Diabetologia 2, 1–13 (1966)

REVIEWS

Review of Current Research

Genetic Differences of Combining Sites of Insulin Antibodies and Importance of C-Terminal Portion of the A Chain to Biological and Immunological Activity of Insulin*

EDWARD R. ARQUILLA, M. D., HENRI OOMS, M. D., and JACK FINN

Department of Pathology, University of California, School of Medicine, Los Angeles, California

Received January 27, 1966

Summary. Using an insoluble insulin complex, it was possible to demonstrate that antibodies to insulin produced in individual animals are directed towards different portions of the insulin molecule. Furthermore, using the antisera from two different inbred strains of guinea pigs and their F_1 and F_2 offspring, evidence is presented for the genetic control of combining site configurations of antibodies to insulin produced in guinea pigs. The importance of different portions of the insulin molecule to biological and immunological activity was investigated. The attachment of ^{125}I to insulin (reportedly to the tyrosines at positions 14 and/or 19 of the A chain) seems to impair both the biological and immunological activity of insulin. The distribution of antibody-bound and free ^{125}I-insulin was found to be different from the distribution of non-labeled insulin. Relatively pure ^{125}I-insulin was separated from non-labeled insulin by acrylamide gel electrophoresis, and was found to have markedly reduced immunological reactivity in the immune hemolysis inhibition assay. It was concluded that antibodies to insulin exist which cannot react with iodinated insulin. Furthermore, when tested in the rat adipose tissue assay, purified ^{125}I-insulin preparations had little or no biological activity. Conversely, mono-substituted fluorescein-labeled insulin (purified on acrylamide gel electrophoresis) appears to retain full immunological and biological competence. Fluorescein is thought to attach to the N-terminal phenylalanine of the B chain and/or to the N-terminal glycine of the A chain. It is concluded that these two residues contribute little to the biological and immunological integrity of insulin. Studies of this nature aid in elucidating the surface configuration of insulin, and thereby may contribute to an understanding of its tertiary structure.

Les différences génétiques des sites de combinaison des anticorps anti-insuline et l'importance de la portion C-terminale de la chaîne A pour l'activité biologique et immunologique de l'insuline.

Résumé. En utilisant un complexe insulinique insoluble il a été possible de démontrer que les anticorps pour l'insuline produits par les animaux considérés individuellement sont dirigés vers différentes parties de la molécule d'insuline. De plus en utilisant les antisérums de 2 souches pures différentes de cobayes et de leur descendance F_1 et F_2 on a pu montrer que les facteurs génétiques contrôlent la configuration des sites de combinaison des anticorps anti-insuline produits par les cobayes. L'importance de différentes parties de la molécule d'insuline en ce qui concerne l'activité biologique et immunologique a été étudiée. La fixation de ^{125}I à l'insuline (que l'on a rapporté s'effectuer sur les tyrosines en position 14 et/ou 19 de la chaîne A) semble diminuer à la fois l'activité biologique et immunologique de l'insuline. La répartition de l'insuline liée aux anticorps et libre a été trouvée différente pour l'insuline-^{125}I et pour l'insuline non marquée. Une insuline-^{125}I relativement pure a été séparée de l'insuline non marquée par électrophorèse sur gel d'acrylamide et a présenté une activité immunologique nettement diminuée dans le dosage par inhibition de l'immune-hémolyse. Il a été conclu qu'il existe des anticorps anti-insuline qui ne peuvent pas réagir avec l'insuline iodée. En outre, les préparations d'insuline-I^{125} purifiée ont peu ou pas d'activité biologique quand on les teste par dosage sur le tissu adipeux de rat. Par contre, l'insuline mono-substituée marquée à la fluorescéine (purifiée par électrophorèse sur gel d'acrylamide) paraît conserver tout son pouvoir immunologique et biologique. On pense que la fluorescéine se fixe à la phénylalanine N-terminale de la chaîne B et/ou à la glycine N-terminale de la chaîne A. On conclut que ces 2 résidus contribuent peu à l'intégrité biologique et immunologique de l'insuline. Des études de ce genre aident à élucider la configuration de surface de l'insuline, et par conséquent peuvent contribuer à comprendre sa structure tertiaire.

Genetische Unterschiede in den Bindungsstellen der Insulinantikörper und die Bedeutung des C-terminalen Anteils der A-Kette für die biologische und immunologische Aktivität des Insulins.

Zusammenfassung. Unter Verwendung eines unlöslichen Insulinkomplexes konnte gezeigt werden, daß die von einem Tier gebildeten Insulinantikörper gegen verschiedene Teile des Insulinmoleküls gerichtet sind. Weiter wurde unter Verwendung von Antiseren zweier verschiedener Inzuchtsstämme von Meerschweinchen und ihrer F_1 und F_2-Nachkommen der Nachweis für die genetische Steuerung der Konfiguration der Bindungsstellen der bei Meerschweinchen gebildeten Antikörper geführt. Es wurde die Bedeutung der verschiedenen Anteile des Insulinmoleküls für die biologische und immunologische Aktivität untersucht. Das Anheften von ^{125}I an das Insulinmolekül (anderen Arbeiten zufolge an das Tyrosin in Stellung 14 und/oder 19 der A-Kette) scheint die biologische und immunologische Aktivität des Insulins zu verändern. Die Verteilung von antikörpergebundenem und freiem ^{125}I-Insulin unterschied sich von der Verteilung des nichtmarkierten Insulins. Relativ reines ^{125}I-Insulin, das mit der Acrylamid-Gel-Elektrophorese von nichtmarkiertem Insulin getrennt wurde, zeigte im immunologischen Hämolysehemmtest deutlich herabgesetzte immunologische Aktivität. Es wurde angenommen, daß es Insulinantikörper gibt, die nicht mit Jodinsulin reagieren können. Außerdem hatte gereinigtes ^{125}I-Insulin wenig oder gar keine biologische Aktivität bei der Bestimmung mit der Methode mit Rattenfettgewebe. Im Gegensatz dazu scheint monosubstituiertes fluorescinmarkiertes Insulin, (gereinigt mit der Acrylamid-Gel-Elektrophorese) die volle immunologische und biologische Wirkung zu behalten. Es wird angenommen, daß Fluorescin an das N des endständigen Phenylalanins der B-Kette und/oder an das N des endständigen Glycins der A-Kette angelagert wird. Es wird geschlossen, daß diese beiden Reste wenig für die biologische oder immunologische Integrität des Insulins ausmachen. Untersuchungen dieser Art tragen dazu bei, die Oberflächenkonfiguration des Insulinmoleküls aufzuklären und können dadurch einen Beitrag zur Kenntnis seiner Tertiärstruktur liefern.

* This study was supported by United States Public Health Service Grant AM 06001.

This paper is based on a talk given before the British Insulin Manufacturers' Colloquium on "The Immunology of Insulin" on September 16, 1965.

Genetic differences of combining sites of insulin antibodies

Several years ago we observed that rabbit antisera to insulin did not neutralize the biological activity of crystalline insulin, whereas guinea pig and human antisera to insulin did [1]. One explanation for these observations was that guinea pigs and humans make antibodies to parts of the insulin molecule which are different from those portions of the insulin molecule to which rabbits make antibodies. The experiments to be presented were designed to test this possibility. Although the evidence does not support such an explanation, it was possible to demonstrate that many rabbits produced antibodies which bound to portions of the insulin molecule to which guinea pigs or other rabbits could not bind. Similarly, occasional guinea pigs produced antibodies which bound to portions of the insulin molecule to which antibodies from rabbits and other guinea pigs could not bind [2].

