Reports

Preserving Fluorescein Isocyanate for Simplified Preparation of Fluorescent Antibody

The extension of the fluorescent antibody technique (*1*) to staining microorganisms and viruses in clinical material (*2*) and the use of such labeled antibodies in a serological procedure (*3*) have made this technique of practical interest to many more laboratories than was previously the case. A major obstacle to the more widespread use of this valuable method has been the problem of preparing the fluorescein-labeled reagent. In this preparation, conversion of amino-fluorescein to fluorescein isocyanate is an important step. Because of the unstable nature of fluorescein isocyanate, the original recommendation was that it be prepared immediately before being mixed with the protein solution to be labeled (*1*). Inasmuch as conversion to the isocyanate requires the use of phosgene gas, which requires special care in handling because of its toxicity, the procedure is not well suited for intermittent performance by small laboratories, which may need to label different solutions at long intervals of time. For this reason, any method of stabilizing the isocyanate so that it can be kept on hand for long periods of time and shipped in a simple manner from one place to another is likely to extend greatly the potential use of fluorescein-labeled antibodies.

One approach to this problem has been to prepare acetone solutions of isocyanate, seal them in glass ampules, and keep them at low temperature. Under these conditions, no significant deterioration occurs for several months, at least (*4*). In this laboratory a different and simpler technique for stabilizing the isocyanate has been used. We have determined that acetone-dioxane solutions of fluorescein isocyanate can be dried onto filter paper or the like, and that the dried reagent stored in a desiccator at room temperature will remain active for at least 7 months (the longest period tested so far).

In order to investigate this method, we used aliquots from an anti-*Toxoplasma gondii* globulin solution whose capacity to stain the homologous species after being labeled with fluorescein had already been established (*5*). In assessing the quality of the labeling which was obtained with the dried isocyanate, homologous organisms were exposed to dilutions of the various labeled aliquots, and the brightness of fluorescence of the organisms was compared visually. The results of several exploratory experiments have led us to adopt the following procedures for preparing and using dried isocyanate.

Fluorescein isocyanate is prepared in the usual manner (*1*) and is dissolved in a quantity of acetone-dioxane (2/1) to yield a solution with the equivalent of 20 mg of amine per milliliter of solution. One milliliter of the solution is delivered into a small flat dish, and a piece of thick, chromatographic paper, 20 mm square, is used to soak up the solution in a uniform manner. Before use, the chromatographic paper is washed in distilled water and then in acetone in order to remove impurities that may cause the isocyanate to deteriorate. The acetone and dioxane we use to dissolve the isocyanate are both specially dried, the acetone by being kept over $CaSO_4$, the dioxane, by having metallic sodium added to it and then by distilling off the water-free solvent. The paper containing the isocyanate is dried in front of a fan for about 1 minute and is then put into a loosely covered glass dish, protected from light. The dish is kept in a desiccator over $CaSO_4$.

Originally, in order to use the dried paper, we prepared a buffered reaction mixture containing acetone and dioxane as well as the protein solution to be labeled. Subsequently, as a result of comparative experiments, it was found that dioxane in final concentration of up to 40 percent in the reaction mixture did not significantly change the staining capacity of the resulting labeled antibody solutions from that obtained in the absence of dioxane. When the concentration of dioxane was increased to 80 percent, a significant degree of protein denaturation occurred, resulting in a distinct lowering of staining capacity. As a result of these observations, we now use a reaction mixture that consists only of the protein solution in saline and 0.5*M* carbonate buffer at *p*H 9. The volume of buffer used is 10 percent of the volume of the protein solution to be labeled. The latter is brought to a concentration of 1 g/100 g by means of a mixture of buffer and saline (*6*).

The actual labeling process is very simple. The required amount of isocyanate is computed in terms of amine on the basis of 0.05 mg of amine per milligram of protein to be labeled. With the volume and size relationships we use, a strip of paper 1.0 by 20 mm contains 1 mg of amine equivalent, making it simple to estimate the size of the strip needed. A piece of paper of the proper size is added to the protein solution, prepared as described above, and maintained in an ice bath. After vigorous shaking of the mixture to make sure that the paper is thoroughly wetted down, the mixture is left overnight on a shaker in the cold and is then removed and dialyzed in the usual manner to remove the unreacted fluorescein derivatives (*1*).

Comparisons of the staining ability of conjugates prepared from dried, stored isocyanate and from freshly made isocyanate dissolved in acetone-dioxane have shown that the two types of conjugates are entirely comparable. We have no information on the fluorescein-protein ratios which result when the two methods are used, but, on a practical basis, the dried isocyanate has yielded conjugates that are as satisfactory as are those prepared in the conventional manner.

One unexpected advantage of this method has been the fact that no visible denaturation of protein occurs with it, whereas there is considerable denaturation when the isocyanate is used in the conventional manner. This observation led us to attempt to react the same antibody solution with fluorescein more than once in order to increase the staining power of the solution. The basis for trying this was the fact that it has been estimated that only 3 to 6 percent of the theoretically possible reactive sites of the protein molecules actually combine with fluorescein isocyanate in the usual labeling procedure (*7*). Previous attempts on our part to react the same antibody solutions more than once with freshly prepared solutions of isocyanate had resulted in great losses of protein by denaturation. However, by using the dried isocyanate it has been possible to double, approximately, the effective staining capacity of a solution by reacting it two or three times with the fluorescein. This is a distinct advantage when one is working with weak antisera or with small quantities of serum, which can thus be made to go farther.

