THE EGG YOLK PLATE REACTION FOR THE PRESUMPTIVE DIAGNOSIS OF CLOSTRIDIUM SPOROGENES AND CERTAIN SPECIES OF THE GANGRENE AND BOTULINUM GROUPS

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The original Nagler (1939) reaction consisted of mixing dilutions of the toxin of *Clostridium perfringens* with human serum and noting the appearance of an opalescence. MacFarlane, Oakley, and Anderson (1941) reported that crude lecithovitellin from egg yolk gave a stronger reaction than serum, and postulated that the reaction was due to the alpha toxin of *C. perfringens*. Crook (1942), van Heyningen (1941), and others have used the tube reaction extensively. The test is now referred to as the Nagler or LV reaction.

In a study of the use of the tube reaction for rapid identification of *C. perfringens*, Hayward (1941) mentioned that cultures of *C. perfringens* on agar containing human serum produced a well-defined opacity extending from the edge of the colony. The reaction was completely inhibited by the addition of homologous antitoxin to the medium. Strains of *Clostridium oedematiens* and the *Clostridium bifermentans–Clostridium sordelli* group also produced similar zones, whereas other species were negative. The reactions of the *C. bifermentans–C. sordelli* group were inhibited by *C. perfringens* antitoxin. In 1943 Hayward presented more extensive studies with the plate reaction and answered the criticisms of Weed and Minton (1943) concerning the nonspecificity of the tube reaction. Hayward concluded that human serum was easier to obtain and was preferable to egg yolk in the plate reaction. It was suggested that 4 per cent concentrations of Evans, Lescher, and Webb peptone could be substituted for the Fildes broth in the medium.

Nagler (1944, 1945) has described a reaction, similar in some respects to the above, for the recognition of *C. oedematiens* (*C. novyi*). The medium used was a peptic digest of ox muscle to which egg yolk and defibrated sheep blood were added. On this medium surface colonies of *C. oedematiens* are surrounded by two distinct zones. The first opaque hemolytic area is surrounded by a dark red zone, referred to as a zone of reduction. A mother-of-pearl luster film covers both the colonies and the zones. In contrast to the foregoing reaction given by type A cultures, a dark red zone without luster was produced by one strain of type B (differentiated, according to Scott, Turner, and Vawter, 1934, and Turner and Eales, 1943, from type A, not on toxin specificity but on cell size and glycerol fermentation) and two of type C (Kraneveld's bacillus of osteomyelitis bacillosa bubalorum) of *C. oedematiens*, and a strain of *Clostridium hemolyticum*. A pearly film, but no dark red zone, was produced by *Clostridium sporogenes*, *Clostridium parasporogenes* and *Clostridium botulinum* (probably *C. parabotulinum* in the Bengston, 1924, terminology). The reaction characteristic of *C. oedematiens* was not inhibited by homologous antitoxin.

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In connection with other studies, it has been our purpose to consider these plate reactions as a means of presumptive recognition of certain clostridia, using adequate numbers of authentic strains of certain species to study the specificity of the lecithinase reactions. We have used human serum at times and confirmed the statements concerning the reactions with it. In plate tests, however, the more intense reactions obtained with the egg yolk appear to be of advantage. With serum the zones of precipitation are considerably less dense, and the luster areas, to be described, are less easily recognized.

Preparation of egg yolk suspension. Scrub and sterilize (dilute $HgCl_2$) the shell of a fresh hen's egg. Aseptically withdraw, by aspiration, the yolk (after the white has first been removed) to a sterile tube which is then closed with a rubber stopper. Add an equal volume of sterile saline and mix by inverting the tube several times. If preserved with merthiolate (1:10,000), the preparation will be usable for 10 to 14 days. Before use, test sterility by plating 1 ml in nutrient agar.

