The Double-Antibody Immunoassay of Insulin

A Standardized Second Antibody Reaction that Eliminates Spurious Results with Human Serum

By

T. A. WELBORN* and T. RUSSELL FRASER

Department of Medicine, Postgraduate Medical School of London

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Summary. 1. Tracer ^{131}I -guinea-pig γ globulin provides a useful check of the efficiency of the insulin-antibody precipitation. It is employed (a) for the accurate preliminary standardization of the precipitating antibody; and (b) during assays to assess completeness of precipitation (in monitor tubes).

2. Similar assays of human serum frequently yield spuriously high results; two responsible factors are (a) sub-optimal second antibody precipitation arising from accentuation by serum of the "prozone phenomenon", and (b) cross-reaction of rabbit anti-guinea-pig serum with human proteins. The former can be overcome by using ideal concentrations of reactants, prolonging incubation, and incorporating a standard amount of heparin, during the second reaction. The latter is minimized by using a rabbit anti-serum made to pure guinea-pig γ globulin.

3. The double antibody method, thus modified, has proved consistently reliable. In the assay of human serum samples diluted 1/10 (i.e. 0.1 ml volumes of serum) optimal precipitation always occurs. Undiluted serum samples (i.e. 1.0 ml volumes) can also be assayed satisfactorily, when the occasional sub-optimal precipitation can be identified and a correction derived from the monitor tubes to yield a valid reading.

Résumé. 1. Une globuline γ de cobaye marquée à ^{131}I utilisée comme traceur fournit un contrôle utile de l'efficacité de la précipitation insuline-anticorps. Elle est employée a) pour la standardisation préliminaire précise de l'anticorps précipitant et b) au cours des dosages pour évaluer si la précipitation est complète (dans des tubes de contrôle).

2. Des dosages semblables de sérum humain donnent fréquemment des résultats faux par excès. Deux facteurs en sont responsables: a) une précipitation sub-optimale par le second anticorps, qui résulte de l'accentuation par le sérum du "phénomène prozone", et b) une réaction croisée du sérum de lapin anti-cobaye avec des protéines humaines. Le premier facteur d'erreur peut être supprimé en utilisant des concentrations idéales de réactants, en prolongeant l'incubation, et en incorporant une quantité standard d'héparine, au cours de la deuxième réaction. Le deuxième facteur d'erreur est réduit au minimum en

utilisant un anti-sérum de lapin obtenu à partir de γ globuline pure de cobaye.

3. La méthode au double anticorps, ainsi modifiée, s'est montrée constamment digne de confiance. Dans le dosage d'échantillons de sérum humain dilués au 1/10 (c'est à dire 0.1 ml de sérum) il se produit toujours une précipitation optimale. Des échantillons de sérum non dilués (c'est à dire 1.0 ml) peuvent également être dosés de façon satisfaisante lorsque la précipitation sub-optimale occasionnelle peut être identifiée et qu'une correction peut être obtenue à partir des tubes de contrôle pour fournir une lecture valable.

Zusammenfassung. 1. Spürdosen von ^{131}I -Meerschweinchen γ globulin bieten eine Möglichkeit zur Prüfung der Wirksamkeit der Insulin-Antikörperpräzipitation. Diese Methode wurde angewendet a) für die genaue vorläufige Standardisierung des präzipitierenden Antikörpers und b) zur Beurteilung der Vollständigkeit der Präzipitation während der Bestimmung (in den Kontrollröhrchen).

2. Ähnliche Untersuchungen des menschlichen Serums führen häufig zu fälschlich hohen Ergebnissen; dafür sind zwei Faktoren verantwortlich: a) eine nicht optimale sekundäre Antikörperpräzipitation, die durch eine Verstärkung des „Prozonphänomens“ durch das Serum entsteht und b) die Kreuzreaktion des gegen Meerschweinchen Serum gerichteten Kaninchenserums mit menschlichen Proteinen. Der erste kann durch Benutzung idealer Konzentrationen der reagierenden Substanzen, verlängerte Inkubationszeit und Zusatz einer Standardmenge von Heparin während der zweiten Reaktion ausgeschaltet werden. Der letztere wird durch Benutzung eines gegen reines Meerschweinchen γ globulin erzeugten Kaninchen-antiserums möglichst klein gehalten.