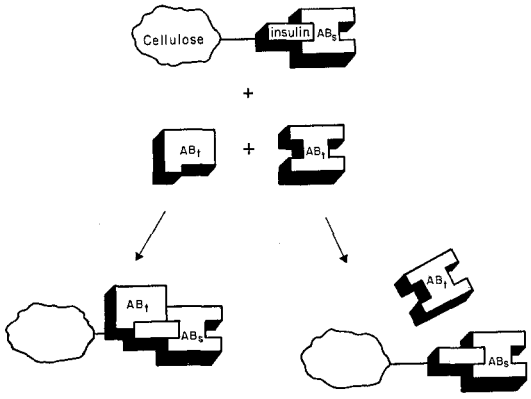


Fig. 1. Binding of various insulin antibodies to insoluble insulin aggregates saturated with antibody

The method (Fig. 1) that we used to demonstrate these differences involved the preparation of an insoluble insulin complex (IC)[1] which was saturated with antibodies (AB_s–IC) from a single saturating antiserum (AB_s). The AB_s–IC was used as a reagent to test for the presence of antibodies in a test antiserum (AB_t) which could react with groupings on insulin with which AB_s could not react. It was assumed that such antibodies in AB_t were directed to sites on the insulin molecule different from those portions of the insulin molecule with which antibodies in AB_s could react, in which case, decreased amounts of insulin antibodies were observed in AB_t following the incubation of AB_t with AB_s-IC. If, however, any given AB_t did not contain antibodies directed to parts of insulin different from the determinants with which the antibodies in AB_s reacted, then no decrease in the amount of insulin antibodies in AB_t was observed following incubation of AB_t with AB_s-IC.

The reliability of such experiments required a method capable of quantitatively measuring the relative amounts of antibodies in two aliquots of the same antiserum. For this purpose the immune hemolytic technique was used. The amount of hemoglobin released following the reaction of antibodies with insulin-coated cells in the presence of excess complement has proven to be an accurate measure of relative antibody concentration. The precision of the method was tested by performing immune hemolytic titrations of five aliquots of antiserum with the same cells on the same day (Table 1). The titration curves were plotted and the area under each curve estimated by planimetry. The standard deviation was found to be $\pm$ 1.5% from the mean of the five samples.

Table 1. *Reproducibility of immune hemolytic titration of antiserum*[1]

Aliquot Number	Dilutions 1/20	1/80	1/320	1/1280
1	.495[2]	.340	.125	.065
2	.480	.330	.115	.070
3	.500	.340	.115	.070
4	.485	.330	.125	.070
5	.500	.330	.115	.075

[1] Antiserum to insulin from rabbit C 5.
[2] Extinction observed at 580 millimicra proportional to hemoglobin released by immune hemolysis for estimation of antibody concentration. The calculated relative antibody concentration of these 5 aliquots had a standard deviation of 1.5%, using the mean as 100%.

In these experiments, an aliquot of non-incubated AB_t was compared to an aliquot of AB_t which had been incubated with AB_s-IC. The percent of antibodies which reacted with exposed determinants on AB_s-IC was calculated from the difference in area of the titration curves of the incubated and non-incubated AB_t aliquots (Fig. 2). An antibody decrease of 10% or more in the incubated AB_t was considered to be definite evidence that antibodies were present in AB_t which combined with sites on insulin different from the sites with which antibodies in AB_s combined.

The validity of these experiments required that the IC be saturated with antibodies from the AB_s. In all experiments, saturation of IC was determined by titrating the third aliquot of AB_s which had been incubated with IC and comparing its antibody level to an aliquot of the AB_s which had not been incubated with IC. In Fig. 3, titration curves demonstrating saturation of two insulin aggregates by antibodies from a rabbit and a guinea pig antiserum respectively are presented. After the first incubation with IC, the antibody titer is markedly decreased. After the third incubation, it

[1] The following abbreviations are used throughout the paper:
IC = insoluble insulin complex
AB_s-IC = antibody saturated insulin complex
AB_t = test antiserum
str 2 = NIH strain 2 histocompatible guinea pigs
str 13 = NIH strain 13 histocompatible guinea pigs
F_1 = str 2 $\times$ str 13 offspring
F_2 = $F_1 \times F_1$ offspring
Rab-GPIG = rabbit anti-guinea pig immune globulin

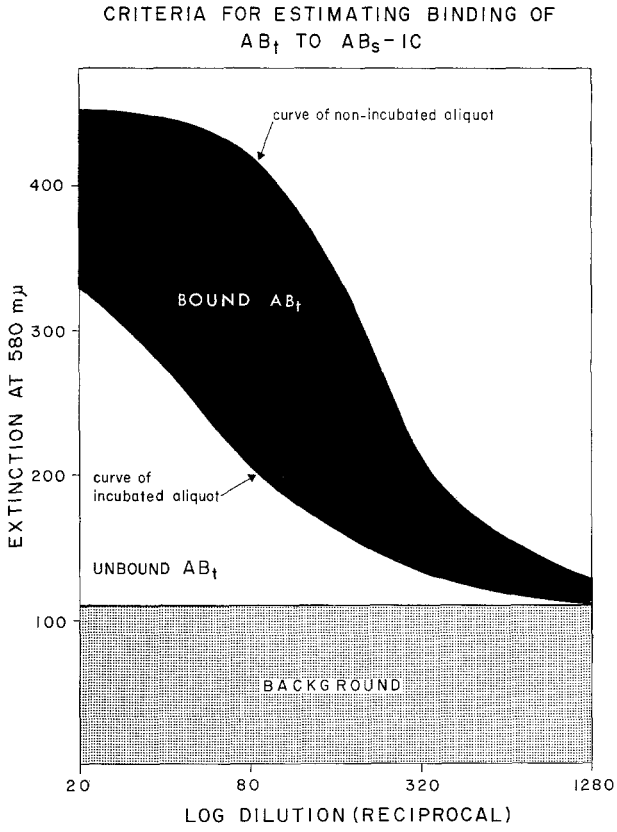


Fig. 2. Criteria for estimating binding of AB_t to AB_s-IC

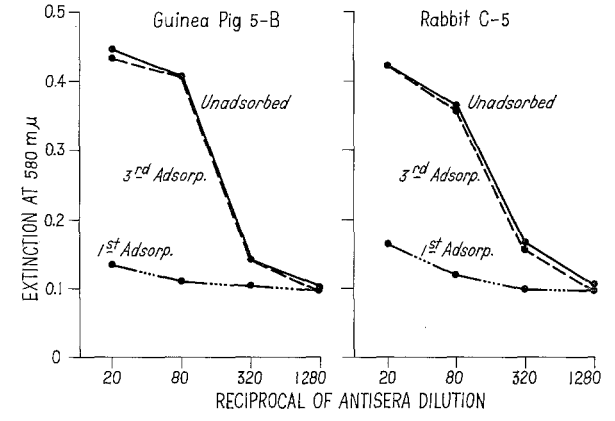


Fig. 3. Saturation of insoluble insulin aggregates with guinea pig and rabbit antibodies

is virtually the same as a non-incubated aliquot of the same antiserum.

We were not able to demonstrate saturation of insulin aggregates in about 5% of the experiments. Occasionally, with very low titered antisera, difficulty in saturating IC with antibodies was encountered. These experiments were not considered valid.

The binding of AB_t to AB_s-IC was attributed to antibodies in the AB_t directed to portions of the insulin molecule with which antibodies in the AB_s could not react. An alternative possibility is the displacement from the AB_s-IC of antibodies with reduced hemolytic activity. Antibodies in AB_t could then react with those portions of the insulin molecule from which such antibodies had been displaced, resulting in a net decrease in hemolytic activity of incubated AB_t. Using I^{131}-labeled insulin antibodies, it was possible to show that AB_s was not displaced from AB_s-IC to a significant degree [2].

Insulin antibody differences were observed when rabbit antisera were tested against insulin aggregates saturated with guinea pig antibodies and vice versa. It was not possible to predict qualitatively or quantitatively which rabbit AB_t possessed antibodies capable of binding to any specific guinea pig aggregate; consequently, no correlation between the capacity of guinea pig antisera to neutralize the biological activity of crystalline insulin and the relative inability of rabbit antisera, was possible.

The results did suggest, however, that antibody variation existed within each of the species. It was subsequently possible to demonstrate that rabbit antisera frequently contained antibodies capable of binding to insulin molecules which had previously been saturated with antibodies from other rabbits (Table 2). In seven of 12 experiments, rabbit AB_t contained antibodies capable of binding with rabbit AB_s-IC. This variability is concerned with the antigenic determinants to which the antibodies are directed. Furthermore, it appears that rabbits produce measurable amounts of antibodies to several antigenic determinants on insulin.

