dye-exclusion test revealed insignificant numbers of dead cells. The brief exposure to unbuffered hypotonic NaCl solution did not impair the cytolytic activity of sensitized lymphocytes on homologous cells *in vitro*.

Summary. Highly purified suspensions of living, immunologically competent lymphocytes were obtained from splenic tissue of mice by destroying the red blood cells with hypotonic saline and separating the nucleated cells by centrifugation. The procedure yielded approximately 40 million lymphocytes from each spleen. Usually 96% (range 90-99%) of the cells were lymphocytes; the rest were other nucleated cells. The viability of lymphocytes was demonstrated by their motility and the exclusion of dye from their cytoplasm. The immunologic competence of these cells was shown by their cytolytic effect on homologous cells *in vitro*.

The able technical assistance of Mrs. Rosemarie Lund is gratefully acknowledged.

1. Parker, R. C., *Methods of Tissue Culture*, Paul B. Hoeber, N. Y., 1961, p282.

2. Rosenau, W., Moon, H. D., *J. Nat. Cancer Inst.*, 1961, v27, 471.

3. Walford, R. L., *Leukocyte Antigens and Antibodies*, Grune and Stratton, N. Y., 1960, p1.

4. Kato, K., *J. Lab. Clin. Med.*, 1941, v26, 703.

5. Davson, H., Danielli, J., *The permeability of natural membranes*, Univ. Press, Cambridge, 1943, p271.

6. Leake, C. D., Pratt, H., *J.A.M.A.*, 1925, v85, 899.

Received September 3, 1963. P.S.E.B.M., 1963, v115.

A Comparison of Insulin Immunoassays.* (28836)

ELLIS SAMOLS AND DAVID BILKUS (Introduced by Sheila Sherlock)
Department of Medicine, Royal Free Hospital, London

All radio-immunoassays for plasma insulin are based on the use of the reaction between insulin-^{131}I and anti-insulin antibody as a tracer system for unlabeled insulin(1). However, different methods have been employed to separate the insulin-^{131}I-antibody complex from residual, "free" insulin. These include paper chromatography(2), salt precipitation (3), and immunoprecipitation(4). Furthermore, different periods of incubation have been utilized(2-4). Since widely divergent results have been obtained using these techniques(2-6), it is clear that some critical factors must exist which are not generally appreciated. In the present investigation some potentially critical factors have been studied: the methods of immunoprecipitation and chromatography have been compared with respect to the effect of interfering with the reaction equilibrium; the effect of dilution of the plasma, and the influence of the presence of *human* antibodies to insulin on the immunoprecipitation method have been assessed; and the immunological potency and stability of several human insulin reference standards over several years has been examined.

Materials. The guinea pig anti-beef antiserum (G.P.A.S.) was prepared by Dr. V. Jones using a Freund's adjuvant and Wellcome beef insulin. Rabbit anti-guinea pig γ-globulin serum (R.A.G.P.S.) was obtained from rabbits immunized to guinea pig γ-globulin emulsified in Freund's adjuvant. The guinea pig γ-globulin was precipitated from guinea pig normal serum at 37°C by addition of sodium sulphate to a final concentration of 13%. The precipitate was twice dissolved in 0.9% NaCl and reprecipitated with 12% sodium sulphate yielding 90% γ-globulin, 10% α_1-β globulin and no albumin on cellulose acetate electrophoresis. The same procedure was used to produce rabbit anti-human γ-globulin (R.A.H.S.). Beef insulin-^{131}I was prepared by adding 5 mc of io-

