Double-Antibody Solid-Phase Radioimmunoassay: A Simplified Phase-Separation Procedure Applied to Various Ligands

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The purpose was to develop a simplified and reliable method of separating free from antibody-bound ligand using a precipitating antibody linked to a cellulose derivative.

Dose-response curves and control sera were set up in parallel for various pituitary and placental polypeptides, steroid hormones, insulin, glucagon, triiodothyronine, thyroxine, angiotensin I, calcitonin, gastrin, cyclic AMP, and digoxin. After first-antibody reactions had reached equilibrium, free and bound ligand were separated using a double-antibody solid-phase system in parallel with conventional methods, including dextran-coated charcoal, double-antibody precipitation, single-antibody solid phase, organic solvents, salt precipitation, and anion-exchange resins. The effect of variations in temperature, incubation time, protein content, pH, and amount of separating material added were studied.

The results showed that separation was complete within 1 hr for small ligand molecules and within 2 hr for larger ones. Dose-response curves and control-sera results closely paralleled those obtained with conventional methods. The method was not affected by moderate variations in incubation variables. Nonspecific binding was less than 3% in all assays, while intra-assay and interassay coefficients of variation were similar to those obtained with conventional phase-separation methods.

It is concluded that the method is a simple and rapid alternative phase-separation system. It has the advantage of being free from common nonspecific intersample variations, and can be applied to any assay system based on rabbit or guinea pig antibodies without preliminary time- or reagent-consuming titration or adjustments to establish optimum phase-separating conditions.

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The basis of radioimmunoassay is the competition between radiolabeled (tracer) and unlabeled ligand for specific antibody-binding sites (*1*). While it is mainly the specificity and affinity of the antiserum, and the specific activity of the tracer, that determine sensitivity and accuracy, the method of separating unbound ("free") from antibody-bound ("bound") ligand plays an important role in determining the reproducibility, speed, and ease of performance of the assay. It is this phase separation that is most affected by minor variations in protein content, pH, temperature, incubation time, and other nonspecific factors (*2*). Many separating techniques have been developed, yet none is ideal. This paper describes our experience with, and simplification of, the double-antibody solid-phase (DASP) technique first described by Den Hollander and Schuurs (*3,4*).

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MATERIALS

Radioimmunoassay kits and components were ob-

tained from various sources and suppliers, while some assay components were manufactured in our laboratory (Table 1). The second antibody-immunosorbent phase-separation material was supplied as a suspension by the manufacturer*.

EQUIPMENT

Automatic diluting pipettes and reagent dispensers were used throughout. All assays were performed in 12- × 75-mm plastic tubes with conically shaped bottoms. The tubes were placed in the special trays of a semiautomated radioimmunoassay system. Centrifugation was performed in a refrigerated centrifuge with a rotor head specifically designed to hold the racks. Counting was done in a 1000-sample triple-head gamma counter capable of accepting the trays. The counter was linked to a computer for data reduction, standard-curve construction, and sample-result generation. For this a modification (*5*) of the computer program developed by Rodbard and Hutt (*6*) was used.

METHODS

For commercial kits, assay procedures as recommended by the suppliers were used, omitting all steps that were part of the phase-separation procedure. All other assays were performed using procedures described in the literature and optimized for the particular reagents available to us (*7,8*). The buffer used for these latter assays was composed of 0.01 *M* sodium phosphate, 0.15 *M* sodium chloride 0.025 *M* EDTA, and 0.25% BSA at pH 7.4. The incubation volume was 0.5 ml, and the first (I_1) and second (I_2) incubation conditions are indicated in Table 1. Labeled prolactin, thyroxine, and immunoglobulin G (IgG) were produced in our laboratory using the chloramine-T method (*9,10*). To compare phase-separation methods, conditions were kept identical until the time of separation; then the usual method and DASP were used in parallel. For the latter procedure, conditions of temperature, pH, ionic environment, and protein concentration were kept identical to those used during the first incubation period of any given assay.

