curred in bottles preserved with trichloroacetic acid prior to acetylene introduction.

Agents of the apparent fixation in the hypolimnia of Lake Mary and Lake Mize would seem to be heterotrophic since ethylene production occurred in anoxic waters where light penetration was negligible. Fixation in the surface waters of these lakes may have been algal, but we detected no blue-green algae in either lake by microscopic examination. Fixation under these environmental conditions has not been previously reported, although low rates of fixation have been noted in surface samples incubated in the dark (*2*, *5*). The immediate ecological significance of these low rates is probably minor; that is, fixation evidently supplies only a small portion of the nitrogen requirements of aquatic microorganisms. However, the biogeochemical significance may be considerable. If the rates of ethylene production in Lake Mary are representative of nitrogen-fixing activity in the lake, the annual fixation of nitrogen in the anoxic region (5 m and below) of this small lake is approximately 4.6 kg (*13*). If fixation is found to be widespread in anoxic lake water and lacustrine sediments, estimates of the role of nitrogen fixation in the global nitrogen budget (*14*) will have to be increased.

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Protein-Bacteriophage Conjugates: Application in Detection of Antibodies and Antigens

Abstract. *Covalent attachment of proteins to bacteriophage yielded modified phage preparations with which it is possible to detect antibodies to proteins at concentrations as low as 0.5 to 2.0 nanograms per milliliter. Similarly, antibodies may be linked covalently to phage, and the resulting antibody-phage conjugate is useful in detecting proteins. An alternative method for quantitative determination of proteins is suggested, in which the inactivation of protein-phage by antibodies to protein is inhibited by the protein tested. With rabbit immunoglobulin G as the protein, as little as 0.3 nanogram per milliliter could be determined.*

Immunospecific inactivation of bacteriophage is the basis of a sensitive method for the detection of antibodies (*1*). Antibodies with specificity directed toward peptides (*2*) and haptens such as the 3-iodo-4-hydroxy-5-nitrophenylacetyl (*3*), penicilloyl (*4*), and 2,4-dinitrophenyl (DNP) (*5*) groups were detected and quantitated by the use of bacteriophage to which the specific molecules were covalently bound. In view of the great interest in the detection of very small amounts of antibodies to proteins, we have now linked proteins to bacteriophage T4 by covalent bonding and shown that the modified phage may be inactivated with antibodies against the protein. Moreover, chemical attachment to bacteriophage of immunospecifically isolated antibodies to protein yielded phage preparations which could be used for detection of protein antigens.

Bacteriophage T4 was grown, purified, and assayed as described (*2*). The proteins used were bovine pancreatic ribonuclease, bovine serum albumin (BSA), and rabbit immunoglobulin G (IgG). Coupling of proteins to the bacteriophage was performed by addition of a solution of the bifunctional reagent tolylene-2,4-diisocyanate (TDIC) (*6*) in dioxane (0.1 ml) to mixtures of bacteriophage and protein in 0.05*M* phosphate buffer, *p*H 7.0, containing 20 μg of gelatin per milliliter (0.6 to 1.4 ml); TDIC is a coupling agent which reacts with amino groups, producing entirely covalently linked conjugates between proteins (*6*). In order to find the optimum conditions, the concentrations of bacteriophage, proteins, and TDIC and the time of reaction were varied. The reaction was performed at 24°C and was terminated by dialysis against the same buffer. Any visible

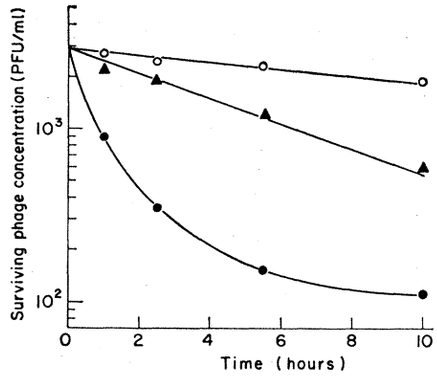

Fig. 1. Inactivation of conjugate consisting of bacteriophage T4 and rabbit immunoglobulin G by a goat antiserum to rabbit immunoglobulin G. The final dilutions of serum in the reaction mixtures were: ●, 1 to 2×10^6; ▲, 1 to 2×10^7. The serum contained 10 mg of antibody per milliliter. ○, Control experiment in which phage was kept in the absence of antiserum; *PFU*, plaque-forming units.

Table 1. Coupling of proteins to bacteriophage T4, and inactivation of the surviving phages with antiserums to proteins.

