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Liquid Scintillation Radioassay in Disposable Microcentrifuge Tubes: Radioimmune Precipitates and Other Applications

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A simple, economical radioassay system employing disposable polypropylene microcentrifuge tubes was developed. Plastic adapters permitted automatic operation in liquid scintillation spectrometers. Counting efficiencies of ^{3}H, ^{14}C, ^{32}P, and ^{125}I in liquid scintillation cocktails and of ^{32}P by Cerenkov radiation (at lower efficiency in absence of added scintillator) were comparable to those in standard vials. Multipurpose use of the microtubes made the system versatile and expedient, e.g., collection of precipitates and radioassay in the same container. Collection of radioimmune precipitates was aided by a carrier inorganic precipitate, $Mg_2P_2O_7$.

Liquid scintillation radioassay is usually done in standard 20-ml vials, although smaller volumes in these vials may frequently be acceptable. Commercially available minivials (7 ml capacity) offer some economic advantage, as do contrived containers designed to fit inside standard glass counting vials (11). The necessity of having to transfer liquid scintillation radioassay samples prepared in other containers to counting vials is a time-consuming task, and may result in inaccuracies due to loss of sample during transfer. Assay of Cerenkov radiation of ^{32}P (and other high energy beta emitters) can be done economically in the liquid scintillation spectrometer in aqueous medium without an added scintillator compound (3); it is possible to recover the sample, but dilution necessary to provide adequate counting volume may make recovery impractical. During the course of performing radioimmune precipitation in plastic microcentrifuge tubes, we investigated direct radioassay by liquid scintillation in the tubes, employing a plastic adapter designed to hold tubes in the spectrometer. We found this to be an expedient and practical means of assay, and the efficiency of counting was comparable to that in large volumes in standard plastic vials. We therefore extended this approach to other applications, including Cerenkov radiation assay of minute samples and assay of acid-insoluble precipitates. We also found the microtubes applicable to liquid scintillation assay of ^{125}I, in the absence of a gamma spectrometer.

Many radioimmunoassay techniques involve precipitation of antigen-antibody complexes with the aid of anti-immune globulin prepared in another species (second antibody technique). The early work of Gerloff et al. (5), and more recent work of Cardiff (2), showed the applicability of this technique to viruses propagated in the presence of radioisotopes (internally labeled) or chemically labeled after purification (externally labeled). Other applications to labeled viral proteins have been presented by Horwitz and Scharff (7). For optimum conditions of precipitate formation, the equivalence zone must be determined, and this is usually done by chemical means after washing the precipitate (7). For this procedure it is necessary to use a relatively large amount of specific antiglobulin. We present a rapid, sensitive, economical method of determining globulin antiglobulin equivalence employing radiolabeled reagents and an inorganic precipitate, $Mg_2P_2O_7$, as carrier. With appropriate reagents, either antigen or antibody may be quantitatively determined. Inorganic carrier precipitation is also applicable to the second antibody technique for radioimmune assay of antiviral sera.

MATERIALS AND METHODS

Microcentrifuge tubes and centrifuge. Polypropylene micro test tubes (Eppendorf 3810) of 1.5-ml capacity (Bio-Rad Laboratories, Richmond, Calif.) or 1.5-ml Bel Art tubes (J. & H. Berge, Inc., South Plainfield, N.J.) were employed. Preliminary tests indicated that even smaller tubes, 0.5-ml capacity, could also serve as scintillation assay containers. Tubes were centrifuged in a Brinkman/Eppendorf centrifuge 3200 (Bio-Rad).

Adapters. Adapters (Fig. 1) to hold microtubes in the liquid scintillation spectrometer were machined