Similar variations were noted (Table 3) amongst insulin antisera produced by guinea pigs. Twenty-one experiments were performed in which 14 different guinea pig AB_t were tested against seven guinea pig AB_s-IC aggregates. Definitive binding of guinea pig AB_t to guinea pig AB_s-IC was noted in three or possibly four of the experiments, as compared to the binding of rabbit AB_t to rabbit AB_s-IC which was observed in seven of 12 experiments. These results emphasize the

Table 2. *Binding of various rabbit antibodies (AB_t) to AB_s-IC saturated by various rabbit antisera*

AB_s (Rabbit)	AB_t (Rabbit)	Percent AB_t bound
	C 20	30.5 ←
	C 29	3.5 ←
C 5	C 30	75.3 ←
	C 31	1.0
	C 33	0.5
C 20	C 5	53.5 ←
	C 5	38.5 ←
C 28	C 29	30.0 ←
	C 30	0.5 ←
	C 31	0.5 ←
C 33	C 5	82.3 ←
	C 28	93.8 ←

← Indicates significant binding.

Table 3. *Binding of various guinea pig antibodies (AB_t) to AB_s-IC saturated by various guinea pig antisera*

AB_s (Guinea pig)	AB_t (Guinea pig)	Percent AB_t bound
1 B	2 B	0.5
	2 B	1.0
1 C	4 C	2.0
	5 c	0.5
	8 B	0.5
2 B	1 B	56.4 ←
	1 C	1.0
	3 B	1.0
2 C	5 B	0.5
	8 B	1.0
5 B	8 B	3.0
	1 D	18.0 ←
	2 D	0.5
6 B	3 D	0.5
	4 D	0.5
	5 D	1.0
	6 D	10.0 (?)
	1 B	2.0
8 B	1 C	18.0 ←
	5 B	0.5
	5 C	0.5

← Indicates significant binding.

greater degree of similarity among insulin antibodies produced by guinea pigs and the relatively marked variability of the insulin antibodies produced by rabbits. Determinants to which insulin antibodies are directed appear to be characteristic for the individual rabbit or guinea pig immunized. At this point, it was postulated that genetic factors direct antibody production toward specific determinants when insulin is the antigen.

We were fortunate to have the cooperation of several investigators[2] in obtaining highly inbred NIH strain 2 and strain 13 guinea pigs. Using these animals it was possible to obtain supporting evidence [3] for the genetic control of insulin antibody production. It appeared that str 2 guinea pigs produced antibodies to portions of beef insulin to which str 13 guinea pigs could not produce antibodies. Since then it has been possible to obtain additional evidence demonstrating that genetic factors control the configuration of the combining site of insulin antibodies produced by guinea pigs [4].

The following criteria were considered necessary to support the postulate that genetic factors control the configuration of the insulin antibody combining sites in guinea pigs:

1. No differences in configuration of antibody-combining site should be demonstrable in guinea pigs of the same strain; i.e., str 2 AB_t should not bind to str 2 AB_s-IC, or str 13 AB_t should not bind to str 13 AB_s-IC.

2. Differences in configuration of antibody-combining site should be demonstrable when antisera from str 2 guinea pigs are compared to antisera from str 13 guinea pigs.

3. Segregation of such differences should be demonstrable when insulin antibodies from hybrid guinea pigs (F_1 and F_2) are compared to insulin antibodies from str 2 and str 13 parents.

Table 4 summarizes experiments in which str 2 antisera were tested against insulin aggregates saturated with str 2 antibodies and str 13 antisera were tested against insulin aggregates saturated with str 13

Table 4. *Similarity in combining sites of insulin antibodies from guinea pigs of the same strain*

Strain 2			Strain 13		
Str. 2 AB_s	Str. 2 AB_t	% AB_t bound	Str. 13 AB_s	Str. 13 AB_t	% AB_t bound
Pool[1]	C 12[3]	0.5	Pool[2]	D 22	0.5
	CB 2	0.5		DB 2	0.5
				DB 3	0.5
C 1	C 2	0.5			
	C 3	0.5	D 2	D 1	0.5
				D 3	0.5
C 9	Pool[1]	0.5			
			D 7	D 6	0.5
C 17	C 18	0.5			
	C 19	0.5	D 15	Pool[2]	0.5
	C 25	0.5			
	C 26	0.5	D 32	D 15	0.5
				D 24	0.5
CB 2	C 12	0.5			
			DB 2	Pool[2]	0.5
CF 1	CD 6	0.5			
	CF 2	9.5	D 16	D 14	0.5
	CG 2	0.5		DK 4	0.5
Total: no Binders in 28 Experiments				DK 5	0.5
				DK 6	0.5

[1] This pool was a combination of approximately equal aliquots of antisera from 7 Str. 2 guinea pigs.
[2] This pool was a combination of approximately equal aliquots of antisera from 5 Str. 13 guinea pigs.
[3] This AB_t was tested twice against the same AB_s-IC.

antibodies. In 13 experiments, none of the str 2 AB_t contained demonstrable antibodies with combining sites different from the antibodies from six str 2 AB_s. Likewise, in 14 experiments, none of the AB_t from str 13 guinea pigs contained significant amounts of insulin antibodies capable of binding to AB_s-IC saturated with insulin antibodies from seven str 13 AB_s. There was one antiserum (CF 2) amongst the str 2 AB_t which had border line amounts (9.5%) of antibodies capable of combining with an insulin aggregate saturated with antibodies produced by a litter mate. The

[2] The authors are grateful to Dr. CHARLES W. MC PHERSON, DVM, Chief, Animal Production Section of National Institutes of Health, Bethesda, Maryland, Dr. ROY WALFORD, Department of Pathology, University of California, School of Medicine, Los Angeles, California and Dr. CHARLES COCHRANE, Division of Experimental Pathology, Scripps Clinic and Research Foundation ,La Jolla, California, for supplying the majority of the animals used in these experiments.

most likely explanation for this observation is the presence of residual heterozygosity amongst our theoretically inbred str 2 animals. No such indication has been noted amongst the str 13 animals.

These experiments indicate that inbred guinea pigs of the same strain produce antibodies toward similar determinants on the insulin molecule. Consequently, it is probable that the configurations of the combining sites of insulin antibodies produced by guinea pigs are similar in animals of the same strain. The demonstration of differences in the configuration of insulin antibody-combining sites when antisera from str 2 guinea pigs were compared to str 13 antisera was complicated. These observations have been presented elsewhere [3].

Table 5. *Combining site overlap of insulin antibodies produced by F_1 offspring compared to antibodies produced by Str 2 and Str 13 guinea pigs*

F_1 AB_t vs Str. 2 AB_s-IC			F_1 AB_t vs Str. 13 AB_s-IC		
Str. 2 AB_s	F_1 AB_t	% AB_t bound	Str. 13 AB_s	F_1 AB_t	% AB_t bound
Pool[1]	40	6.0	Pool[2]	40	0.5
	41	0.5		41	0.5
	43	3.5		43	0.5
	44	0.5		44	0.5
	45	0.5		45	0.5
	46	0.5		46	38.2←
	47	0.5		47	0.5
	48	0.5		48	0.5
	53	0.5		53	0.5
CB 2	41	0.5	DB 2	41	0.5
	44	0.5		44	0.5
	45	0.5		45	0.5
	46	0.5		46	59.0←
				48	0.5
CF 1	40	11.0←			
	50	7.8	DK 1	41	0.5
				44	60.5←
CG 2	41	0.5		49	0.5
	44	0.5		51	47.6←
	48	0.5		52	35.1←
Total: 7 binders in 42 experiments			DK 2	41	9.5
				44	20.9←
				48	0.5
				50	8.9
				52	0.5

← Indicates Significant binding.

[1] This pool was a combination of approximately equal aliquots of antisera from 7 Str. 2 guinea pigs.

[2] This pool was a combination of approximately equal aliquots of antisera from 5 Str. 13 guinea pigs.

Table 5 summarizes the results obtained from experiments designed to demonstrate configurational differences of combining sites of insulin antibodies produced by F_1 offspring compared to antibodies produced by str 2 and str 13 guinea pigs. In all, 42 experiments were performed. In seven of the 42 experiments, F_1 hybrid AB_t contained antibodies which had combining site configurations different from the configurations of the combining sites of insulin antibodies produced by str 2 or str 13 guinea pigs. Eighteen experiments testing F_1 AB_t against IC saturated with str 2 antisera and 24 experiments testing F_1 AB_t against IC saturated with str 13 antisera were performed.

