Immunoassay of Insulin with Insulin-Antibody Precipitate

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(*Received* 30 *January* 1963)

Micromethods for the assay of insulin based on the reaction between ^{131}I-labelled insulin and insulin antibody have been described by Yalow & Berson (1960) and Grodsky & Forsham (1960). In principle they depend on the use of insulin antibody to obtain samples of insulin from mixtures prepared by adding standard or unknown solutions of unlabelled insulin to a fixed amount of ^{131}I-labelled insulin and thereby to compare the specific activities of the mixtures (which will diminish as the amount of unlabelled insulin increases). In principle, therefore, these methods depend on isotope dilution. Since most insulin–antibody complexes are soluble, it has been necessary to devise some method of separating free insulin from antibody-bound insulin. In the method of Yalow & Berson (1960) this was achieved by electrophoresis with solvent flow (chromatoelectrophoresis), whereas Grodsky & Forsham (1960) employed fractional precipitation with salts. The assay of insulin was achieved empirically by comparing the activities of standard and unknown solutions of the hormone.

In the methods described in the present paper, use has been made of the fact that insulin antibody or the complex it forms with insulin may be precipitated with anti-γ-globulin serum, as first described by Sköm & Talmage (1958). In the method initially developed this precipitation reaction was used to separate the antibody-bound insulin from the free insulin. When it was found that insulin antibody that had been precipitated with anti-γ-globulin serum was still capable of reacting with insulin, new assay methods were developed with pre-precipitated insulin antibody. The methods described in the present paper appear to have the advantages over those previously described of greater simplicity and rapidity, and of achieving comparable sensitivity with preparations of ^{131}I-labelled insulin of much lower specific activity. Preliminary accounts of these methods have been published (Hales & Randle, 1962, 1963).

EXPERIMENTAL

Materials and methods

Insulin. Ox insulin (recrystallized six times) and pig insulin were given by Boots Pure Drug Co. Ltd., Nottingham, by Burroughs Wellcome. Beckenham, Kent, and Eli Lilly and Co., Indianopolis, U.S.A. Human insulin was prepared from autopsy pancreas by an unpublished method of Dr L. F. Smith. Prepared in this way, it ran as a single band on ascending chromatography in butan-2-ol–aq. 1% (v/v) acetic acid (1:1, v/v) (Fenton, 1959). The potency of human insulin by rat-diaphragm bioassay (Randle, 1956) was indistinguishable from that of ox insulin [stated potency 22·3 i.u./mg. (95% confidence limits in the range 7·4–66·9 i.u./mg.)]. Standard solutions of insulin for immunoassay were prepared by diluting a stock solution of 0·2 mg./ml. (in 3·3 mM-HCl) with buffer [40 mM-sodium phosphate, pH 7·4, containing bovine plasma albumin (1 mg./ml.), merthiolate (0·6 mM) and NaCl (0·9%)].

^{131}I-labelled insulin. [^{131}I]Iodide was obtained from The Radiochemical Centre, Amersham, Bucks. in the form of a solution obtained by distilling $^{131}I_2$ into 20 mM-NaOH (catalogue no. 1 BS/3) at the highest available specific activity (in general 1 drop of a solution containing 2–8 mc in a small stoppered container). Insulin was iodinated with ICl by Springell's (1961) modification of MacFarlane's method. The [^{131}I]iodide as supplied was diluted with 20 μl. of ICl solution prepared as follows. To 2·5 ml. of a solution containing NaI (0·4 M) and $NaIO_3$ (0·2 M) was added dropwise with shaking (in a 10 ml. stoppered cylinder) 2·4 ml. of conc. HCl (until the black precipitate disappeared); 1 ml. of CCl_4 and 1 drop of 0·1 M-NaI were added together with water to give a total volume of 11 ml. This stock solution of ICl was kept at 4° and diluted for iodination (1 ml. of stock ICl solution added to 22·85 ml. of 2 M-NaCl, 2·06 ml. of N-NaOH and 22·84 ml. of water, and the whole diluted fourfold with 60 mM-glycine–0·1 N-HCl–0·24 M-NaCl). To the [^{131}I]iodide diluted with ICl was added 50 μg. of crystalline ox insulin in 10 μl. of glycine buffer (for composition see above). After 5 min. the contents of the iodination mixture were transferred to 1 ml. of 8 M-NaI solution in 24/32 in. Visking Seamless cellulose tubing (Hudes Merchandising Corp. Ltd., London, W. 1) and dialysed against five changes each of about 600 ml. of 3 mN-HCl for a total of 28 hr.

The purity of the preparation was checked by mixture with unlabelled insulin followed by ascending chromatography (Fenton, 1959) and by zone electrophoresis for 3 hr. at 300 v on Whatman 3 MM chromatography paper in barbitone buffer, pH 8·6 (Flynn & de Mayo, 1951), containing urea (7 M). Chromatograms and electrophoretograms were stained for protein with bromocresol green (Fenton, 1959) and radioautographs prepared on Ilford X-ray film (Industrial G). In most instances a single band of radioactivity was detected which was coincident with the band of protein staining. The specific activity (on assuming 100% recovery of insulin) was 5–20 mc/mg.

Guinea-pig anti-insulin serum. Antisera to human, ox and pig insulins were prepared in guinea pigs by the method of Robinson & Wright (1961).

Rabbit anti-(guinea-pig γ-globulin) serum. Crude guinea-pig γ-globulin was obtained from normal guinea-pig serum

by precipitation with $(NH_4)_2SO_4$ [1 vol. of sat. $(NH_4)_2SO_4$ plus 2 vol. of serum] followed by dialysis against water and freeze-drying. For the production of antisera 10 mg. was dissolved in 1 ml. of 0·9% NaCl and emulsified with an equal volume of complete Freund's adjuvant. Weekly injections (subcutaneously into the back) were made into rabbits for 1 month, followed by weekly injections of 10 mg. of globulin in 1 ml. of 0·9% NaCl. After a total of 6 weeks the animals were bled by cardiac puncture. A precipitin test was carried out with 0·2 ml. of doubling dilutions of the antiserum and 0·1 ml. of a solution of crude guinea-pig γ-globulin (1 mg./ml.) in 0·9% NaCl. A satisfactory antiserum gives a visible precipitate in this system at dilutions of 1:64 or more. Further blood samples were taken at weekly intervals, and after this an additional injection of γ-globulin was given.