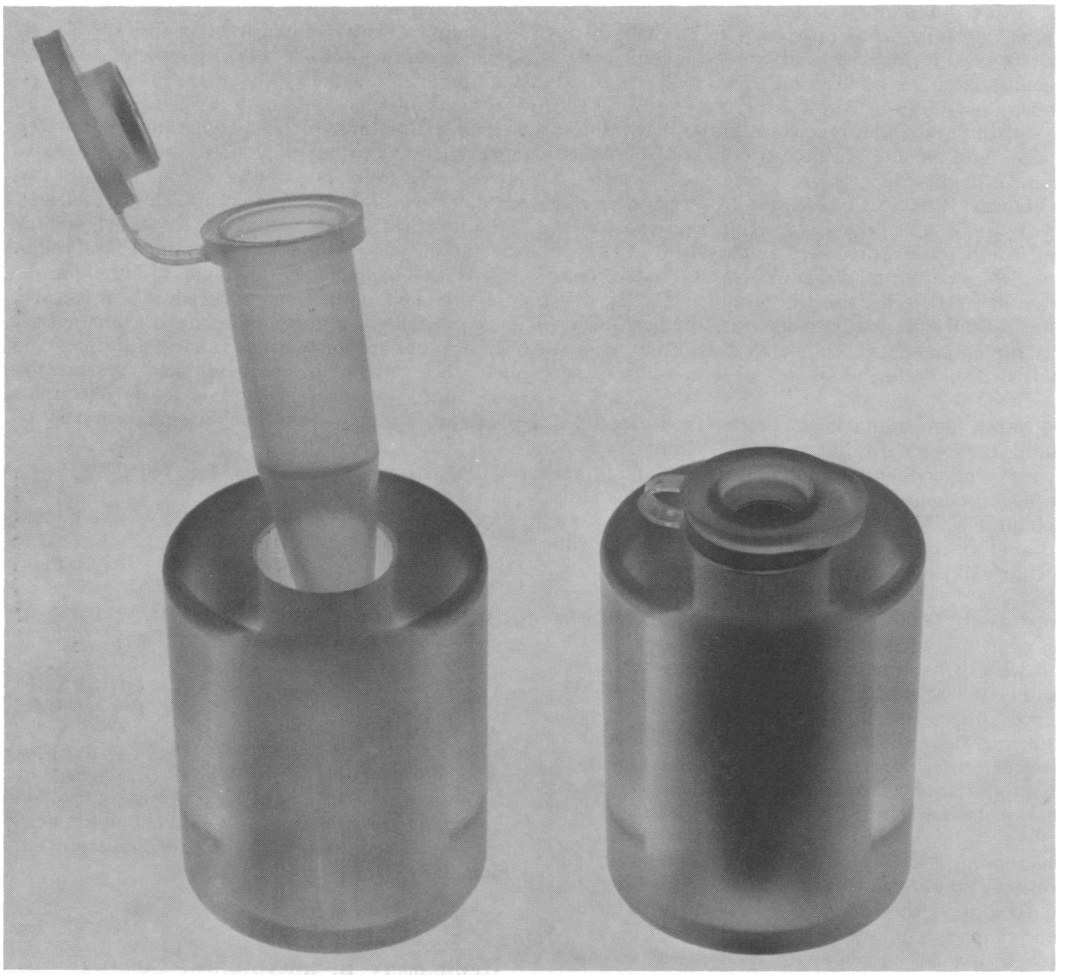

FIG. 1. *Photographic illustration of microtubes (1.5 ml capacity) and Plexiglas adapters designed to hold microtubes in liquid scintillation spectrometer. (Microtube on right was filled with ink for photographic enhancement.)*

from a 1 and ⅛ inch Plexiglas rod. The external dimensions were 36.2 mm length by 27.3 mm diameter; the edges were rounded or beveled to prevent jamming in the sample-changing apparatus of the spectrometer. The center hole, 11.1 mm in diameter (7/16 inch drill), was drilled to provide a loose fit for the microtubes which protruded less than 1 mm from the bottom of the adapter. In the Packard model 4322 spectrometer which was employed in these studies, optimum efficiency was obtained with the tubes seated as deeply as possible in the adapters. To simplify construction, the adapters were not polished, but left with slightly rough, nearly transparent surfaces; comparison with a polished adapter showed little difference in counting efficiency.

Liquid scintillation cocktail. Aquasol Universal L. S. C. Cocktail (New England Nuclear Corp., Boston, Mass.) was routinely employed in these studies. Other cocktails could also be employed in the microtubes.

Radioisotopes. The radioisotopes used were: [^{3}H]formaldehyde, 1% solution in water, specific activity 100 mCi/mmol (New England Nuclear Corp.); [^{14}C]formaldehyde, 1% solution, specific activity 10 mCi/mmol (New England Nuclear Corp.); iodine-125, protein iodination grade (reductant free), as NaI in 0.1 N NaOH, carrier free (New England Nuclear Corp.); and [^{32}P]orthophosphate, carrier free (Schwarz-Mann, Orangeburg, N.Y.).