Double-antibody solid-phase separation procedure. The solid-phase immunosorbent is supplied as a suspension in vials containing 5.5 ml each. The contents of a vial (whether anti-rabbit or anti-guinea pig) are sufficient to precipitate completely 1 μg of the appropriate (rabbit or guinea-pig) IgG, the amount contained in approximately 5 μl of neat (undiluted) antiserum. From this information the fraction of the vial content needed to precipitate all the IgG contained in each tube of any assay system can readily be calculated (see Addendum). The vial content was diluted with assay buffer so that the appropriate amount of immunosorbent was contained in 0.2 ml. This was added at the end of I_1 using a Cornwall syringe while the material was kept suspended by continuous magnetic stirring. The vortex action set up by the addition of the material to the tube caused adequate mixing and obviated the need for shaking of the trays. The tubes were then incubated (I_2) at I_1 temperature conditions for 1 hr (for small-ligand molecules, such as digoxin and thyroid hormones), or 2 hr (for larger ligands, such as pituitary and placental polypeptides). At the end of incubation, 2.0 ml of normal saline containing 0.1% Tween 20 wetting agent were added, and the tubes centrifuged in their trays for 5 min at 1000 *g*, after which they were decanted and the precipitate counted.

Determination of within- and between-assay coefficients of variation. Dose-response curves were constructed for all ligands using DASP to separate free from bound ligand. In addition, 36 tubes were assayed using a control serum† with a target value near the most sensitive part of the standard curve to obtain the within-assay coefficient of variation (CV). Nonspecific binding (NSB) was determined in tubes containing all components except the specific antiserum. For several assays the control sera were assayed in successive runs to obtain the between-assay CV.

Comparison of dose-response curves using different phase-separating procedures. Dose-response curves for several different ligands were constructed in duplicate, with one of them being phase-separated by one of the usual methods and the other by DASP. Thirty-six tubes containing a control serum with a target value near the most sensitive part of the standard curve were also assayed to obtain the within-assay CV. Standard curves were constructed and compared for parallelism, and the performance of each assay was assessed.

Effect of time on phase separation. Six trays of 36 tubes each, containing rabbit anti-human thyrotropin antibody, thyrotropin tracer, and human serum, were incubated (I_1) followed by the addition of immunosorbent. The second incubation time (I_2) was varied from 5 to 240 min. The procedure was repeated for prolactin, thyroxine, triiodothyronine, digoxin, angiotensin I, and insulin. For the remaining ligands, DASP separation was compared after incubation times of 0.5, 1, 2, 3, and 4 hr. The mean and CV of each time condition was then calculated.

Effect of nonspecific variables on phase separation. Seven trays containing 36 tubes each were set up as described above but using the insulin assay system and with the following modifications:

Tray	Modification
1	basal (contains 0.1 ml serum, i.e., usual condition)
2	excess immunosorbent (120% of amount used in tray 1)
3	increased temperature (from 4 to 20°C)
4	decreased serum proteins (contains 0.075 ml serum)