Medium. In substitution for the digest media suggested by Hayward (1941, 1943) and Nagler (1945) the following medium, prepared from commonly available ingredients, is recommended: proteose peptone no. 2, 40 g; Na_2HPO_4, 5 g; KH_2PO_4, 1 g; NaCl, 2 g; $MgSO_4$, 0.1 g; glucose, 2 g; agar, 25 g; and distilled water, 1,000 ml. Adjust pH to 7.6 and sterilize for 20 minutes at 240 F. Add 10 ml of yolk suspension to each 90 ml of medium and, for best results, pour 15 ml in 100-mm diameter plates. Streak plates in such a manner that well-isolated colonies will be obtained. Incubate plates, in anaerobic system of preference, for 48 to 72 hours. Positive results, particularly with *C. perfringens*, may appear earlier.

Other agar media tested included beef heart infusion, liver infusion, casamino acids, N-Z case, trypticase, proteose peptone, proteose peptone no. 3, bacto peptone, thio-peptone, nutri-peptone, yeast extract, sodium caseinate, amigen, and peptic digest of beef liver and beef muscle. The peptones and similar products were tested, with the salts listed above, etc., in 1, 2, and 4 per cent concentrations. In all instances the 4 per cent concentration was superior to the other two. Without giving the results in detail, we may mention that the heart infusion, the peptic digests, and certain peptones (trypticase, bacto peptone, proteose peptone, and proteose peptone no. 3) gave sufficiently good results to indicate that they might be used with the plate reaction. In general, it appears that any medium which supports satisfactory growth and provides suitable conditions for toxin production probably could be used.

Species Identification Reactions

Clostridium perfringens (C. welchii). The colonies are round and smooth (rough variants excepted) and are surrounded by a wide (8-mm) zone of opaque white precipitate (figure 1, numbers 1, 2, 3, and 4). There is no luster, and after the colonies have stood at room temperature in the air for several hours, extra zones appear at the edge of the previous zone with the final outer edge more
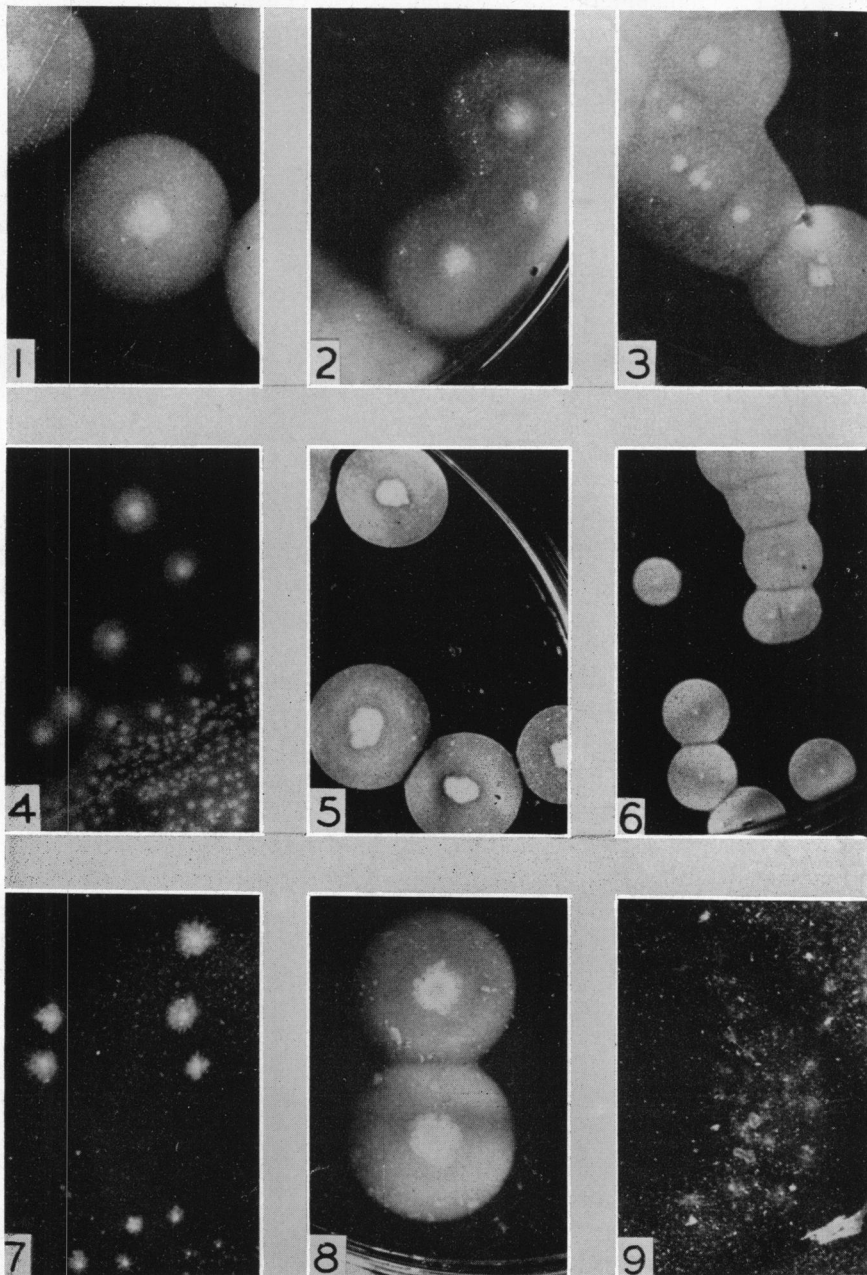
FIG. 1. COLONIES OF VARIOUS CLOSTRIDIA ON EGG YOLK AGAR

