A MODIFICATION OF THE KLIGLER LEAD ACETATE MEDIUM

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The development of a single medium which will afford a maximum amount of information in a minimum time has been the aim of bacteriologists for many years. This has been especially desirable for the differentiation of the colon-typhoid-dysentery group. The need for such a medium and the limited success of the proposed procedures is evidenced by the numerous and varied plating methods described for the isolation of typhoid and dysentery bacilli.

In 1911 Russell (1911) devised a differential medium for use in test tubes. This medium was composed of nutrient agar to which he added 1 per cent lactose, 0.1 per cent glucose and a sufficient amount of 5 per cent aqueous solution of litmus to give the medium a distinct purple violet color. The object of the Russell medium was to obtain a rapid differentiation between the lactose fermenting and non lactose fermenting gram negative bacilli. The introduction of this medium marked a distinct step in the advancement of our knowledge of the gram negative aerogenic bacilli. Today in most of the laboratories of this country it has been adapted, either in its original or modified form, as one of the standard laboratory culture mediums.

In 1916 Kligler (1916) described a medium which gave even more information than did that of Russell. The essential differences between the two were that Kligler omitted the glucose, added 0.05 per cent lead acetate and substituted Andrade's indicator for the litmus in the Russell medium. At the conclusion of this article and in a subsequent one Kligler (1918) stated that

this medium might be successfully combined with the Russell double sugar tube. By the use of the Kligler medium, one is able not only to differentiate between the members of the colon and paratyphoid groups but also to differentiate, with a fair degree of accuracy, between paratyphoid A and B and between typhoid and dysentery strains. Soon after the appearance of Kligler's first article, Jordan and Victorson (1917) published the results of their work on the differential value of a lead acetate medium.

Kligler recommends the use either of meat infusion agar or beef extract agar as a basis for this medium, although he states that sharper reactions are, as a rule, obtained with the meat infusion agar. The agar is adjusted to pH 7.4 or made neutral to Andrade's indicator, 1 per cent by volume of this indicator is added, and the medium is tubed in 5 cc. quantities and sterilized. A lactose-glucose solution containing 20 per cent lactose and 2 per cent glucose is sterilized separately and 0.25 cc. added in a sterile manner to each tube. Both the lactose and the lead acetate solutions may be added before slanting and as soon as the agar is cooled to about 60°C. If this is not done the lead flocculates the pepton.

This medium has been used fairly extensively and has proven in many ways to be valuable for differential purposes. It has not, however, received as widespread use for teaching purposes and in public health laboratories as its usefulness warrants; and in all probability this is due to the time consumed in its preparation and the difficulty in obtaining a clear medium. The investigations herein reported were made in an attempt to overcome these disadvantages.

Experiments were made with regard to (1) various indicators; (2) the optimum temperature for mixing the ingredients of the medium and (3) the best method for its sterilization.

INDICATORS

The question of a more suitable indicator has been taken up by Wight (1925) who reported the use of multiple indicators in the preparation of his "Rainbow Medium." We tried this

modification and found it to be only slightly more valuable in our hands than the original Kligler medium with the Andrade indicator.

We then tested the following indicators singly and in various combinations: Andrade's, phenol red, brom thymol blue and thymol blue. The following formula was used throughout as a basis for the medium.

Bacto-beef extract	5 grams
Pepton (P.D.)	10 grams
Sodium chloride (B. & A.)	5 grams
Agar shreds	15 grams
Tap water	1000 cc.

The agar was first thoroughly washed in running water and was then heated in the required amount of water, until it was dissolved, after which the other ingredients were added. The reaction was adjusted to pH 7.4 and the medium was boiled for from five to eight minutes. At the end of the heating, the reaction was readjusted to pH 7.4 and the sediment allowed to settle to the bottom of the container. The clear supernatant agar was then decanted into another container and was accurately divided into quantities of 100 cc. and sterilized by heating at 10 pounds pressure for twenty minutes.

To each of five flasks containing 100 cc. of the above sterile medium were added 1 gm. of lactose, 0.1 gram of glucose, and 0.05 gram of lead acetate ($Pb(C_2H_3O_2)_2$ Baker's analyzed), the medium having been cooled previously to 50°C. Indicators were then added in the following proportions:

		cc.
Flask 1.	Andrade's indicator	1
Flask 2.	0.02 per cent aqueous solution phenol red	5
Flask 3.	0.04 per cent aqueous solution thymol blue	5
Flask 4.	0.02 per cent aqueous solution phenol red	5
	0.02 per cent aqueous solution brom thymol blue	5
Flask 5.	0.02 per cent aqueous solution phenol red	5
	0.02 per cent aqueous solution brom thymol blue	5
	Andrade's indicator	1

Batches of the above samples were tubed and sterilized at 3 pounds pressure for fifteen minutes, slanted and cooled. This

amount of heat proved sufficient for complete sterilization. Tubes representing the five flasks of medium were then inoculated with different strains of *B. typhosus; B. dysenteriae* Shiga; *B. dysenteriae* Hiss-Russell; *B. dysenteriae* Flexner; *B. paratyphosus* A; *B. paratyphosus* B; *B. aertrycke; B. enteritidis* (Gaertner); *B. typhi-murium; B. morgani; B. proteus; B. fecalis-alkaligenes; B. coli-communis; B. Friedlander; B. acidi-lactici* and *B. lactis-aerogenes.* These tubes were incubated at 37.5°C. and readings were made at the end of twenty-four hours and again at the end of forty-eight hours.