TABLE 1. REAGENTS, SUPPLIERS, ASSAY CONDITIONS, AND MODIFICATIONS

Ligand	Reagents and suppliers				Assay conditions and modifications
	Standard	Tracer	Receptor	Separation procedure	
ACTH	1.	ACTH kit (c)		DCC (a)	Kit protocol
	2.	ACTH kit		Anti-rabbit DASP* Organon (u)	Kit protocol; I_2* = 1 hr at 4°C
Angiotensin I	1.	Plasma renin activity kit (v)		DCC	Kit protocol
	2.	Plasma renin activity kit (v)		Anti-rabbit DASP	Kit protocol; I_2 = 1 hr at 4°C
Calcitonin	1. (h)	I-125 calcitonin (own product)	Rabbit anticalcitonin (w)	DCC	I_1* = 120 hr at 4°C; I_2 = 10 min at 4°C
	2. (h)	I-125 calcitonin (own product)	Rabbit anticalcitonin (w)	Anti-rabbit DASP	I_1 = 120 hr at 4°C; I_2 = 2 hr at 4°C
Cyclic AMP*	1.	CAMP kit (j)		Ammonium sulphate	Kit protocol
	2.			Anti-rabbit DASP	Kit protocol; I_2 = 1 hr at 4°C
Cortisol	1.	Gammacoat cortisol kit (i)		SASP*	Kit protocol
	2. (i)	I-125 Cortisol (i)	Rabbit anticortisol (b)	Anti-rabbit DASP	I_1 = 2 hr at 4°C; I_2 = 1 hr at 4°C
Digoxin	1.	Digoxin kit (v)		DCC	Kit protocol
	2.	Digoxin kit (v)		Anti-rabbit DASP	I_2 = 1 hr at 4°C
HFSH*	1. LER-907 ref. prep. (q)	I-125 FSH (f)	Rabbit anti-FSH (q)	Anti-rabbit DAB (*,d)	I_1 = 48 hr at 20°C; I_2 = 24 hr at 20°C
	2. LER-907 ref. prep. (q)	I-125 FSH (f)	Rabbit anti-FSH (q)	Anti-rabbit DASP	I_2 = 2 hr at 20°C
Gastrin	1.	Gastrin immutope kit (y)		Anion exchange resin	Kit protocol
	2.	Gastrin immutope kit (y)		Anti-rabbit DASP	I_2 = 1 hr at 4°C
Glucagon	1.	Pancreatic glucagon kit (s)		Alcohol	Kit protocol
	2.	Pancreatic glucagon kit (s)		Anti-rabbit DASP	I_2 = 2 hr at 4°C
HCG*	1. LER-907 ref. prep. (q)	I-125 HCG (f)	Rabbit anti-LH (q)	Dioxane	I_1 = 48 hr at 20°C
	2. LER-907 ref. prep. (q)	I-125 HCG (f)	Rabbit anti-LH (q)	Anti-rabbit DASP	I_2 = 2 hr at 20°C
HGH*	1. HGH ref. prep. (q)	I-125 HGH (v)	Guinea pig anti-HGH (q)	Anti-guinea pig DAB (n)	I_1 = 24 hr at 4°C; I_2 = 24 hr at 4°C
	2. HGH ref. prep. (q)	I-125 HGH (v)	Guinea pig anti-HGH (q)	Anti-guinea pig DASP (u)	I_2 = 2 hr at 4°C
HPL*	1.	HPL kit (c)		Alcohol	Kit protocol
	2.	HPL kit (c)		Anti-rabbit DASP	I_2 = 2 hr at 20°C
Insulin	1. (v)	I-125 Insulin	Guinea pig anti-insulin (z)	Anti-guinea pig DAB	I_1 = 24 hr at 4°C; I_2 = 24 hr of 4°C
	2. (v)	I-125 Insulin	Guinea pig anti-insulin (z)	Anti-guinea pig DASP	I_2 = 2 hr at 4°C
HLH*	1. LER-907 ref. prep. (q)	I-125 LH (f)	Rabbit anti-LH (q)	Anti-rabbit DAB	I_1 = 48 hr at 20°C; I_2 = 24 hr at 20°C
	2. LER-907 ref. prep. (q)	I-125 LH (f)	Rabbit anti-LH (q)	Anti-rabbit DASP	I_2 = 2 hr at 4°C
Prolactin	1. PRL ref. prep. (q)	I-125 prolactin (own product)	Rabbit anti-prolactin (m)	Anti-rabbit DASP	I_1 = 120 hr at 4°C; I_2 = 2 hr at 4°C
Testosterone	1. (w)	I-125 testost. (f)	Rabbit anti-testost. (r)	Anti-rabbit DASP	I_1 = 3 hr at 20°C; I_2 = 1 hr at 20°C

Table 1 (*continued*).

Thyroxine	1.	Immophase thyroxine kit (k)		SASP	Kit protocol
	2. (k)	I-125 T_4 (own product)	Rabbit anti-T_4 (q)	Anti-rabbit DASP	I_1 = 2 hr at 4°C; I_2 = 1 hr at 4°C
	3.	Tetrasorb kit (b)		Anion exchange resin sponge	Kit protocol
Triiodothyronine	1. (e)	I-125 T_3 (o)	Rabbit anti-T_3 (g)	Anti-rabbit DAB	I_1 = 2 hr at 4°C; I_2 = 24 hr at 4°C
	2. PRL ref. prep. (e)	I-125 T_3 (o)	Rabbit anti-T_4 (q)	Anti-rabbit DASP	I_2 = 1 hr at 4°C
HTSH*	1. TSH ref. prep. (p)	I-125 TSH (f)	Rabbit anti-TSH (q)	Anti-rabbit DAB	I_1 = 96 hr at 4°C; I_2 = 24 hr at 4°C
	2. TSH ref. prep. (p)	I-125 TSH (f)	Rabbit anti-TSH (q)	Anti-rabbit DASP	I_2 = 2 hr at 4°C
	3.	(t)		DAB with PEG (a)	Kit protocol

