RAPID METHODS FOR THE DETECTION OF GELATIN HYDROLYSIS[1, 2]

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The need for quicker methods of identifying bacterial cultures is reflected in the large number of rapid biochemical methods that have been described in recent years. Procedures have varied widely although the principles have usually been the same, namely, the use of large inocula of young organisms, small amounts of media, ideal incubation conditions, and sensitive tests for changes that have occurred in the substrates. This research on methods of detecting gelatin hydrolysis was done to evaluate and improve rapid methods. The aims have been to select reliable methods and thereby save the time of the laboratory worker, and to devise micromethods for detecting changes in the substrate in order to conserve materials.

MATERIALS AND METHODS

Three basic procedures for detecting hydrolysis of gelatin have been investigated: (a) Testing with ninhydrin for amino acids produced during hydrolysis (Levinson and Sevag, 1954); (b) precipitating unhydrolyzed gelatin in an agar-gelatin medium so as to show hydrolysis by the presence of a cleared area around the bacterial cultures. Acid mercuric chloride ($HgCl_2$, 15 g; concentrated HCl, 20 ml; distilled water, 100 ml) and 1.0 per cent solution of tannic acid (Frazier, 1926), and 20 per cent sulfosalycilic acid (Chapman, 1952) were tested as precipitating agents. Also, a method of incorporating ammonium sulfate and sodium chloride in the medium (Chapman, 1948) was tried. (c) The liquefaction of discs of formalin-denatured gelatin (Kohn, 1953). Various modifications of these procedures have been tried. Details of the most useful ones will be given in the section on Results.

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The various procedures have been tested with a group of cultures selected to represent both rapid and slow gelatin liquefiers. The cultures of Enterobacteriaceae used were received from the Communicable Disease Center, Chamblee, Georgia.

RESULTS

Ninhydrin method. The best results were obtained with a 1.5 per cent gelatin medium containing 10 ppm of manganese sulfate. The medium was dispensed in 13 by 100 mm tubes and carefully sterilized to avoid overheating. Tubes of sterile medium were preheated in a 37 C water bath and then inoculated with 0.1-ml amounts of cell suspensions prepared by carefully harvesting the growth from 4-hr nutrient agar slant cultures in 1.5-ml amounts of physiological saline. It is necessary to avoid large clumps of organisms and particles of agar. After incubation periods of 2 to 24 hr in the water bath, gelatin hydrolysis may be determined by adding 0.5 ml of 0.1 per cent aqueous ninhydrin solution (freshly prepared or stored in the refrigerator for not more than 2 weeks) and heating the tube in an 80 C water bath for up to 3 hr. The appearance of a bluish-purple color indicates hydrolysis.

Positive tests have been obtained consistently with gelatin-hydrolyzing cultures but it has not been possible to avoid some false positive reactions. Although saline plus cell suspensions, and uninoculated gelatin medium gave consistently negative results, occasional cultures of non-gelatin-hydrolyzing organisms in the gelatin medium gave positive results. This may be explained by the extreme sensitivity or the lack of specificity of the ninhydrin reagent. These false positive reactions limit the usefulness of the procedure to that of a screening test.

Methods using gelatin-precipitating agents. Studies of the effects of varying different factors led to the development of four procedures that have given useful results. These procedures incorporate features of methods used by Frazier (1926), Chapman (1948, 1952), Oakley *et al.*

(1948), Clarke and Cowan (1952), and Clarke (1953).

(1) Plate modification of Frazier (1926) method:—Plates are poured with 15-ml quantities of medium (tryptose, 20 g; beef extract, 3 g; agar, 15 g; $MnSO_4$, 10 ppm; gelatin, 10 g; distilled water, 1000 ml; pH 7.0). The plates are dried overnight under Coors porcelain tops and are then spot-inoculated with 3-mm loopfuls of actively growing cultures on tryptose-beef extract agar slants. After suitable periods of incubation (at 37 C in this study), which may vary from 1 to 96 hr with different strains of organisms, the plates are developed by wiping off the site of inoculation with a cotton swab soaked in the acid mercuric chloride indicator of Frazier (1926) or 20 per cent sulfosalycylic acid (Chapman, 1952). Plates that are to be incubated for over 48 hr should have snugly-fitting sterile filter paper discs placed inside the tops of the plates and, after 48 hr, the discs should be moistened daily with sterile distilled water.

