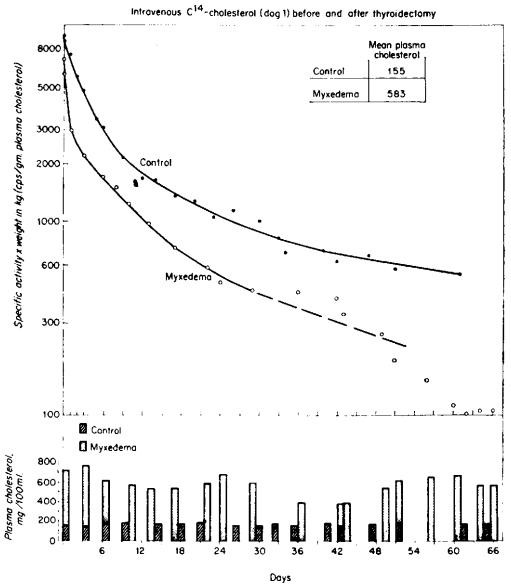

FIG. 4. Disappearance of C^{14} from plasma of dog 1 before and after thyroidectomy.

for measuring plasma cholesterol-C^{14} after intravenous injection of this sterol; it consists of liquid scintillation counting of an alcohol-acetone extract of the plasma. The distribution of cholesterol-4-C^{14} in 2 normal dogs was calculated from a 60- to 70-day study of plasmatic radioactivity, assuming a 4-compartment mammillary system. Excretory rate constants of 1.5 and 1.6% per day of the retained dose were derived. Preliminary estimates of the "exchangeable cholesterol pool" were made. The size of this pool appeared to increase as serum cholesterol rose, either as the result of a high fat diet or of thyroidectomy.

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Received September 21, 1962. P.S.E.B.M., 1963, v112.

Identification of Toxinogenic *C. diphtheriae* with Fluorescent Antitoxin: Demonstration of Its Nonspecificity.* (27990)

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Two recent reports have described the use of the immunofluorescence technic for identification of toxinogenic strains of *Corynebacterium diphtheriae*(1,2). These published procedures involve staining of diphtheria organisms with fluorescein-conjugated, commercially available diphtheria antitoxin. While working with this technic in our laboratory certain findings appeared which raised questions as to its specificity. These findings form the basis of this report.

Methods. Bacteria studied. Eleven culture isolates of *Corynebacterium diphtheriae* consisting of gravis, mitis and intermedius types, and one culture isolate of *Corynebacterium xerosis* were obtained from the Bacteriological Laboratories, Maryland State Department of Health, and from the American Type Culture Collection. Two culture isolates of diphtheroids and single culture iso-

* Supported by contract with Army Chemical Corps, Biological Laboratories, Fort Detrick, Md. and by training grant, Nat. Inst. of Allergy and Infect. Dis.

lates of *Proteus mirabilis, Bacillus subtilis, Staphylococcus aureus, Streptococcus fecalis* and an unidentified yeast were obtained from Johns Hopkins Hospital Bacteriology Laboratories.

Growth of bacteria. Corynebacterial cultures were maintained on Loeffler slants. Prior to use each microorganism was subcultured onto Tellurite agar and blood agar, and its morphology verified by gram staining. Isolated colonies were transferred to flasks containing infusion-free broth for diphtheria toxin production as described by Bayne Jones(3). This was prepared in acid washed glassware and used in volumes adjusted to give a surface-to-volume ratio of 0.58. Cultures were incubated in this medium for from 2 to 7 days at 34° to 35°C. Other bacteria were maintained on nutrient agar slants and grown in nutrient broth, but were otherwise handled identically with the *Corynebacteria.*

Preparation of organism for staining. Corynebacteria were separated from the infusion-free broth by centrifugation, washed, and resuspended to a turbid suspension in nutrient broth. Smears on clean glass slides were made from this suspension and allowed to air dry at room temperature for 15 minutes. These smears were either used immediately for fluorescent staining, or were stored at −20°C for no longer than 7 days prior to use. Other bacteria were obtained from nutrient broth and handled identically.

Determination of virulence. Each of the *Corynebacteria* and diphtheroids used was examined for toxin production by 2 methods. A 0.1 ml aliquot of the bacterial suspension obtained from the infusion-free broth was injected intradermally into each of 2 guinea pigs, one of which had received 500 units of diphtheria antitoxin intraperitoneally on the previous day and the other of which received 20 units of antitoxin intraperitoneally at the time of intradermal challenge. Skin reactions were read daily for 5 days. Each culture was checked at least 3, and at most 5, times by this method, each test utilizing organisms from a different infusion-free broth incubation. In addition, aliquots of this broth were removed after 2 and 7 days of incubation, cleared by centrifugation, and sterilized by membrane filtration. Sterility of these culture filtrates was established by inoculation on blood agar. One-tenth milliliter aliquots of these culture filtrates were injected intradermally in a rabbit, and skin reactions were read at 3 and 5 days. Controls, including dermal reactivity to diphtheria toxin and to constituents of the broth, were included in testing each rabbit. A number of the *C. diphtheria* cultures were also incubated in modified Mueller's medium supplied by the Massachusetts Biologic Laboratories, without and with increments in iron concentration added as ferrous sulfate. Though toxin production of some known toxinogenic strains could be enhanced in Mueller's medium, no strain considered atoxinogenic from infusion-free broth culture was shown to produce toxin in Mueller's medium.