3. Die so modifizierte Doppel-Antikörpermethode hat sich als vollständig zuverlässig erwiesen. Bei Untersuchungen des menschlichen Serums in einer Verdünnung von 1/10 (d.h. 0.1 ml Serummenge) kommt es immer zu einer optimalen Präzipitation. Auch unverdünnte Serumproben (d.h. 1.0 ml Serummenge) können zufriedenstellend untersucht werden, wenn die gelegentliche nicht optimale Präzipitation erkannt wird und, um eine gültige Ablesung zu bekommen, eine Korrektur von den Kontrollröhrchen abgeleitet werden kann.

The immunological assay of serum insulin is now established as a sensitive and useful procedure^{17,7}. The unlabelled insulin in a sample is measured by the extent of its interference with the combination between a specific insulin antibody and tracer ^{131}I -labelled insulin. After incubation, various techniques are used

to separate the free from the antibody-bound insulin, for instance, paper electrophoresis¹⁷, or Sephadex chromatography⁴, but also the addition of a second antibody that precipitates the insulin-antibody complex¹⁰. These techniques can give comparable results¹. Obviously the double-antibody method relies on the consistent efficiency of the second antibody reaction and doubts about this have led some observers to prefer other methods. Indeed, various authors have

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reported that with some sera spuriously high insulin assay values have occurred probably because of incomplete second antibody precipitation^{10,9,5}, while other published results may also have suffered from the same source of error³. No method of assessing completeness of insulin-antibody precipitation has yet been described. This paper reports such a method, evolved after assessment of some of the previous sources of error, and including modifications that can eliminate them.

Methods

Our double-antibody insulin assay follows a technique modified from that of MORGAN and LAZAROW¹⁰. This uses for the first antibody reaction guinea-pig anti-(ox)-insulin serum (GPAI serum) which is subsequently precipitated by rabbit anti-guinea-pig serum (RAGP serum). Since the GPAI serum is diluted to the range of 10^{-4} to 10^{-6} and these minute quantities of antibody will not precipitate reliably, is additional "carrier" guinea-pig serum (GP serum) regularly added so that the RAGP serum then forms an easily visible precipitate. After centrifuging, the supernatant can then be decanted. To an assay or standard sample of 1.0 ml are added the reactants which give a final volume of 1.4 ml. Other technical details are given in the Appendix and Fig. 5.

Errors in the double-antibody reaction

1. Non-specific effects of some human sera on the second antibody reaction

In our early trial assays, it was found that while satisfactory standard curves were repeatedly obtained with concentrations of ox insulin ranging from 5 to 200 μ U/ml, with about half of the assayed sera (mostly from normal subjects) consistent assay results were

Fig. 1. Assays of 11 sera undiluted and at various dilutions down to 1/10 (old assay procedure). Note reproducible values over whole range of dilutions in Group A (6 sera); but gross inconsistencies in Group B (5 sera), with excessively high values at increasing serum concentrations

Fig. 2. Immuno-electrophoretic comparison of RAGP antiserum (old assay) and RAGP γ antiserum (new assay). Each was plated against guinea-pig serum and human sera. Note: (1) the crude RAGP antiserum not only gives precipitin lines against all components of GP serum but also against α_2 , β globulins in 2 of 3 human sera tested; while (2) the more specific RAGP γ antiserum shows a single dense band against GP γ and a thin line against human γ -globulin

not obtained over a range of dilutions from unity (1.0 ml of serum) to under $1/10$ (0.1 ml serum) (see Fig. 1). In the more concentrated samples, these "inhibitory" sera gave enormously elevated values, while in some cases the results were suspiciously high even in the diluted range. Thus, while both first and second antibodies reacted consistently with buffer solutions of insulin, interference occurred in the presence of some human sera. The following experiments were made therefore to assess this artefact and to evolve a means of preventing it.