* *Abbreviations:* CAMP = cyclic adenosine monophosphate; DAB = double antibody; DASP = double-antibody solid-phase; DCC = dextran-coated charcoal; HCG = human chorionic gonadotrophin; HFSH = human follicular stimulating hormone; HGH = human growth hormone; HLH = human luteinizing hormone; HPL = human placental lactogen; HTSH = human thyrotrophic hormone; I_1 = first incubation period; I_2 = second incubation period; PEG = polyethylene glycol; Ref. prep. = reference preparation; SASP = single-antibody solid-phase. *Sources of reagents:* (b) Abbott Labs, Chicago, IL; (c) Amersham/Searle Corp, Oakville, Ont, Canada; (d) Dr. David Armstrong, London, Ont, Canada; (e) Bio-Rad Labs, Richmond, CA; (f) Bio-Ria, Montreal, Quebec, Canada; (g) Dr. C. Burke, Oxford, UK; (h) Ciba/Geigy Ltd, Basle, Switz; (i) Clinical Assays Inc, Cambridge, MA; (j) Collaborative Research Inc, Waltham, MA; (k) Corning Glass Works, Medfield, MA; (l) Diagnostics Biochem Canada Inc, London, Ont, Canada; (m) Dr. H. Friesen, Winnipeg, Man, Canada; (n) Hyland Division of Travenol Labs Inc, Costa Mesa, CA; (o) Industrial Nuclear Co., Incorporated, St. Louis, MO; (p) Medical Research Council, National Institute for Medical Research, Holly Hill, London UK; (q) National Pituitary Agency, Baltimore, MD; (r) New England Nuclear Corp, Worcester, MA; (s) Novo Research Institute, Novo Alle, Bagsvaerd, Denmark; (t) Nuclear Medical Laboratories Incorporated, Dallas, TX; (u) Organon Canada Ltd, West Hill, Ont, Canada; (v) Schwarz/Mann, Div of Becton, Dickinson and Co. Canada, Ltd, Mississauga, Ont, Canada; (w) Sigma Chemical Company, St. Louis, MO; (x) Dr. F. Singer, Los Angeles, CA; (y) E.R. Squibb and Sons Inc, Princeton, NJ; (z) Dr. P. Wright, Indianapolis, IN.

5 increased serum proteins (contains 0.12 ml serum)
6 contains no serum
7 tracer only

The mean and CV were calculated for each tray and the mean expressed as a percentage of the basal value, except for trays 4 and 5. Results of these two were expressed as a percentage of the expected amount of ligand contained in tubes containing 25% less, or 20% more, human serum than tray 1. As tray 6 contained no human serum, its mean was arbitrarily set at 100%, which allowed it to be compared with the tubes containing serum. Phase separation was as described above, noting the variation in tray 2, to which 20% extra immunosorbent was added, and the increased temperature (20°C) in tray 3. The seventh tray was set up containing tracer only, and served as a measure of pipetting and counting errors.

Effect of pH on phase separation. To circumvent the effect of pH variation on the interaction between ligand and first antibody, this experiment was performed using rabbit immunoglobulin and anti-rabbit immunosorbent. The rabbit IgG was labeled with iodine-125 (9), diluted with unlabeled IgG, and incubated with anti-rabbit immunosorbent in groups of 6 tubes for 1 hr at room temperature. A total of 11 sets of six tubes each were used, with acidities ranging from pH 4.5 to 11.

RESULTS

Nonspecific precipitation using DASP was less than 2% in most assays and did not exceed 3% in any assay. Standard curves were generally superimposable regardless of the phase-separation method used. The only exceptions were the single-antibody solid-phase (SASP) assays (cortisol and thyroxine): the antisera—and hence their mean affinities and standard-curve slopes—in the SASP and corresponding DASP were different. Table 2 shows the within- and between-assay CVs of the ligands assayed. The former is less than 5% (with the exception of testosterone, 7.8%) and compares favorably with that found for a variety of other phase-separation methods (Table 3).