Preparation of fluorescein labelled antitoxin. Equine diphtheria antitoxin was supplied by the Wyeth Laboratories, Inc., and by the Massachusetts Dept. of Public Health Biologic Laboratories. Aliquots of each were precipitated 3 times with 50% ammonium sulfate and the globulin was conjugated with fluorescein isothiocyanate by classical procedures(4). The milligram ratio of fluorescein isothiocyanate to protein was 1:40. Unconjugated fluorescein was removed by prolonged dialysis in pH 7.4 buffered saline at 5°C. Conjugation of unfractionated antitoxin was similarly performed. A portion of ammonium sulfate precipitated Wyeth antitoxin was suspended in a saturated sodium chloride solution overnight at 5°C and then separated by centrifugation. Excess sodium chloride was removed by dialysis and the resulting protein solution conjugated with fluorescein isothiocyanate as above. Such a procedure has been reported to increase the specificity of antitoxin solutions(5). Antitoxic activity of all conjugates was assayed by the Fraser modification of the Romer technic(6) using a standard antitoxin from National Inst. of Health. The antitoxic activity of all conjugates was adjusted by dilution with buffered saline to 1000 L+ units per ml.

Absorption of antitoxin conjugates. Organisms of one diphtheroid (culture C-1) and one atoxinogenic strain of *C. diphtheriae*

(culture D-11) were separated from infusion-free broth by centrifugation. The organisms were washed once with nutrient broth and mixed with equal volumes of various fluorescein-labelled antitoxin preparations. These suspensions were held at 37°C for one hour, separated by centrifugation, and the supernatant sterilized by membrane filtration. Some conjugates were subjected to multiple absorptions in this manner. In addition, an aliquot of whole Massachusetts Biologic Laboratories conjugated antitoxin was absorbed with a preparation of diphtheria toxin supplied by the same laboratory. One volume of antitoxin was mixed with 4 volumes of toxin and incubated at 37°C for 30 minutes. The solution was centrifuged, membrane filtered and used without further dilution for fluorescent staining. This amount of toxin was in excess of antitoxin as judged by skin testing in the rabbit. By calculation from L+ strengths of the 2 solutions, there was approximately 2 times the amount of toxin necessary to neutralize antitoxin activity of the conjugate. It was also shown that this absorbed conjugate would no longer stain toxin gelled in agar by a technic for detection of soluble antigen under investigation in this laboratory(7).

Fluorescent staining. Bacterial smears were fixed in acetone, absolute methanol or absolute ethanol, or stained unfixed. Only the direct staining technic was used on the smears, and staining times of from 15 to 60 minutes were tried. A variety of dilutions of conjugated antitoxin were used, and a 1:5 dilution with buffered saline gave most satisfactory results. Following staining, slides were washed in buffered saline for two 5 minute intervals. They were then drained and mounted in buffered glycerol. The fluorescence microscope used had a 200 watt mercury vapor light source with a Corning 5840 exciter filter and Wratten 2A barrier filters. The following sytem was used for visual quantitation of intensity of fluorescent staining:

no specific fluorescence	0
soft fluorescence of most organisms	+
soft fluorescence of most organisms with occasional organisms brightly fluorescent	++
bright fluorescence of all organisms	+++

Results. Table I summarizes the results of these experiments. Six of 11 strains of *C. diphtheria* were judged toxinogenic by both tests used. Mitis, intermedius and gravis types were found in both virulent and avirulent groups. As mentioned, use of a modified Mueller's medium with various iron concentrations, while altering the quantity of toxin produced, in no instance revealed toxin production by a strain previously considered atoxinogenic. It was therefore shown that about one-half of the *C. diphtheriae* strains studied produced no toxin by *in vivo* or *in vitro* testing. Four of these strains were also judged atoxinogenic by the supplying laboratory; their judgment on the fifth is not known.

The results of staining the various bacteria with fluorescent antitoxin are presented in Table I. No significant differences in staining resulting from alterations in method of fixation, staining time, or dilutions of antitoxin within the limits tried were seen. Ammonium sulfate precipitable fractions of the 2 antitoxins gave results qualitatively identical to their parent antitoxin, though intensity of staining was slightly less with fractionated material. Absorption studies gave identical results with both Wyeth and Massachusetts Biologic Laboratory antitoxins. The data in Table I, therefore, are representative of those obtained from the various procedures and fractions tried.

