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Enrichment of Loeffler's Medium with Glycerol

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During the early years of World War II diphtheria became fairly prevalent in the high, mountainous regions near Rio de Janeiro, Brazil. Under the direction of Dr. Bretz, the Public Health Laboratory in Petropolis, some 20 km. from the capitol, was faced with the necessity of rapidly arranging laboratory facilities to aid in diagnosis, epidemiology, and control of the disease.

The preparation of Loeffler's medium presented a serious problem because there was no available source of the necessary quantities of serum. In cast about for an appropriate substitute, it occurred to Dr. Bretz that Petragnani's medium (minus the dye) might serve, as it was in good supply, made of readily available materials, and of what might be supposed to be adequate nutrient substances. A small quantity of the medium was therefore prepared without dye and, after slanting and sterilizing was inoculated with a number of different strains of *Corynebacterium diphtheriae* in pure culture. The organisms grew luxuriantly, in 18 hours at 37° C. The results of microscopic examination far exceeded expectations. The organisms, especially when stained by the Albert-Layburn method, presented all of the well-known, distinctive pleomorphism of *C. diphtheriae*, and the bars and granules were exceedingly large, distinct, and numerous. Other organisms, common in throat cultures (diphtheroids, cocci, and others), were also cultivated on the medium and found to be readily distinguishable from true diphtheria bacilli. In mixed cultures they presented no difficulty or confusion in microscopic diagnosis. This medium and the Albert-Layburn stain were, therefore, adopted for use at Petropolis in the diagnosis of diphtheria, and for some time proved very valuable. The modified Petragnani's medium appeared to be as good as Loeffler's medium.

In 1944 the senior author had an opportunity to continue his studies in Baltimore at the Johns Hopkins School of Hygiene and Public Health, Department of Bacteriology. Large numbers of cultures, both pure and "field," were available for study, as well as an abundance of patients since an epidemic was in progress in a nearby school. It soon became evident that Petragnani's medium, in conjunction with the Albert-Layburn stain, was a valuable diagnostic tool. This medium, however, is expensive and time consuming in preparation.

Because of the usually good morphological differentiation of *C. diphtheriae* on the Petragnani medium, a series of simple experiments was under-

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taken to determine which of the ingredients in Petragnani's medium might be playing the principal role in producing the excellent results observed. Difco heart-infusion agar was used as a basal medium. By itself, this induces in *C. diphtheriae* little of the distinctive morphology so useful in diphtheriology and supports only a minimal growth of the organisms. To this medium the different ingredients used in Petragnani's medium were added, each ingredient in a separate lot of medium, in the proportions used in Petragnani's formula or as nearly so as was practicable. The ingredients thus tested were whole milk, potato starch, diced potato, eggs, and glycerine (c. p.). Several lots of media were also prepared with combinations of glycerol and potatoes. The media were dispensed, autoclaved, and cooled as slants.

Groups of each were then inoculated with cultures of *C. diphtheriae* and diphtheroids (*C. xerose*, *C. pseudodiphtheriticum*). After incubation at 37° C. for 18 to 24 hours smears were prepared in duplicate from each culture for microscopic examination. One of each pair of smears was stained with methylene blue, the other by the Albert-Layburn method as given below:

Formula for Albert-Layburn Stain

Solution I

Toluidin blue	0.15 gm.
Malachite green	0.20 gm.
Glacial acetic acid	1.00 cc.
Alcohol 95 percent	2.00 cc.
Distilled water	100.00 cc.

Autoclave 15 lb. 15 minutes.
Let stand 24 hours, filter.

Solution II

Iodine crystals	2.0 gm.
Potassium iodide	3.0 gm.
Distilled water	300.0 cc.

1. Fix smear by heat.
2. Flood with solution No. 1 for 3 to 5 minutes.
3. Wash in tap water.
4. Flood with solution No. 2 for 1 minute.
5. Wash, blot dry, and examine.