Varying the time of incubation for DASP separation (I_2) showed that separation was virtually complete in 1 hr for small molecules such as thyroid hormones, digoxin, and angiotensin I, while ligands of larger molecular weight—such as insulin, HTSH, prolactin, and growth hormone—showed a further increase in precipitation up

TABLE 2. WITHIN- AND BETWEEN-ASSAY COEFFICIENTS OF VARIATION USING SECOND-ANTIBODY SOLID-PHASE PRECIPITATION

Ligand	Within-assay CV (%)	Between-assay CV (%)
HGH	2.7	8.1
HLH	3.2	11.1
HFSH	2.0	10.5
HTSH	2.3	9.1
ACTH	4.8	13.4
Prolactin	2.7	6.3
Triiodothyronine	3.2	7.0
Thyroxine	2.4	5.7
Digoxin	1.4	6.1
Cortisol	2.8	—
Testosterone	7.8	—
HCG	3.2	11.1
HPL	2.0	—
Insulin	2.6	6.2
Glucagon	4.6	—
Gastrin	3.8	—
Calcitonin M	4.0	9.7
Angiotensin I	2.3	11.7
Cyclic AMP	3.9	—

to 2 hr (Figs. 1 and 2). The largest CV at any incubation time was less than 5% (with the exception of testosterone) and nonspecific binding was less than 2%.

The effect of various nonspecific factors on the phase-separating ability of DASP is demonstrated in Fig. 3. Note that the CV (1.2%) for tracer dispensing and counting (tray 7) is the same as when an incubation and separation using DASP was done in buffer (tray 6). Increasing the amount of immunosorbent by 20% (tray 2) gave a mean insulin value of 98.6% of the basal value (tray 1), while increasing the temperature from 4 to 20°C (tray 3) gave a mean of 99.4% of the basal value. Decreased serum protein (tray 4) and increased serum protein (tray 5) resulted in values of 102.4 and 99.4%, respectively, of the calculated amount. The CV for all trays was 3.6% or less.

The effect of pH changes on precipitation of labeled IgG by DASP is shown in Fig. 4. Over the pH range 4.5 to 9 there was no difference, but some decrease in precipitation occurred at pH 10 and 11.

DISCUSSION

With the introduction of radioimmunoassays for a wide range of ligands, a variety of phase-separation methods have been developed. Although each method has its advocates and detractors, two methods in particular are of interest. The advantage of the double-antibody (DAB) method is that it can be used for all assays where the antiligand (first antibody) has been raised in the same animal species (*2*). However, for matrix formation and precipitation to occur, relatively large quantities of carrier gamma globulin and precipitating (second) antibody are required. In addition, nonspecific interferences are a frequent and troublesome occurrence. Hales and Randle have described ineffective precipitation and falsely high ligand values due to gamma globulins in the assay sample (*11*). Complement also inhibits the precipitating reaction, again resulting in falsely high ligand values (*12*). Heparin is anticomplementary in the assay and is a further source of possible error (*13*). Variations in protein concentration, pH, and

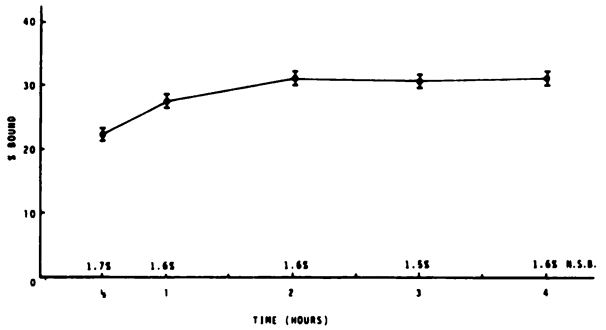

FIG. 1. Effect of time on DASP separation in insulin assay. Anti-insulin is guinea-pig gamma globulin and precipitating immunosorbent was cellulose-bound anti-guinea-pig gamma-globulin sheep gamma globulin. Results were the mean (± s.d.) of 36 tubes for each incubation time. No increase in separation (precipitation) occurred beyond 2 hr. Nonspecific binding is indicated.

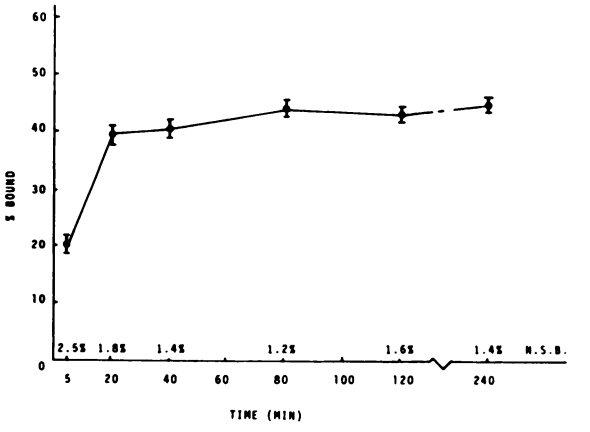

FIG. 2. Effect of time on DASP separation in thyrotropin assay. Anti-HTSH antiserum used was rabbit gamma globulin and precipitating immunosorbent was cellulose-bound anti-rabbit gamma globulin sheep gamma globulin. Results at each time were mean (± s.d.) of 36 tubes. No further precipitation occurred beyond 80 min. Nonspecific binding is indicated for each incubation time.