Inoculation of the plates from liquid-medium cultures or cell suspensions may lead to "halos" around the areas of inoculation due to reduction of the gelatin content of the medium from dilution with the liquid. These must not be confused with clearing due to gelatin hydrolysis.

Adjustment of the medium to pH 8.0 gave better results with a strain of *Staphylococcus aureus* but poorer results with some other organisms.

Manganese sulfate was added to the medium after it had been shown to give more rapid results, as suggested by the work of Levinson and Sevag (1954). The addition of cysteine or the substitution of cysteine and iron for the manganese, as suggested by the works of Weil and Kochalty (1937) and Kochalty *et al.* (1938), did not result in a more rapid test. Likewise, the addition of 0.01 M calcium sulfate or its substitution for the manganese sulfate as suggested by the work of Lautrop (1956*b*), did not result in a more rapid test.

(2) Tube modification of the Frazier (1926) method:—The same medium is used as in the plate modification. It is dispensed in 1.0-ml amounts in 10 by 75 mm tubes. The tubes are preheated in a 37 C water bath and then inoculated by layering 0.2-ml amounts of dense cell suspensions on the surface. The cell suspensions are prepared by harvesting the growths from 4.0- to 4.5-hr slant cultures on tryptose-beef extract agar in 1.0-ml amounts of saline. After suitable periods of incubation in the water bath, 2 or 3 drops of the acid mercuric chloride "developer" are added to each tube to be tested. A clear area below the meniscus is indicative of gelatin hydrolysis. A slightly lighter area may result from reduction of the gelatin content at the surface of the medium by dilution with the liquid of the inoculum. To avoid misinterpretation, a control tube to which 0.2 ml of sterile saline has been added should be used. When the tubes are to be incubated beyond 48 hr, 0.1-ml quantities of saline should be added daily, after 48 hr, or the tubes should be closed with stoppers or screw caps.

If 0.1 per cent cysteine is added to the medium and lead acetate papers are inserted into the mouths of the tubes, hydrogen sulfide production can be detected in 1 to 8 hr without interference with the demonstration of gelatin hydrolysis.

(3) Plate method using an ammonium sulfate-sodium chloride medium:—In studies with staphylococci, Chapman (1948) avoided the use of a developing solution by incorporating ammonium sulfate along with the sodium chloride in Stone's medium. The medium was cloudy and clear areas developed around the colonies of gelatin utilizers. By changing the basic medium and the proportion of ammonium sulfate and sodium chloride, we have been able to adapt the principle of this medium for use with organisms other than staphylococci.

The medium contains: gelatin, 5 g; agar, 10 g; NaCl, 9.0 g; $MnSO_4$, 10 ppm; $(NH_4)_2 SO_4$, 120 g; and distilled water, 1000 ml. The gelatin, NaCl, $MnSO_4$, and agar are first put into solution by heating. The ammonium sulfate is added, whereupon the medium becomes opaque-white, due to the precipitated gelatin. The pH of the medium is adjusted to 7.0 and it is dispensed into tubes in 11- to 12-ml amounts. It is sterilized by autoclaving for 10 min at 10 pounds pressure. In pouring the plates the agar should be decanted from any heavy precipitate that may have formed in the bottoms of the tubes during autoclaving.

The plates were inoculated as were the plates with the modified Frazier method. Apparently, hydrolysis of the gelatin is the result of preformed enzymes since the cultures fail to grow on the medium. Peculiarly, the addition of tryptose and beef extract to the medium produced a medium that gave some false negative results.