2. Cross-reaction between human serum and RAGP serum

When the assay tubes of certain normal human sera were observed to yield a more bulky precipitate (2- to 3-fold), a cross reaction was suspected. Therefore samples of these human sera were labelled with 131 I of which trace amounts were added in 1 ml of buffer to assay tubes along with the usual amounts of second antibody reagent. After incubating, centrifuging and decanting, it was found that 30–60% of the human labelled protein was precipitated by the RAGP serum; while in the similarly treated control tubes set up without GP serum or RAGP serum only 3% was found. Further evidence of this cross-reaction was obtained by immuno-electrophoretic studies. Certain of these "inhibitory" human sera were plated against RAGP serum which revealed precipitation arcs opposite the α_2 and β globulins and lipoproteins of human sera (Fig. 2). Although such cross-reaction may not directly interfere with the specific antibody reactions in the assay, the larger precipitates could bind non-specifically some of the free insulin- 131 I. Therefore, to minimize this source of error, a more specific second antibody was prepared (i.e. one made to pure guinea-

pig gamma-globulin instead of whole GP serum — see Appendix). When this was similarly assessed by immuno-electrophoresis, a trace precipitated opposite the gamma-globulin but not opposite any other human serum protein (Fig. 2). This serum has been used as the second antibody in all subsequent assays and in the experiments reported below; the results indicate that its minimal cross-reaction has not impaired the consistency of the second antibody reaction.

3. "Prozone phenomenon"* accentuated by the presence of human serum

For ideal second antibody precipitation, it is obvious that the final concentrations of guinea-pig gamma-globulin ($\text{GP}\gamma$) and rabbit anti-guinea-pig gamma-globulin serum ($\text{RAGP}\gamma$) must be those found to give maximal precipitation, i.e. at the "prozone peak"¹². Such optimal concentrations are usually

Figure 3a.

Fig. 3a. Completeness of second antibody reaction: effect of varying "carrier" GP γ concentration in monitor tubes with tracer ¹³¹I-GP γ (in the absence of heparin and after incubation 18 hrs). Note: (1) in the buffer tubes a wide range of GP γ gives optimal precipitation, while (2) in tubes with 0.1 ml human serum, precipitation is impaired in all tubes even at the more narrow "prozone peak".

(Tube additions shown are those also used for Figures 3b, 3c and 4)

Figure 3b

Fig. 3b. Completeness of second antibody reaction; effect of human serum added in varying volumes (when concentration of GP γ "carrier" is optimal but with no added heparin). Note progressive impairment of precipitation with increasing amounts of human serum. 18 hrs incubation

* In antigen-antibody precipitin systems there is a narrow "zone of equivalence" of concentrations of the reactants at which optimal precipitation occurs, and beyond which antigen or antibody excess impairs precipitation. This is the "prozone phenomenon".

Figure 3c

Fig. 3c. Completeness of second antibody reaction; effect of human serum (0.1 ml) (incubated for varying times with a range of concentrations of "carrier" GP γ without heparin.) Note serial improvement in precipitation with more prolonged incubation; but still sub-optimal precipitation at "prozone peak" even after 72 hrs

determined by measuring the recovery of ¹³¹I-insulin which precipitates in buffer solutions, after first and second antibody reactions. However, adequate preliminary tests should rather measure guinea-pig gamma-globulin precipitation, and also take account of the effect of human serum on the second antibody reaction. Therefore we have studied the completeness of the precipitation with trace amounts of ¹³¹I-GP γ (about 1% of the total GP γ present). Assessing the effect of varying concentrations of "carrier" GP γ , in the presence or absence of human serum, it has been found repeatedly that in buffer solutions a broad "prozone peak" of optimal precipitation is obtained over a range of "carrier" GP γ concentrations (Fig. 3a); however in similar tubes containing 0.1 ml of human serum precipitation was impaired, and the "prozone peak" critically narrowed. Increasing the volume of serum added causes progressive impairment of precipitation (Fig. 3b). These experiments employ an 18 hr incubation, and were performed in the absence of heparin.