We now feel confident that the F_1 offspring may have combining site configurations which are different from the inbred parents. More specifically, it appears that the differences in the configurations of the antibody combining sites of the F_1 antisera are demonstrated more frequently and markedly when IC is saturated with str 13 antisera. Six of 24 F_1 AB_t contained significant quantities of antibodies which combined with sites on insulin to which str 13 AB_s could not combine, whereas only one of 18 F_1 hybrid AB_t had significant quantities of antibodies which could combine with insulin sites left exposed when str 2 antibodies were used to saturate.

In Table 6, we see that when antisera from F_2 hybrids ($F_1 \times F_1$) were tested, combining site differences were more frequent (15 of 36 experiments) than when the F_1 antisera were tested (seven of 42 experiments). Unlike the antibodies produced by F_1 hybrids, the frequency and magnitude of F_2 antibody combining site configurational differences was about the same

Table 6. *Configurational differences of combining sites of insulin antibodies produced by F_2 animals compared to antibodies produced by Str 2 and Str 13 guinea pigs*

F_2 AB_t vs Str. 2 AB_s-IC			F_2 AB_t vs Str. 13 AB_s-IC		
Str. 2 AB_s	F_2 AB_t	% AB_t bound	Str. 13 AB_s	F_2 AB_t	% AB_t bound
Pool[1]	62	63.0←	Pool[2]	62	42.2←
	63	0.5		63	3.5
	64	49.0←		65	0.5
	65	0.5			
	66	0.5	D 15	61	1.6
	67	0.5		62	60.8
				63	2.2
C 14	61	39.5←		64	99.0←
	62	80.1←			
	63	0.5	D 25	61	2.6
	64	71.1←		62	75.1←
				63	2.0
CF 1	49—85	0.5		64	88.7←
	52—91	0.5			
Total: 15 binders in 36 Experiments			DB 2	64	39.0←
			DK 1	63	0.5
				64	24.0←
DK 2	49—78	30.0←			
	52—82	0.5			
DK 3	49—68	0.5			
	49—77	14.3←			
	49—78	32.0←			
	50—76	0.5			
	51—73	0.5			
	51—74	35.0←			
	52—82	0.5			
	52—83	0.5			

← Indicates significant binding.

[1] This pool was a combination of approximately equal aliquots of antisera from 7 Str. 2 guinea pigs.

[2] This pool was a combination of approximately equal aliquots of antisera from 5 Str. 13 guinea pigs.

when tested against either str 2 AB_s (five of 12 experiments) or str 13 AB_s (ten of 24 experiments).

The evidence presented up to this point shows: 1) no configurational differences of antibody combining sites amongst guinea pigs of the same strain; 2) configurational differences of insulin antibody combining sites between str 2 and str 13 guinea pigs; 3) segregation of these differences in F_1 and F_2 guinea pigs. It is therefore concluded that genetic factors control the configuration of the insulin antibody combining sites in guinea pigs. Since insulin antibody combining sites from F_2 guinea pigs can have configurations that are different from the configurations of the insulin antibodies produced in either of the two inbred parental lines, we feel that it is probable that more than one gene and not multiple alleles at a given gene locus are controlling the configuration of the combining sites of antibodies produced against insulin by guinea pigs. Such a possibility is consistent with the latest proposed model of γG antibody which suggests that the configuration of the antibody combining site of γG is formed by sequences of amino acids in contiguous portions of both the light (λ) and heavy (γ) chains [7, 8]. Consequently, it is possible that at least two genes are involved in dictating the configuration of the antibody combining site of γG immunoglobulin.

Importance of the C-terminal portion of the A chain to the biological and immunological activity of insulin

The specific portions of the insulin molecule with which insulin antibodies combine are not known. Such information would help identify amino acid residues on the surface of the insulin molecule and consequently help elucidate the various configurations of the combining sites of insulin antibodies.

LANDSTEINER [10] has demonstrated that halogens substituted onto haptens interfere with antibody-hapten combination. We therefore designed experiments to test whether insulin labeled with radioactive iodine reacts to the same degree with antibodies as does insulin which is not so labeled. In these studies, we have assumed that any demonstrated decrease in the reaction between antibody and iodinated insulin, as compared with the reaction between antibody and *non*-labeled insulin, was due to distortion of antigenic determinants by the attached iodine.

Two series of experiments were performed. The first series compares the distribution of free and antibody-bound non-labeled insulin with the distribution of free and antibody-bound I^{125}-insulin. To be valid, such a comparison requires that the free and bound insulin be measured by a method *not* dependent upon the distribution of radioactivity. We have used an insulin assay based on immune hemolysis inhibition and compared it to the HALES and RANDLE modification of the radioimmune assay [9].

If antibodies combine with both the I^{125}-insulin and crystalline insulin to the same degree, then the free and bound insulin assayed radioimmunologically should equal the free and bound insulin assayed by immune hemolysis inhibition. If, however, binding of antibodies with I^{125}-insulin is impaired by the iodine, then the radioimmune assay should show more free insulin and less bound to antibodies than measured by the immune hemolysis inhibition assay.

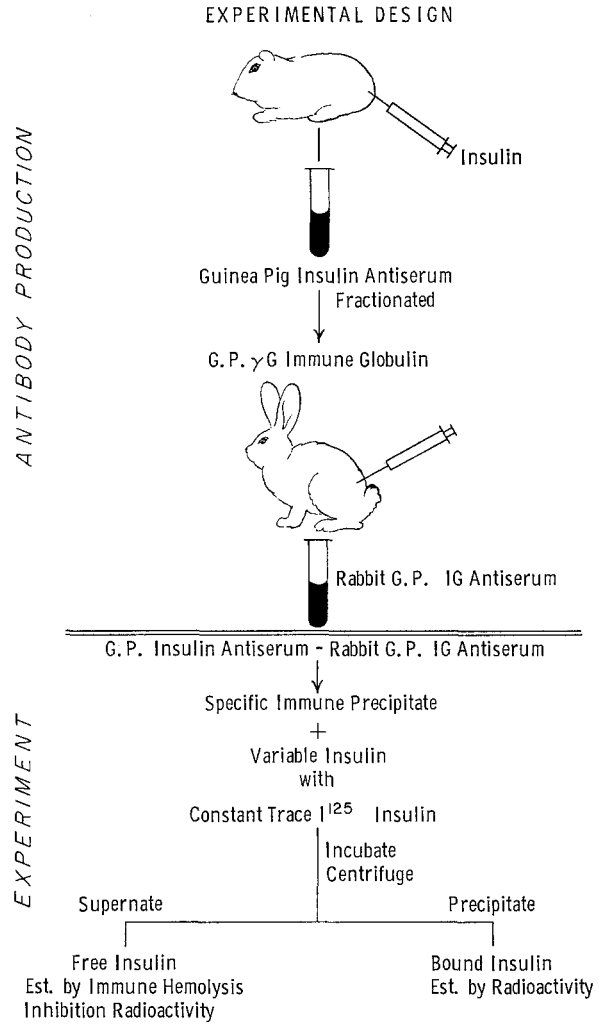


Fig. 4. Experimental design for comparison of the radioimmune insulin assay to the immune hemolysis inhibition assay

The experimental approach used for this first series of experiments is represented schematically in Fig. 4. Pooled guinea pig antiserum to beef insulin was fractionated by column chromatography using the method of YAGI and PRESSMAN [14]. The gamma globulin fraction obtained was used as an antigen to prepare rabbit antiserum to guinea pig gamma globulin. A specific immune precipitate similar to the one used by HALES and RANDLE was then prepared by adding this rabbit antiserum to guinea pig insulin antiserum. To the specific immune precipitate a constant amount of I^{125}-insulin and variable amounts of

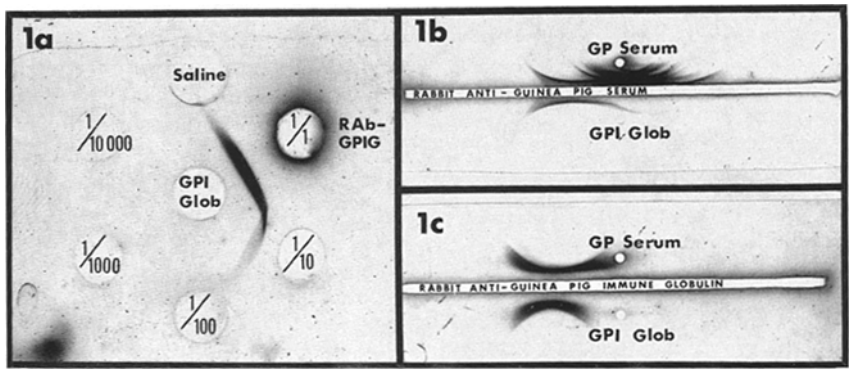


Fig. 5. Immunological homogeneity of guinea pig gamma globulin (GPIG) prepared from pooled insulin antiserum

non-labeled insulin were added. The amount of free insulin not bound to the specific immune precipitate, present in the supernate, was measured by both immune hemolysis inhibition and radioactivity.