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dine^{131}† to 5 μg Lilly beef insulin in 0.16 M borate buffer pH 8.0 using chloramine-T as the oxidizing agent(7). Preparations of labeled insulin with specific activities of 600-650 μc/μg were possible because of the consistent efficiency (60-65%) of utilization of the iodine at 23°C. Low temperatures (<10°C) impaired the efficiency of iodination. Although the initial 'damaged' fraction(2) was less than 10% after dialysis against distilled water for 2 hours, the insulin-^{131}I was further purified on cellulose columns(2), reducing the degradation products to 0-3%. The 'damaged' insulin-^{131}I was assessed by chromatography(2), and also by immunoprecipitation as the proportion of radioactivity which could not be bound by concentrated (1:500) G.P.A.S. Results were the same by either method. The insulins used were Lilly crystalline beef insulin (lot 719106) 25.2 U/mg, Wellcome‡ once crystallized human insulin 21.9 international units (i.u.)/mg by mouse convulsion assay [limits of error (p = 0.95) = 19.5-24.5 i.u./mg], Fisher§ crude human insulin, Cambridge 'pure' human insulin‖ and Lilly¶ crude human insulin (lot 493-10GP-90); 6.1 U/mg by rabbit assay [95% confidence limits ± 7.2%].

Biological and immunological units. By definition the biological international unit is 1/24 mg, supplied by a beef-pork reference standard from the Division of Biological Standards, Mill Hill, London. Dr. M. A. Root informed us that Lilly biological units, not stated as such, are international units [however see (3,6)]. As the insulin-antibody reaction may be species-specific, and as immunological reactivity is not necessarily equivalent to biological activity(6), the "immunological" unit for human insulin in this study is a hypothetical entity, obtained by transposing the biological potency of the Wellcome crystalline human insulin reference standard.

Methods. I. Insulin-^{131}I (2-4 μU), unlabeled insulin and/or plasma (usually 1:10) were added to 11 × 51 mm glass tubes. G.P.A.S. (1:500, 1:170,000 or 1:220,000) was added *last*. Dilutions of all reagents were prepared in veronal buffer, 0.1 ionic strength, pH 8.6, containing 0.125% serum albumin. The total reaction mixture was always 0.5 ml and all concentrations in the text are expressed as the final concentration of this reaction mixture (plasma insulin concentration when expressed as μU per ml plasma is = μU per ml × dilution factor). After incubation at 4°C for periods of 3 hours to 5 days, the bound insulin-^{131}I was separated by chromatography or immunoprecipitation.

II. Unlabeled insulin was first allowed to react with G.P.A.S. for 18 hours at 4°C. Only then was the insulin-^{131}I added. The reaction was terminated by immunoprecipitation 3 hours (R1) and 18 hours (R2) later for G.P.A.S. 1:220,000; 18 hours (R3) and 24 hours (R4) later for G.P.A.S. 1:170,000 (Fig. 1). *Chromatographic separation* has been described in detail(2,8). Batches of Whatman 3 MM papers vary in suitability for immunoassay. By courtesy of Mr. Angel Reeve, Batch No. 12066 was selected.

Immunoprecipitation. In principle the soluble bound insulin-^{131}I complex is precipitated with an anti-γ-globulin serum(9). Normal guinea pig serum (usually 0.02 ml) was added to dilute guinea pig anti-sera (1:170,000-1:220,000), but not to the concentrated guinea pig anti-serum (1:500), before adding the RAGPS (usually 0.1 ml). A visible precipitate was immediately evident, and the tubes were incubated for 2 hours at 4°C, previously determined as a time adequate for equilibrium. In some studies dissociation of the bound insulin-^{131}I complex was induced by incubating the precipitate for 2 hours at 37°C. The precipitate was separated by centrifuging at 4° for 20 minutes at 3000 r.p.m. The supernatant fluid was discarded, remov-

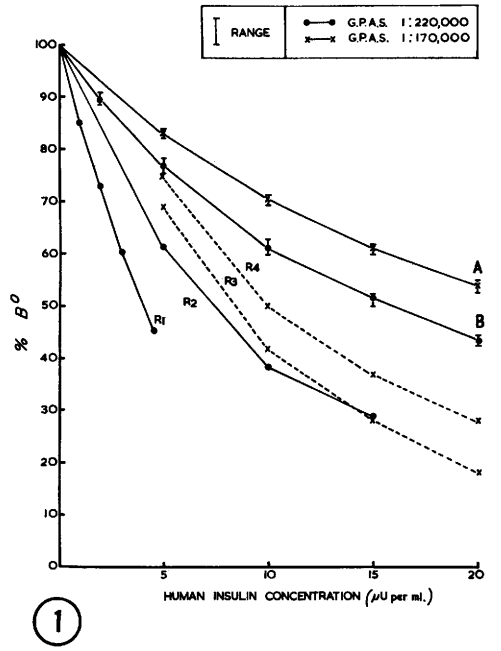
† Catalogue No. I BS-3, Radiochemical Centre, Amersham, England.