TABLE 3. COMPARISON OF WITHIN-ASSAY COEFFICIENTS OF VARIATION USING VARIOUS PHASE-SEPARATION METHODS

Ligand	Method	CV (%)	CV (%)
HTSH	Double antibody	3.4	DASP 2.3
HLH	Double antibody	3.6	DASP 3.2
HFSH	Double antibody	2.4	DASP 2.0
HGH	Double antibody	4.5	DASP 2.7
Insulin	Double antibody	3.3	DASP 2.6
Triiodothyronine	Double antibody	7.5	DASP 3.2
Digoxin	Dextr. coat. charc.	3.8	DASP 1.4
ACTH	Dextr. coat. charc.	4.3	DASP 4.8
Calcitonin M	Dextr. coat. charc.	4.1	DASP 4.0
Angiotensin I	Dextr. coat. charc.	2.1	DASP 2.3
Gastrin	Anion-exchange resin	3.3	DASP 3.8
Thyroxine	Anion-exchange resin sponge	6.1	DASP 2.4
Glucagon	Alcohol precipitation	4.2	DASP 4.6
HPL	Alcohol precipitation	2.1	DASP 2.0
HCG	Dioxane precipitation	2.7	DASP 3.2
Cyclic AMP	Ammon. sulph. precipitation	3.6	DASP 3.9
Cortisol	Single-ab. solid phase	3.3	DASP 2.8
Thyroxine	Single-ab. solid phase	1.8	DASP 2.4
HTSH	Double ab. with PEG	4.7	DASP 2.3

osmolarity also interfere with the precipitating reaction (*14*). Finally, prolonged incubation (as is necessary with some precipitating antibodies) increases the likelihood of degradation of iodinated ligand due to proteolytic enzymes in the sample to be assayed (*15*). Steps must be taken to minimize these unwanted effects, and optimal conditions for the formation of the precipitating immune complex must be assessed frequently.

Precipitation procedures using organic solvents or salts are rapid and inexpensive, but they require careful optimization and are prone to considerable nonspecific binding due to coprecipitation of the free ligand. In addition whole serum must be added to ensure precipitation of the immune complex (*16,17*).

Nonspecific adsorption of small molecules to charcoal, exchange resins, and similar materials are widely used because of their economy, ease of separation, and speed.

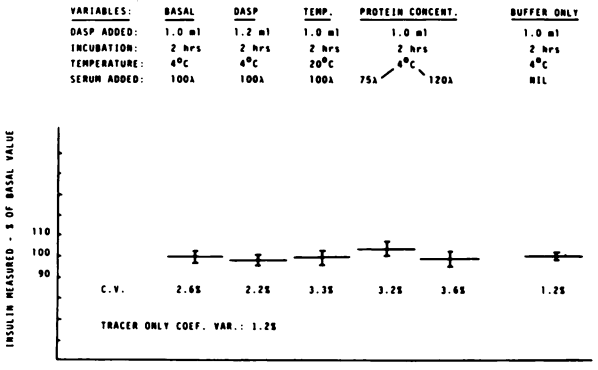

FIG. 3. Effect of variation in nonspecific factors on separation of free from bound insulin. Each result represents mean (± s.d.) of 36 tubes. Variation in incubation condition is indicated above results, which are expressed as percentage of basal value (excluding buffer only). There is no significant effect of any of factors on precipitation.

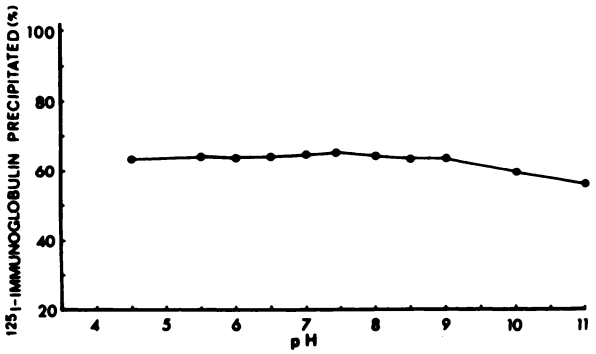

FIG. 4. Effect of pH on precipitation of rabbit gamma globulin by immunosorbent. Each point is mean of six determinations. There is no appreciable effect in pH range 4.5 to 9, but some decrease at higher alkalinity.