In Fig. 5, the immunological characteristics of the reagents used to prepare the specific immune precipitate are demonstrated: 1a. is a photograph of an agar double diffusion test of the guinea pig γG rich in insulin antibodies (in the center well) against varying dilutions of rabbit anti-guinea pig γG antiserum; 1b. is an immunoelectrophoretic analysis which shows that the guinea pig γG globulin developed a single component in the γG globulin region when tested against rabbit antiserum to whole guinea pig serum; 1c. demonstrates that the rabbit antibody induced with the anti-insulin guinea pig γG developed a single precipitin arc characteristic of γG immunoglobulin when tested against the whole guinea pig serum. It therefore is probable that the specific immune precipitate consisted mainly of guinea pig γG immunoglobulin containing insulin antibodies and rabbit anti-guinea pig γG.

In order to perform these experiments, it was necessary to eliminate contaminating material from I^{125}-insulin. Such material did not precipitate with TCA and reacted poorly with excess insulin antibodies. Purification was accomplished by passing the labeled insulin through a Sephadex G 75 column in the presence of bovine serum albumin at neutral pH. In this manner, it was possible to obtain a sample of I^{125}-labeled insulin, 95% of which combined with *excess* amounts of insulin antiserum. The stock I^{125}-insulin so prepared contained 0.3 μg of insulin per ml as measured by the immune hemolysis inhibition assay.

The immune hemolysis inhibition assay is presented graphically in Fig. 6. Hemolytic titration curves were prepared for controls, insulin standards and test samples. The percent inhibition of immune hemolysis for each was calculated from the areas of the respective titration curves. The standard insulin assay curve was plotted from the percent inhibition of immune hemolysis and the log of the added insulin concentrations. The estimation of insulin in test samples was determined from the standard curve. The regression line had a range between 0.01 and 0.1 μg of insulin, a slope of 0.75, and a standard deviation of ± 6.1. The index of precision (λ) was 0.08.

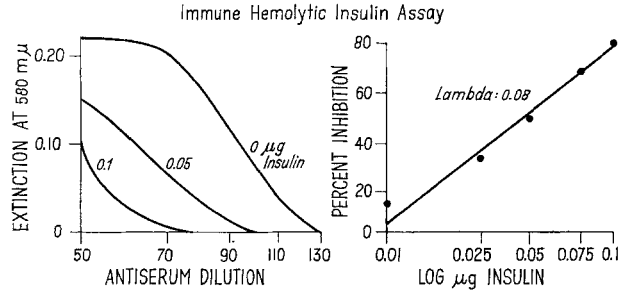


Fig. 6. Immune hemolytic insulin assay

An example of the radioimmune insulin assay procedure, as used by us, is shown in Fig. 7. All determinations were based on the amount of I^{125}-insulin displaced by added insulin. The amount of trace I^{125}-insulin bound when no insulin was added was assumed to represent 100% binding by antibody. The mean percent I^{125}-insulin displaced, plotted against the log insulin added resulted in a straight line and an index of precision of 0.05. The method and index of precision are similar to that previously reported by HALES and RANDLE [9].

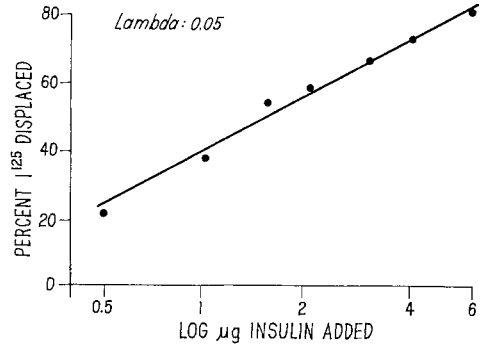


Fig. 7. Radioimmune insulin assay

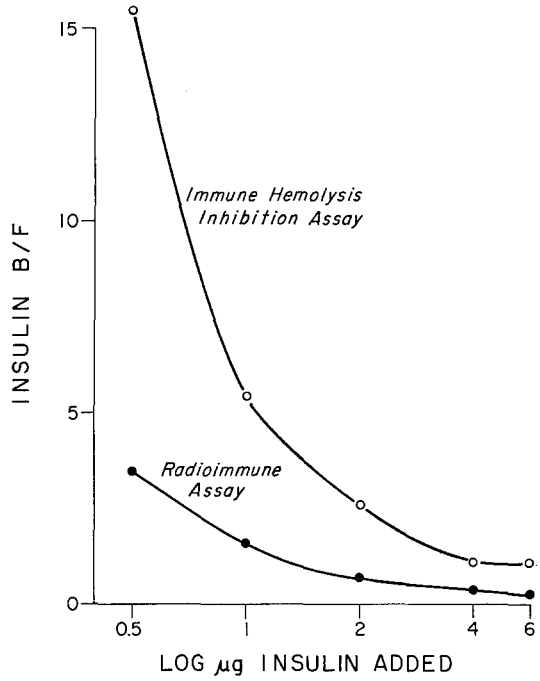


Fig. 8. Comparison of insulin bound to free (B/F) ratios measured by radioactivity and immune hemolysis inhibition*

* B/F ratios determined from the mean values obtained from three typical experiments

In Fig. 8, the ratios of bound to free insulin, as measured by the radioimmune assay and by immune hemolysis inhibition, are plotted against the log of added insulin. In all experiments and at each level of added insulin, less insulin was free and more was bound to antibody when measured by immune hemolysis inhibition than when measured by the radioimmune assay. The distribution was much more distorted at the low levels of added insulin than at the higher levels. This disparity is attributed to the presence of antibodies with reduced ability to combine with I^{125}-insulin. Such antibodies would combine with non-labeled insulin much more efficiently than with I^{125}-insulin, resulting in a more marked disparity between bound and free insulin at the low levels of added insulin (region of antibody excess). These observations may explain why other workers have found it necessary to use high antibody dilutions (antigen excess) in the radioimmune assay in order to insure relatively linear displacement of I^{125}-insulin with the addition of non-labeled insulin.

The antiserum used in the above experiments was found to have a neutralizing capacity of between 0.5 and 0.75 units of insulin per ml serum (measured in rat adipose tissue). In a typical experiment, 0.02 ml of this antiserum was used, and the lowest level of insulin added was 0.5 μg (12.5 mU). This is equivalent to 0.6 units of insulin per ml of antiserum. When measured in the immune hemolysis inhibition assay, a mean of 0.47 μg of insulin (or 94% of added insulin) was bound to the specific immune precipitate, which agrees well with the neutralizing capacity of the antiserum. On the other hand, the radioimmune assay indicated that 20% of the 0.5 μg added insulin was not bound to the specific immune precipitate. These correlations further emphasize the heterogenous nature of the I^{125}-insulin. They also emphasize the *inability* of considerable amounts of antibodies in the antiserum to react with I^{125}-insulin.

We have recently prepared specific immune precipitates with strain 2 and strain 13 antisera, and tested the displacement of the I^{125}-insulin from such specific immune precipitates by non-labeled insulin. Considerably more non-labeled insulin was necessary to displace I^{125}-insulin from the specific immune precipitate prepared with the str 13 antiserum than was necessary to displace I^{125}-insulin from the specific immune precipitate prepared with the str 2 antiserum (Fig. 9). These observations are additional evidence

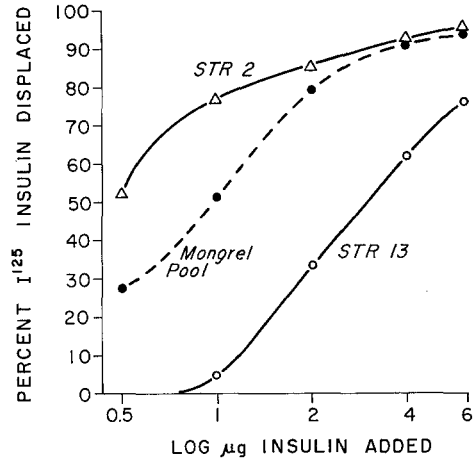


Fig. 9. Variations in insulin radioimmune assay standard curves with various guinea pig antisera*

* Specific immune precipitates contained equivalent amounts of insulin antibody as determined by hemaglutination and immune hemolysis.

that insulin antibodies produced by str 2 animals are different from the antibodies produced by str 13 animals. It appears that str 13 animals make more antibodies which cannot react with I^{125}-insulin than do the str 2 animals.