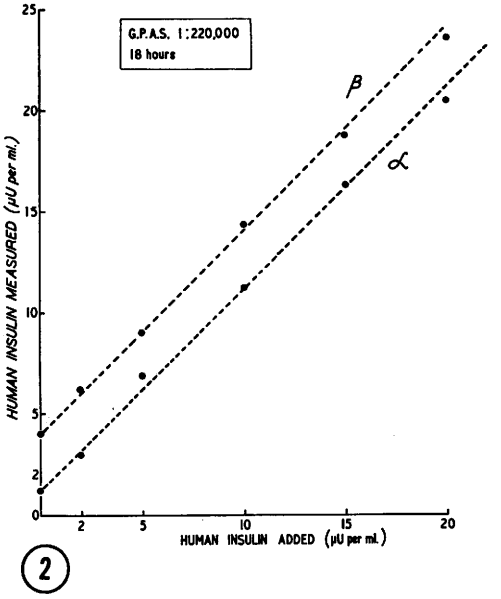
‡ Dr. G. H. Smith, Wellcome Research Laboratories.

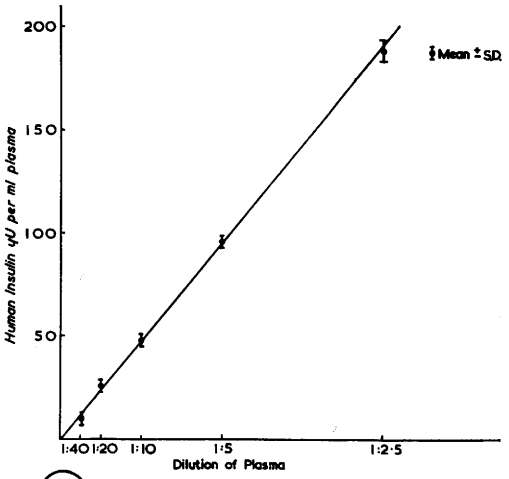
§ Dr. A. M. Fisher, Connaught Research Laboratories.

‖ Prof. F. G. Young, Cambridge.

¶ Dr. M. A. Root, Lilly Research Laboratories.







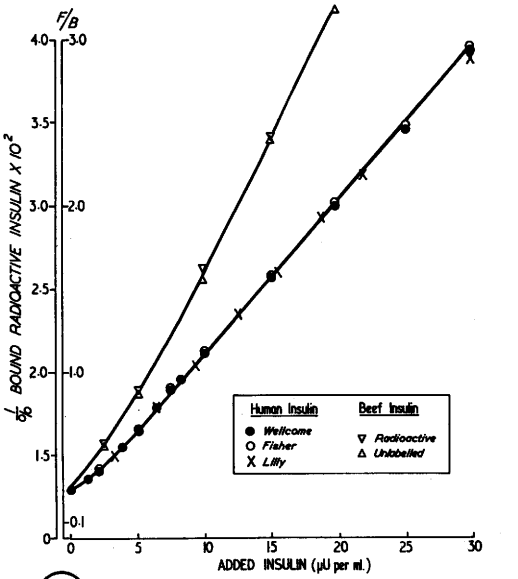


FIG. 1. Standard curves. A. Incubation system I. 2-4 μU insulin-^{131}I. Range for 30 separate curves over one year. Incubation period 18 hr (usually) to 5 days. B. Incubation system I. 3-4 μU insulin-^{131}I. Range for 60 separate curves over one year. Incubation period 18 hr (usually) to 5 days. R1-4. Incubation system II. See Methods.

FIG. 2. Recovery of human insulin added to plasma (1:10). Initially plasma α assayed 12 μU/ml plasma and β 40 μ U/ml plasma.

FIG. 3. Effect of dilution of plasma on insulin.

