainly frequently exceeded in insulin-resistant subjects given large therapeutic doses of insulin.

In almost all cases, more beef insulin than human insulin was bound at the same insulin con-

centration by the same antiserum (Figure 3, Table II) but in some cases the differences were small after correction for the fraction of radioactivity presumed unavailable for binding to antibody. However, in several subjects (M.N., H.L. and Ge) the binding of human insulin was significantly lower than the binding of beef insulin at the same concentration. In all subjects the concentrations employed were generally far below the maximal binding capacities of the antisera so that frequently most of the insulin was bound. At higher concentrations more marked differences might have become evident.

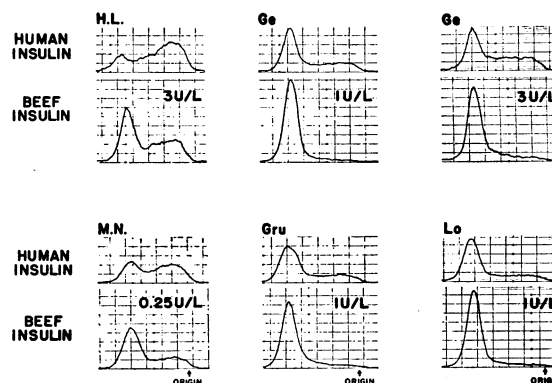


FIG. 3. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS OF INSULIN-ANTISERUM MIXTURES INCUBATED FOR 15 MINUTES AT 37° C.

## DISCUSSION

Studies such as have been reported here should become considerably easier to perform when pure human insulin becomes available. However, it is evident that the crude preparation employed is adequate for the demonstration of the binding of human insulin to human antibeef-pork insulin antibodies and for general quantitative estimation of the extent of such binding. In previous studies on the competitive inhibition offered by unlabeled human insulin to the binding of  $I^{131}$ -labeled animal insulins (1), only a weak inhibitory effect of human insulin was observed with antiserum H.L. In the present study this finding is confirmed by the direct demonstration of a weak reaction with human insulin- $I^{131}$ , but such marked differences between human insulin and beef insulin are seen not to hold uniformly in all subjects. In other experiments, not reported here, in which unlabeled human insulin and unlabeled beef insulin each competed with beef insulin- $I^{131}$ , it was observed that the competitive reaction with human insulin is strong where the direct reaction of human insulin- $I^{131}$  is strong and that a weak competitive reaction is confirmed by a weak direct reaction, as in Subject H.L.

The differences in behavior of various antisera toward human and beef insulin help explain certain clinical phenomena. For example, Subject H.L., whose clinical course is described in greater detail elsewhere (3), maintained essentially the same moderately good control of his diabetes in the absence or presence of insulin therapy (55 U protamine zinc insulin daily). It can be concluded that the strong binding of beef insulin to his antibodies deprived him of the benefit of this treatment, whereas the much weaker binding of human insulin permitted the advantageous utilization of his own endogenously secreted human insulin. Also, Subject M.N., who took insulin therapy for about 1 year but who has for the past 4 years maintained excellent control without insulin treatment, showed a stronger reaction with beef insulin than with human insulin, although his insulin-binding capacity was in the range found in nonresistant subjects. Furthermore, experience has shown that some insulin-resistant patients may be well controlled on sulfonylurea drugs. Presumably the endogenous in-

ulin secreted in response to stimulation by the sulfonylureas reacts less well with antibody than do the administered animal insulins. Another case in point is the report of Lowell (12) that 30 U of human insulin was capable of reducing the blood sugar in a patient resistant to a similar dose of beef-pork insulin.

The reaction of human insulin with human antisera to beef-pork insulin does not, of course, imply that human insulin is antigenic in man. In fact, we have never observed any evidence of binding of insulin in sera of patients who never received animal insulin. The reaction simply indicates that once the antigenicity of animal insulins has resulted in the production of antibodies, these antibodies are then able to react also with the closely similar human insulin. Since insulin-antibody complexes are of small molecular size, remain soluble (3), and do not appear to be sensitizing, allergic manifestations of such complex formation are not exhibited.<sup>6</sup> Such might not be the case were the antigenic characteristics of insulin somewhat different. It appears not beyond the realm of possibility that certain autoimmune states may originate by immunization with foreign antigens (through ingestion or by entry through other portals) which chemically resemble certain endogenous proteins.

## SUMMARY AND CONCLUSIONS

1. The preparation of  $I^{131}$ -labeled human insulin from a lot of human insulin containing approximately 25 per cent insulin by weight and its purification from labeled contaminants are described.
2. The reaction of human insulin- $I^{131}$  with insulin-binding antibodies in the sera of human subjects treated with commercial mixtures of animal insulins is demonstrated directly.
3. Comparison of the binding of human insulin and beef insulin in antisera from eight insulin-resistant and nonresistant diabetic subjects revealed a lesser affinity of antibody for human than for beef insulin in most cases, but considerable variability in this respect was encountered among different antisera.

<sup>6</sup> Allergy to insulin is an expression of reaction with sensitizing antibody (13), an antibody quite distinct from "blocking" antibody (13) and the insulin-binding antibody reported here (14).

4. It is suggested that the ability of certain diabetic patients (who show evidence of insulin resistance to exogenous insulin) to tolerate withdrawal of insulin therapy, with or without the aid of sulfonylurea agents, is related to the relatively weak binding to antibody of endogenously secreted human insulin.

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