It was possible to demonstrate more directly the reduced ability of antibodies to react with I^{125}-labeled insulin. In essence the experiments consisted of immune hemolysis inhibition with I^{125}-insulin, subsequent precipitation of antibodies and antibody-I^{125}-insulin complexes, and measurement of free and antibody-bound I^{125}-insulin (Fig. 10). The radioactivity in the supernate after precipitation was found to be due to I^{125}-insulin which could not react with antibodies causing lysis of insulin-cells. These supernates were tested for biological activity with the rat epididymal fat pad and also for the presence of immunologically intact I^{125}-insulin.

The initial experiments involved inhibition of immune hemolysis using five different dilutions of

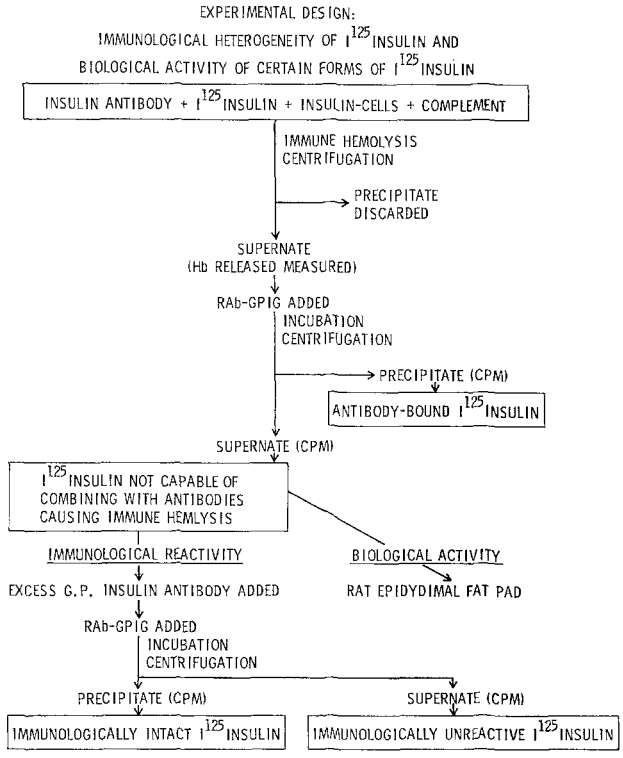


Fig. 10. Experimental design — immunological heterogeneity of I^{125}-insulin and biological activity of certain forms of I^{125}-insulin

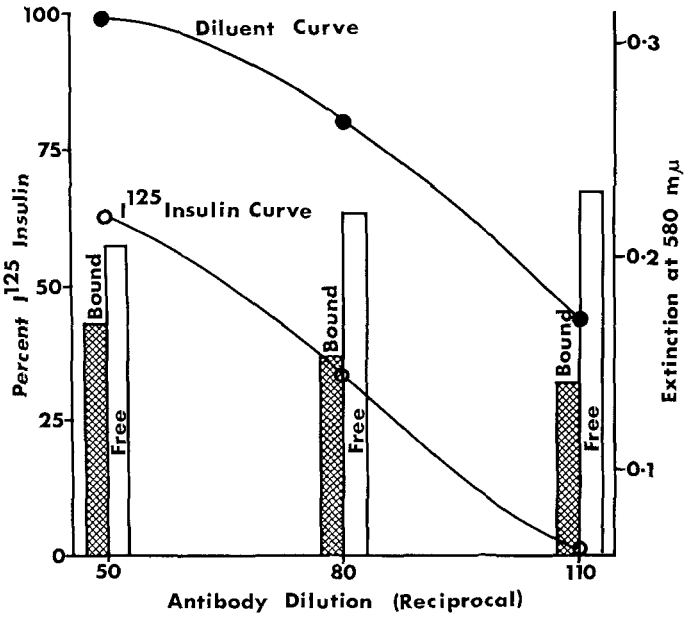


Fig. 11. Antibody bound and free I^{125}-insulin at various antiserum dilutions following immune hemolysis reaction

antiserum. In Fig. 11, the observations made at three of the antiserum dilutions (50, 80 and 110) are presented. The degree of hemolysis at each point is expressed as the extinction at 580 mμ. The I^{125}-insulin caused significant inhibition of immune hemolysis, as evidenced by the comparison of the I^{125}-insulin titration curve with the diluent titration curve. However, at all points on the I^{125}-insulin titration curve, antibodies were present in sufficient excess to cause hemolysis of insulin-cells above that noted at background levels.

Precipitation of antibodies and antibody-insulin complexes from the immune hemolysis supernate was then performed by adding excess amounts of rabbit antiserum to guinea pig γG globulin. The free I^{125}-insulin and antibody-bound I^{125}-insulin were estimated from the radioactivity in the supernate and precipitate. The bars shown in Fig. 11 demonstrate that significant amounts of free I^{125}-insulin were found at all dilutions of antisera. Yet, at the lowest dilution of antiserum, there was a sufficient excess of antibodies to cause 70% of the hemolysis seen when diluent alone was added. Approximately 60% of the I^{125}-insulin added was found *not* to be bound to antibody. Consequently, it is probable that a species of I^{125}-insulin existed that could not combine with antibodies capable of causing immune hemolysis of insulin-cells. Furthermore, at the other antiserum dilutions, there is an indication that very few of the antibodies in the antiserum pool used were capable of combining with I^{125}-insulin. A greater than two-fold decrease in antibody (from 50 to 110 fold dilution) was accompanied by a relatively small increase of free I^{125}-insulin (57 to 68%). It seems reasonable to conclude that a relatively large portion of the stock I^{125}-insulin preparation which was employed could not combine with antibodies which caused lysis of cells coated with non-labeled insulin.

It was possible that the radioactivity not bound to antibodies represented I^{125}-insulin which was no longer immunologically intact because of alteration or degradation during immune hemolysis. It was also possible that antibodies were capable of inducing immune hemolysis in the presence of insulin. Experiments testing the immune hemolysis supernate for immunologically intact I^{125}-insulin and also for biological insulin activity were therefore performed.

In these experiments, an example of which is presented in Table 7, only one dilution (50 fold) of the pooled insulin antiserum was used. The lysis noted when no insulin was added to antiserum was considered to be 100% immune hemolysis. The immune hemolysis inhibition (36%) by 0.1 μg of insulin was equal to the inhibition caused by 0.1 μg of I^{125}-insulin. Sufficient antibodies, in excess of added insulin, were therefore present in these experiments to cause 64% of the hemolysis of insulin-cells noted with antiserum alone.

After the degree of immune hemolysis was determined, excess rabbit antiserum to guinea pig γG globulin was added to the cell-free supernates. The percent free and antibody-bound I^{125}-insulin was determined from the radioactivity in the supernate and immune

Table 7. *Inhibition of immune hemolysis by I^{125}-Insulin*

Sample		Ext. 580 mμ[1]	Immune Hemolysis	Cpm in precipitate (Ab-Bound I^{125}-insulin)[2]	Cpm in supernate (free I^{125}-insulin)[3]
Diluent	(a)	0.26	100%	—	—
	(b)	0.27	100	—	—
0.1 μg	(c)	0.17	64	—	—
Insulin	(d)	0.17	64	—	—
0.1 μg I^{125}-	(e)	0.16	64	58.5%	41.5%
Insulin	(f)	0.17	64	60.0	40.0

[1] Proportional to hemoglobin released at this dilution (50) of antiserum to insulin.
[2] After Addition of Rab-GPIG to immune hemolysis supernates.
[3] Guinea pig antiserum to insulin added in excess to supernates and subsequent addition of Rab-GPIG resulted in a precipitate which contained 87% of the free I^{125}-Insulin.

precipitate respectively. About 40% of the I^{125}-insulin remained free following the immune hemolysis inhibition reaction (Table 7). Insulin antibodies were present in sufficient excess to cause 64% of the hemolysis noted in the control. Consequently, a form of I^{125}-insulin which could not react with certain of the antibodies capable of causing immune hemolysis of the insulin-cells existed in substantial quantities. Most (87%) of this form of I^{125}-insulin was immunologically reactive when precipitated with excess insulin antiserum and subsequent addition of rabbit anti-guinea pig γG globulin.

