Solid-Phase Radioimmunoassay of Protein Biosynthesis

Abstract. *Solid-phase radioimmunoassay was used for measuring the rate of radioisotope incorporation into a single protein species. Radioactive antigen was measured by its binding to specific antiserum covalently linked to insoluble bromoacetylcellulose. The insoluble antigen-antibody complex was collected on filters for counting. The assay is performed in about 4 hours as compared with several days for methods based on the precipitin reaction.*

Immunochemical procedures for detesting radioactively labeled specific proteins have been used advantageously in protein biosynthesis studies, usually in tissue culture systems (*1*). The standard immunochemical method, coprecipitation of radioactive antigen with antibody and unlabeled antigen at equivalence, has several disadvantages. (i) It requires 0.1 to 1.0 mg of antiserum globulin. (ii) Purified nonradioactive antigen is needed as a coprecipitant. (iii) Assays based on the precipitin reaction require several days because most investigators have allowed at least 12 hours to ensure complete precipitation and because a separate precipitation must be done to measure nonspecific binding of radioactive protein to the antigen-antibody precipitate. This report describes a solid-phase radioimmunoassay for specific protein biosynthesis, which is performed in about 4 hours and requires no coprecipitant and as little as 5 μg of antiserum globulin.

Reagents were prepared as follows. Three different preparations of rabbit antiserum against pooled, purified immunoglobulin G (IgG) from normal human serum were absorbed with IgM and IgA myeloma proteins of different light-chain specificities and were monospecific by immunoelectrophoresis and Ouchterlony analysis with various purified myeloma proteins. The globulin fraction was obtained by precipitation of antiserums or of serums from nonimmunized rabbits with equal volumes of 28 percent Na_2SO_4 and coupled to bromoacetylcellulose (BAC) (*2*). The IgG from pooled normal human serum was purified by DEAE-cellulose chromatography. This IgG and normal mouse serum was labeled with approximately one molecule of ^{125}I per molecule of protein (*3*).

For the solid-phase radioimmunoassay, sufficient bovine serum albumin and barbital-buffered saline, *p*H 7.4 (NaCl 8.5 g/liter and sodium barbital 1.09 g/liter) were added to siliconized glass test tubes so that the final salt and albumin concentrations were 0.14*M* and 3 percent, respectively, after later addition of sample. The incubation volume in these experiments was 0.5 ml, but the results were unchanged with incubation volume of up to at least 2.0 ml. The BAC-antiserum was added as specified. After being incubated for 20 minutes at 37°C, the sample was added and incubation was resumed at 37°C. After 3 hours, the reaction mixture was diluted with 2 ml of 0.14*M* NaCl containing 10 percent of fetal bovine serum and poured onto glass fiber filters (Millipore) under suction. The incubation tube was rinsed three times with 2 ml of the NaCl-serum mixture, and the rinse fluid was poured on the filter. The filters were dried and placed flat on the bottom of scintillation vials for counting (15 ml of Liquifluor).

To measure self-absorption of radioactivity due to the insoluble BAC-antiserum and the filter, the washed complex of antigen and BAC-antiserum was dissolved in strong alkali (NCS, Nuclear-Chicago) for counting. Self-absorption, with carbon-14, was 36 percent.

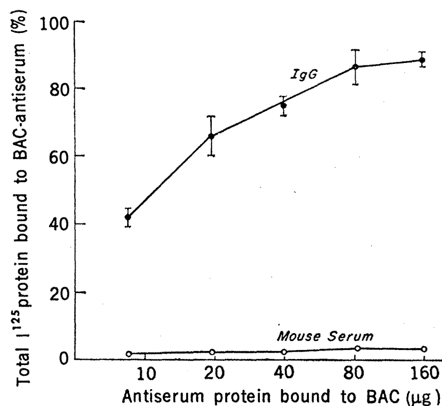

Fig. 1. Effect of changing BAC-antiserum concentration. ^{125}I-Labeled mouse serum (80 ng) and [^{125}I]IgG (70 ng) were combined with increasing amounts of BAC-antiserum in the binding assay described in the text. Maximum binding of [^{125}I]IgG was 59.5 ng, comprising 85 percent of the ^{125}I-labeled protein IgG preparation. The supernatant, containing the unbound radioactive protein, was incubated with 80 μg of fresh BAC-anti-IgG. Only 1.4 ng of [^{125}I]IgG became bound in the second incubation. Apparently, the radioactive protein in the [^{125}I]IgG preparation which did not bind to BAC-anti-IgG was antigenically inactive. This may have been the result of denaturation during the labeling procedure. Of the labeled protein capable of binding to BAC-anti-IgG, 98 percent was bound during the first incubation.