In general, toxinogenic strains stained more intensely than did atoxinogenic ones, but with each procedure some staining of atoxinogenic strains occurred. No manipulation of staining conditions or staining reagents allowed clear differentiation between toxinogenic and atoxinogenic organisms. Various absorptions of staining antitoxin, or use of a saline soluble antitoxin fraction, did not improve this differentiation. There was persistent staining of some organisms following absorption of antitoxin with excess toxin, probably due to the presence of non-antitoxic

TABLE I.

Bacteria	Virulence: Toxin production in liquid medium	Virulence: Guinea pig testing	Fluorescent antitoxin staining†: Mass. Biol. Lab. antitoxin	Wyeth Labs. antitoxin	"Saline-soluble" antitoxin	Antitoxin absorbed with C-1	Antitoxin absorbed with D-11	Antitoxin absorbed with toxin
Corynebacteria diphtheriae								
D-1 (*mitis*)	+	+	+	+	0			
D-2 (*gravis*)	+	+	++	++	+	+		
D-3 (*gravis*)	+	+	+++	++	++			
D-4 (*intermedius*)	+	+	+++	++		++		+
D-5 (*mitis*)	+	+	+++	++	+			
D-6 (*gravis*)	+	+	+++	++	0			
D-7 (*mitis*)	0	0	+++	++	+	+	+	
D-8 (*mitis*)	0	0	++	+	0			+
D-9 (*gravis*)	0	0	+	0				
D-10 (*intermedius*)	0	0	+	+				
D-11 (*mitis*)	0	0	++	+	+	+	0	0
Xerosis								
X-1	0	0	++	+	+			
"Diphtheroids"*								
C-1	0	0	++	+		0		0
C-2	0	0	0	0	0			
Other families								
Proteus mirabilis				0				
Bacillus subtilis				++				
Staphylococcus aureus				0				
Streptococcus fecalis				0				
Yeast				+				

* Unidentified corynebacteriae isolated from patient urines. † See text for full explanation.

antibodies whose corresponding antigen was not present in the toxin solution used for absorption. Table I also indicates that positive staining of *C. xerosis,* one of the 2 diptheroids, *B. subtilis* and a yeast occurred with use of labelled Wyeth antitoxin.

Discussion. These results indicate that staining of *C. diphtheria* by fluorescein-labelled diphtheria antitoxin is not specific for toxinogenecity. Any attempt to relate specificity of staining to intensity of staining is thwarted by overlap in staining intensity between toxinogenic and atoxinogenic groups. Staining of organisms other than *Corynebacteria* compounds this lack of specificity. The test as described, giving false positive rather than false negative results, makes the safer clinical error. Risk associated with clinical use of diphtheria antitoxin(8), however, requires the most desirable laboratory test to be free of positive as well as of negative errors.

Results presented here are in general agreement with those of Jones and Moody(1) who encountered "weak to moderate" staining of all 6 atoxinogenic *C. diphtheriae* tested, as well as staining of some streptococci and staphylococci. These results, however, are in disagreement with those of Whitaker *et al.* (2) who noted no staining of those "diptheroids and nontoxigenic diphtheria organisms tested," though toxinogenic strains were readily identified. These workers report identification of virulent diphtheria organisms by fluorescent staining of pharyngeal smears in cases where cultural identification of virulent or avirulent *C. diphtheriae* was not accomplished. This finding raises question as to the specificity of their staining technic.

If the disagreement between our data and those of Whitaker *et al.* is due to use of different lots of antitoxin, careful screening of each lot would be necessary before its use. The differences might be due to different sources of bacteriologic material in the 2 studies. *C. diphtheriae* in the present study, however, were obtained from media allowing toxin production by all toxinogenic strains.

Furthermore, one would expect false negative rather than false positive staining of bacteria grown under sub-optimal conditions, in view of the fastidious requirements by diphtheria organisms for toxin production.

It is well recognized(9) that antibodies against "accessory diphtheria antigens" are produced by immunization of horses with any but the purest preparations of diphtheria toxoid, and gel diffusion studies have confirmed the presence of multiple antibodies in the antitoxins used in this study. We believe that such accessory antibodies significantly influence staining of microorganisms with fluorescein-labelled antitoxin, and account for its failure selectively to identify toxinogenic *C. diphtheriae.* Antitoxin is prepared by immunization of horses with a culture filtrate toxoid from a given strain of diphtheria. Hewitt(10) has shown serological cross reactions between various strains of *C. diphtheriae,* and Gundersen(11) has shown that this immunological relationship can extend to other members of the genus. Serological studies such as those of Soda and Cleverdon(12) indicate the presence of antigens common to *Corynebacteria* and other bacterial families. Such cross reactivity in addition to "natural antibody" in the immunized horse undoubtedly explains staining of the variety of *Corynebacteria* and other microorganisms seen in this study.