Micropipettes. Various micropipettes or pipetting devices were employed, as appropriate. These included: Biopette automatic pipette, 0.2-ml capacity with adapters to deliver 25, 50, 75, and 150 μliters (Schwarz-Mann); Centaur micropipette, 5 and 10 μliters (Cole-Parmer, Chicago, Ill.); Drummond dialamatic microdispenser, 25-μliter capacity in 1-μliter increments (Drummond Scientific, Broomall, Pa.); and various sizes of Drummond disposable capillary pipettes.

Gamma globulin. Commercially prepared (Pen-

tex, Miles Laboratories, Inc.) rabbit and human gamma globulins were purchased from Calbiochem LaJolla, Calif. Monkey and lamb globulins were precipitated from whole serum with half saturated ammonium sulfate.

Anti-immunoglobulins. Goat antisera to rabbit, human, and monkey gamma globulin were obtained from Antibodies, Inc., Davis, Calif.

Viruses. Poliovirus type 2, strain P-712, was propagated in 1-5C-4 cell monolayers (8); labeling with ^{3}H or ^{32}P and purification were as described elsewhere (13). Influenza virus, strain WSN_H (15) was propagated in 1-5C-4 cells; the procedure for labeling with ^{32}P was similar to that for poliovirus, and purification was similar to that described by Nayak (10) except that sucrose gradient solutions were in water rather than D_2O.

Protein determinations. Protein concentrations were estimated according to the method of Lowry et al. (9) using bovine serum albumin as a standard, or by measurements of absorbance at 280 nm (gamma globulin $E_{1cm}^{1\%} = 15.4$).

Labeling of gamma globulins. Labeling with [^{14}C]formaldehyde was as described by Rice and Means (12), with resulting specific activities of approximately 1,000 counts per min per μg. Essentially the same procedure was used with [^{3}H]formaldehyde, but with higher resulting specific activities, approximately 10,000 counts per min per μg, reflecting the higher starting specific activity of [^{3}H]formaldehyde. Labeling with ^{125}I was a modification of the method of Greenwood et al. (6): with continuous magnetic stirring, 73 μg of protein in 25 μliters of 0.5 M phosphate buffer, pH 7.5, was mixed with 50 μCi of ^{125}I in 5 μliters; 20 s after the addition of 50 μg of chloromine-T in 25 μliters, 120 μg of sodium metabisulfite in 50 μliters was added, followed by 1 mg of KI in 10 μliters; the preparation was dialyzed against four changes of phosphate-buffered saline (PBS) and it yielded an activity of approximately 600,000 counts per min per μg.

Procedure for quantitative precipitin reaction in microtubes. The reaction mixture contained lamb globulin (0.75 mg/ml) to eliminate adsorbance of components to the wall of microtubes. A 25-μliter amount of antigen (radioactive labeled and unlabeled gamma globulin) and 25 μliters of an appropriate dilution (usually 1:25) of antibody (goat antisera to gamma globulin) were mixed in a preweighed microtube and incubated at 37 C for 2 to 2.5 h. The reaction mixture was diluted with 200 μliters of PBS containing 0.01 M $Na_4P_2O_7$, followed by addition of 10 μliters of 0.5 M $MgCl_2$. Samples were mixed on a Vortex mixer, and the $Mg_2P_2O_7$ precipitate which instantly formed was sedimented in the microcentrifuge. After removal of most of the supernatant, the remainder was measured by weighing (to 1-mg accuracy). The precipitate was dissolved in 25 μliters of formic acid (80%), mixed with 1 ml of Aquasol, and transferred to an adapter for liquid scintillation radioassay.

Calculation of precipitated radioactivity. Two alternative methods were used for calculation of precipitated radioactivity in those procedures where residual supernatant was weighed. The residual supernatant was only a small fraction (usually 3 to 20 μliters, assuming density = 1.0) of the total volume (the sum of all reactants); the mass of the precipitate was negligible for the purpose of calculation. In method (i) the bulk of the supernatant was counted separately in a standard vial. It was necessary to determine the relative counting efficiencies in the two containers with appropriate cocktails and additives. In method (ii) the supernatant was discarded, and the calculation was dependent upon assay of total radioactivity in a sample of the input material in the appropriate solubilizer and cocktail in a microtube. Thus, pipetting accuracy was more critical in method (ii). Appropriate background counts (different in microtubes and vials) were determined, and calculations, aided by simple programs for an electronic calculator, were based on the following formulas:

$$\text{(i) } CPM_{ppt} = CPM_{mt} - \frac{W}{V-W}(R)(CPM_{sup})$$

$$\text{and, \% in precipitate} = (100)\frac{CPM_{ppt}}{CPM_{mt} + (R)(CPM_{sup})}$$

$$\text{(ii) } CPM_{ppt} = CPM_{mt} - \frac{W}{V}(CPM_{tot} - CPM_{mt})$$

$$\text{and, \% in precipitate} = (100)\frac{CPM_{ppt}}{CPM_{tot}}$$

where CPM = counts/min (corrected for background) for precipitate (*ppt*), microtube (*mt*), supernatant (*sup*) and total input (*tot*); W, weight of residual supernatant in milligrams; V, total volume in microliters; and R, counting efficiency ratio of microtube to supernatant vial.

RESULTS

Radioassay in microtubes. Counting efficiencies of the low energy β-emitting isotopes ^{3}H and ^{14}C, and of ^{32}P Cerenkov radiation in microcentrifuge tubes, were nearly identical to those employing a 10 times greater volume in polyethylene vials (Table 1). Background counts (not shown) with microtubes were lower than with standard vials, presumably due to smaller target volume for stray radiation. The slightly lower relative efficiencies with high energy ^{32}P and γ-emitting ^{125}I may be attributed to the greater portion of the primary radiation escaping undetected from the smaller tubes. The effect of quenching measured by the addition of water (to 10% by volume) varied with the isotope (Table 1); it was not appreciably different in standard vials (not shown). Quenching varied, of course, with the nature as well as with the quantity of additives. Small volumes of acids (HCl, acetic acid) and alkali (NaOH) could be employed as solubilizers with only moderate quenching. Formic acid, which

TABLE 1. *Counting efficiencies in microtubes; comparison with standard polyethylene vials, and quenching by water*

Scintillation medium[a]	Isotope	Counting efficiency in microtube (%)[b]	Counts/min in microtube[c] ÷ counts/min in vial	Counts/min in Aquasol + 10% H_2O per counts/min in Aquasol
Aquasol	^3H	50	0.99	0.76
Aquasol	^{14}C	~75	0.98	0.97
Aquasol	^{125}I	>80	0.84	0.88
Aquasol	^{32}P	>90	0.94	0.86
Phosphate-buffered saline	^{32}P	~25	1.02	—

[a] Volume, 1 ml.

[b] Counts per minute × 100/theoretical disintegrations per minute. [^{3}H]toluene internal standard was employed for ^{3}H; all others were approximated from stock solutions.

[c] Duplicate samples (containing 10 μliters of water or less) pipetted into 1 ml of scintillation medium in a microtube or 10 ml in a standard vial.

was found to dissolve $Mg_2P_2O_7$ precipitates more readily than HCl or acetic acid, did quench to a greater extent; counting efficiency for ^{3}H in 1 ml of Aquasol plus 25 μliters of HCOOH was 34%. Relative counting efficiencies were affected to a slight extent by instrument settings. Decreasing the volume below 1 ml slightly increased the counting efficiency of ^{3}H, but for practical reasons 1 ml of Aquasol was used for routine purposes. Thus, careful standardization was necessary for precise comparisons of samples in different containers or different media.

The counting efficiency of Cerenkov radiation was virtually constant irrespective of sample volume in the range tested (10 μliters to 1 ml). Thus, even small samples may be expeditiously monitored in microtubes. The suitability of using microtubes for collection of gradient fractions and their subsequent rapid radioassay by detection of Cerenkov radiation is apparent; neither removal of samples for counting nor use of scintillation fluid is necessary. An example of a sucrose gradient sedimentation of ^{32}P-labeled influenza virus monitored by Cerenkov radiation is shown in Fig. 2. Gradient fractions were collected and assayed directly in microtubes.