Magnification in all cases is approximately 3 ×.   The luster areas described in the text do not show. No. 1, *Clostridium perfringens* type A. No. 2, *Clostridium perfringens* type B. No. 3, *Clostridium perfringens* type C. No. 4, *Clostridium perfringens* type D. This illustrates the smaller zone of precipitation sometimes encountered with this species. No. 5, *Clostridium novyi* type A. No. 6, *Clostridium novyi* type B. No. 7, *Clostridium tertium*. No. 8, *Clostridium sordelli*. No. 9, *Clostridium tetani*.

intense than the remainder of the area, but the line of demarcation is not a sharp one. The reaction is given by all four toxin types (Oakley, 1943). Each of 50 strains which we have tested give the typical picture, although there is some variation in the size of the zone of precipitation.

Clostridium novyi (*C. oedematiens*) type A. The reactions were given by each of 61 strains classed as *C. novyi* by other reactions. Colonies are smooth, with irregular edges, and show a precipitation zone under the colony and in a regular circle to a radial distance of 4 mm (figure 1, number 5). The precipitation is more intense than with *C. perfringens*, and the edge of the zone is sharply defined. The characteristic feature is an irridescent luster area, marked by radial linear striations, covering the colony and extending beyond over the surface of the agar in a regular circular zone to a radial distance of about 2 mm, only partially covering the precipitation zone. After a further period, an additional zone of intense luster appears, which is banded by a less intense area. Additional concentric zones of precipitation may appear around the original. In three cultures a slightly different type of reaction was observed immediately after the plates were removed from the anaerobic environment. With these, the colony is more or less rough and larger, and the luster zone is narrow and follows the contour of the colony. The radial striations are less marked. The precipitation extends beyond the luster in a regular circle. After an additional period, the reaction is similar to that described for the others.

Clostridium novyi (*C. oedematiens*) type B. Small irregular, transparent colonies produce a wide (8-mm) regular circle of precipitation under and beyond the colony. The edge of the zone is sharply defined (figure 1, number 6). No luster is evident immediately or later. After the plates have been exposed for several hours, a larger zone of precipitation is present, with the original zone outlined by a heavy ring of precipitation. Eight strains were tested, and all gave the reaction described.

The Clostridium sordelli–Clostridium bifermentans group. Small-to-medium-sized colonies, which are slightly raised and shiny and have rough edges, produce no luster but a wide zone of precipitation (figure 1, number 8). The reaction is practically indistinguishable from that of *C. perfringens*. Later, one or more additional zones of precipitation may appear beyond the original zone. These reactions were given by 36 strains. It is of importance that the same reaction is given by the nontoxic *C. bifermentans* type as by the toxic *C. sordelli* type. This and other evidence obtained in collaboration with Helen Michael indicates that the production of a precipitate from egg yolk does not parallel the production of the lethal toxin by *C. sordelli*.