All technical papers and comments on them are published in this section. Manuscripts should be typed double-spaced and be submitted in duplicate. In length, they should be limited to the equivalent of 1200 words; this includes the space occupied by illustrative or tabular material, references and notes, and the author(s)' name(s) and affiliation(s). Illustrative material should be limited to one table *or* one figure. All explanatory notes, including acknowledgments and authorization for publication, and literature references are to be numbered consecutively, keyed into the text proper, and placed at the end of the article under the heading "References and Notes." For fuller details see "Suggestions to Contributors" in Science 125, 16 (4 Jan. 1957).

The method used for relabeling is essentially a repetition of the original process. After the unreacted fluorescein derivatives have been dialyzed away, the labeled protein is simply shaken overnight again with the proper amount of dried isocyanate. Since the protein solution is already in approximately 1 gram percent concentration, no more saline is added. However, buffer is again added (10 percent of the original volume of the globulin).

In our opinion, the main advantages to be obtained from the use of dried isocyanate in the manner described above are (i) that the isocyanate can be prepared centrally in either commercial or noncommercial laboratories and can then be sent out to smaller research or diagnostic laboratories for actual use, and (ii) that an antiserum can be made to stain more intensely by relabeling, without danger of loss of protein content as a result of denaturation.

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References and Notes

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31 July 1957

Mortality of Aquatic Insects and Fishes Caused by Use of Latex Tubing in Experimental Apparatus

A commonly used brand of latex rubber tubing was employed in the construction of apparatus for experiments on the dissolved oxygen requirements of certain aquatic insects under varying conditions of flow. A unit of the apparatus consisted of a glass tube through which water was continually recirculated by means of a small centrifugal pump. About 10 ft of ½-in. latex tubing provided the connections between the tube, a 5-gal bottle in which the dissolved gas content of the water was adjusted, and the pump. The test animals were held in the tube in a capsule of stainless-steel screen. The total volume of water in use at any one time was about 20 lit. During any one experiment there was no exchange of the water. Test water temperatures were about 20°C.

Table 1. Mortality associated with latex tubing in standing water tests.

Test No.	Animal	No.	Test conditions	Animals dead in		
				24 hr	48 hr	72 hr
1a	*A. pacifica*	10	Latex tubing		3	7
1b	*A. pacifica*	10	(control)		0	0
2a	*A. californica*	10	Latex tubing		5	6
2b	*A. californica*	10	(control)		0	0
3a	*L. reticulatus*	10	Latex tubing	0	0	5
3b	*L. reticulatus*	10	(control)	0	0	0
4a	*O. tshawytscha*	10	Latex tubing	10		
4b	*O. tshawytscha*	10	(control)	0		
5a	*O. tshawytscha*	10	Latex tubing	10		
5b	*O. tshawytscha*	10	(control)	0		
6	*A. californica*	10	Plastic tubing		0	0

In numerous trials it proved to be impossible, with the use of this apparatus, to keep more than a small percentage of the nymphs of *Acroneuria pacifica* and *Acroneuria californica* (Plecoptera) alive and in good condition, even under "control" conditions, for periods of several days. Usually, within 24 hours, some mortality occurred, and all or most of the surviving nymphs were in sluggish condition. Generally, in 2 or 3 days, about half of the nymphs were dead or moribund.

Subsequent testing of individual parts of the apparatus for harmful effects on the nymphs indicated that only the latex tubing could be responsible for the trouble. Confirmatory tests were conducted, in 1-gal glass jars, in which the two species of *Acroneuria,* fingerling king salmon (*Oncorhynchus tshawytscha*), and immature guppies (*Lebistes reticulatus*) were used. In these experiments a piece of latex tubing several feet long was coiled in the test jars filled with water. The animals were then introduced into the test water, which was kept well aerated and in continuous circulation by rising air bubbles. Appropriate controls were used. As Table 1 shows, no losses occurred among the control animals, but in all cases there was considerable mortality among the test animals, of all species, in the jars that contained the tubing. A piece of plastic tubing was used in a similar jar test. No deaths occurred, and the stonefly nymphs used appeared to be normally active after 3 days.

When about 1500 ml of water per minute flowed continuously and without recirculation or reuse through 10 ft of the latex tubing and into a glass tube containing stonefly nymphs, no difficulty was experienced in keeping the animals alive and in good condition for several days.

As a result of these experiments, the original apparatus was reconstructed and the plastic tubing, which had been found to be apparently harmless, was used; in the course of tests performed thereafter, with this apparatus, no unexpected mortality of stonefly nymphs occurred.

Although widely used and apparently entirely suitable for most experimental purposes, latex rubber tubing evidently should be used with caution in experiments involving the recirculation of water in closed systems where there is no continuous exchange of water and where species of animals sensitive to the constituents of the tubing are utilized. Even though mortality might not result from such use of the tubing, the results of sensitive physiological tests might be markedly influenced.

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Synthesis of "On-Off" and "Off" Responses in a Visual-Neural System

The most distinctive feature of the discharge of impulses in the vertebrate optic nerve in response to a light stimulus is the marked activity elicited by changes in the level of illumination. The early records of Adrian and Matthews from the whole optic nerve (*1*) demonstrate a strong burst of activity when the light is turned on, a continuing discharge at a lower rate as long as the light remains on, and, upon the cessation of light, a renewed burst which gradually subsides.

Hartline (*2*) has shown that this composite response results from individual fibers whose activity differs markedly: some fibers discharge regularly as long as the light shines; others discharge only briefly when the light is turned on and again when it is turned off, with no activity during steady illumination; still others respond only when the light is turned off. These complex responses, observed in third-order neurones, have been ascribed by Hartline (*2*) and Granit (*3*)