Determination of equivalence employing labeled antigen. The equivalence test employed a series of microtubes containing increasing quantities of antigen (labeled and unlabeled) and a constant amount of antibody. If

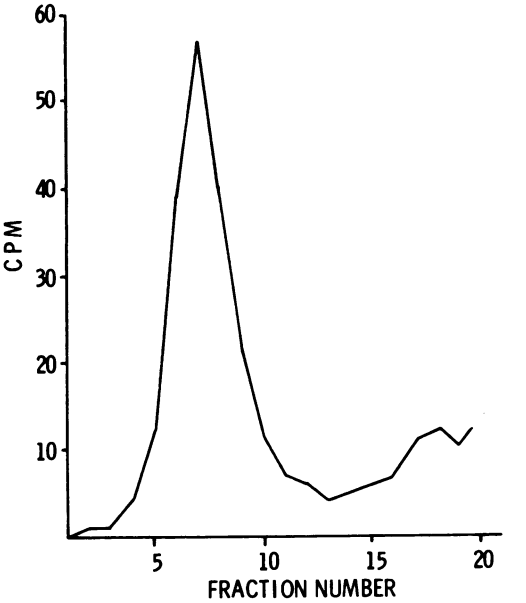

FIG. 2. *Cerenkov radioassay in microtubes after sucrose density gradient sedimentation of ^{32}P-labeled influenza virus. Gradient fractions were collected dropwise (by bottom puncture) into microtubes and counted directly without addition of scintillation cocktail. Centrifugation conditions: 1.0-ml sample of clarified tissue culture fluid from WSN_H-infected 1-5C-4 cells, plus ^{32}P-labeled sucrose-purified WSN_H tracer, was layered over a 3.6-ml linear 15 to 55% (wt/wt) sucrose gradient in NTE buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA) and centrifuged 45,000 rpm at 4 C for 2.5 h in a Spinco SW50L rotor. Fractions were four drops each (~225 μliters).*

the percent of radioactivity precipitated was plotted against the amount of antigen added, the curve approached 100% in the zone of antibody excess and fell off in the zone of antigen excess. Accurate determination of equivalence was difficult from this type of plot. If, however, the quantity of antigen precipitated (percent precipitated × quantity added ÷ 100) was plotted versus quantity of antigen added, a sharp peak was obtained, which represented the maximum amount of precipitable antigen and thus the theoretical equivalence point. Equivalence was determined for three different anti-Ig sera (Fig. 3). Goat antirabbit Ig employed at levels of 1 μliter (Fig. 3a) and 0.1 μliter (Fig. 3b) showed maximum precipitation at 3.3 and 0.4 μg of rabbit globulin, respectively. Rabbit (Fig. 3a and b) and human (Fig. 3c) gamma globulin were commercially purified, and as expected approximately 100% was precipitated; the amount precipitated was also approximately that expected from the nominal ti-