Clostridium hemolyticum. This species gives a punctiform colony with a wide area of intense precipitation surrounding the colony.

Clostridium sporogenes. The precipitate is deposited under the colony and rarely spreads beyond (figure 2, number 12). A slight luster covers the colony but does not extend beyond. In the usual type of colony the rhizoids may extend beyond the luster and precipitate. After an additional period, the edge of the precipitate is marked by a zone of more dense precipitation. In some

cases there is a slight clearing of the medium in a narrow band beyond the colony edges; this may be surrounded by a zone of faint precipitation.   This reaction
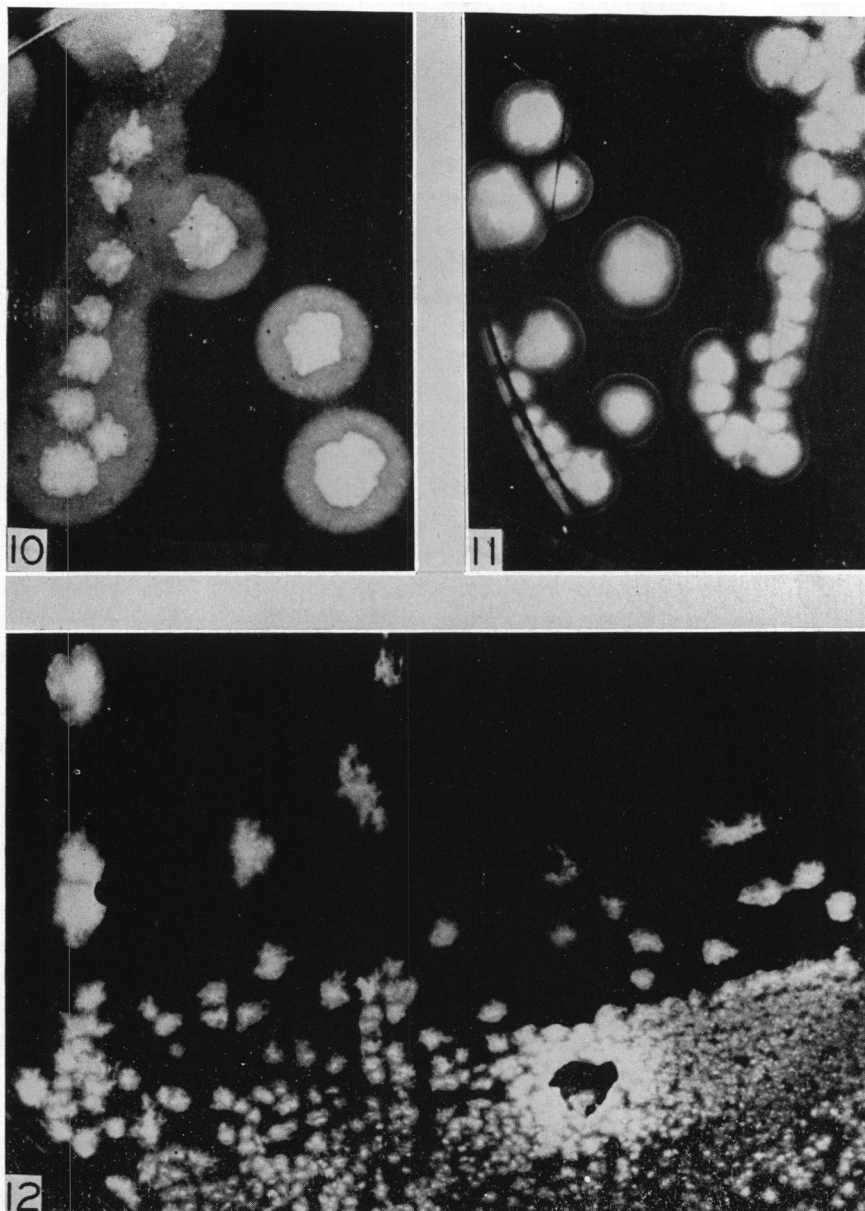