It is probable, therefore, that only a small proportion of the antibodies in the antiserum had combining site configurations capable of binding to this form of I^{125}-insulin. Immunological heterogeneity of I^{125}-insulin is implicit in this explanation of the observed results. However, the possibility that antibodies are capable of inducing immune hemolysis in the presence of intact free insulin, although not likely, should also be considered. Experiments were therefore designed to test the biological insulin activity of the supernate from immune hemolysis inhibition reactions performed with insulin and I^{125}-insulin as described in the preceding section. In addition, the biological activity of the I^{125}-insulin not capable of inhibiting immune hemolysis but immunologically intact was also tested.

The free I^{125}-insulin which did not inhibit immune hemolysis had no demonstrable biological insulin effect (Table 8). On the basis of radioactivity, approximately 500 μU of insulin were present in these samples of I^{125}-insulin added to the fat pad. The mean maximal response noted with 500 μU of crystalline insulin was 83%. The immune hemolysis inhibition supernates to which 0.1 μg of non-labeled insulin was added and from which antibodies and antibody-insulin complexes were removed with Rab-GPIG, also contained no demonstrable biological insulin activity.

These results demonstrated that I^{125}-insulin not capable of inhibiting immune hemolysis was biologically non-reactive when tested with the rat epididymal fat pad. The results further demonstrated that no free insulin which was biologically active remained following the immune hemolysis reaction. These results

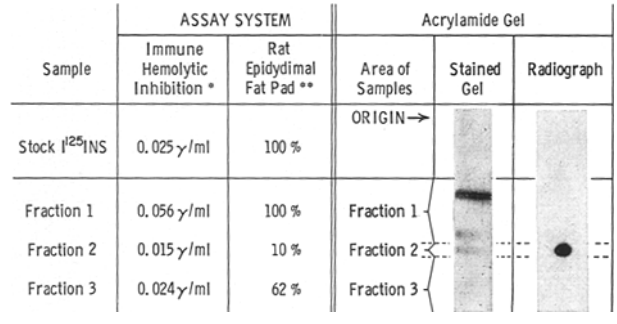
Table 8. *Biological activity of I^{125}-insulin not capable of inhibiting immune hemolysis*

Sample	% Maximum Response Exp. #1	Exp. #2	Mean % Maximum Response
Medium control	37:24	39:25	31.25 ± 7.9
500 μU Insulin	79:79	83:93	83.5 ± 11.5
0.1 μg Ins. Sup.	—	33:26[1]	29.5 ± 5.0
0.1 μg I^{125}-Ins. Sup.	26:00	00:00[2]	6.5 ± 13.0

[1] Aliquots of samples (c) and (d) tested for immune hemolysis inhibition, see Table 7, to which Rab-GPIG was added.
[2] Aliquotes of samples (e) and (f) respectively, see Table 7.

substantiate the immunological heterogeneity of the I^{125}-insulin employed in these experiments. This species of I^{125}-insulin not capable of inhibiting immune hemolysis, when separated from other forms of insulin, lacks biological insulin activity as measured by the rat epididymal fat pad method.

Recently we have been able to separate this form of I^{125}-insulin from much of the non-labeled insulin using discontinuous acrylamide gel electrophoresis. Fig. 12 is a summary of one experiment in which the



* Sample volumes were adjusted with VBS-Albumin until their counting rates/ml were comparable (± 2%) to the counting rate of Stock I^{125}Insulin at a concentration of 0.025 γ/ml.

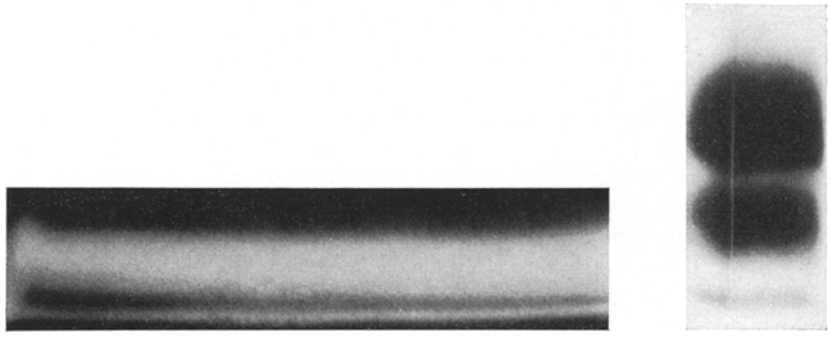
** Results express biological activity of 0.01 γ (250 μU) of each sample measured by immune hemolysis inhibition, as a percentage of maximal stimulation by 4 γ (100 mU) Insulin.

Fig. 12. Heterogeneity of I^{125}-insulin demonstrable by acrylamide gel descontinuous electrophoresis

I^{125}-insulin was submitted to electrophoresis and a subsequent radioautograph of the gel was made. It is evident that the bulk of the I^{125} is associated with fraction 2. Fraction 1 and fraction 3 probably contain large quantities of nonlabeled insulin, since they were biologically (column 3, Fig. 12) and immunologically active (column 2, Fig. 12). Fraction 2, which contains the majority of the I^{125}-insulin was not biologically active and possessed only partial (3/5) immunological activity.

whether large groups, such as fluorescein, would interfere with the binding of antibody with insulin.

Employing acrylamide gel electrophoresis, it was possible to separate a uniformly-migrating species of fluorescein-labeled insulin from nonlabeled insulin. Fig. 13 is a photograph demonstrating the block acrylamide gel electrophoresis of fluorescein-labeled insulin. The fluorescein-labeled insulin migrates as two distinct components. The slower moving fluorescent band has been eluted from the acrylamide gel, and its immuno-



Preparative hemolysis inhibition of crystalline and purified fluorescein-conjugated insulin

Fig. 13.

These results further substantiate the biological and immunological heterogeneity of I^{125}-insulin preparations. They also suggest that the majority of the I^{125}-insulin, when labeled at one atom per molecule, is not biologically active.

Previous reports [5] in which the *in vivo* half-life of insulin was determined using I^{131}-labeled insulin may have to be re-evaluated. In view of the results presented here, it is probable that a sizable amount of the I^{131}-insulin employed by these investigators was biologically inert. It is doubtful that the *in vivo* half-life of a material which is immunologically similar to insulin only in some respects and biologically inactive, is the same as biologically-active insulin. It may therefore be erroneous to consider the catabolism of iodinated insulin to be the same as insulin.

These results do *not* invalidate the radioimmune assays employing iodine-labeled insulin. In those situations, labeled insulin is used as an indicator and the assay is standardized with crystalline insulin.

The results do indicate that iodine interferes with the binding of I^{125}-insulin to many of the antibodies in insulin antisera. It is therefore probable that the antibodies not capable of binding with iodinated insulin are probably not capable of participating in the radioimmune insulin assays.