Blood samples. Rabbit and guinea-pig sera or antisera were separated by centrifuging from blood that had been allowed to clot for 16 hr. at 4°. Blood samples were obtained by cardiac puncture under ether anaesthesia. For assay of insulin, human blood was collected from an antecubital vein and rat blood was drawn from the inferior vena cava under Nembutal anaesthesia. Crystalline heparin (0·2 mg./ml.) (given by Evans Medical Ltd., Liverpool) was added to prevent coagulation, and plasma was separated by centrifuging (plasma was preferred to serum because haemolysis is less frequent and the latter interferes with the immunoassay of insulin).

Other materials. Bovine plasma albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd. Tris (Sigma 121) was obtained through V. A. Howe and Co. Ltd., 46 Pembridge Road, London, W. 11. Other chemicals were of analytical grade except for the NaI and $NaIO_3$,H_2O, which were laboratory reagents from British Drug Houses Ltd.

Storage. Antisera were diluted with 0·04 M-tris–HCl buffer or 0·04 M-phosphate buffer, pH 7·4, containing bovine plasma albumin (1 mg./ml.) and merthiolate (0·6 mM), and stored at −10° in 5 ml. lots (or whatever was appropriate for a single experiment). This method of storage was necessary because repeated freezing and thawing leads to loss of potency. Solutions of human insulin in 3·3 mM-HCl stored at −10° lost activity slowly and it was advisable to limit storage to 1 month. Plasma samples for immunoassay have been stored at −10° without the addition of preservative without loss of activity.

PRINCIPLE AND PROCEDURE

Principle of isotope dilution and immunological assay

If a given volume of a solution of ^{131}I-labelled insulin, of concentration $2i_0$ and specific activity x, is mixed with an equal volume of buffer or a solution of unlabelled insulin, of concentration $2i$, then the concentration of ^{131}I-labelled insulin will be i_0 in each case, but the specific activity will be reduced in the presence of unlabelled insulin to $xi_0/(i_0+i)$. It follows that the change in specific activity will be a function of i and that the latter may be estimated by reference to known standard solutions of insulin if the changes in specific activity can be measured. This seems to be the basis of immunological assays for insulin in which insulin antibody is used merely to obtain a sample of insulin. Assay of radioactivity in the insulin–antibody complex then provides an estimate of the specific activity of the original insulin sample provided that the antibody extracts only part of the insulin pool (i.e. conditions must be such that insulin is in excess of its antibody). The theoretical relationship for the principle of isotope dilution as applied to this system is as follows:

Let C_0 be the radioactivity of insulin–antibody complex when i is zero. Let C_i be the radioactivity of insulin–antibody complex when the concentration of unlabelled insulin is i. Let I_0 and I be the amounts of ^{131}I-labelled insulin bound by antibody under these conditions.

Then $$\frac{I_0}{I} = \frac{i_0+i}{i_0} = \frac{i}{i_0}+1$$

and $C_0 = xI_0$ and $C_i = xI$ and $C_0/C_i = I_0/I$.

Then $$\frac{C_0}{C_i} = i.\frac{1}{i_0}+1.$$

It follows that C_0/C_i will be linearly related to i, that C_0/C_i will be unity when i is zero, that $i_0 = -i$ when C_0/C_i is zero and that the slope of the line is $1/i_0$ (see Fig. 1). The theoretical relationship given above will only hold in practice if the affinity of antibody for ^{131}I-labelled insulin and unlabelled insulin is the same and if the amount of insulin bound by antibody is independent of the concentration of insulin. Departures from this theoretical relationship in practice will not, however, interfere with the practical assay of insulin provided that the latter is made by reference to standard solutions of the hormone.

Procedures for immunological assay of insulin

Three procedures for the immunoassay of insulin have been developed and the choice of a particular procedure depends on the purpose for which it is required. In the first (method A) ^{131}I-labelled insulin plus or minus standard or unknown solutions of unlabelled insulin is incubated with insulin antibody, and the insulin–antibody complex is

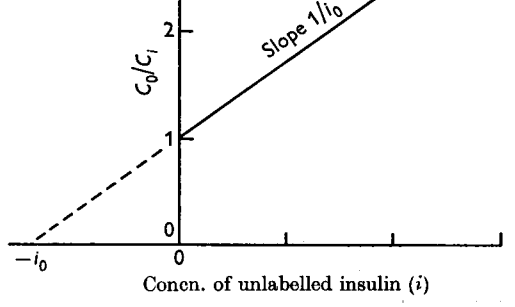

Fig. 1. Theoretical relationship between the ratio of the radioactivities in the insulin–antibody complex in the absence and presence of unlabelled insulin and the concentration of unlabelled insulin. Experimental details are given in the text. C_0 and C_i, radioactivities in the insulin–antibody precipitate in the absence and presence of unlabelled insulin respectively; i, concn. of unlabelled insulin.

subsequently precipitated with anti-γ-globulin serum (precipitating antiserum), filtered and assayed for radioactivity. It is quicker than the other methods but less satisfactory for the assay of insulin in plasma because γ-globulin may interfere with precipitation of the insulin–antibody complex by cross-reacting with the precipitating antiserum (unless high concentrations of the latter are used). In the second (method B) the insulin antibody is first precipitated by incubation with precipitating antiserum, then incubated with ^{131}I-labelled insulin plus or minus standard or unknown solutions of unlabelled insulin and finally filtered and assayed for radioactivity. In the third (method C) the procedure is as for method B but the precipitated antibody is incubated with standard or unknown solutions of unlabelled insulin for a short period before the addition of ^{131}I-labelled insulin. This modification of method B increases the sensitivity of the assay. Methods B and C are the methods of choice for assay of insulin in plasma because γ-globulin in plasma is not introduced until after the precipitation of insulin antibody, and consequently cannot interfere by cross-reacting with the precipitating antiserum.