Protein coupled to bacteriophage	Protein (mg/ml)	Phage* (O.D.)	TDIC (mg/ml)	Dioxane (%)	Reaction time (hours)	Surviving phage† (%)	Antibody detected‡ (ng/ml)
Ribonuclease	7	100	0.1	7	1	1.2	2
	12	100	0.1	7	1	30	3
BSA	11	70	2.3	14	2	0.05	2
	13	70	2.3	14	2	0.003	2
Rabbit IgG	9	70	0.2	14	2	0.6	0.5

* A solution with an optical density (260 nm; 1-cm path length) of 1.0 contained 10^{11} plaque-forming units per milliliter. † The percentage of phage surviving the coupling process was calculated from the number of plaque-forming units and the optical density of the modified phage preparation. ‡ This was the lowest concentration of antibody detected from the dilution of serum (of known antibody content), which gives 50 percent inactivation of the protein-phage conjugate after reaction for 10 hours at 37°C.

precipitates were removed by centrifugation at low speed. The protein-bacteriophage conjugate remaining in solution was separated from the lighter unreacted protein by successive centrifugations for 1 hour at 20,000*g*, the residue being resuspended in the buffer.

Bacteriophage preparations which were reacted with TDIC in the absence of protein were almost completely inactivated, probably because of the capacity of the bifunctional reagent to attack two adjacent sites on the particle. Increase in protein concentration up to a certain amount protected the bacteriophage from such inactivation. Further increase in protein concentration resulted in a high extent of inactivation. Rise in TDIC concentration during the coupling process caused increased inactivation of the bacteriophage. The bacteriophage concentrations used during the coupling were relatively very high (Table 1).

The reaction conditions at which the highest sensitivity for the detection of antibodies by the modified phage was achieved are summarized in Table 1. Antiserums to the protein which was coupled to the bacteriophage were reacted with the modified phage preparations at increasing dilutions for different periods of time. The concentration of antibodies in the serums was evaluated by quantitative precipitin analysis. Normal serum had no effect on the modified phages, nor did the antiserums to protein inactivate unmodified bacteriophage T4. Goat antibody to rabbit immunoglobulin G was purified by isolation on an immunoadsorbent prepared from the IgG and bromoacetyl cellulose (*7*); it was as effective as the antiserum from which it was derived in inactivating the IgG-phage conjugate. The use of protein-phage conjugates permits the detection of amounts of antibodies to protein as low as 0.5 ng/ml (see Table 1).

The rates of inactivation of the IgG-bacteriophage conjugate (Table 1) by a goat antiserum against the IgG, at two different dilutions, are shown in Fig. 1. Above 95 percent inactivation, the rate levels off, probably owing to the heterogeneity of the protein-phage conjugate as even a 100-fold higher concentration of antibody left some phage capable of forming plaques. Methods known to enhance the rate of bacteriophage inactivation, such as plating by the "decision" technique (*8*), addition of antibodies against the immunoglobulin G used for inactivation (*9*), and lowering of the ionic strength (*10*) did not significantly alter the kinetics of the reaction.

As the rabbit IgG was successfully bound chemically to the bacteriophage, we have similarly bound to bacteriophage T4 immunospecifically isolated (*7*) rabbit antibodies to DNP. The resulting antibody-phage conjugate (0.3 percent of the phage survived) was reacted with different concentrations of DNP-BSA (7 moles of DNP per mole of BSA), without decrease in viable phage. Addition of an excess of antibodies to DNP to this system caused significant inactivation of the modified phage. Thus, for example, when 0.05 μg of the DNP derivative of BSA was added to 1 ml of the antibodies to DNP conjugated with phage (1600 plaque-forming units) and left to react for 10 hours at 37°C, followed by addition of 20 μg of antibodies to DNP (kept for an additional hour), 70 percent of the conjugated phages were inactivated. It seems, therefore, that, by making use of our technique, it should be possible to detect and determine quantitatively proteins.

An alternative method for the detection of proteins depends on inhibition of phage inactivation. Haptens may inhibit the inactivation of hapten-phage conjugates by means of antibodies to haptens (*3, 11*). We have now successfully inhibited with rabbit IgG the inactivation of the IgG-phage by means of antibodies to rabbit IgG. Thus, 0.2 ml of the IgG (1 ng/ml) was mixed with 0.2 ml of goat antiserum to IgG diluted to contain 2 ng of antibody per milliliter and left for 3 hours at 37°C. A conjugate of rabbit IgG and phage (0.2 ml containing 500 plaque-forming units) was added, and the mixture was kept for an additional 20 hours at 37°C. At the same time, an experiment was carried out in which buffer alone replaced the IgG solution. The modified phage was inactivated to the extent of 70 percent in the absence of the IgG, whereas only 40 percent of the phage was inactivated in the presence of the IgG (0.2 ng/ml). Higher concentrations of the IgG in the reaction mixtures inhibited the inactivation of the IgG-phage to a greater extent. Total inhibition was achieved with 30 ng of the IgG per milliliter.

In conclusion, the extension of the chemical modification of bacteriophage to protein conjugates allows for sensitive assays for the detection of antibodies to proteins, as well as for the quantitative determination of the proteins themselves.

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