FIG. 2

No. 10, *Clostridium botulinum* types C, D, or E.   No. 11, *Clostridium parabotulinum* type A or B.   No. 12, *Clostridium sporogenes*.

has been given by approximately 100 strains.   A slight variation from this has been noted with approximately 50 strains for which a tentative designation

to this species has been made on other grounds. In these, the colony is more regular, and the luster and the precipitate do not extend beyond the colony. In three cultures, which may, however, not be *C. sporogenes*, we have observed no reaction.

Clostridium parabotulinum types A and B (Bengston, 1924, terminology). The raised, irregular-edged colonies are covered with a luster which extends in a regular circle slightly beyond the colony edge. An area of precipitation lies under the colony and to the edge of the luster zone (figure 2, number 11). A slight clearing of the medium beyond the precipitation may be noted. Although the radial striations are present, they are not so marked as in the *C. novyi* reaction. At a later period, there may be a slight precipitate beyond the luster zone, but this is indistinct. The reaction is given by nontoxic strains, identifiable by physiological and agglutination reactions, as well as by the toxic cultures.

Clostridium botulinum type B. The colonies are flat and spreading with irregular edges. The reaction is essentially the same as for *C. parabotulinum* although the reaction zones tend to be wider. Three strains have been studied.

Clostridium botulinum types C, D, and E. In these cultures the flat, irregular-edged colonies are surrounded by a wide zone of precipitation as shown in figure 2, number 10. There is also a narrow luster zone which follows the contour of the colony edge. The regular circle of precipitation extends well beyond the luster. The edge of the precipitation is not so sharply defined as with *C. novyi*. Later, an additional luster zone appears and somewhat indistinct zones of precipitation may be formed. These reactions were given by two strains of type C and one strain each of types D and E.

Other species. Strains of the following species of *Clostridium* have failed to show a luster, a zone or precipitation, or any other identifying reaction on the medium other than the usual colonial morphology: *C. tetani* (figure 1, number 9), *C. septicum* and *C. tertium* (figure 1, number 7), *C. histolyticum, C. capitovalis, C. chauvoei, C. cochlearium, C. butyricum* (physiologically closely allied to *C. perfringens*), and *C. acetobutylicum.* For the latter two organisms the glucose concentration in the medium was increased to 1 per cent. Crook (1942), using the tube reaction, obtained negative results with *C. septicum, C. histolyticum,* and *C. tetani,* and also with one strain of *C. botulinum.* With the latter species (*C. parabotulinum* of the American literature) we have obtained positive reactions with the plate technique but negative results with the tube method. Also, although Crook reported positive results with *C. chauvoei,* we have found this organism to be negative in the plate test.

We have obtained positive results with various aerobic organisms, particularly *Actinomyces, Aspergillus,* and certain members of the genus *Bacillus.* In the *Bacillus* group positive results (precipitation but no luster) were obtained with strains designated *B. lacticola, B. tumifaciens, B. ellenbachensis, B. megatherium, B. cereus, B. mycoides,* and several unidentified cultures which appeared as contaminants, but negative results with *B. graveolens, B. subtilis, B. circulans, B. brevis, B. anthracis, B. ruminatus, B. rotans, B. alvei, B. globigii, B. aterrimus,* and *B. silvaticus.*

Inhibition of Reactions by Antiserum

It appears that no statements concerning this question will be of value until more information is available based upon the use of sera of known antilecithinase content. Commercially prepared antitoxin should not be used, without assay, to inhibit the reaction of a newly isolated strain in the divided plate technique in which one-half of the plate is spread with antiserum to inhibit the characteristic reaction (Hayward, 1943, 1945; War Wounds Committee, 1943). The antilecithinase properties of a serum may not bear a positive correlation to the antilethal value, and it is presumed that the latter value has been the one considered in the standardization of antitoxic sera. Certain samples of commercial sera seem to be almost completely lacking in antilecithinase properties. This may explain the failure of antitoxin inhibition of the *C. oedematiens* reaction on blood-egg-yolk agar reported by Nagler (1944, 1945). Also, as Hayward (1943) has pointed out, the antitoxin of *C. perfringens* inhibits the reaction of the *C. sordelli–C. bifermentans* group. Our results confirm this and reveal evidence of other examples of this phenomenon.