Practical details

Concentration of ^{131}I-labelled insulin. The concentration of ^{131}I-labelled insulin used represents a compromise between the need to have sufficient radioactivity for counting and the need for unlabelled insulin to form a reasonable proportion of the mixture. It is convenient to add 250 $\mu\mu$g. of ^{131}I-labelled insulin, which (at 12 mC/mg.) yields a final count of about 1000/min. in a Nuclear–Chicago gas-flow counter (efficiency approx. 30%) when no unlabelled insulin is added and which permits the detection of 250 $\mu\mu$g. of human insulin/ml. (approx. 6×10^{-6} i.u./ml.).

Concentration of anti-insulin serum. In method A, 0·1 ml. of ^{131}I-labelled insulin solution [diluted to 2·5 μmg./ml. with 40 mM-phosphate buffer, pH 7·4, containing bovine plasma albumin (1 mg./ml.) and merthiolate (0·6 mM)], 0·1 ml. of 0·9% NaCl and 0·1 ml. of different dilutions of anti-insulin serum (doubling dilutions between 1:1000 and 1:32 000 made with phosphate buffer containing albumin and merthiolate) were mixed in tubes (5 cm. × 1 cm.) and incubated at 4° for 4 hr. Precipitating antiserum (0·1 ml. of 1:2 to 1:16 dilutions was suitable, depending on the activity of a particular precipitating antiserum) was added and incubation continued for a further 18 hr. The precipitated insulin–antibody complex was then collected by filtration on 2 cm. Oxoid cellulose acetate membranes (Oxo Ltd., London) mounted on a Pyrex hydrosol microanalysis filter-holder (Millipore Filter Corp., Bedford, Mass.; catalogue no. XX 10 025 00, obtained through V. A. Howe and Co. Ltd.). The tubes were washed twice with 0·75 ml. of 4% (w/v) bovine plasma albumin in 40 mM-phosphate buffer, pH 7·4, with the aid of a Pasteur pipette and the washings filtered. The addition of albumin to the wash fluid prevented the retention of free ^{131}I-labelled insulin by the cellulose acetate membranes (pig plasma clarified by filtration through kieselguhr provides a cheaper and apparently satisfactory alternative to bovine plasma albumin). The efficiency of the washing procedure was controlled by the inclusion of a tube in which insulin antiserum was replaced by phosphate–albumin–merthiolate solution. The membranes were then fixed on to unwaxed cardboard bottle-top liners by Dunlop rubber solution, dried for 1 hr. at 75° and assayed for radioactivity in a Nuclear–Chicago gas-flow counter (1000 counts being recorded). To check the recovery of radioactive insulin in the insulin–antibody precipitate, 0·1 ml. of ^{131}I-labelled insulin solution was plated and counted at the same time.

In methods B and C, 0·1 ml. of a mixture of equal volumes of dilutions of anti-insulin serum (see above) and of 1:15 precipitating antiserum was incubated at 4° for 16–24 hr. in tubes (5 cm. × 1 cm.). To each was then added 0·1 ml. of ^{131}I-labelled insulin solution (containing 250 $\mu\mu$g.) and 0·1 ml. of phosphate–albumin–merthiolate–NaCl, and after mixing incubation was continued for a further 22 hr. The contents of each tube were then filtered and the radioactivity was assayed as described above.

In each method the concentration of anti-insulin serum which removed approximately half the total radioactivity was selected for insulin assay.

Assay of insulin. In method A, ^{131}I-labelled insulin (250 $\mu\mu$g. in 0·1 ml.) was mixed with 0·1 ml. of standard or unknown solutions of insulin or phosphate–albumin–merthiolate–NaCl, and diluted antiserum (0·1 ml.) added with mixing. The subsequent procedure was as described in the preceding section. In method B, 0·1 ml. of ^{131}I-labelled insulin (250 $\mu\mu$g.) and 0·1 ml. of phosphate–albumin–merthiolate–NaCl or standard or unknown solutions of unlabelled insulin were added after 16–24 hr. of precipitation of the insulin antibody with precipitating antiserum; the procedure was otherwise as outlined in the preceding section. In method C, 0·1 ml. of phosphate–albumin–merthiolate–NaCl or standard or unknown solutions of unlabelled insulin were added 16–24 hr. after the mixing of the insulin antibody with precipitating antiserum. The contents of the tubes were mixed and incubated for 6 hr. at 4°. The ^{131}I-labelled insulin (250 $\mu\mu$g. in 0·1 ml.) was then added and, after mixing of the solutions, incubation was continued at 4° for a further 16 hr. The procedure was then as given in the preceding section.

RESULTS

Effect of different experimental conditions on recovery of ^{131}I-labelled insulin. The effect of the concentration of anti-insulin serum on the recovery of ^{131}I-labelled insulin in a typical experiment is shown in Fig. 2. The results shown were obtained with method A but essentially similar results are obtained with methods B and C. With antiserum in excess (usually at dilutions between 1:1000 and 1:4000) 90–96% of the radioactivity was recovered in the precipitate in each experiment, from which it is concluded that precipitation of the insulin–antibody complex by anti-γ-globulin serum is substantially complete. With further dilution of the anti-insulin serum there was a diminution in the fraction of ^{131}I-labelled insulin recovered in the precipitate. Satisfactory recovery of radioactivity in the precipitate has been achieved in all methods without the addition of further (carrier) guinea-pig γ-globulin, and indeed the latter did not improve the recovery of radioactivity.

The effect of different concentrations of rabbit anti-(guinea-pig γ-globulin) serum on the removal of ^{131}I-labelled insulin at different concentrations of anti-insulin serum in method A is shown in Fig. 3. With the particular precipitating antiserum employed the recoveries of radioactivity were comparable at dilutions of 1:12 and 1:24 but diminished at a dilution of 1:48. In other experiments it has been found that removal of ^{131}I-labelled insulin is diminished when undiluted precipitating antiserum is used. It is suggested that this is due to the action of unlabelled insulin in the precipitating antiserum, since a similar effect is seen if unlabelled insulin is added to the precipitating antiserum. This is presumably due to continuing reaction between insulin and insulin antibody during precipitation of the latter with anti-γ-globulin serum, an observation that led to the development of methods B and C. This effect does not, however, interfere with the assay of insulin by method A, since it influences the recovery of radioactivity with standard, unknown and control solutions. The recovery of radioactivity in method A was not influenced by pH over the range 6·9–7·8, nor by heparin at the concentration at which it was added to blood samples. The ionic strength of the medium has been found to affect the recovery of radioactivity in the precipitate, the recovery being diminished at higher ionic strengths. For this reason standard insulin solutions are made up in 0·9% sodium chloride to approximate to the ionic strength of plasma.