Phenol red used alone proved to be the most satisfactory indicator for the change in pH of the medium and interfered least in the reading of the lead sulphide reaction. The development of acidity produced a canary yellow color, alkalinity a pink, and neutrality no change from the original color of the medium. Organisms producing hydrogen sulphide showed browning of the surface which usually extended along the line of the stab. With this indicator increased acidity caused the yellow color to become lighter in intensity and the browning was more easily distinguished; whereas with the Andrade indicator increased acidity produced a more intense red and obscured somewhat the lead sulphide reaction. This improvement was strikingly demonstrated with cultures of *B. typhosus* which gave a red or acid butt and an unchanged slant with Andrade indicator and a yellow or acid butt and a pink or alkaline slant with phenol red.

Kligler in referring to the work of Sacquépée and Chevrel (1905) and of Burnet and Weissenbach (1915) on the use of lead acetate for differentiation said, "Neither of these authors say anything about the nature of the lead acetate reaction. At first thought one would expect it to be a reaction between the H_2S liberated and the lead. If that were so, sugar exerting a sparing effect on the utilization of pepton, should inhibit or completely interfere with the reaction. This was found not to be the case. The experiments indicated that the reaction involved is probably a reduction of the lead acetate to lead oxide (PbO_2) which is brown." However he did not state the nature of the experiments by which he reached this conclusion. It was our belief that the reaction was

due to the formation of lead sulphide. To determine this point, strips of white filter paper saturated with lead acetate solution were suspended inside the upper end of the tubes inoculated with *B. typhosus* and the other strains of organisms mentioned above. In every case cultures which showed browning of the lead acetate medium also showed browning of the lead acetate paper, whereas cultures causing no change in the medium produced no change in the paper. The positive papers, when tested chemically, gave positive reactions for lead sulphide and negative reactions for lead oxide (Prescott and Johnson, 1918). We, therefore, believe the browning in both instances to be due to the same process, i.e., the production of hydrogen sulphide by the growing organisms and its interaction with the lead acetate to form lead sulphide.

PREPARATION AND STERILIZATION

The other two points of interest in the problem, namely, the optimum temperature for mixing the ingredients of the medium and the most satisfactory method for sterilizing it can be considered in the same general discussion. It is well known that the indicators and the carbohydrates can be added to a medium at any temperature, without producing unfavorable results. Kligler found that his best results were obtained by adding separately the carbohydrates and lead acetate solution to agar which had been tubed, sterilized and cooled to about 60°C. It is obvious that if it were possible to mix all of the ingredients of the medium in a flask, then tube and sterilize it, one would have a valuable differential medium which could be prepared as easily and simply as extract agar. With this object in view, experiments were carried out as follows:

In each of three flasks were placed 500 cc. of the melted nutrient agar containing 5 grams lactose, 0.5 gram glucose and 25 cc. of 0.02 per cent solution phenol red, prepared according to the formula given above. To each of these flasks 0.25 gram of lead acetate, either crystalline or in solution, was added at the following temperatures and the corresponding changes noted.

Flask No. 1. Temperature 100°C. Distinct flocculation.
Flask No. 2. Temperature 75°C. Slight flocculation.
Flask No. 3. Temperature 50°C. No flocculation.

The medium in each of these flasks was then tubed through sterile funnels into sterile tubes in 5 cc. amounts and sterilized in the following manner. One-third of the tubes from each of the flasks was heated in the autoclave at 5 pounds pressure for twenty minutes; one-third at 10 pounds pressure for fifteen minutes and the remaining third at 15 pounds pressure for fifteen minutes. All tubes were slanted with butts sufficiently deep for making good stab inoculations. It was noted that sterilization was complete in each instance and had not increased the turbidity of the medium.

The various sets of tubes were then inoculated with each of the 16 strains of organisms used in our other experiments, incubated for twenty-four hours, and the reactions in all tests could be read with a fair degree of accuracy. However, the most satisfactory reactions were obtained in those tubes to which the lead acetate had been added at 50°C., irrespective of the temperature used for sterilization.

SUMMARY

The essential points noted by us in the foregoing experiments are:

1. Phenol red gives more satisfactory results as an indicator than Andrade's indicator which is used in the Kligler medium.

2. It is not necessary to add the sterile lead acetate to melted sterile agar tubes, but all of the ingredients of the medium can be mixed in bulk at a temperature of 50°C. or lower thus preventing flocculation. The lead acetate can be added either in crystals or in solution. The medium can then be tubed and sterilized by heating in the autoclave at 5 pounds pressure for twenty minutes.

3. No damage occurs and no flocculation takes place, even though sterilization be accomplished by heating at 15 pounds pressure for fifteen minutes.

4. The browning of the lead acetate medium is due to the for-

mation of lead sulphide and in the absence of this medium, the production of H_2S by organisms may be determined by suspending in the neck of the culture tubes strips of white filter paper saturated with lead acetate solution.

5. The preparation of the medium thus simplified makes it thoroughly practical for use in teaching laboratories and in public health diagnostic laboratories.

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