Further studies with markedly "inhibitory" human sera revealed that these effects can be minimized but not eliminated by prolonging the incubation (Fig. 3c); but while prolongation of incubation to 24, 48 and 72

Fig. 4. Completeness of second antibody reaction; comparison of effect of heparinized plasma (0.1 ml) and serum (0.1 ml) from same subject. Note that heparin accelerates the reaction and enables optimal precipitation over a broad "prozone peak", both at 4 and 72 hrs. incubation

Table 1. *Completeness of the second antibody reaction with buffer or serum: effect of added heparin. A minimum amount of 10–50 i.u. per tube avoids incomplete precipitation in the presence of serum after 16–24 hrs. (but not at 3 hrs.)*

	Percent of ¹³¹ I-GP γ globulin precipitated (in monitor tubes)								
Heparin (i.u./tube) →	0 i.u.	10 i.u.	50 i.u.	1000 i.u.	0 i.u.	1 i.u.	10 i.u.	50 i.u.	1000 i.u.
Assay Sample →	Buffer 1 ml (i.e. "optimal" precipitation)				Normal serum 0.1–0.2 ml + buffer to 1 ml*				
Experiment I									
Time 3 hrs	91%	—	91%	—	23%	25%	81%	85%	—
24 hrs	95%	—	94%	—	88%	(79%)	92%	93%	—
Experiment II									
Time 16 hrs	80%	81%	82%	83%	64	—	81%	82%	82%

hrs progressively improves the precipitation, the reaction is still less complete than in buffer alone, even at the narrowly defined "prozone peak".

4. Addition of heparin with the second antibody to eliminate the interference from human serum

Several experiments have shown that added heparin counteracts these delaying and "prozone"-narrowing effects of added human serum on the second antibody reaction. Fig. 4 shows the results of one of these. Note that with heparin, maximal precipitation is obtained within 4 hrs, whereas without heparin it is still sub-optimal even after 72 hrs. Heparin also considerably improved the accuracy of duplicate estimations of serum (as also indicated in Fig. 4). Thus for a number of reasons heparin is a useful addition to the assay system.

Further observations (Table 1) revealed that a certain minimum quantity of heparin is required per tube to produce optimal precipitation, and so our routine procedure now involves adding 25 i.u. per tube along with one of the second antibody reagents.

Recommended modifications in the assay procedure

To ensure consistent and reliable second antibody precipitation, these findings suggest that the double-antibody procedure should include:

I. Preliminary purification of guinea-pig gamma-globulin which is used for (a) the production of a precipitating rabbit anti-GP γ serum, and also (b) the preparation of ^{131}I -labelled GP γ . The latter is used with serum samples in parallel monitor tubes (that contain no ^{131}I -insulin) to compare the equivalence

of GP γ precipitation with the optimal precipitation that occurs in buffer samples. If the serum samples impair the second antibody reaction, the monitor tubes will reveal the sub-optimal precipitation: a true measurement of antibody-bound ^{131}I -insulin can then be obtained by multiplying the ^{131}I -insulin precipitate value by a factor that corrects for the observed impairment of GP γ precipitation. Table 2 illustrates this procedure and its validity.

II. Heparin in standard amounts (e.g. 25 i.u.) should be added to all assay and monitor tubes, along with one of the second antibody reactants.

ASSAY TUBES

MONITOR TUBES

Used to identify + correct for possible suboptimal precipitation.

* 0.2 ml serum in Experiment I.
0.1 ml serum in Experiment II.

Fig. 5. Flow sheet for a routine assay

Table 2. *Methods of correction of insulin assay values, used when monitor tubes indicate sub-optimal second antibody precipitation (old assay procedure with no heparin). Corrected values are consistent at various dilutions*

Serum assayed		Measured Precipitates		True ^{125}I -insulin (% bound) =	Insulin Assay Result ($\mu\text{U}/\text{ml}$) (from standard curve)	
Subject	Volume (ml)	insulin- ^{125}I (%) bound	"Correction factor" (i.e. monitor tubes) optimal GP γ ^{125}I Ppt $\times$ subjects GP γ ^{125}I Ppt		(Uncorrected)	Corrected
I	0.1	81.0	$\times \frac{96.0}{96.0}$	= 81.0	(20)	20
	0.5	71.1	$\times \frac{96.0}{94.9}$	= 72.4	(29)	25
	1.0	47.0	$\times \frac{96.0}{76.0}$	= 59.7	(39)	20
II	0.1	67.8	$\times \frac{88.2}{85.8}$	= 69.9	(9)	7
	0.5	18.4	$\times \frac{88.2}{29.8}$	= 54.8	(>40)	6

III. Incubation of the second antibody reaction should be sufficiently prolonged to enable complete precipitation even in the presence of human serum. We have found that 24 hrs is both adequate and convenient.