(4) Tube method using ammonium sulfate-sodium chloride medium:—The same medium is used as in the plate modification. It is dispensed

TABLE 1

Hydrolysis of gelatin as demonstrated by methods using gelatin-precipitating agents

Organism	Hydrolysis Time					
	Standard test at 20 C; stab method		Modified Frazier method		Test with $(NH_4)_2SO_4$-NaCl medium	
	Plain gelatin	Nutrient gelatin	Plate method	Tube method	Plate method	Tube method
	hr		*hr*		*hr*	
Salmonella abortus-bovis	48	48	1	4	(−96)	5
S. schleissheim	48	48	1	2	5.5	5
S. texas	48	48	1	2	4.5	4.5
	weeks					
S. dar-es-salaam	8	3	24	72	24	8
S. riogrande	8	5	72	96	72	72
S. weslaco	8	6	72	96	96	48
	hr					
Pseudomonas sp.	48	48	1	2	3	3
Proteus sp.	48	48	1	2	5.5	5
	weeks					
Cloaca (*Aerobacter cloacae*)						
1	8	6	72	96	72	72
2	8	3	24	48	72	48
3	7	2	24	48	24	24
4	8	4	24	72	48	24
5	8	4	48	72	72	48
Arizona (*Paracolobactrum arizonae*)						
1	9	6	72	96	96	72
2	8	4	48	72	72	48
3	7	3	24	72	48	48
4	8	4	48	72	48	72
5	9	6	48	72	96	72

Results with *Salmonella dublin*, *S. london*, *S. newport*, *S. oranienburg*, *S. typhosa* and 5 strains of *Klebsiella* were negative after 3 months by the standard test (stab method) and after 96 hr by the other methods.

into tubes and inoculated as in the tube modification of the Frazier method. Positive results are indicated by clear areas which extend down the tube below the inoculum. Again a control is necessary to avoid misinterpretation of the "halo" effect and measures must be taken to prevent excessive dehydration of the medium when the tubes are incubated beyond 48 hr. The medium may be used for the simultaneous determination of hydrogen sulfide production if 0.03 per cent sodium thiosulfate or 0.1 per cent cysteine is added and lead acetate papers were placed in the mouths of the tubes.

The results of a typical experiment with representative slow and rapid gelatin liquefying cultures, using the four procedures that have been described, are given in table 1. These results have been repeated with only slight variations in times. It can be seen that all the procedures gave reliable results within 1 to 6 hr for the rapid-liquefying cultures and 24 to 96 hr for the slow liquefiers, except that the strain of *Salmonella abortus-bovis* gave negative results by the plate method with the ammonium sulfate-sodium chloride medium. These results are difficult to explain since the same culture hydrolyzed gelatin when the tube method was used with the same medium. The plate modification of the Frazier method and the tube method with the ammonium sulfate-sodium chloride medium gave the most rapid results. The former was more rapid with the rapid liquefiers but the times were about the same with the slow liquefiers.

In addition to the results that are shown in the table, all four procedures have given rapid and reliable results with one or more strains of *Staphy-*

TABLE 2

Hydrolysis of gelatin as demonstrated by Lautrop's modification of Kohn's method

Organism	Hydrolysis Time	
	Toluene-free suspensions	Toluene-treated suspensions
	hr	
Salmonella abortus-bovis	48	48
S. schleissheim	48	48
S. texas	18	36
S. dar-es-salaam	120	120
S. riogrande	120	120
S. weslaco	120	120
Pseudomonas sp.	2	8
Proteus sp.	6	36
Cloaca (*Aerobacter cloacae*)		
1	120	96
2	96	72
3	48	48
4	72	72
5	96	96
Arizona (*Paracolobactrum arizonae*)		
1	120	96
2	72	72
3	72	72
4	96	96
5	72	96

Results with *Salmonella dublin*, *S. london*, *S. newport*, *S. oranienburg*, *S. typhosa* and 5 strains of *Klebsiella* were negative in 168 hr.

lococcus aureus, *Serratia marcescens*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Vibrio comma*, *Vibrio metschnikovii*, *Bacillus anthracis*, *Bacillus megaterium*, *Bacillus cereus*, and *Bacillus subtilis*.

Methods using formalin-denatured gelatin. Using discs prepared as described by Kohn (1953), this method gave reliable results with nutrient agar slant and nutrient broth cultures. More rapid results were obtained with the slant cultures than with the broth cultures. Except with *Serratia marcescens*, results were obtained more rapidly with this procedure than with the routine stab culture procedures, but not nearly as rapidly as with the methods using gelatin-precipitating agents.