More intense staining of toxinogenic strains by labelled antitoxin could be due to staining of toxin at or on the surface of the organism, in addition to staining of accessory antigens on the bacterial wall. It is possible, however, that staining of *C. diphtheriae* by fluorescent antitoxin is independent of the presence of toxin, and that more intense staining of toxinogenic strains results from their closer antigenic similarity. It is known (13) that expression of toxinogenicity by phage is possible in only certain strains of *Corynebacteria.* Furthermore, evidence closely links the appearance of free toxin in liquid media with lysis of diphtheria organisms, and Barksdale has noted the absence of evidence indicating the presence of toxin in actively growing cells(14). Stainable diphtheria toxin may not, therefore, be present at or on the surface of intact, viable diphtheria organisms. If so, attempts to identify toxin on the surface of these organisms as a basis for determining toxinogenicity will be unrewarding.

Summary and conclusions. 1. Immunofluorescent staining of various *Corynebacteria* and other microorganisms with fluorescein labelled diphtheria antitoxin resulted in staining of toxinogenic and atoxinogenic strains of *C. diphtheriae,* as well as of certain other *Corynebacteria* and unrelated microorganisms. 2. Specificity of this staining for identification of toxinogenic *C. diphtheriae* could not be improved by alterations in conditions of staining, or by chemical manipulation of antitoxin solutions. 3. This lack of specificity is felt to be due to multiple non-antitoxic (accessory) antibodies present in the antitoxin preparations which cross-react with a variety of bacterial antigens. 4. It is suggested that stainable diphtheria toxin may not be present at or on the surface of viable, intact diphtheria organisms, and specific identification of toxinogenic *C. diphtheriae* by fluorescent antitoxin may not be possible. 5. On the basis of data presented here, this technic as currently described would seem to offer little more than a gram stain to the diagnostic bacteriology of diphtheria.

The authors wish to express their appreciation to the Massachusetts Dept. of Public Health Biologic Laboratories and to Wyeth Laboratories, Inc., for generous supplies of toxin, antitoxin and media, to Dr. Elizabeth Petran of Maryland State Health Dept. for help in obtaining culture material, and to Miss Carol Ross for valuable technical assistance.

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Received September 26, 1962. P.S.E.B.M., 1963, v112.

A Study of the Chlorpromazine Induced Decreased Liver Glutathione Reaction.* (27991)

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Bartlett and Register(1) reported that administration of chlorpromazine to rats induced a lowering of liver non-protein sulfhydryl (NPSH). The decreased liver NPSH reaction is also elicited by a number of nonspecific stressors(2-4). Register and Bartlett(5) have shown that injection of epinephrine into rats produced the decreased liver NPSH reaction. Earlier work in these laboratories(6) has shown that in addition to epinephrine, administration of the psychotomimetic agents lysergic acid diethylamide, mescaline and serotonin produces a lowering of rat liver NPSH. The major portion of total NPSH in tissue is composed of reduced glutathione and changes in tissue concentrations of NPSH are due to changes in concentrations of reduced glutathione(7).

This investigation concerns the mechanism through which chlorpromazine decreases liver glutathione.

Methods. Determination of liver concentrations of glutathione was made by the amperometric titration procedure of Benesch and Benesch(8) employed in our laboratories(4,6, 9 and 10).

One group of 10 female Holtzman rats (150-200 g) received intraperitoneal injections of 10 mg/kg of chlorpromazine while 2 other groups received saline. Twenty-four hours after the first injections the chlorpromazine treated rats received a second injection of 10 mg/kg of chlorpromazine. The 2 remaining groups were administered 10 mg/kg of chlorpromazine and an equal volume of saline, respectively. The rats were sacrificed 4 hours after the final injection for liver glutathione determination. In a second experiment 6 rats were injected with 10 mg/kg of chlorpromazine while a second group received saline. The rats were sacrificed 8 hours after the injections and liver glutathione levels determined.

Rats adrenalectomized 24 hours prior to the experiment were maintained at room temperature, received standard Wayne Lab Blox and 1% sodium chloride *ad libitum*. Twelve rats received 10 mg/kg of chlorpromazine and 10 received saline. Four hours after the injections the rats were sacrificed for liver glutathione determinations.

Hypophysectomized rats§ were used immediately upon receipt and required no special treatment. Approximately 8 hours after surgery 6 of the rats received injections of 10 mg/kg of chlorpromazine and a second group of operated rats were subjected to bilateral hind leg rubber band tourniquet stress. The tourniquets were removed after 3½ hours. The rats were sacrificed 4 hours after administration of chlorpromazine or application of the tourniquets.

* Supported, in part, by grant from Nat. Inst. of Mental Health.

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