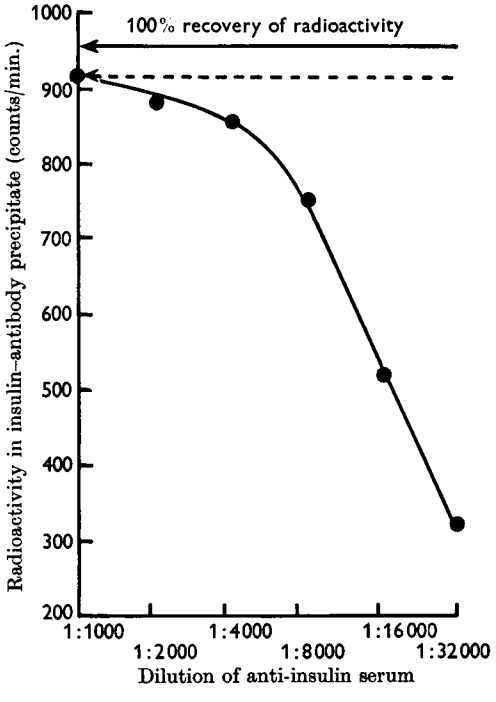

Fig. 2. Effect of the concentration of antiserum on the recovery of ^{131}I-labelled insulin. Experimental details are given in the text.

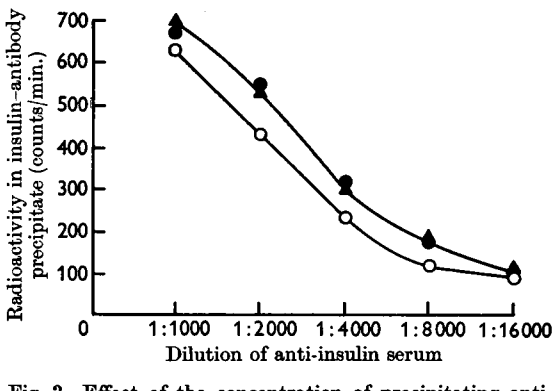

Fig. 3. Effect of the concentration of precipitating antiserum on the recovery of ^{131}I-labelled insulin. Experimental details are given in the text. Dilution of precipitating antiserum: ○, 1:48; ●, 1:24; ▲, 1:12.

With the object of limiting the total period of incubation to about 24 hr. in method A the effect on the radioactivity of the precipitate of adding the precipitating antiserum at different times during the 24 hr. was investigated under conditions used in the assay of insulin (i.e. with insulin in excess of its antibody). When precipitating antiserum was added after 8 hr. the radioactivity of the precipitate was about 25% greater than when it was added immediately. The rate of increase of radioactivity/hr. was about 10% in the first hour and 2% for each hour thereafter up to the eighth hour (Fig. 4). Hence it seemed advisable in method A to wait for at least 1 hr. before adding precipitating antiserum, and for convenience a period of 4 hr. has been used as a routine followed by 18 hr. of precipitation.

In the development of method A, it was found that at least 14 hr. is needed for precipitation of the

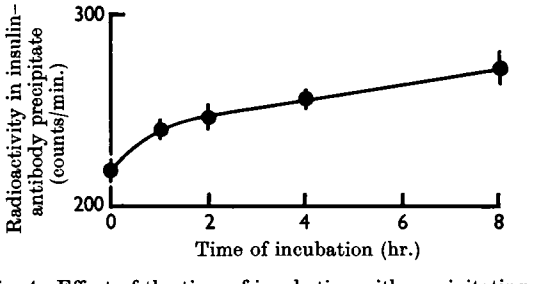

Fig. 4. Effect of the time of incubation with precipitating antiserum on the recovery of ^{131}I-labelled insulin in the insulin–antibody precipitate. Experimental details are given in the text. The time allowed for precipitation was 24 hr. minus the time of incubation shown on the abscissa.

insulin complexed with antibody by anti-γ-globulin serum as judged by the recovery of radioactivity under assay conditions (i.e. with insulin in excess of antibody). In developing methods B and C, therefore, 16–24 hr. of incubation was allowed for precipitation. Longer periods of incubation are unsuitable since less insulin is bound by antibody when 36 or 48 hr. of precipitation is used. Aggregation of the precipitate into larger particles with longer periods of precipitation may explain the decreased recovery of insulin observed. The effect of the period of incubation of precipitated insulin antibody with ^{131}-labelled insulin on the recovery of the latter in the precipitate in methods B and C is shown in Fig. 5. The removal of insulin was substantially complete at 24 hr. In practice it has been convenient to use a period of 22 hr.

Effect of different concentrations of unlabelled insulin on the recovery of ^{131}I*-labelled insulin.* The effect of increasing concentration of human, ox and pig insulins on the recovery of ^{131}I-labelled ox insulin bound by guinea-pig anti-(human insulin) serum in method A is shown in Fig. 6. The curve is in the general form obtained in their methods by Yalow & Berson (1960) and Grodsky & Forsham (1960). With this particular antiserum there was little difference between the curves obtained with human, ox or pig insulins. We do not know whether the lack of discrimination between these different insulins is a general property of antisera to human insulin. With antisera to ox and pig insulins, Berson & Yalow (1962) found that some antisera discriminate widely between ox and human insulins whereas others show little discrimination.