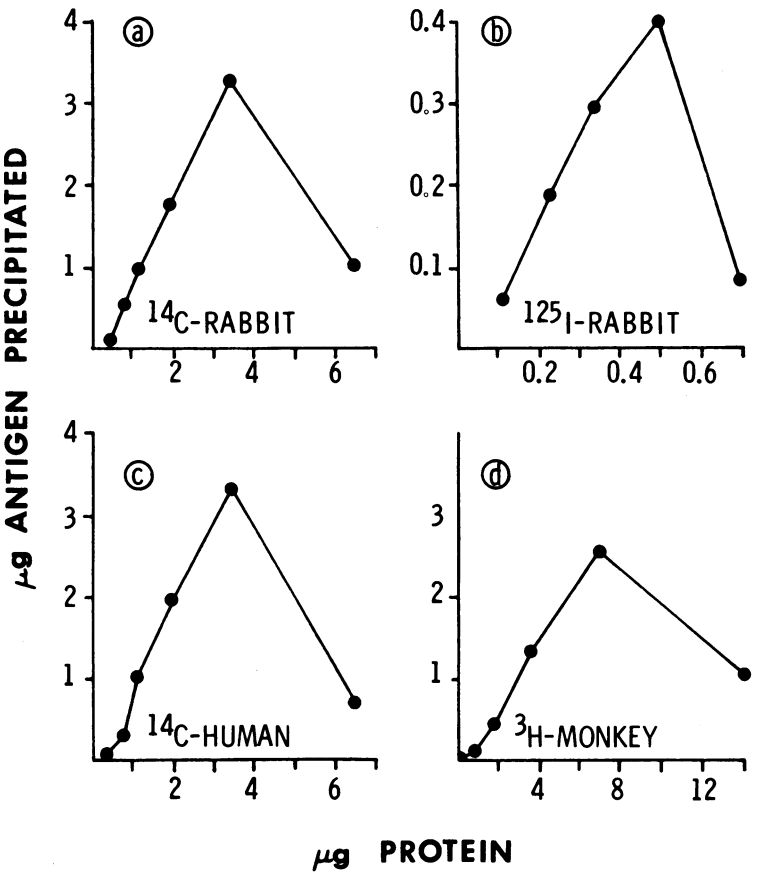

FIG. 3. *Determinations of Ig-anti-Ig equivalence by quantitative precipitin reaction in microtubes. Trace amounts of labeled globulin were mixed with corresponding unlabeled globulin, followed by appropriate anti-Ig serum. Procedures and calculations are described in text. (a) Varying amounts of rabbit γ-globulin mixed with 0.43 μg (~700 counts/min) of ^{14}C-rabbit γ-globulin precipitated with 25 μliters of a 1:25 dilution of goat anti-rabbit Ig. (b) Varying quantities of a mixture of 3.26 μg of rabbit γ-globulin and 0.18 μg (~105,000 counts/min) of ^{125}I-rabbit γ-globulin in 125 μliters of lamb globulin precipitated with 25 μliters of a 1:250 dilution of goat anti-rabbit Ig. (c) Varying amounts of human γ-globulin mixed with 0.43 μg (~700 counts/min) of ^{14}C-human γ-globulin precipitated with 25 μliters of a 1:25 dilution of goat anti-human Ig. (d) Varying amounts of monkey globulin mixed with 0.03 μg (~500 counts/min) of ^{3}H-monkey globulin precipitated with 25 μliters of a 1:25 dilution of goat anti-monkey Ig.*

ter stated by the antiserum supplier. Monkey gamma globulin (Fig. 3d) was prepared from a crude $(NH_4)_2SO_4$ precipitate, and only a maximum of 39% of the radioactivity was precipitated corresponding to 2.6 μg of protein. Presumably, the remaining 61% of the radioactivity was not bound to Ig. Since all proteins in the crude mixture may not have been labeled to the same specific activity, this result served only as an approximation of the Ig content of this mixture.

Minimal quantities of antigens applicable to Ig-anti-Ig precipitins were limited by specific activities obtainable with ^{3}H and ^{14}C. With much higher specific activities obtained with ^{125}I, it was possible to test decreasing quantities of antigen and antibody at equivalence (Fig. 4). The fraction of antigen precipitated fell off below 0.7 μg. Nevertheless, as shown in Fig. 3b, it was possible to estimate equivalence employing antigen quantities of less than 1 μg.

Radioimmune precipitation with antiviral sera. The second antibody procedure (5) for assay of antiviral sera with labeled virus appeared adaptable to direct radioassay in microtubes. Our initial exploratory experiments were performed with hyperimmune rabbit antiserum to poliovirus type 2 in the presence of added normal rabbit serum. Cardiff (2) reported more sensitive and reliable results could

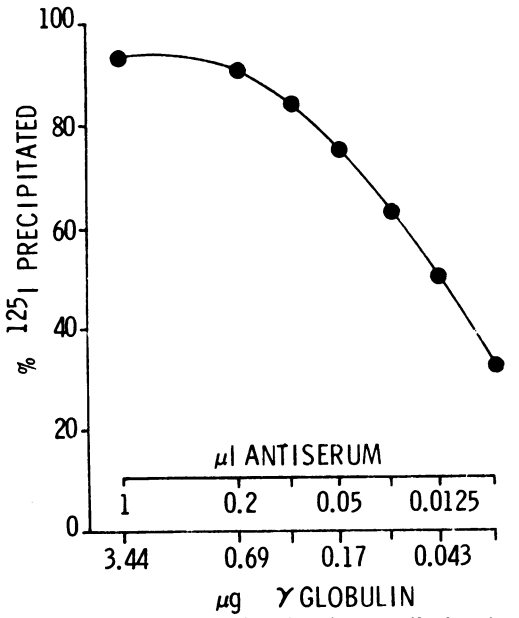

FIG. 4. *Effect of quantity of antigen-antibody mixture at equivalence upon recovery in precipitate. The reaction mixture contained antiserum corresponding to the usual level, 1 μliter, and dilutions thereof (first step fivefold, and twofold thereafter). It also contained a mixture of unlabeled and ^{125}I-labeled rabbit gamma globulin diluted separately in a similar series to maintain the ratio at equivalence. Final volumes, incubation, etc., were the usual conditions indicated in Materials and Methods.*