Specificity of the Reaction

The LV reaction was originally thought to be specific for *C. perfringens*, though this concept has been dispelled by the results of Hayward (1941, 1943) and Crook (1942), and by the foregoing results. One may logically question the value of this reaction in view of the nonspecificity. It appears that the plate test, and under certain conditions (specific serum inhibitions or studies with pure cultures of known species designation) the tube reaction, may prove of considerable value. The reactions listed above have been obtained constantly and with a sufficient number (when available) of strains of the given species as to leave little doubt that they may be considered typical.

It is true that certain species give similar plate reactions—group I: *C. perfringens*, *C. sorelli–C. bifermentans*, *C. hemolyticum*, and *C. novyi* type B; group II: *C. parabotulinum* types A and B and *C. botulinum* type B; and group III: *C. novyi* type A and types C, D, and E of *C. botulinum*. This does not, in our opinion, destroy the value of the reaction; because if the species designation of an unknown strain is narrowed to the members of a given group, the differentiation of the species comprising the group would be easy by physiological reactions and in some instances almost unnecessary if the origin of the strain in question is known. In addition, the following characters on the plate medium are of value in the recognition of the named species. The reaction of *C. novyi* type B may be distinguished from others in group I by the clearly defined edge of the precipitation zone. On standing, the outer extra zone is separated from the original by a heavy narrow area of precipitate. The colony of *C. hemolyticum* is more raised, more regular, and smaller than those of the other species in relation to the reaction zone. The colonies of the *C. sordelli* group tend to be flatter and more transparent and irregular than the usual smooth type of *C. perfringens* colony. The reactions of *C. sordelli*, on the whole, seem to be wider than those of the nontoxic *C. bifermentans*, but, within the group, some strains of

C. sordelli give zones that are narrower than the widest zone of *C. bifermentans*. Within group II, the colonies of *C. botulinum* type B tend to be less raised and the reaction zones relatively wider than those of *C. parabotulinum*. The colonies of *C. botulinum* type B resemble those of the other *C. botulinum* types, even though the reaction is very much like that of *C. parabotulinum*. The luster area of the types C, D, and E of *C. botulinum* is narrow and follows the contour of the flat, irregular colony. In the typical *C. novyi* type A reaction, the luster area is wide and circular. A few strains of *C. novyi* showed narrower luster zones, which followed the colony edge and closely resembled the reactions of the *C. botulinum* types.

DISCUSSION

The egg yolk agar plate reaction would appear to be of considerable value as a presumptive species reaction in clinical and other laboratories. In the food research laboratory the differentiation of *C. sporogenes* and *C. parabotulinum* is difficult, and the reactions described above may be of great value in the early recognition of *C. parabotulinum* from samples involved in botulism. In the clinical laboratory it appears possible that the reaction will be of aid in the rapid identification of certain of the gas gangrene organisms. Although it is possible that the reactions may be elicited by colonies obtained by streaking directly from wound exudates, it may be necessary to enrich such samples in suitable liquid media such as Brewer's thioglycolate broth and to inoculate egg yolk agar plates after 4 to 6 hours of incubation.

ACKNOWLEDGMENT

SUMMARY

A peptone base medium and an egg yolk supplement are described for use in plate culture demonstration of the LV (lecithovitellin) or Nagler reaction. By use of this medium presumptive identification of the following is possible: *Clostridium perfringens* (*C. welchii*), *C. novyi* (*C. oedematiens*), *C. sordelli–C. bifermentans*, *C. hemolyticum*, *C. botulinum*, *C. parabotulinum*, and *C. sporogenes*.

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