The method works well for small molecules at low concentrations, but is less satisfactory for larger molecules, especially at higher concentrations (*2*). Variations in the protein concentration of the sample readily affect the method, and significant coprecipitation of the bound fraction is a problem with ligands of larger molecular weight. Finally, the nonspecific but high affinity that charcoal exhibits for small free ligand molecules results in a competition for the ligand already bound to the specific receptor. With increasing contact time, therefore, some of the bound ligand may be "stripped off" the receptor (*2*). Furthermore, any factor affecting the affinity constant of the specific receptor may disturb this competition and result in altered separation characteristics (*18*). To prevent erroneous results rigid adherence to optimal conditions is therefore mandatory.

The single-antibody solid-phase separating method is free of the problems mentioned above and has the additional advantages of speed and ease of handling (*2*). For every different ligand, however, the first antibody must be coupled to an insoluble substance. This method thus lacks versatility. In addition, the amount of insolubilized first antibody added to each assay tube must be kept constant to ensure precision.

The double-antibody solid-phase separation combines the speed and ease of handling of solid-phase systems and the versatility of the double-antibody technique. The material used in separating free from bound ligand is produced by chemically linking the gamma globulin of the second antibody to a cellulose derivative (*19*). This makes the complex sufficiently large and heavy for spontaneous precipitation. Precipitation of first antibody-bound ligand occurs after it binds to the cellulose-linked anti-first-antibody gamma globulin. The separation method as originally described involved the use of continuous end-over-end mixing and long incubation period (*3,4*). Using a greatly simplified technique, we have been able to shorten the procedure and generally adapt the method for use with a variety of radioligand assays in a clinical radioassay laboratory, without the need for any special equipment. As it does not depend on matrix formation, no carrier protein is needed, and there is no interference with matrix formation by proteins and, theoretically, anticoagulants, such as are seen in the double-antibody separation technique. In practice this is borne out by the freedom from the effects of nonspecific variables—such as temperature, variations in protein concentration, time, and pH—on the precipitation of the bound ligand (Figs. 1-4). That, together with the use of the immunosorbent material in excess of what is needed for complete precipitation, result in an extremely reproducible phase-separation method capable of withstanding reasonable variations in separation conditions. Thus with this method, separation of free from bound ligand is less likely to introduce errors in the final value obtained. A further advantage of the method is that the amount needed in any assay being developed can be determined from the titer of the antiserum used without time-consuming titrations and optimization of conditions for phase separation (see Addendum).

In summary, we have developed a rapid and simple phase-separation procedure using a second antibody linked to a solid phase. The method as applied to a wide variety of ligands was found to be reliable, easy to perform, free of interference by common nonspecific factors, and efficient in separating free from bound ligand. Its low nonspecific binding and excellent reproducibility, as measured by within- and between-assay coefficients of variation, were excellent and compared favorably with a variety of commonly used phase-separation methods.

FOOTNOTE

* Organon Canada Ltd.
† Lederle Diagnostics.

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ADDENDUM

The number of assay tubes that can be phase-separated with one vial of immunosorbent as supplied by the manufacturer may be estimated by the following formula: divide the reciprocal of the titer of the antiserum added to the assay tube by one fifth of the volume of the antiserum added, expressed in microliters: that is,

$$\frac{1}{\text{titer}} \div \frac{\text{volume added}}{5}$$

Sufficient assay buffer is then added to the phase-separating material so that the final volume of the immunosorbent solution added to each tube is 0.2 ml.

Example: If 100 μl of rabbit anti-TSH serum with a titer of 1/2,000 is added per assay tube, then one vial of immunosorbent is sufficient for $\frac{2,000}{1} \div \frac{100}{5} = 100$ tubes. The volume of the material is then brought up to 20 ml, and 0.2 ml added to each assay tube.

We have noticed that while all batches of double-antibody solid-phase have a certain minimum separating ability specified by the manufacturer, some batches have an effectiveness much greater than stated and will allow the separation of several times the stated number of tubes. To achieve maximum cost effectiveness, therefore, it is advantageous to test each batch.

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