be obtained if the Ig-anti-Ig precipitate was formed at equivalence. Our preliminary results, employing [^{32}P]poliovirus, were essentially in agreement with Cardiff's. However, as an alternative to determination of equivalence for each unknown serum, we investigated the feasibility of $Mg_2P_2O_7$ carrier precipitation for collection of the virus-antibody-antiglobulin complex. Presence of a protein such as lamb globulin, and approximate volumes of 0.25 ml during the virus-antibody reaction and 0.6 ml during precipitation, were important factors in minimization of nonspecific adsorption or trapping of radioactive virus. Results of a screening test for antibodies to type 2 poliovirus showed a correlation between radioimmune precipitation and neutralization titers (Table 2). This test employed 10 μliters of goat anti-human serum, estimated to be in the region of antibody excess for the 1.67-μliter quantities of human sera.

Miscellaneous applications. Perchloric or trichloroacetic acid precipitates of radioactive proteins, nucleic acids, or viruses formed in the presence of an appropriate unlabeled carrier, such as bovine albumin or yeast nucleic acid,

TABLE 2. *Poliovirus radioimmune precipitation in microtubes: screening test of human sera and comparison with neutralizing antibodies*

Serum no.[a]	RIP (%)[b]	Neutralization titer (reciprocal of dilution)[c]
9	91	3,000
10	90	~3,000
11[d]	72	~3,000
3	34	150
13[d]	16	<30
2	15	<30
6	12	<30

[a] Stored (−20 C) sera from laboratory personnel and diagnostic sera (kindly provided by E. H. Lennette, California Department of Public Health).

[b] Purified [^{32}P]poliovirus was diluted in PBS containing 0.75 mg of lamb globulin per ml and clarified in the microcentrifuge. A 50-μliter amount (650 counts/min) of virus was dispensed into each microtube containing 10 μliters of a 1:6 dilution of serum. A 200-μliter amount of PBS was added and mixed. After 1 h of incubation at 37 C, 0.3 ml of a 1:30 dilution of goat antihuman Ig serum was added and incubation continued for 1.5 h. The inorganic precipitate was formed by addition of 50 μliters of 0.08 M $Na_4P_2O_7$ and 25 μliters of 0.4 M $MgCl_2$. Precipitates were collected by centrifugation, dissolved in 25 μliters of formic acid, and counted after addition of 1 ml of Aquasol; supernatants were discarded. The percent of radioactivity precipitated was calculated by method (ii) described in Materials and Methods.

[c] Neutralizing antibodies were assayed (after 2 h of incubation of serum plus virus) in Vero or 1-5C-4 monolayers, testing varying concentrations of serum against approximately 50 plaque-forming units of virus. A 50% reduction from plaque count of virus controls was considered the neutralization titer.

[d] Complement-fixation titer was <1:8 according to data from California Department of Public Health.

were collected, dissolved in a small volume of 1 N NaOH, and assayed for radioactivity in microtubes. In those instances where acid-soluble radioactivity was not excessive (iodinated gamma globulin after dialysis, partially purified virus, etc.) residual supernatant was weighed and the appropriate correction was applied. When acid-soluble radioactivity was in great excess of acid-insoluble (fluid or crude extracts from infected cells, etc.), it was necessary to wash the precipitates with perchloric or trichloroacetic acid and recentrifuge the precipitate.

Chemical fractionation techniques coupled with radioassay in microtubes were investigated. A ^{32}P-labeled influenza virus preparation (similar to that shown in Fig. 2) was extracted with chloroform-methanol according to Folch et al. (4) in a microtube. The upper aqueous phase was transferred to a separate tube, followed by a

wash, and the organic phase remained in the original tube; both were assayed by Cerenkov counting. (Efficiency of Cerenkov counting may be different in aqueous and nonaqueous media.) Of the input radioactivity, 73% was recovered in the organic phase representing phospholipid, and 23%, presumably ribonucleic acid (RNA), was recovered in the aqueous phase. Preliminary experiments also indicated the feasibility of using microtubes for analysis of RNA, deoxyribonucleic acid (DNA), and protein by methods described by Shatkin (14).

