A COMPARISON OF LEAD, BISMUTH, AND IRON AS DETECTORS OF HYDROGEN SULPHIDE PRODUCED BY BACTERIA

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Received for publication January 10, 1934

Several methods have been used to detect hydrogen sulphide produced by bacteria. Filter papers impregnated with lead carbonate or lead acetate have been utilized ever since Gayon (1877) demonstrated the formation of hydrogen sulphide from albuminous material by bacterial action, and Schardinger (1894) applied the test to the sanitary examination of water. Sasaki and Otsuka (1912), and Tanner (1917) treated the lead acetate papers with glycerol. The addition of lead acetate or lead carbonate to the nutrient media has been especially advocated by Kligler (1917), Jordan and Victorson (1917), Thompson (1921), Bailey and Lacy (1927), and others. Wilson (1923) proposed the use of ferric chloride media to detect sulphides. After trying nickel, iron, lead, and manganese salts Levine and coworkers (1932) concluded that ferric citrate was one of the best indicators. Pacheco and Mello (1932) recommended bismuth carbonate agar which they found to be more sensitive and less toxic than iron or lead media.

Because difficulty has been encountered in testing a large number of recently isolated bacteria for their hydrogen sulphide producing properties as an aid in characterizing them for identification, and because the Committee on the Pure Culture Study of Bacteria (1933) is not ready to recommend any one method for such determination the following studies were undertaken.

EXPERIMENTAL

A comparison of different salts of bismuth, iron, lead, nickel, manganese, copper and tin showed that only the first three merited

further consideration as indicators of small quantities of hydrogen sulphide in nutrient agar. The sensitivity of these minerals for detecting the presence of sulphide was tested in a medium consisting of 30 grams Bacto-peptone, 2.0 grams beef extract, 2.0 grams sodium chloride, 1.0 gram dibasic potassium phosphate, and 15 grams agar per liter. Just prior to the test various amounts of sodium sulphide were added to tubes of the molten media at 45°C. together with the mineral salts. The results were read after the media had cooled and solidified.

It was found that the smallest quantity of sulphide indicated by bismuth salts is 0.25 millimol sulphide S, 0.2 millimol by lead salts, and 0.1 millimol sulphide S by ferrous iron compounds. The minimum concentration of these cations required to produce a perceptible coloration in media containing 0.5 millimol sulphide S is 0.1 millimol ferrous iron, 0.2 millimol lead, or 0.5 millimol bismuth. Twice this concentration of each cation gives more satisfactory contrasts. Although, chemically, ferric iron is a poor sulphide indicator it is considered because, regardless of whether small quantities of ferric or ferrous iron are added to nutrient media, following heat sterilization and the growth of microörganisms both forms of iron will be present in concentrations which vary with the oxidation-reduction potential of the substrata.

The effect of these minerals on the multiplication of microörganisms was tested in the medium described above. After the addition of different quantities of iron, bismuth and lead salts, the reaction was adjusted to pH 7.2. The media were used to plate appropriate dilutions of sewage effluent. Table 1 shows the number of bacteria which developed from comparable quantities of sewage.

Not only was there a diminution in the number of colonies which developed on the media containing the higher concentrations of iron, lead, and bismuth but there was also a decided decrease in the size of the colonies which did appear. From the data of several experiments similar to those presented in table 1 it was found that, in general, concentrations of iron up to 4.0 millimols ferric iron or 2.0 millimols ferrous iron did not inhibit the multiplication of sewage bacteria. In fact, small quantities

of iron stimulated bacterial growth. It made little difference whether the chloride, sulphate, citrate or acetate of iron was used, provided the H-ion concentration was properly adjusted.

As little as 0.2 millimol lead retarded the growth of some sewage bacteria (see table 2). Less than this quantity of lead was not tried because it had previously been demonstrated that at least

TABLE 1

Number of bacteria from a sample of diluted sewage which developed on media containing various concentrations of iron, lead, or bismuth

MILLIMOLS CATION	FERROUS CHLORIDE	FERRIC CHLORIDE	LEAD ACETATE	BISMUTH SUBCARBONATE
None	197	197	197	197
0.2	194	200	181	184
0.5	203	192	103	161
1.0	212	228	76	126
2.0	216	206	21	88
4.0	170	219	3	52
7.5	38	124	0	17
10.0	0	75	0	0

TABLE 2

Number of sewage bacteria developing on media containing varying quantities of lead acetate expressed as millimols lead