The result of plotting C_0/C_i against i for an experiment with anti-(human insulin) serum, unlabelled human insulin and ^{131}I-labelled ox insulin with method A is shown in Fig. 7. In conformity with the principle of isotope dilution there is a linear relationship between C_0/C_i and i, and C_0/C_i is 1·0 when i is zero (statistical analysis showed no significant deviation from linearity). A similar type of relationship was observed in a further experiment (not shown) with anti-(ox-insulin) serum and unlabelled and ^{131}I-labelled ox insulins. The linear relationship was observed under these conditions over a range of concentrations of unlabelled insulin from 5 to 320 microunits/ml.; at concentrations greater than 320 microunits/ml. the relationship ceased to be linear. In discussing the theoretical relationship for isotope dilution it was pointed out that this would only hold in relation to the immunoassay if the amount of insulin bound by antibody is independent of the concen-

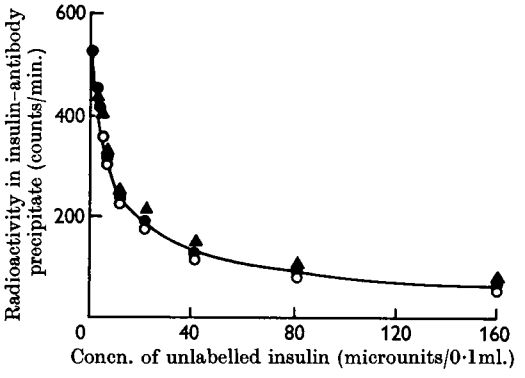

Fig. 6. Effect of unlabelled human (●), pig (▲) and ox (○) insulins on the recovery of ^{131}I-labelled insulin in the insulin–antibody precipitate. Method A was used for the assay of insulin. Experimental details are given in the text.

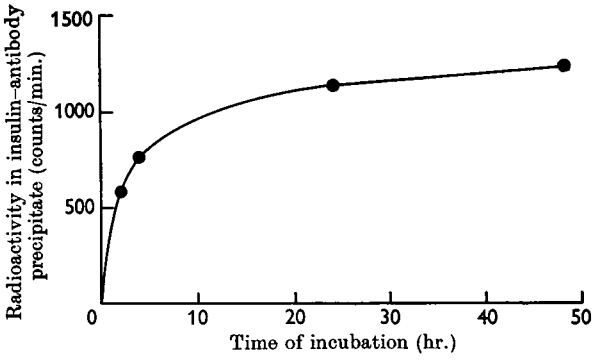

Fig. 5. Effect of the time of incubation with the insulin–antibody precipitate on the recovery of ^{131}I-labelled insulin in the insulin–antibody precipitate. Experimental details are given in the text. Anti-insulin serum and the precipitating antiserum were mixed 22 hr. before incubation commenced.

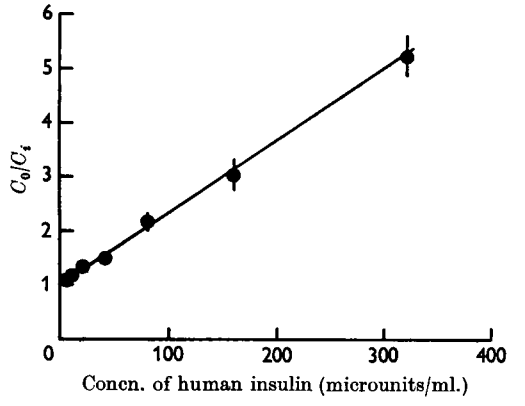

Fig. 7. Effect of unlabelled human insulin on the recovery of ^{131}I-labelled insulin in the insulin–antibody precipitate. Method A was used for the assay of insulin. Experimental details are given in the text. For definitions of C_0 and C_i see Fig. 1. Values are given ± S.E.M.

tration of insulin. This has been tested by studying the effect of increasing concentrations of ^{131}I-labelled insulin (250–4000 $\mu\mu$g. added in 0·1 ml.) on the recovery of radioactivity in the insulin–antibody precipitate. The results of such an experiment with method A are shown in Fig. 8. The radioactivity of the precipitate increased as the concentration of ^{131}I-labelled insulin was increased and reached a plateau at a concentration of about 2000 $\mu\mu$g. added in 0·1 ml. In the assay of insulin in human plasma it is necessary to limit the addition of ^{131}I-labelled insulin to 250 $\mu\mu$g. in 0·1 ml., and it seems unlikely therefore that the theoretical relationship for isotope dilution will hold under these conditions. This does not, however, interfere with the use of the relationship between C_0/C_i and i for the construction of a standard curve and such a linear relationship is a great advantage. This linear relationship has also been observed with methods B and C (see Fig. 11).

The effect of antisera to different insulins on the radioactivity of the insulin–antibody complex at different concentrations of unlabelled human insulin is shown in Fig. 9. Method A was used and the experiments were made simultaneously with the same solutions of unlabelled human insulin and ^{131}I-labelled ox insulin. The gradient of the reduction of radioactivity as a function of the concentration of unlabelled human insulin (plotted on a logarithmic scale) is much steeper with the anti-(human insulin) serum than with either anti-(ox insulin) or anti-(pig insulin) sera, particularly at the lowest insulin concentrations. This might suggest that the most sensitive assay of human insulin can be achieved with anti-(human insulin) serum. The relationship between C_0/C_i and i for anti-(human insulin) and anti-(ox insulin) sera is shown in Fig. 10, and though both relationships are linear the slope for anti-(human insulin) serum is much steeper than that for anti-(ox insulin) serum.

A comparison between methods B and C is shown in Fig. 11. In method C (in which the unlabelled

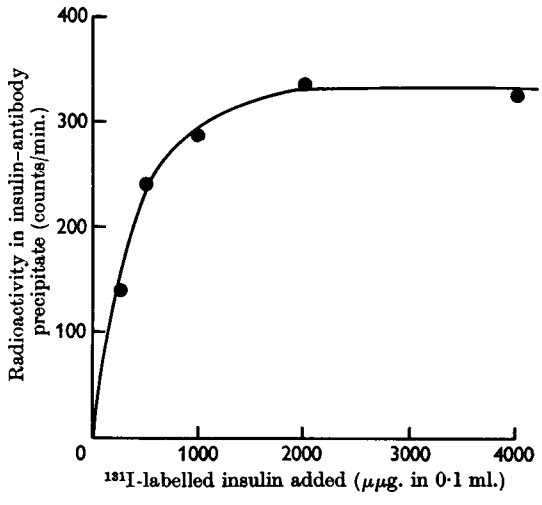

Fig. 8. Effect of the concentration of ^{131}I-labelled insulin on its recovery in the insulin–antibody precipitate. Method A was used for the assay of insulin. Experimental details are given in the text.