FIG. 4. Comparison of insulins. F/B = ratio: $\frac{\text{"free" insulin-}^{131}\text{I}}{\text{bound insulin-}^{131}\text{I}}$

TABLE I. Chromatography and Precipitation. Incubation System I. G. P. A. S. 1:170,000. Insulin-^{131}I 4 μU per ml.

Human insulin, μU/ml	Bound insulin-^{131}I, % 18 hr Chrom 4°C	18 hr Ppte 4°C	18 hr Ppte 37°C	5 days Chrom 4°C	5 days Ppte 4°C	5 days Ppte 37°C	% B° *All* Chrom	% B° *All* Ppte
0	61.5	61.5	44.9	78.0	79.0	58.5	100	
5	51.9	51.3	37.8	66.1	65.9	48.5	82.9–84.7	
10	44.1	43.4	31.6	56.0	55.8	40.5	69.2–71.8	
15	37.9	37.2	26.9	47.9	47.8	35.1	59.9–61.6	
20	32.9	32.8	24.2	42.9	41.9	31.2	53.0–55.0	

ing virtually all of the non-bound insulin. This procedure required less than 10 minutes for 68 tubes. Finally the precipitate was washed once with 1 ml of veronal buffer containing 0.125% human serum albumin. The precipitate was counted in a well-type scintillation counter. The undamaged, immunologically reactive insulin-^{131}I, was measured in control tubes containing concentrated (1:500) G.P.A.S., and amounted to 97-100% of the total radioactivity added. With dilute G.P.A.S. and no unlabeled insulin the precipitate gave counts in excess of 20,000/100 seconds. This was reduced to 300 c/100 seconds by addition of saturating amounts of unlabeled beef insulin (200 mU/ml). Bound insulin-^{131}I was represented by the total counts in the precipitate minus the counts in the tube containing saturating amounts of beef insulin.

Results and discussion. Comparison of immunoprecipitation and chromatography. The bound insulin-^{131}I% (bound insulin-^{131}I/total undamaged insulin $\times$ 100) decreases as insulin concentration increases (Table I). Clearly chromatography and precipitation are simple technical variants, as the bound insulin-^{131}I% is the same when insulin concentration, temperature and time of incubation are equivalent. The agreement between chromatography and precipitation was also excellent when the same experiment was performed with G.P.A.S. 1:220,000; and when relatively low specific activity (20 μc/μg) insulin-^{131}I was used. This agreement is also of interest in view of the report that guinea pig anti-beef insulin sera invariably contain 2 types of anti-insulin antibodies, distinctly different in their chromatographic behavior on DEAE cellulose columns and electrophoretic mobility(10).

Interference with equilibrium. Incubation system I. As equilibrium was reached only after 4-5 days, the absolute amounts of bound insulin-^{131}I increased between 18 hours and 5 days (Table I). Yet for each given concentration of unlabeled insulin, the ratio: bound insulin-^{131}I/bound insulin-^{131}I in the absence of unlabeled insulin $\times$ 100 (%B°) was a constant. This finding is of interest as association between insulin and antibody is a second order reaction. The constancy of %B° for each particular concentration of unlabeled insulin was invariable between 18 hours and 5 days for G.P.A.S. 1:170,000 and G.P.A.S. 1:220,000, but was not observed if incubation was interrupted after only 3 hours. Dissociation of insulin-antibody complexes induced by precipitation at 37°C instead of 4°C, reduced the absolute amount of bound insulin-^{131}I (Table I) but the %B° for each concentration of unlabeled insulin was still constant. Exponential dissociation, a first order reaction, may explain this constancy. With G.P.A.S. 1:500 reduction of bound insulin-^{131}I at 37°C was negligible (0-1%) showing that the decrease in bound insulin-^{131}I in dilute anti-sera after warming was not caused by dissociation of the globulin-anti γ-globulin complex. Separate studies showed that association of insulin-antibody complexes was minimal during the period of centrifugation at 4°C which followed precipitation at 37°C. In all, these results clearly show that incubation periods from 18 hours onwards should be satisfactory in this system if the bound insulin-^{131}I is separated within

a short time in both standard and unknown mixtures.

