TABLE 1. *Comparison of fluorescent staining titers of rabbit antiglobulins conjugated with four fluorochromes*

Organism for production of antiglobulin	Organism tested for fluorescent staining	Fluorescent staining titer*			
		FITC	RB-200	RITC	DANS
Corynebacterium diphtheriae strains 5 and 7	*C. diphtheriae* strain 7	1:128	1:64	1:32	1:8
C. diphtheriae strains 5 and 7	*C. diphtheriae* strain 8	1:64	1:16	1:16	1:8
Escherichia coli O119:B14	*E. coli* O119:B14	1:512	1:32	1:16	1:8

* Conjugates for comparison of titer were produced from samples of the same antiserum fractions and were of equal final volumes. Abbreviations: FITC, fluorescein isothiocyanate; RB-200, lissamine rhodamine B; RITC, tetramethylrhodamine isothiocyanate; and DANS, 1-dimethylaminonapthalene-5-sulfonic acid.

volumes of each conjugate to be compared were collected from the columns.

Staining of antigens with the conjugates for *E. coli* was carried out as described by Thomason et al. (Bull. World Health Organ. **25**:137, 1961); the technique of Moody and Jones (J. Bacteriol. **86**:285, 1963) was employed for staining with conjugates for the diptheria bacillus. For microscopic observation of the stained antigens, an Osram HBO-200 light source was used in conjunction with a cardioid dark-ground condenser, a 3 mm BG-12 primary filter, and a 2 mm OG-1 secondary filter.

Results (Table 1) revealed that highest specific staining titers were produced by FITC conjugates, with DANS conjugates exhibiting the lowest titers. Smears from pure cultures of two diptheroids and smears of normal throat flora from six individuals exhibited no troublesome nonspecific staining with any conjugates for the diptheria bacillus. Likewise, all labeled antiglobulins for *E. coli* O119:B14 failed to stain the two heterologous *E. coli* serotypes tested.

Results of this study clearly showed the superiority of FITC for preparation of immunofluorescent conjugates used to stain bacterial smears. The technical conditions for conjugation with each reagent and the optical filters employed were selected on the basis of conditions and optics recommended in the literature. Improvement of conjugation methods and filter combinations for rhodamine and DANS may increase the effectiveness of these dyes.

METHOD TO FACILITATE THE ISOLATION OF *CLOSTRIDIUM BOTULINUM* TYPE E

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During the investigation of three outbreaks of type E botulism in the United States in 1963, difficulty was encountered in obtaining pure culture isolates of the causative organism from foodstuff in which type E toxin was demonstrated.

The spores of *Clostridium botulinum* type E (hereafter referred to as type E spores) are known to be almost as heat-sensitive as the vegetative cells of numerous contaminants, precluding the use of heat to reduce the extraneous microflora. Thus, the isolation of this species from toxic foodstuffs, grossly contaminated with facultative microorganisms, is difficult. Preliminary studies were made to determine whether the chemical resistance of type E spores could be employed to separate them from the contaminating vegetative microflora. A 50% concentration of ethanol was chosen, since it was found to be germicidal for vegetative cells, without adversely affecting type

E spores. This concentration is obtained by simple mixing of equal parts of absolute ethanol and the enrichment culture prepared as follows.

When the presence of type E toxin was demonstrated in the suspect fish by titrations with mice, portions of the sample were cultured in duplicate tubes of cooked meat medium (CMM) prepared from fresh liver and Trypticase-peptone-glucose (TPG) broth (Schmidt, Nank, and Lechowich, J. Food Sci. **27**:77, 1962), and were incubated for 3 days at 30 C. After incubation, 2-ml samples of the enrichment culture were placed in sterile test tubes and were mixed with an equal volume of absolute ethanol. This mixture was allowed to stand at 25 C for 1 hr with occasional mixing. The culture-alcohol mixture was streaked on liver veal agar plates containing 4% sterile egg yolk and was subcultured into TPG broth. Plates were incubated in Case Anaero jars under a nitrogen atmosphere for 48 hr at 35 C.

After incubation, the plates were examined for characteristic colonies of *C. botulinum* type E. The opalescence zones (McClung and Toabe, J. Bacteriol. **53**:139, 1947) produced by type E colonies aided colony recognition, especially if other sporeforming organisms were present. When typical colonies predominated, the corresponding TPG broth subcultures were assayed for type E toxin. The assaying of TPG broth subculture, rather than reculturing of the individual colonies from the plates, eliminated the possibility of selecting a nontoxic variant frequently encountered in toxic subcultures.

Pure culture isolates were rapidly obtained by this procedure from 40 of 41 individual packages of smoked whitefish chubs containing type E toxin, including those associated with victims of the outbreak in the Tennessee-Alabama-Kentucky area in September-October 1963. In addition, pure cultures were obtained from canned tuna fish (Johnston, Feldman, and Sullivan, Public Health Rept. U.S. **78**:561, 1963) and the smoked whole whitefish associated with the outbreak of type E botulism in Kalamazoo, Mich., in September 1963. When the enrichment cultures were streaked on plates without alcohol treatment, the overgrowth of facultative microorganisms prevented the recovery of *C. botulinum* type E.

Direct plating of the macerated fish without treatment was not successful for isolating the species, whereas alcohol treatment of the fish often permitted isolation by direct plating. In a few cases, difficulty was encountered when clostridia or other sporeformers were also present. It is felt, however, that the ability of *C. botulinum* type E to sporulate more readily than many other sporeformers in TPG enrichment broth will aid in its selection by means of the alcohol treatment. Studies are under way on the applicability of this method for the direct recovery of small numbers of *C. botulinum* type E spores from foodstuffs, water, mud, soil, and other possible sources, in environmental surveys.

ORGANIC NUTRIENTS REQUIRED FOR GROWTH AND SPORULATION OF *BACILLUS CEREUS*

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Although a number of chemically defined media for the cultivation of various strains of *Bacillus cereus* have been described (Foster and Heiligman, J. Bacteriol. **57**:639, 1949; Williams and Harper, J. Bacteriol. **61**:551, 1951; Sergeant et al., J. Bacteriol. **74**:728, 1957; Lundgren and Beskid, Can. J. Microbiol. **6**:135, 1960), none was found suitable for shaker cultures of *B. cereus* strain T. The medium used almost exclusively for the cultivation of this strain is the G medium, which is a weakly buffered glucose-yeast extract-mineral salts solution. Preliminary studies revealed that the requirement for yeast extract in the G medium could be satisfied with 0.2% Vitamin Free Casamino Acids (Difco). Among the amino acids in Casamino Acids, six were found to be essential or stimulatory for growth and sporulation of *B. cereus* strain T. The chemically defined medium finally derived from these studies which supports both growth and sporula-