IV. The usual preliminary standardization of second antibody reactants should be performed carefully, in the presence of human serum and the absence of heparin, and over a period of 4–8 hours; when the zone of optimal precipitation will be narrowed, thus enabling the precise determination of the ideal concentrations of GP serum "carrier" required (see Appendix and Fig. 3a).

The use of this modified assay

Over the last 9 months, this has been our routine procedure, and 29 of 30 assay-days have had consistent second antibody reactions; only one assay was discarded and this because of faulty standardization of the reactants. Up to 100 samples can conveniently be assayed in duplicates by a single operator.

With the usual 0.1 ml serum assays, 100 human samples have been tested using the monitor tubes; this has revealed the percentage of GP γ precipitated in the serum tubes to be usually within 1% (and always within 3%) of that of the buffer tubes. In a recent series of assays performed by two independent operators we have included 0.1 ml of a standard serum sample in order to assess the reproducibility of the method from day to day; in 10 assays by one worker the standard's value ranged from 53 to 64 $\mu\text{U}/\text{ml}$ of insulin (58.1 mean $\pm$ 4.2 S.D.; coefficient of variation 7.2%) and in 5 assays by the other worker, it ranged from 49 to 66 $\mu\text{U}/\text{ml}$ (59.6 mean $\pm$ 7.0 S.D.; coefficient of variation 12%). Using this assay procedure, we have reported elsewhere the range of normal serum insulin values during an oral glucose tolerance test¹⁶.

Further, with this modified procedure, from a small experience it appears possible to assay reliably less diluted serum, i.e. up to 1 ml serum in the final assay volume of 1.4 ml. Table 3 shows the results from assays of various dilutions of 12 sera; these gave consistent insulin values despite the varying dilutions (in those sera within the range of sensitivity of the standard curve), and in 9 of the 10 serum samples monitored for gamma-globulin precipitation, no correction of the insulin precipitate values was required, even in the undiluted samples. Also, in the assay of these larger

Table 3. *Assays of 12 human sera undiluted and at various dilutions down to 1/10 (by new assay procedure with 25 i.u. heparin per tube and incubating the second antibody for 24 hrs.). Note: (1) for serum samples 1–7 consistent assay values at all dilutions; and probably also for samples 8–12 whose less diluted samples were beyond the accurate sensitive range of the standard curve; (2) while monitor ^{125}I -GP tubes were run for 10 of the samples, only one (No. 11) showed impaired precipitation with the more concentrated serum*

Human serum sample	Insulin value ($\mu\text{U}/\text{ml}$) at various volumes of serum (made up to 1 ml.)				GP γ - ^{125}I precipitation at various volumes of serum (made up to 1 ml.)**			
	0.1	0.2	0.5	1.0	0.1	0.2	0.5	1.0
1	26	30	27	23	99%	99%	97%	99%
2	(>6*)	5	6	6	—	—	—	—
3	8	8	11	13	—	—	—	—
4	19	16	15	21	—	—	99%	99%
5	43	40	40	34	97%	97%	99%	99%
6	60	65	67	(>60*)	98%	98%	100%	99%
7	34	—	28	37	104%	—	106%	105%
8	96	—	(>80*)	(>40*)	103%	—	105%	103%
9	145	—	(>80*)	(>40*)	102%	—	95%	99%
10	185	—	(>80*)	(>40*)	104%	—	104%	103%
11	355	—	(>80*)	(>40*)	102%	—	92%	82%
12	(>400*)	—	(>80*)	(>40*)	102%	—	100%	98%

* Beyond sensitive range of standard curve.

** Expressed as % of optimal precipitation of GP- ^{125}I (as found in buffer tubes).

volumes of serum using the more specific second antibody (RAGP γ serum), control tubes set up *without* GPAI serum have shown that the non-specific binding of ^{131}I -insulin in the precipitate tube is constantly within the usual range of 2 to 4% (Table 4).