It required only a short series of such experiments to show clearly that, so far as the morphology of *C. diphtheriae* was concerned, glycerol is the effective agent in Petragnani's medium. The methylene blue stained smears were, in our opinion, as effective as those stained with the Albert-Layburn stain although the latter greatly emphasized the granules. In spite of these results it was not considered desirable to adopt a glycerol-agar medium at once for routine diagnostic purposes. This possibility may be made the basis of a future, more extensive, study.

A series of tests was made in which glycerol was added to fluid Loeffler's medium in concentrations ranging from 0.5 percent to 10 percent before dispensing and sterilizing the mixture. Glycerol was also added to Pai's medium (1) in 8 percent concentration. Microscopic examination of various strains of *C. diphtheriae* and other bacteria cultivated on these mixtures showed that the extent of granule formation and distinctiveness of morphology were both directly related to the presence and quantity of glycerol, regardless of the basal medium. Both Albert-Layburn and methylene blue stains were used. The test organisms included 52 strains of *C. diphtheriae*, 25 diphtheroids, 15 strains of Group A streptococci, and 18 strains of *Staphylococcus aureus*. A portion of one of the tabulations serves to show the nature of the findings (table 1). The optimal concentration of glycerol appeared to be near 8 percent. A suggested explanation of the effect of the glycerine was that the corynebacteria metabolize it in some way. Although previous investigators (2) have shown that *C. diphtheriae* does not regularly ferment (acidify) glycerol, further tests were arranged to confirm that point in the present study. Thirty pure cultures of *C. diphtheriae* from among those used in this study were inoculated into Difco heart-infusion broth containing 1 percent glycerol and held at 37° C. The cultures showed excellent growth but only slight and varying degrees of acidity developed, and then only after several days of incubation. If the glycerol was metabolized, the end products were not markedly acid.

After the return of Dr. Bretz to Brazil, the study of glycerol-enriched Pai medium was continued in the Communicable Disease Center in Atlanta, Ga. The Diphtheria Laboratory in CDC, under the direction of Dr. Elizabeth I. Parsons, has now adopted the addition of glycerol to Pai's medium in 8 percent concentration as a routine procedure. Only methylene blue stain is used, since the glycerolized medium yields superior morphological results, obviating the necessity of using a special granule stain. The study of glycerolized Loeffler's medium for diagnostic work in this laboratory has not yet been extensive since relatively few origi-

Table 1

COMPARISON OF MORPHOLOGICAL DISTINCTIVENESS (META CHROMATIC GRANULE FORMATION) OF "C. DIPHTHERIAE" AND SOME OTHER ORGANISMS WHEN CULTIVATED ON MEDIA CONTAINING GLYCEROL.

Cultures Examined	Loeffler's Medium with Glycerol (Percent)						Pai Medium with 8 Percent Glycerol
	0	0.5	1.0	4.0	8.0	10.0	
*C. diphtheriae**							
416	+/−	+/−	+/−	++	+++	+++	++++
426	+/−	+/−	+/−	++	++++	++++	++++
973	+/−	+/−	+/−	++	++++	+++	+++
992	+/−	+	+	++	++++	++	++++
991	+/−	+	+	++	++++	++	++++
976	+/−	+	+	++	++++	++++	+++
*Streptococcus pyogenes***							
Kes.	−				−		−
Les.	−				−		−
Ro 130 b	−				+/−		+/−
Ro 127	−				+/−		+/−
*Diphtheroids***							
1	+/−	+/−	+/−	+/−	+	+	+/−
2	+/−	−	−	+/−	+/−	+/−	−
3	+/−	+	+	+	+	+	+
4	+/−	−	++	+	++	+	++
5	+/−	+	+	+	++	+	++
*Staphylococcus aureus***							
All. Me. 6	−				−		−
Aur. K 73	−				−		−
Aur. Mo. 7	−				−		−
Bel. ear	−				−		−

*Plus and plus-minus signs indicate ease with which *C. diphtheriae* is recognized and distinguished from other organisms by its morphology as compared with growth on plain Loeffler's medium.
** Plus, minus, and plus-minus signs indicate degree of granule formation and of resemblance to *C. diphtheriae*.

nal diagnostic cultures are made in this laboratory. The Pai's medium is used mainly for pure culture study.

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