Incubation system II. In system II, in contrast to system I, the %B° for given insulin concentrations was not constant until equilibrium had been reached. Shorter periods of incubation with insulin-^{131}I produced a more acute decrease in the %B° (Fig. 1, curves $R_1 \rightarrow R_2 \rightarrow B$; $R_3 \rightarrow R_4 \rightarrow A$). Similar results were obtained by chromatography. Therefore, this method of distorting equilibrium increases the sensitivity of the assay for a given dilution of G.P.A.S. As sensitivity is also increased by dilution of G.P.A.S., (system I, Fig. 1, of A→B) the sensitivity of system II using G.P.A.S. 1:440,000, and 2 μU of insulin-^{131}I is very great (< 0.001 μU insulin per ml). However, such sensitivity is not necessary for assay of plasma insulin in man.

Immunoprecipitation for assay in plasma. Immunoprecipitation was usually performed after 18 hours' incubation, and was reproducible over the period of a year (Fig. 1, curves A and B. The accuracy of a single curve is 2 μU per ml S.D. $\pm$ 0.26, 10 μU per ml S.D. $\pm$ 0.35 for 10 replicates each. Recovery of human insulin added to plasma was virtually quantitative (Fig. 2). Repeated assays in the same plasma for several months agreed with an average S.D. $\pm$ 10% over a range from 10-200 μU per ml plasma. Comparison of 15 plasmas using different conditions for chromatography (G.P.A.S. 1:300,000, incubation period 3-5 days) and precipitation (G.P.A.S. 1:170,000 or 1:220,000, incubation period 18-20 hours) agreed with an average S.D. of $\pm$ 12%. In addition comparisons showed that it was possible to assay plasma insulin in a total period of 2 hours, although sensitivity was impaired.

Dilution of plasma did not affect the assay (Fig. 3). In rat plasma an 'inhibition' at high concentrations of plasma and a 'plateau' at low plasma concentrations has been reported(4), producing spuriously high insulin levels in either range. This "dilution effect" was not produced in human plasma by altering our system to use borate buffer, pH 8.5, and rabbit anti-guinea pig whole serum, as described by Morgan and Lazarow(4). We could only achieve an "inhibition" in human plasma by suboptimal precipitation with RAGPS. In this case precipitation was adequate to produce an acceptable standard curve in the absence of plasma, but incomplete in tubes containing plasma. The resultant falsely "high" plasma insulin levels may be misleading, as relative changes in plasma insulin levels, for example, a rise after glucose, are still assayed. Separate studies showed that a similar effect could be produced by human γ-globulin (see *Methods*), but not by human albumin, suggesting that suboptimal precipitation in plasma is caused by cross-reaction between plasma γ-globulin and RAGPS, impairing the precipitation of guinea pig γ-globulin.

TABLE II. Tubes containing 2 μU Insulin-^{131}I, G. P. A. S. 1:220,000 $\pm$ Human Antiserum or Normal Human Serum 1:5 Incubated for 18 Hours at 4°C. Then 0.1 ml guinea pig normal serum (1:50) and 0.1 ml R. A. G. P. S. added.

Serum	R.A.H.S., ml	"Bound insulin-^{131}I," %
—	—	67.2
—	4	67.2
Human antiserum—1	—	< .05
"	4	96.2
Human antiserum—2	—	< .1
"	4	96.8
Normal human serum	—	60.3
"	4	60.4

Optimal precipitation for each pool of RAGPS was easily determined by titrating RAGPS against dilute and concentrated GPAS, measuring bound insulin-^{131}I in the presence and absence of plasma. In this precipitating system both antibody (RAGPS) excess and antigen (γ-globulin) excess caused sub-optimal precipitation.