Table 4. *Minimal and consistent non-specific "co-precipitation" of ^{131}I -insulin (in control tubes set up without GPAI serum). Note that this precipitation remains low in all tubes*

Percentage of ^{131}I -insulin precipitated in control tubes			
Buffer	0.1 ml serum	0.5 ml serum	1.0 ml serum
2.7% (N = 4)	2.7% (N = 5)	3.5% (N = 3)	3.5% (N = 3)

Discussion

For immunoassays, no method yet available for separating the bound and free hormone is perfect. The original chromatoelectrophoretic separation not only avoids the complications of a second antibody, but also identifies non-specific binding of the labelled hormone ("protein damage") separately from the antibody-bound and free fractions; "damage" when present is seen between the two main peaks of radioactivity¹⁷. However only small volumes of the incubated mixture can be put on the paper so that high specific activity is necessary and the apparatus is elaborate. The Sephadex separation⁴ which may have similar advantages has yet to be fully assessed, and also requires elaborate apparatus. GRODSKY and FORSHAM⁶ extract serum samples with acid ethanol in order to avoid "protein" error; but this again involves extensive technical manipulation, and also presumes that "total extractable" insulin is the same as serum insulin. We have found that ethanol precipitation as a substitute for the second antibody is unsatisfactory for the measurement of insulin in serum samples, because it co-precipitates much free ^{131}I -insulin non-specifically¹⁴.

The double antibody assay is technically the simplest. However, our findings, as well as those of others^{10, 9, 5}, clearly indicate the need for modifications to prevent any assay sample interfering with the second antibody reaction. HALES and RANDLE⁷ have suggested that pre-precipitation of GPAI serum by RAGP γ anti-serum will avoid cross-reaction with human plasma proteins, but reports^{2, 15} indicate that the method is not free from interference by certain human plasma samples, although the addition of EDTA seems to overcome this. It seems evident that errors common to other double antibody methods also apply despite pre-precipitation. Their method separates the doubly-bound labelled hormone from the free by microfiltration, rather than by the more simple and eminently satisfactory separation by centrifuging and decanting as described by MORGAN and LAZAROW¹⁰.

The latter assay, when suitably modified, may be the most amenable to automation.

MORGAN and LAZAROW have suggested that complement may be the factor in the assayed serum which inhibits the second antibody reaction to a varying extent, and have shown that adding EDTA in appropriate concentration can abolish the interference of undiluted rat plasma¹¹. Our studies performed concurrently demonstrate that heparin, which is also anti-complementary, similarly overcomes the inhibition from human serum, by accelerating the second antibody reaction and broadening the zone of optimal precipitation. On these grounds, it may appear practicable to assay plasma from heparinized samples rather than serum. However, we have found that heparin has a small inhibiting effect on the first antibody combination; which could be covered by including heparin in both the standard curve tubes as well as the plasma sample tubes, in a certain minimum quantity. We find it convenient to add heparin in a standard concentration with one of the second antibody reagents; it is thus included in all assay and monitor tubes.

When due regard is given to the critical factors involved in the second antibody reaction, this method can measure reliably the insulin in human serum. Our data indicate that the assay of sera diluted $1/_{10}$ (i.e. 0.1 ml) or less is constantly free from uncertain second antibody precipitation, when the modified assay is used. Further, a monitor tube device using ^{131}I -GP gamma-globulin can be used to check the completeness of the second antibody reaction, particularly with more concentrated serum samples; this enables the calculation of a corrected value for "bound" ^{131}I -insulin whenever precipitation of GP γ is found incomplete. "Protein damage" is not a significant source of error in this assay system, for equivalent values are obtained when assaying from 0.1 ml to 1.0 ml of serum. This ability to measure insulin in undiluted serum (1.0 ml) with reasonable accuracy is important as a validation of the method. Further, while less necessary for insulin estimation, the ability to assay undiluted serum may be helpful in similar assays of other hormones, e.g. growth hormone and parathormone, whose levels appear to be difficult to measure in small volumes of normal sera.

Appendix

Reagents Used for the Double-Antibody Assay of Insulin

1. Borate buffer pH 8.6⁹ with 0.2% bovine albumin fraction V (Armour) is used as the standard diluent for serum samples and reagents.

2. Standard insulin. Aliquots of a pure (bovine, pork or human) insulin preparation at a concentration of 1000–2000 $\mu\text{U/ml}$ are stored at -20°C . When required for assay, one aliquot is serially diluted to give standards of (60), 40, 20, 10, 5, 2.5, 1.25 (6.3) micro-

units per ml; and duplicate assay tubes received 1.0 ml of these.