Nonspecific binding to BAC-antiserum was measured directly by saturating antigen binding sites by prior incubation with excess nonradioactive antigen before the radioactive sample was added. Because binding of antigen to BAC-antiserum was virtually irreversible, only nonspecific binding of radioactive protein could occur under these conditions. For routine use, nonspecific binding to BAC-antiserum was measured by the radioactivity bound to a complex of rabbit γ-globulin and BAC; this complex was calibrated by comparing the radioactivity bound to it with that bound to BAC-antiserum in the presence of excess antigen.

Initial experiments were designed to find the best assay conditions for binding of antigen to BAC-antiserum and to decrease to a minimum the binding of all other proteins. These conditions were established from a model system based on the reaction of human [^{125}I]-IgG with rabbit antiserum to human IgG coupled to BAC (BAC-anti-IgG). Nonspecific protein binding to BAC-anti-IgG was assayed with the use of ^{125}I-labeled mouse serum as a source of protein that would contain a minimum of antigens cross-reacting with rabbit or human IgG. Specific binding at 37°C was complete after 2 hours, whereas nonspecific binding was complete after several minutes. Bovine serum albumin (30 mg/ml) decreased nonspecific binding without affecting specific binding.

The components of the assay system and the incubation conditions were varied one at a time in order to determine their effect on binding of specific antigen ([^{125}I]IgG) and nonspecific protein (^{125}I-labeled mouse serum) to BAC-anti-IgG. After 3 hours of incubation, binding of IgG was maximum at 37° and 45°C, but it was only 60 percent of maximum at 4°C; binding is optimum from *p*H 5 to 8. Urea (2*M* and 4*M*) inhibited specific binding of IgG, while salt (up to 1.2*M* NaCl) had no significant effect. The effect of urea, ionic strength, *p*H, and temperature on nonspecific binding of mouse serum to BAC-anti-IgG were the same as on specific binding of [^{125}I]IgG.

Stability of the binding was tested by isolation of the complex of BAC-anti-IgG with [^{125}I]IgG and subsequent incubation with a 500-fold ex-

cess of unlabeled IgG for 3 hours at 37°C. This treatment removed only 15 percent of the bound [^{125}I]IgG. When the complex was incubated at 37°C for 24 hours without excess unlabeled IgG, no labeled IgG could be eluted. However, 80 percent of labeled IgG could be eluted from BAC-anti-IgG by incubation in 0.1*M* acetic acid at 37°C for 3 hours (*2*).

The amount of BAC-antiserum to be used in solid-phase radioimmunoassay was determined by a titration curve, with radioactive antigen in severalfold excess over that anticipated in routine assay conditions (Fig. 1). The titration curves obtained with the use of [^{14}C]IgG antigen from tissue culture fluid reached a plateau at 10 μg of antiserum globulin.

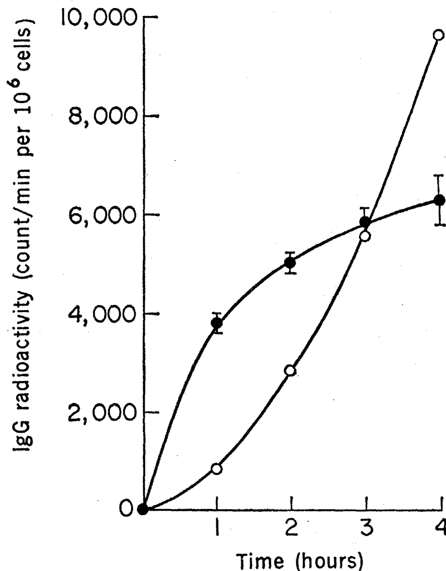

Fig. 2. Kinetics of synthesis and secretion of IgG. Cells from human lymphoid cell line IM-9 growing in logarithmic phase were centrifuged at 500*g* for 10 minutes; the cell pellet was washed once in Eagle minimum essential medium containing 1 percent Ficoll but lacking leucine. The cell pellet, which contained 5 × 10^6 viable cells, was suspended in 1 ml of the same medium containing [^{14}C]leucine (2 μc/ml; 45 mc/mmole) and incubated at 37°C. At the specified time, the supernatant was removed and centrifuged at 500*g* for 10 minutes. The supernatant was dialyzed against three changes of 500 ml of 0.14*M* NaCl and then centrifuged at 15,000*g* for 10 minutes. The cells were removed from the bottom of the petri dish by repeated pipetting up and down and were washed twice in cold, Eagle balanced salt solution. Cytoplasm extracts were prepared with 0.5 percent NP40 (Shell Chemical Co.) (*8*) and centrifuged for 90 minutes at 100,000*g*. Portions of supernatant fluid (in duplicate) and cytoplasm extract (in triplicate) were assayed for binding to BAC-anti-IgG and BAC-rabbit γ-globulin (5 μg of antiserum globulin). The antigen-BAC-antibody complex from the cytoplasm extracts was washed twice in 2 ml of 0.14*M* NaCl containing 10 percent fetal bovine serum before plating. The standard deviation of the assays on the supernatant was too small to be represented accurately on the graph. Solid circles, cytoplasm extract; open circles, extracellular fluid.