SEWAGE SAMPLE NUMBER	MILLIMOLS LEAD					
	None	0.2	0.5	1.0	2.0	4.0
14	236	206	158	103	32	2
22	304	272	196	114	26	8
23	43	34	19	9	0	0
26	128	83	74	41	5	2
27	260	198	164	122	19	0
28	87	70	53	46	11	3

0.2 millimol was required to detect sulphides in peptone media. The toxicity of the lead was increased when the concentration of peptone was decreased. The presence of either glucose or lactose had no apparent effect on the bacteriostatic action of the lead. Equivalent quantitities of lead were equally toxic regardless of whether the carbonate, basic acetate or neutral acetate was used.

Since bismuth salts are insoluble, media which contained either

the subcarbonate or nitrate of this mineral were chalky, making it difficult to count the colonies which grew on such media. However, it was obvious that 1.0 millimol bismuth retarded colony development and that 4.0 millimols inhibited the growth of over half of the sewage bacteria. Similar effects were observed from the use of equi-molar quantities of bismuth as either the subcarbonate or nitrate in the lower concentrations, but the nitrate was more toxic when concentrations in excess of 4.0 millimols bismuth were present.

Next, the effect of iron, lead, and bismuth salts on the multiplication of several pure cultures was investigated. The latter consisted of 20 common bacteria including members of the colon-aerogenes group, spore-forming rods and cocci, all of which had been maintained in the laboratory for several years, and a similar number of very recently isolated marine bacteria. Media similar to those described above were poured into Petri dishes and the plates were divided into eight sectors with a wax pencil. The sectors were uniformly streaked with a standard loopful of the test organisms. Suspensions of the organisms were prepared by emulsifying a loopful of a twenty-four-hour-old agar slant culture in 5.0 cc. physiological saline solution. Thus, each medium was seeded with approximately the same number of cells. The inoculated plates were incubated at 30°C. and examined for comparative growth after twenty-four hours. Table 3 illustrates the kind of record which was kept.

To summarize briefly the results, it was found that the recently isolated bacteria were much more sensitive to lead, iron, and bismuth salts than were the old laboratory cultures. Although their growth was definitely retarded most of the former multiplied to some extent on 0.2 millimolar lead media. Some of the recently isolated cultures failed to grow on 0.5 millimolar lead media, less than half of them developed in the presence of 1.0 millimol lead even after four days, and none of these young strains tolerated 2.0 millimols lead. The growth of a few of the old stock cultures, lightly inoculated as in this experiment, was retarded by 0.2 millimolar lead media, but all of them multiplied after four days on 0.5 millimolar lead media. The majority of the

old strains did not multiply in the presence of 2.0 millimols lead. These findings corroborate the results obtained from plating sewage bacteria and warrant the conclusion that while most bacteria will grow on media containing 0.5 millimol lead, the multiplication of many of them is abnormal. For comparison, 0.5 millimol lead is approximately equivalent to 0.02 per cent lead acetate [$Pb(CH_3COO)_2 \cdot 3H_2O$].

Concentrations of ferric or ferrous iron up to 2.0 millimols seemed to accelerate bacterial multiplication and all of the pure cultures tolerated 4.0 millimols of these cations. Most of the

TABLE 3

Comparative growth of bacteria after twenty-four hours' incubation on media containing varying quantities of lead as lead acetate

TEST ORGANISM	MILLIMOLS LEAD					
	None	0.2	0.5	1.0	2.0	4.0
Bact. paratyphosus (pigeon)	xxxx	xxxx	xx	x	—	—
Bact. aertrycke	xxxx	xxxx	xxxx	xx	x	—
Bact. coli-communis	xxxx	xxxx	xxxx	xxx	x	x
Strept. fecalis	xxxx	xx	xx	—	—	—
Staph. albus	xxxx	xxx	x	—	—	—
Sarcina lutea	xxxx	xxxx	xxxx	xx	x	—
Micr. tetragenus	xxxx	xxx	—	—	—	—
B. megatherium	xxxx	xxx	xx	x	—	—
Marine 501	xxxx	—	—	—	—	—
Marine 516	xxxx	xxx	x	—	—	—
Marine 534	xxxx	xx	x	—	—	—
Marine 568	xxxx	x	x	—	—	—

cultures grew in the presence of 7.5 millimols iron. The development of none of the pure cultures was inhibited by 1.0 millimol bismuth, but the growth of some was retarded by twice this amount.