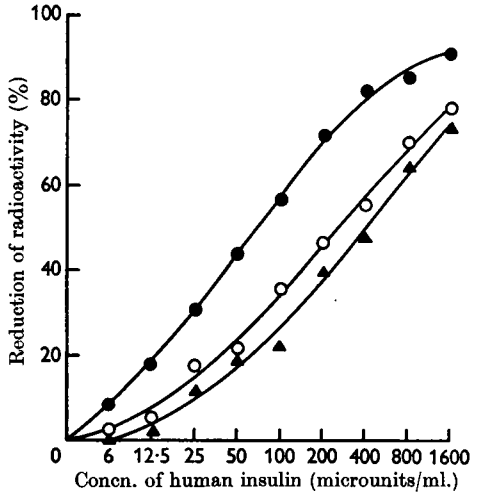

Fig. 9. Comparison of antisera to human (●), pig (▲) and ox (○) insulins. Method A was used for the assay of insulin. Experimental details are given in the text.

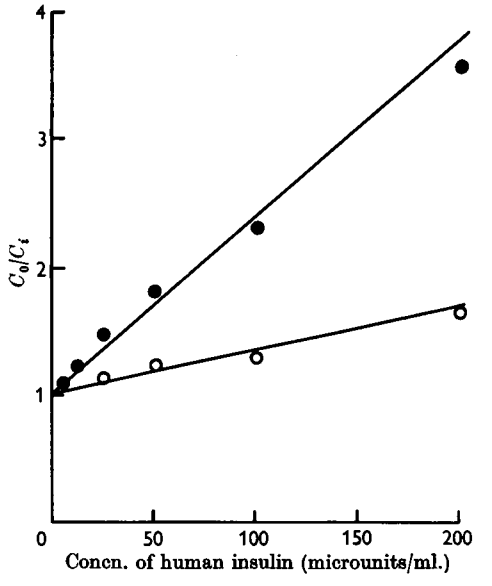

Fig. 10. Comparison of antisera to human (●) and ox (○) insulins. Method A was used for the assay of insulin. Experimental details are given in the text. For definitions of C_0 and C_i see Fig. 1.

human insulin is added 6 hr. before the ^{131}I-labelled ox insulin) the reduction of the radioactivity of the insulin–antibody precipitate was greater at the lower concentrations of unlabelled human insulin than with method B, and, moreover, the slope of the reduction of radioactivity as a function of unlabelled insulin was greater in method C. Method C thus provides a more sensitive assay for insulin than method B.

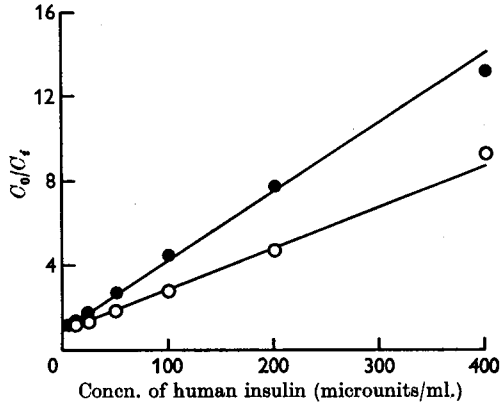

Fig. 11. Comparison of the sensitivities of methods B (○) and C (●) for the assay of insulin. Experimental details are given in the text. For definitions of C_0 and C_i see Fig. 1.

Assay of insulin in blood plasma. Each of these methods may be used to determine insulin in blood plasma provided that the standard insulin used is from the same species of animal (because of differences in reactivity of insulins from different species). In practice it is convenient to use a reference standard of ox insulin calibrated against human insulin (because of scarcity of the latter) in assaying insulin in human blood plasma. This has been done in many of the experiments described below.

When plasma from five normal people was assayed for insulin with method A and with the use of a high concentration of precipitating antiserum (dilution less than 1:12), a satisfactory assay was achieved. The plasma insulin concentration was (in microunits/ml., given as means with the ranges in parentheses): after starvation, 16 (6–25); and 30, 60 and 150 min. after the oral administration of glucose (50 g.), 64 (35–88), 65 (43–120) and 22 (6–32) respectively. When five samples of human plasma with high insulin activity were assayed at three dilutions (undiluted, and diluted 1:2 and 1:4) the calculated insulin contents at dilutions of 1:2 and 1:4 were (means ± S.E.M.) 105 ± 8·6 % and 87 ± 18 % of that of the undiluted samples (304 microunits/ml.). There was no significant difference between the estimates obtained from the three dilutions. The estimates of insulin concentrations in normal human plasma (see Table 1) are comparable

Table 1. *Insulin concentration of plasma from normal people and from normal or alloxan-diabetic rats*

Experimental details are given in the text. Human blood was collected from an antecubital vein, rat blood from the inferior vena cava under Nembutal anaesthesia. Values for rat plasma are given as equivalents of human insulin since standard rat insulin was not available.

Source of blood plasma	Method of assay	Concn. of plasma insulin (microunits/ml.) Starved	Fed	30 min. after glucose	60 min. after glucose	150 min. after glucose
Normal people (given 50 g. of glucose)	A					
1		17	—	35	43	37
2		25	—	85	50	32
3		19	—	88	120	25
4		12	—	50	50	12
5		6	—	60	62	<6
Normal people (given 100 g. of glucose)	A					
1		10	—	35	20	25
2		27	—	250	45	27
3		12	—	140	37	25
4		12	—	45	45	5
5		22	—	320	100	22
Normal people (given 100 g. of glucose)	C	—	—	60, 128, 140, 124	—	—
Normal rats	A	45, 30, 60, <6, 50, 15, 20	—	—	—	—
Normal rats	A	—	120, 140, 140, 140, 130	—	—	—
Alloxan-diabetic rats	A	—	0, 0, 0, 12, 0, 0	—	—	—