Recently we have labeled insulin with fluorescein isothiocyanate. It is thought that the isothiocyanate group couples with free amino groups: the N-terminal phenylalanine of the B chain; N-terminal glycine of the A chain; and the lysine at position 29 of the B chain. These conjugates were prepared to determine logical and biological characteristics have been studied. The non-labeled insulin migrates at a slower rate than either of the two species of fluorescein labeled insulins, which is what one would expect in view of the changes in charge coincident with fluorescein isothiocyanate substitution. The material which has been separated is very interesting. As studied by immune hemolysis inhibition (Fig. 14), it can be seen that this species of fluorescein-labeled insulin has immunological char-

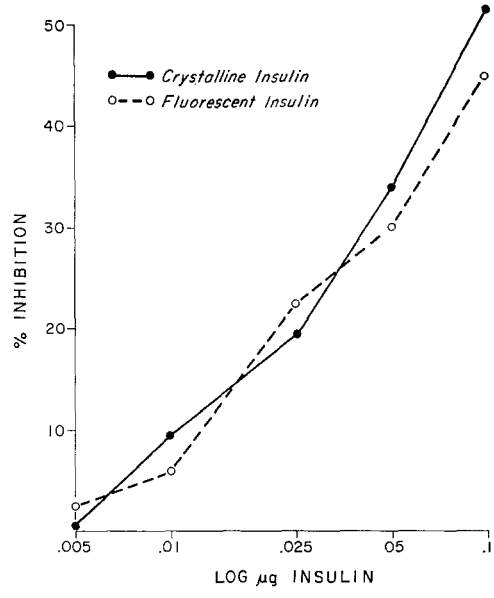


Fig. 14. Immune hemolysis inhibition of crystalline and purified fluorescein-conjugated insulin

acteristics similar to crystalline insulin. When specific immune precipitates were prepared, this material also behaved similarly to crystalline insulin. Therefore, we have not been able to discern any distortion of the immunological characteristics of this particular species of fluorescein-labeled insulin. Furthermore, in 13 experiments, no differences in biological activity between fluorescein-labeled insulin and crystalline insulin could be demonstrated using the rat epididymal fat pad method (Table 9). Consequently, this particular species of fluorescein-labeled insulin does not seem to be significantly distorted to affect its immunological characteristics and its biological activity upon rat adipose tissue.

Table 9. *Biological activity of purified fluorescein-conjugated insulin*

Sample	Mean % of maximum[1]	Standard deviation[2]
Crystalline insulin	69.9	± 32
Fluorescent insulin	64.8	± 25

[1] Biological activity of crystalline and fluorescent insulin (250 μU) in the rat epidydimal fat pad by the method of Ball & Merrill (Endocrinology 69:596, 1961).

[2] Derived from 10 experiments with crystalline insulin and 13 experiments with fluorescein-conjugated insulin.

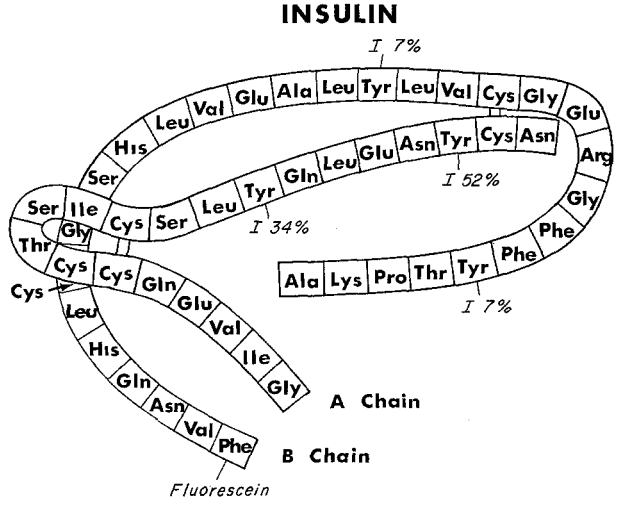


Fig. 15. Relative iodination of tyrosines in insulin

Fig. 15 is a two dimensional schematic representation of the amino acid sequence of beef insulin demonstrating the positions where iodine substitution occurs when insulin is labeled with 0.8 atoms of iodine per molecule. This study was performed by VAN DOESBURG and HAVINGA [13]. Most (90%) of the iodine attaches either to the tyrosines at positions 19 and/or 14 of the A chain. It is therefore likely that most of the iodine-labeled insulin used in the experiments which were presented in this communication, had iodine attached to the tyrosines at position 14 and/or 19. WILSON, DIXON and WARDLAW [12] have presented evidence that antibodies directed to the A chain determinants are more effective in neutralizing biological insulin activity than antibodies directed to B chain determinants.

Since most of the iodine in labeled insulin is attached to the tyrosines at positions 14 and/or 19 of the A chain, it seems probable that those antibodies incapable of reacting with iodinated insulin are directed toward determinants involving these tyrosines. Therefore, it would also be probable that these are the same antibodies which are effective in neutralizing the biological activity of insulin. Those antibodies which could combine with iodine-labeled insulin are probably antibodies which are less effective in neutralizing biological insulin activity.

Recently, CARPENTER [6] demonstrated changes in optical rotatory dispersion and loss of biological activity when the C-terminal asparagine has been removed from insulin. We have demonstrated that I^{125}-insulin, labeled at one atom of iodine per molecule, when separated from non-labeled insulin, has markedly reduced immunological activity and no demonstrable biological activity.

TIETZE [11] has demonstrated that the N-terminal phenylalanine of the B chain is most readily labeled when insulin is conjugated with fluorescein isothiocyanate (Fig. 15). Since no reduced immunological activity or biological activity in rat adipose tissue was apparent in our samples of separated fluorescein-labeled insulin, it is possible that distortion of the N-terminal portion of the B chain does not demonstrably affect immunological and biological insulin activity.

We therefore postulate that the integrity of the C-terminal portion (A 14 to A 21, inclusive) of the A chain is very important to biological activity, surface configuration and maintenance of the antigenic characteristics of the insulin molecule.

Using the approach of substituting materials on the surface of the insulin molecule and studying the biological and immunological characteristics of the labeled insulin, it is hoped that information can be obtained concerning the surface configuration of the insulin molecule as related to biological activity and antigenic characteristics.

Acknowledgement. We wish to thank Dr. OTTO BEHRENS, Eli Lilly Company, Indianapolis, Indiana and Dr. J. SCHLICHTKRULL, Novo Industri A/S, Copenhagen, Denmark, for the generous supply of crystalline beef insulin.

References

[1] ARQUILLA, E.R., J. ALEXANDER, E. PTACEK and E. LOOSLI: Studies on Insulin Neutralization by Antisera from Various Species. I. Methodology and Assay of Insulin with Intact Anesthetized Rats. Diabetes **11**, 412–418 (1962).

[2] —, and J. FINN: Insulin Antibody Variations in Rabbits and Guinea Pigs and Multiple Antigenic Determinants on Insulin. J. exp. Med. **118**, 55—71 (1963).
[3] — — Genetic Differences in Antibody Production Toward Determinants on Insulin. Science **142**, 400—401 (1963).
[4] — — Genetic Control of Combining Sites of Insulin Antibodies Produced by Guinea Pigs. J. exp. Med. **122**, 771—784 (1965).
[5] BERSON, S.A., R.S. YALOW, A. BAUMANN, M.A. ROTHSCHILD and NEWERLY K.: Insulin-I^{131} Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin Treated Subjects. J. clin. Invest. **35**, 170 (1956).
[6] CARPENTER, F.H.: Personal Communication.
[7] EDELMAN, G.M., and J.A. GALLY: A Model for the 7s Antibody Molecule. Proc. nat. Acad. Sci. U.S. **51**, 846 (1964).
[8] FOUGEREAU, M., and G.M. EDELMAN: Corroboration of Recent Models of the γG Immunoglobulin Molecule. J. exp. Med. **121**, 373 (1965).
[9] HALES, C.N., and P.J. RANDLE: Immunoassay of Insulin with Insulin Antibody Precipitate. Biochem. J. **88**, 137 (1963).
[10] LANDSTEINER, K.: The Specificity of Serological Reactions, Revised Edition, N.Y. Dover Publications, p. 182, 1962.
[11] TIETZE, F., G.E. MORTIMORE and N.R. LOMAX: Preparation and Properties of Fluorescent Insulin Derivatives. Biochim. biophys. Acta **59**, 336 (1962).
[12] WILSON, S., G.H. DIXON and A.C. WARDLAW: Resynthesis of Cod Insulin from its Polypeptide Chains and the Preparation of Cod-Ox "Hybrid" Insulins. Biochim. biophys. Acta **62**, 483 (1962).
[13] VAN DOESBURGH, J.T.S. de V., and E. HAVINGA: The Reactivities Towards Iodine of the Four Tyrosine Residues of Insulin. Biochim. biophys. Acta **82**, 96 (1964).
[14] YAGI, Y., P. MAIER and D. PRESSMAN: Two Different Anti-insulin Antibodies in Guinea Pig Antisera. J. Immunol. **89**, 442 (1962).

Dr. EDWARD R. ARQUILLA
Department of Pathology
University of California
School of Medicine
Los Angeles/California U.S.A.