Although the addition of iron, lead, and bismuth salts to appropriate media have all been found to detect, with limitations, the hydrogen sulphide produced by some bacteria, such tests are not applicable to certain pigmented bacteria and others which normally darken the media because this coloration masks the color of the metallic sulphides which may be formed. Such microör-

ganisms are not uncommon. Under these conditions the use of filter paper strips impregnated with the proper mineral salt is desirable. A comparison of different salts of manganese, tin, copper, iron, lead, bismuth, silver, mercury, and nickel, proved that while papers impregnated with some of these substances indicate the presence of hydrogen sulphide, lead acetate papers are superior, being more specific and more sensitive. In practice, strips of white filter paper measuring 5 by 50 mm. were soaked in a saturated solution of lead acetate, steam-sterilized in plugged tubes and dried in an oven at 120°C.

Sensitivity tests showed that lead acetate papers detect as little as 0.01 millimol sulphide S in peptone media. It will be recalled that at least 10 times this concentration of sulphide was required to darken perceptibly similar media containing the most efficacious mineral indicator, viz., ferrous iron. When much meat extract and other colored ingredients were present in the media, the lead acetate papers were found to be from 10 to 100 times (depending upon the coloration and reaction of the medium) more sensitive than iron, lead, or bismuth media for detecting traces of hydrogen sulphide. Treating lead acetate papers with glycerol to increase the hydroscopicity as practiced by some investigators has not been found to improve their usefulness. The importance of extreme sensitivity of the test for hydrogen sulphide production will be appreciated when one notes the small quantity of reduceable sulphur available in most peptones, and the minute amount of hydrogen sulphide produced by some bacteria, even from an adequate source. We have found Bacto-tryptone to be more suitable for the test than any other commercial peptone tried from a viewpoint of available sulphur content, growth-promoting properties, and uniformity of results.

CONCLUSIONS

There is a very narrow margin of safety between the concentration of lead salt in media which is required to detect sulphides and that which inhibits the multiplication of bacteria. While there is a vast difference in the lead-sensitivity of different bacteria, for those herein considered the ratio of the required con-

centration of lead to the average inhibitive concentration is 1:2. There is a larger margin of safety for certain lead-tolerant bacteria and particularly for old strains adapted to laboratory culture, but on the other hand, some lead-sensitive bacteria are inhibited by the least amount of lead acetate which is required to give a distinct color change with sulphides. In classifying 434 representatives of the Brucella group according to their ability to produce hydrogen sulphide ZoBell and Meyer (1932) found both lead acetate and lead carbonate to be entirely unsuitable as indicators because as little as 0.2 millimol lead inhibited the multiplication of some of these microörganisms. There was so much difference in the lead tolerance of different Brucellas that their cultivation on lead acetate agar became a differential test for their ability to tolerate lead rather than a test for their ability to produce hydrogen sulphide.

The average ratio of the required concentration of ferrous or ferric iron in nutrient media to the inhibitive concentration is about 1:40. Since the chalky appearance of bismuth media obscures the signs of bacterial multiplication, it is more difficult to estimate the ratio of the required to the inhibitive concentration, but it is about 1:5. Though comparative rather than absolute, these ratios indicate the superiority of iron salts as sulphide indicators. In practice we have found iron media much more satisfactory than either lead or bismuth. The use of 0.05 per cent ferric ammonium citrate or 0.03 per cent ferrous acetate is recommended in media containing at least 3.0 per cent peptone.

However, for testing new and unknown pure cultures of bacteria for their hydrogen-sulphide-producing properties as a means of characterizing them for identification and classification, the time-honored lead acetate paper method seems even better than ferrous iron media. Papers are more quantitative since the blackened area of the papers can be more readily estimated than the degree of darkening of iron media. Redfield (1912) states that "it is possible with a fair degree of accuracy to determine the equivalent of hydrogen sulphide for each millimeter of blackening of lead acetate paper strips." For recording the actual results the exposed lead acetate papers can be labeled, mounted in series and photographed or filed for future reference.

In advocating lead acetate papers, cognizance is taken of the wide application of the use of differential media containing lead, iron, or bismuth for such special tests as distinguishing between members of the typhoid-paratyphoid, colon-aerogenes or other special groups of bacteria. It is not our intention to criticize these tests because they have already proved their usefulness. Furthermore for studying the sulphur metabolism of microörganisms, there is no substitute for quantitative procedures such as those described by Almy and James (1926) and others.

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