Immunoprecipitation and human antisera. Two human anti-beef insulin sera (1, 2 in Table II) had maximal beef insulin binding capacities of > 2000 U/liter, and effectively competed with the dilute GPAS for the available insulin-^{131}I. The absence of significant precipitation in this system (Table II) of human beef insulin-binding γ-globulin by R.G.P.A.S. does not exclude some cross-reaction between human γ-globulin and

R.G.P.A.S., as the concentration of human serum in the reaction mixtures was relatively high (50 × the concentration of guinea pig serum). In practice human antisera with high or low insulin binding capacities (< 20 U/liter) competed for insulin-^{131}I and reduced the apparent "bound insulin-^{131}I" to give a spuriously high insulin level. Dilution of human antisera "confirmed" the falsely high insulin levels, indicating that the guinea pig γ-globulin-RAGPS precipitating system is at least partially species-specific. These human antisera gave less than zero or falsely low insulin levels by chromatography, as the total bound insulin-^{131}I (bound to human antibodies + guinea pig antibodies) was, in fact, increased. Such antibodies were obvious on screening by chromatography or electrophoresis in the absence of G.P.A.S. (2), or by precipitation with R.A.H.S.(9).

Comparison of insulins. Using Wellcome crystalline human insulin (21.9 i.u./mg) as a reference standard, the Fisher crude human insulin assayed 6.0 "immunological" units/mg and the Lilly crude human insulin 6.65 "immunological" units by chromatography and immunoprecipitation (Fig. 4). Standard Wellcome human insulin curves (A and B in Fig. 1) were also used for repeated comparisons of the Fisher human insulin over a period of a year, demonstrating the excellent immunological stability of these human insulins. On this reference the mean fasting plasma insulin levels in 100 normal subjects were 19 μU per ml, S.D. ± 7.5, in good agreement with the mean fasting plasma insulin level of 21μU per ml reported by Yalow and Berson(2), who used Fisher human insulin, regarded as 6.0 u/mg, as a standard.

Our samples of Cambridge pure human insulin rapidly lost immunological reactivity. Deterioration was also suggested by the appearance of > 50% of Cambridge insulin-^{131}I, compared with < 5% of Wellcome human insulin-^{131}I, as 'degraded' insulin-^{131}I after comparable labeling with iodine ^{131} (50 μc/μg). That the Cambridge human insulin was relatively pure initially may be inferred by the similarity of early Cambridge/Fisher comparisons(6,8) with the present Wellcome/Fisher human insulin comparison.

The immunological reactivity of high specific activity (650 μc/μg) and unlabeled Lilly beef insulin was identical (Fig. 4). The human insulin competes less strongly than beef insulin against the binding of beef insulin-^{131}I by anti-beef insulin antibodies, illustrating the importance of immunological species-specificity in insulin immunoassay. The relative degree of cross-reaction between beef and human insulin was approximately similar over the range of insulin concentrations tested [however, see(6)]. The shape of the curves in Fig. 4 has been explained (11), and similar approximately linear curves are obtained by plotting the reciprocals of the values for bound insulin-^{131}I% or %B° in Table I and Fig. 1 against insulin concentration.

Summary. Radio-immunoassay of insulin gives the same results whether immunoprecipitation or chromatography is used. The effects on the assay systems of interference with equilibrium, and the influence of human anti-insulin sera on immunoprecipitation are described. It is suggested that present radio-immunoassays should give similar plasma insulin levels in man if similar reference standards insulins are used. The immunological potency of several human insulins in general use is compared.

1. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., Newerly, K., *J. Clin. Invest.*, 1956, v35, 170.

2. Yalow, R. S., Berson, S. A., *ibid.*, 1960, v39, 1157.

3. Grodsky, G. H., Forsham, P. H., *ibid.*, 1961, v40, 799.

4. Morgan, C. R., Lazarow, A., *Diabetes,* 1963, v12, 115.

5. Spellacy, W. N., Goetz, F. C., *New Eng. J. Med.*, 1963, v268, 988.

6. Yalow, R. S., Berson, S. A., *J. Clin. Invest.*, 1961, v40, 2190.

7. Hunter, W. M., Greenwood, F. C., *Nature,* 1962, v194, 495.

8. Samols, E., Ryder, J. A., *J. Clin. Invest.*, 1961, v40, 2092.

9. Skom, J. H., Talmage, D. W., *ibid.*, 1958, v37, 783.

10. Yagi, Y., Maier, P., Pressman, D., *J. Immunol.*, 1962, v89, 442.

11. Ekins, R., Samols, E., *Lancet,* 1963, v2, 202.

Received September 3, 1963. P.S.E.B.M., 1964, v115.