with those obtained by Yalow & Berson (1960). When insulin was assayed in the same samples of plasma from five normal people with method A by using a low concentration of precipitating antiserum (1:32) a satisfactory assay was not achieved. The calculated insulin content was substantially higher (mean values: after starvation, 84 instead of 15 microunits/ml.; and 30, 60 and 150 min. after the oral administration of glucose, 170, 160 and 90 instead of 64, 65 and 21 microunits/ml. respectively; see Table 1). Moreover, when five samples of plasma were assayed undiluted, and diluted 1:2 and 1:4, the calculated insulin concentrations at dilutions of 1:2 and 1:4 were $168 \pm 15\%$ and $330 \pm 18{\cdot}1\%$ respectively of that of undiluted plasma (equivalent to 150 microunits of human insulin/ml. of plasma). These observations showed that, at low concentrations of precipitating antiserum, dilution of human plasma did not lead to the expected fall in the insulin activity of the latter. It is suggested that this is due to interference by human γ-globulin, which at low concentrations of rabbit anti-(guinea-pig γ-globulin) serum (precipitating antiserum) removes enough of the latter by cross-reaction to render incomplete subsequent precipitation of the insulin–antibody complex. This leads to diminished radioactivity of the insulin–antibody precipitate and thereby to spuriously high values for plasma insulin activity, which, because of the high concentration of γ-globulin, are not decreased by dilution of the plasma. In support of this idea it has been found that the complex between guinea-pig anti-(human insulin) serum and ^{131}I-labelled ox insulin can also be completely precipitated by anti-(human γ-globulin) serum. Precipitation of human γ-globulin and human plasma by rabbit anti-(guinea-pig γ-globulin) serum has been demonstrated by immunodiffusion in agar gel.

The recovery of human insulin added to human plasma (by using method A and a high concentration of precipitating antiserum) was tested in three experiments. The recoveries of insulin (25, 50 and 100 microunits respectively) added to 1 ml. of plasma assayed to contain 41 microunits/ml. were 106, 99 and 113%. Method A has also been applied to the assay of insulin in blood plasma collected from the inferior vena cava of fed normal and alloxan-diabetic rats (48 hr. after 60 mg. of alloxan/kg. had been given intravenously). In fed normal rats (mean plasma glucose concentration, 147 mg./100 ml.) the plasma insulin concentration (expressed as microunits of human insulin equivalent/ml.) was 130 ± 13 (mean $\pm$ S.E.M. for four rats). In plasma from five of six alloxan-diabetic rats (mean plasma glucose concentration, 1261 mg./100 ml.) no insulin could be detected (less than 6 microunits/ml. being the lower limit of the assay in this experiment). We conclude that method A can be used to assay insulin in plasma but that γ-globulin can interfere if precipitating antiserum is not in excess.

Possible interference by γ-globulin in plasma can be completely avoided if as in methods B and C the insulin antibody is first precipitated with precipitating antiserum and then used for the assay of insulin. Complete lack of interference by γ-globulin is shown by the observation that the radioactivity of the precipitate is unaltered when ^{131}I-labelled insulin is added in undiluted guinea-pig serum instead of in phosphate buffer (insulin in guinea-pig serum does not react significantly because of the very low affinity of the antibody for guinea-pig insulin, as shown by using a sample of pure guinea-pig insulin provided by Dr L. F. Smith). With method C the mean insulin concentrations in samples of plasma from four normal people 30 min. after the oral administration of glucose (100 g.) were calculated to be 93, 112, 124 and 128 microunits/ml. when plasma samples were assayed undiluted and at dilutions of 1:2, 1:4 and 1:8 respectively. These values are similar to those obtained with method A and by Yalow & Berson (1960).

Sources of error. The accuracy with which a given concentration of insulin can be determined depends on the error associated with the experimental manipulations and assay of radioactivity and on the form and slope of the standard curve. The errors associated with the latter (assuming constant experimental error) are least when it is a straight line and when the slope is steepest.

The method of plating used (i.e. filtration through cellulose acetate membranes) gives the expected reproducibility in the assay of radioactivity of the precipitate as shown by repeated counts on a large number of samples. The error of the assay of radioactivity in the precipitate after duplicate sampling of an unknown insulin solution was about twice that of duplicate counting of single samples (the ratio of the standard errors was 2·05 for 14 pairs in each group). Experimental manipulations and counting thus contribute about equally to the overall error of the measured effect of a given concentration of insulin. Where great sensitivity is not required the accuracy may conveniently be enhanced by increasing the concentration of ^{131}I-labelled insulin and recording more counts. The ease of the manipulations involved in these methods also makes it practicable to perform a large number of replicate determinations and thereby to increase the accuracy. Since the counting error at a given total count is a fixed proportion of the count rate and since the lowest insulin concentrations are represented by the highest count rates, this error translated into units of insulin

will be greatest at the lowest concentrations. To reduce this error a larger total of counts may be recorded on these samples. The error in determining the insulin concentration will be lowest where there is the greatest change in counts relative to change in insulin concentration and where the change in count rate represents a large proportion of the count rate. With methods A and B and the antiserum that we have used this appears to hold when the concentration of unlabelled insulin is 100–400% of that of ^{131}I-labelled insulin.

Since C_0/C_i is linearly related to the concentration of unlabelled insulin over a reasonable range and C_0/C_i is a fixed point (1·0) when the latter is zero, the line can be most accurately fixed by measuring C_0/C_i for a suitable concentration of insulin (see above) with an additional point to check the linearity of the relationship. Since the parameter of response, C_0/C_i, is linearly related to dose, i, the relationship is similar to that of many microbiological assays and the Common Zero five-point assay design (two doses of standard, two of unknown and a common fixed point at zero dose) could be used with advantage (see Emmens, 1948). By using this design and human insulin solutions with method A it has been calculated that the 95% confidence limits with duplicate determinations on two doses of standard and two doses of unknown insulin solutions were $\pm 15\%$ of the estimate.

The accuracy of this method can be conveniently compared with those of biological assay by calculating λ (S.D. of points about the regression line divided by the slope of the regression line). This has been done for two standard curves obtained with method A by using the linear portion of a plot of radioactivity in the precipitate against log (insulin concentration) (since bioassays for insulin involve a semi-logarithmic plot). The calculated values for λ were 0·056 and 0·016 as compared with 0·2 to 0·4 for rat-diaphragm bioassay (Randle, 1956) and 0·25 to 0·32 for rat-epididymal-fat-pad bioassay (Shepp *et al.* 1960). The accuracy of the immunological method is thus superior to that of these biological assays.