The use of solid-phase radioimmunoassay for measuring newly synthesized intracellular IgG and its secretion is depicted in Fig. 2. The cell line used for these experiments is IM-9 in which IgG comprises 40 percent of the secreted protein and 7 percent of the intracellular protein (*4*). Newly synthesized IgG was secreted long before the specific activity of the intracellular IgG became a constant. When the proportion of sought-for product is small, as when crude extracts of cytoplasm are assayed, it is advisable to separate the antigen-BAC-antibody complex by centrifugation (2000*g* for 15 minutes) before it is poured on the filters for counting.

This step eliminates most of the soluble radioactive proteins which otherwise bind to the filters and increase the background. Nonspecific binding in this experiment was 0.3 percent of the total radioactivity in the cytoplasm extracts and 0.4 percent for the secreted proteins. In later experiments, nonspecific binding of radioactivity to BAC-rabbit γ-globulin and to the filters was decreased still further when bovine γ-globulin (20 mg/ml) was used in the incubation and in the wash fluild. Solid-phase radioimmunoassay has also been used to detect radioactive human IgG in eluates from acrylamide gels (*4*) and to measure synthesis of immunoglobulins of various heavy- and light-chain types secreted by mouse myeloma cells in tissue culture (*5*).

Regulation of synthesis of proteins has been studied by assay of the sought-for radioactive protein with the use of immunochemical procedures, and this method should continue to have widespread application in viral, bacterial, and animal cell systems both in whole cells and in cell-free extracts. However, its use has not been widespread, owing at least in part to cumbersome methods involving the precipitin reaction. For measuring radioactive biosynthetic products, the advantages of solid-phase radioimmunoassay are several. (i) The assay takes only 4 hours, most of which is incubation time. (ii) Only 5 to 10 μg of the γ-globulin fraction of the antiserum is required for each assay, at least as used to detect immunoglobulin secreted by human and mouse cells in tissue culture. (iii) Antiserum bound to BAC is stable for at least 1 year if stored as a slurry in the refrigerator (*6*). (iv) Purified nonradioactive antigen is not required as a coprecipitant. (v) Binding of antigen is independent of antigen or BAC-antiserum concentration over a wide range provided that BAC-antiserum is in excess. Thus effluents from chromatography or ultracentrifugation may often be analyzed directly without preliminary concentration (*5*). (vi) In contrast to their effects on the precipitin reaction (*7*), *p*H and ionic strength are much less significant factors in the binding of antigen in solid-phase radioimmunoassay. Thus, compared to the precipitin reaction, the solid-phase radioimmunoassay requires less critical conditions. It also is more suitable for measurement of specific protein in crude cytoplasm extracts because it reduces to a minimum nonspecific binding.

HAROLD C. SOX, JR.*
Immunology Branch, National Cancer Institute, Bethesda, Maryland 20014

BEHZAD MOHIT
Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda 20014

References and Notes

1. J. H. Vaughn, A. H. Dutton, R. W. Dutton, M. George, R. Q. Marston, *J. Immunol.* **84**, 258 (1960); F. T. Kenney and W. L. Albritton, *Proc. Nat. Acad. Sci. U.S.* **54**, 1693 (1965); A. R. Williamson and B. A. Askonas, *J. Mol. Biol.* **23**, 201 (1967); J. L. Fahey and I. Finegold, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 283 (1967); M. Kern and R. M. Swenson, *Proc. Nat. Acad. Sci. U.S.* **59**, 546 (1968); D. K. Granner, S. I. Hayashi, E. B. Thompson, G. M. Tomkins, *J. Mol. Biol.* **35**, 291 (1968).
2. J. B. Robbins, J. Haimovich, M. Sela, *Immunochemistry* **4**, 11 (1967).
3. A. S. MacFarlane, *Mammalian Protein Metabolism*, H. N. Munro and J. B. Allison, Eds. (Academic Press, New York, 1964), vol. 1, p. 331.
4. H. C. Sox, Jr., unpublished observations.
5. B. Mohit, unpublished observations.
6. D. L. Mann, H. Granger, J. L. Fahey, *J. Immunol.* **102**, 618 (1969).
7. E. Kabat and M. Mayer, *Experimental Immunochemistry* (Thomas, Springfield, Ill., 1961), pp. 42–45.
8. T. W. Borun, M. D. Scharff, E. Robbins, *Biochim. Biophys. Acta* **149**, 302 (1967).
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* Present address: Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire.

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