3. ^{131}I -insulin. Code No. 1B, 38 supplied by the Radiochemical Centre, Amersham (in vials containing 1 ml of a 0.1 $\mu\text{G}/\text{ml}$ solution of labelled bovine insulin, specific activity 10 $\mu\text{C}/\mu\text{G}$). On arrival, the batch is subdivided into 0.1 ml aliquots and deep frozen; for each assay 0.1 ml is made up to 8 ml total volume, which suffices for 80 tubes.

4. Anti-insulin sera have been developed in guinea-pigs by the method of WRIGHT¹³, from which suitable antisera are selected as suggested by YALOW¹⁷. For their standardization, ethanol precipitation (i.e. addition of ethanol to final concentration 80%) can be conveniently used instead of the second antibody¹⁴.

5. Guinea-pig gamma-globulin ($\text{GP}\gamma$) is prepared from whole serum, previously dialyzed overnight against 0.015 M phosphate buffer, pH 7.0; 10–20 ml is applied to a 30×3 cm anion exchange column (DEAE-Sephadex A-50, Pharmacia) that has been prepared in the recommended manner and equilibrated with the same buffer. This column binds all major serum protein components except gamma globulin (and occasionally haemoglobin and siderophilin) which is collected in the eluate. The eluate is concentrated and an aliquot tested to confirm that it is electrophoretically pure. Hyperimmune guinea-pigs, such as those previously inoculated with mycobacteria will give at least double the usual yield of $\text{GP}\gamma$.

6. ^{131}I - $\text{GP}\gamma$: Approximately 20 μg of pure $\text{GP}\gamma$ is labelled by the method of HUNTER and GREENWOOD⁸ and then purified by passage through a small DEAE-Sephadex A-50 column previously equilibrated with 0.015 M phosphate buffer, pH 7.0. The specific RAGP γ antiserum will usually precipitate 80–98% of the final product under optimal conditions. The most suitable labelling has been achieved by combining 20 μg of $\text{GP}\gamma$ with 10 μg of bovine albumin (the latter appears to protect against radiation damage; it is subsequently bound on the DEAE column).

7. Rabbit anti- $\text{GP}\gamma$ antisera. After homogenizing with FREUND's adjuvant, pure $\text{GP}\gamma$ has been injected subcutaneously into rabbits at monthly intervals for at least 3 months. Each animal has received 5–10 mg $\text{GP}\gamma$ per injection, given into the four areas drained by axillary and inguinal lymphatic systems. The animals are bled 40 ml by ear vein 7–20 days after the last injection; suitable sera are pooled, divided into aliquots and deep frozen.

Preliminary standardization of second antibody reactants

RAGP γ antisera prepared as specified can usually be diluted $1/4$ or $1/5$. Serial dilutions of "carrier" $\text{GP}\gamma$ serum (e.g. $1/25$, $1/50$, $1/100$, $1/200$, $1/400$) are set up, and 0.1 ml of these various concentrations is added to duplicate assay tubes containing 0.9 ml of buffer. Then in order are added 0.1 ml of (1) a normal human serum, (2) "tracer" ^{131}I - $\text{GP}\gamma$, (3) RAGP γ , antiserum appro-

priately diluted. The reaction is incubated for 4–8 hours at 4°C , then the tubes are centrifuged and precipitate and supernatant counted. The tubes containing the maximal precipitation of ^{131}I - $\text{GP}\gamma$ indicate the optimal concentration of "carrier" guinea-pig serum to be used for each batch of RAGP γ antiserum. The ratio of concentrations of RAGP γ and "carrier" $\text{GP}\gamma$ for optimal precipitation, appears to be constant: thus if undiluted RAGP γ gives maximal precipitation with ($1/50$) "carrier" $\text{GP}\gamma$ serum; then ($1/4$) RAGP γ will give maximal precipitation with ($1/200$) "carrier" $\text{GP}\gamma$ serum (independent of total volume).

It is often necessary to "narrow down" the precise concentration of GP serum required by a further standardization procedure (e.g. at $1/50$, $1/60$, $1/70$, $1/80$, $1/100$ concentrations of GP serum). Note — heparin is always omitted from this preliminary testing in order to accentuate the "prozone phenomenon" and thus obtain a precise peak.

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Dr. T.A. WELBORN
Department of Medicine
Postgraduate Medical School of London
London/England