We investigated the feasibility of pelleting virus in microtubes in the preparative ultracentrifuge, followed by direct radioassay. Microtubes filled almost to the cap floated in a nearly vertical orientation in a solution of density comparable to that inside the tube; under these conditions the tubes withstood the centrifugal field in a Spinco SW 25.1 rotor at 25,000 rpm. Duplicate 5-μliter portions (approximately 2,000 counts/min) of a purified [^{3}H]poliovirus preparation were mixed with medium containing 0.1% bovine serum albumin in microtubes and centrifuged 2 h at 25,000 rpm. The pellets (not visible) were suspended by Vortex mixing in 20 to 30 mg of residual supernatant, mixed with Aquasol, and counted, with recoveries of 96 and 99.8% of radioactivity.

DISCUSSION

The use of microtubes as a versatile container, having special merit in radioassay applications, has been presented. Microtubes provide a simple, efficient, and economic means to assay radioactivity; they are especially adaptable to samples prepared by centrifugation. Although the cost differential of microtubes compared to plastic scintillation vials is small, the advantages of expediency and economy are apparent when the following are considered. The necessity to transfer samples to another container for counting is eliminated, and the quantity of scintillation medium is greatly reduced. A common vessel which can be utilized for collection, assay (without dilution), storage, and recovery has distinct value for counting by Cerenkov radiation. Adapters to hold microtubes in liquid scintillation spectrometers are easily constructed and reusable. However, if adapters are not available, counting can be accomplished by mounting microtubes inside standard counting vials. The microcentrifuge designed to hold microtubes attains 15,000 rpm, enabling rapid collection of precipitates. Microtubes can be used in other centrifuges as well, for example, in adapters for tubes (10 by 75 mm) in the Servall centrifuge.

The counting technique described should be adaptable to any liquid scintillation counting system employing standard vials. In addition to the Packard 4322, we have tested the technique in the Packard 314 and the Beckman LS-250. In the latter instrument, for automatic sample changing operation, it was necessary to interrupt the sample detector light beam, which was done easily with an opaque paper cover. If a gamma spectrometer is not available, assay of gamma isotopes, such as ^{125}I, can be accomplished with high efficiency by liquid scintillation.

Determination of equivalence is one of the useful applications described. Although it is possible to collect precipitates without the inorganic carrier, it is more difficult to visualize the precipitate and remove the supernatant. Presence of carrier also aids in collection of finely divided precipitates. The same technique is applicable to determination of either antigen or antibody employing appropriate radiolabeled reagents. For antigen determination a trace quantity of purified labeled antigen is mixed with varying volumes of the sample containing antigen to be assayed, and precipitated with antibody prestandardized with purified antigen. By plotting percent precipitated times volume of sample versus volume of sample, an equivalence peak (similar to those in Fig. 3) is obtained from which the quantity of unknown antigen can be determined. An alternate procedure, inherently less precise, employs a single precipitation for each sample and comparison with a standard curve of percent precipitated versus micrograms of antigen in the region of antigen excess. We have employed this alternate procedure for preliminary determination of γ-globulin in 14 human sera; precipitates formed from less than 1 μliter of serum (plus a trace quantity of labeled human γ-globulin) fell within the expected range for nonpathologic sera.

Antibody content of an unknown serum may be compared with a known serum employing labeled antigen in the equivalence method. If the antigen-antibody ratio is known by independent determination, the absolute concentration of antibody may be calculated. An alternative method of estimating precipitable antibody content, applicable to sera in which the antibody content may vary widely, is mixing constant amounts of labeled antigen with a dilution series of the antiserum. This is less precise than the equivalence method, but the equivalence method may be applied subsequently.

Microtubes have also proven useful for evaluating labeled antibodies by precipitation of

unlabeled antigen. In preliminary experiments anti-rabbit Ig antibodies were purified from goat serum by the method of Avrameas and Ternynck (1) and labeled by [^{3}H]formaldehyde coupling or by chloromine-T iodination with ^{125}I. The ^{3}H product was 70% precipitable, but the iodinated product was only about 30% precipitable. We interpreted this as greater structural damage to the antibody in the more drastic iodination procedure.

We have not fully evaluated the second-antibody radioimmune precipitin assay in microtubes, nor have we fully explored all the miscellaneous potential applications for microtubes, but this preliminary survey of various applications verifies their usefulness, especially in the rapid, direct radioassay system described. Their possible usefulness in competition assays (2), using labeled antigen in competition with unlabeled antigen, is also visualized.

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