General applicability. The present studies were concerned with the use of the precipitation of insulin antibody for the assay of insulin. It seems reasonable to suggest that these methods would be applicable to the specific microassay of any protein which can be purified and labelled with a suitable radioactive isotope, which is antigenic and where precipitation of the antibody with anti-γ-globulin serum does not interfere with the combination with antigen or vice versa. An assay for growth hormone involving some of these principles has been described by Utiger, Parker & Daughaday (1962). These methods appear to be simpler and to be capable of achieving comparable sensitivity with that described by Yalow & Berson (1960) despite the use of radioactive insulin of much lower specific activity, and may thus be more readily applicable where the handling of large quantities of radioactive iodine presents difficulties.

SUMMARY

1. Three new methods for immunoassay of insulin with guinea-pig anti-(human insulin) serum, ^{131}I-labelled ox insulin and rabbit anti-γ-globulin serum are described. In method A anti-insulin serum is used to obtain samples of insulin from solutions containing a fixed amount of ^{131}I-labelled insulin mixed with standard or unknown solutions of unlabelled insulin, the insulin–antibody complex being precipitated with anti-γ-globulin serum and the precipitate separated by filtration. Assay of radioactivity in the precipitate then permits the determination of insulin by reference to a standard curve. In method B insulin antibody is first precipitated with anti-γ-globulin serum and the precipitate used to sample insulin mixtures. Method C is a modification of method B in which greater sensitivity is achieved by incubating the precipitated insulin antibody with unlabelled insulin before introducing the ^{131}I-labelled insulin.

2. Plasma γ-globulin can interfere in method A if anti-γ-globulin serum is not in excess but it cannot interfere in methods B or C. The latter are therefore preferred for the assay of insulin in blood plasma.

3. Methods A and B are in principle based on isotope dilution, but in practice this principle is not obeyed because the amount of insulin bound by antibody varies with the insulin concentration.

4. With ^{131}I-labelled insulin of specific activity 5–20 mc/mg., 6×10^{-6} i.u. (6 microunits) of human insulin/ml. can be detected with these methods.

5. The plasma insulin concentration in five normal people was (in microunits/ml.): after starvation, 16; and 30, 60 and 150 min. after the oral administration of glucose (50 or 100 g.), 64 and 158, 65 and 49, and 22 and 21 respectively.

We thank Dr C. Giles of the Department of Pathology, City General Hospital, Stoke-on-Trent, and Dr L. F. Smith, Dr G. H. Smith and Dr J. C. Boursnell for their help with the preparation of human insulin and the labelling of ox insulin; Mr M. J. Brittain for skilled technical assistance; and the British Insulin Manufacturers and British Diabetic Association for grants that contributed to the cost of these investigations. We thank Professor F. G. Young for his interest and encouragement.

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An Improved Method of Separating Amino Acids as *N*-2,4-Dinitrophenyl Derivatives

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(*Received* 7 *January* 1963)

The separation of DNP-amino acids is most frequently done by chromatography, either on a column or on paper. Column chromatography on wet silica gel with eluents such as chloroform, butanol in chloroform, propanol in cyclohexane, benzene etc., was introduced by Sanger (1945). The method was later modified by buffering the stationary phase (Blackburn, 1949*a*, *b*; Middlebrook, 1949) and by employing kieselguhr as the supporting medium (Bell *et al.* 1949; Perrone, 1951); Mills (1952) used dry kieselguhr containing traces of ammonium carbonate with a series of chloroform–ethyl methyl ketone–water mixtures. In such systems the bands of the DNP-amino acids are fairly broad and often overlap (Callow & Work, 1952), so that usually two or more columns are needed to isolate any one compound from a complicated mixture.

Two-dimensional paper chromatography will resolve most of the DNP-amino acids in a protein hydrolysate (Levy, 1954; Kubota, 1961), but losses due to irreversible adsorption on the paper occur. Further, R_F values tend to depend on the load applied, and certain of the solvent mixtures readily change in composition with temperature.

Work in this Laboratory called for the determination of the amount and specific radioactivity of valine. This has led to a general method of separating the amino acids, as dinitrophenyl derivatives, which is an improvement on those currently used. A preliminary report has been given (Matheson, 1962).

MATERIALS AND METHODS

Chemicals. DNP-L-proline, bis(DNP)-L-lysine, DNP-L-valine, DNP-DL-serine, bis(DNP)-L-tyrosine, DNP-L-threonine and DNP-DL-glutamic acid were prepared by the method of Rao & Sober (1954). DNP-DL-alanine, DNP-DL-phenylalanine, DNP-glycine, DNP-DL-aspartic acid, DNP-α-DL-aminobutyric acid and DNP-γ-aminobutyric acid were given by Dr F. J. Bealing, and DNP-DL-methionine and DNP-DL-leucine by Dr R. L. M. Synge. DNP-hydroxy-L-proline was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; dinitrophenol was supplied by British Drug Houses Ltd. and ethyl acetate (analytical reagent grade) by Hopkin and Williams Ltd.

Kieselguhr. Hyflo Super-Cel (Johns–Manville Co.) (about 200 g.) was stirred for 0·5–1 hr. with enough 3N-HCl to give a freely flowing suspension. The greenish yellow liquid was filtered off and the residue was treated with more HCl. The washing procedure was repeated until no more colour was extracted. Four or five washes usually sufficed. The material was then washed with water until the pH of the washings rose to about that of the water. The resulting cake of Hyflo Super-Cel was broken up and dried thoroughly overnight at 105°. The powder was sieved mechanically and that passing through 100-mesh (B.S. 410/1943) was collected.

Buffers. (*a*) 0·1M-Tris–maleic acid buffer: 250 ml. of 'tris acid maleate' (24·2 g. of tris base plus 23·2 g. of maleic acid/l.) was mixed with 125 ml. of 0·2M-tris base and made up to 500 ml.; to each 100 ml. of this mixture was added 3·5 g. of sodium chloride, which brought the pH to about 5·4.

(*b*) 0·1M-Sodium phosphate–salt buffer: 26·3 ml. of 0·2M-NaH_2PO_4 was mixed with 223·7 ml. of 0·2M-Na_2HPO_4 and diluted to 500 ml.; to each 100 ml. was added 3·5 g. of sodium chloride, giving a final pH of 7·4.