The Greene and Larks (1955) rapid modification of the Kohn method was modified by reducing the gelatin content of the discs to 5 per cent, which produced more rapid results. This method gave reliable results in from 1.5 to 18 hr with a group of rapid-liquefying cultures. In some cases it was as rapid as the methods with gelatin-precipitating agents. Strains of *Bacillus anthracis* could be distinguished from those of other liquefying bacilli by their requirement of 15 to 18 hr for the demonstration of gelatin hydrolysis as compared to 2 to 6 hr. This distinction could not be made with the slightly more rapid methods with gelatin-precipitating agents.

With the addition of 0.1 per cent cysteine and 10 ppm of manganese sulfate to the peptone water and the placing of lead acetate papers in the tubes, it has been possible to detect hydrogen sulfide production rapidly (15 to 45 min) without affecting the results of the gelatin hydrolysis test.

The Lautrop (1956*a*, *b*) method was tested using cell suspensions with and without toluene. The results are shown in table 2. For comparative purposes reference may be made to table 1 which includes results with the routine stab culture method and with the methods with gelatin-precipitating agents. The results are entirely reliable but the method is not as rapid as those with gelatin-precipitating agents, requiring 18 to 48 hr for the rapid-liquefying cultures as compared to 1.0 to 5.5 hr, and 48 to 120 hr for the slow-liquefying cultures as compared with 24 to 96 hr. The results with the toluene-treated cells do not appear to warrant running the extra set of determinations.

DISCUSSION

All the methods for the detection of gelatin hydrolysis using gelatin-precipitating agents or formalin-denatured gelatin which have been described have given accurate results, except for the plate method using the ammonium sulfate-sodium chloride medium, which failed with one culture. For most purposes the plate modification of Frazier's method appears to be the method of choice. It is the most rapid of the methods and is relatively simple. The results are clear-cut and easily read. On the other hand, the tube method with the ammonium sulfate-sodium chloride medium might be chosen to avoid the use of a developing solution and the inoculation of replicate cultures when results are desired at different incubation times. The tube method has always given reliable results and is almost as rapid as the plate modification of Frazier's method.

Under certain conditions, one of the modifica-

tions of the Kohn method might be convenient. Since we used the modification of Greene and Larks' method and the Lautrop method with different groups of cultures, it is not possible to compare them with respect to rapidity. With the modification of the Greene and Larks method, however, time is saved by the use of a smaller volume of cell suspension.

If the simultaneous determination of hydrogen sulfide production from cysteine is desired, the tube modification of the Frazier method, the tube method with the ammonium sulfate-sodium chloride medium, or the modified Greene and Larks method may be used. To determine hydrogen sulfide production from sodium thiosulfate it is necessary to use the method with the ammonium sulfate-sodium chloride medium.

SUMMARY

A ninhydrin method, four procedures in which a gelatin-precipitating agent is used, and three modifications of Kohn's method have been investigated for the rapid determination of gelatin hydrolysis. A plate modification of Frazier's method in which the cultures are spot-inoculated on a nutrient gelatin-agar medium and the plate is developed after incubation with an acid mercuric chloride solution has given the most rapid results, showing gelatin hydrolysis after one hour with rapid liquefiers and after 24 to 74 hr with slow liquefiers that require up to 9 weeks with the routine stab culture method. A tube method using an ammonium sulfate-sodium chloride medium has given results almost as rapidly. Both methods have given accurate results. Lautrop's method and a modification of Greene and Larks' method have also given reliable results, but not quite as rapidly. The production of hydrogen sulfide from cysteine or sodium thiosulfate may be determined simultaneously with gelatin hydrolysis using the tube method with the ammonium sulfate-sodium chloride medium by adding the sulfur source to the medium and placing lead acetate papers in the tubes. Hydrogen sulfide production from cysteine may also be determined with the tube modification of the Frazier method or the Greene and Larks method.

REFERENCES

CHAPMAN, G. H. 1948 An improved Stone medium for the isolation and testing of food-poisoning staphylococci. Food Research, **13,** 100–105.

CHAPMAN, G. H. 1952 A simple method for making multiple tests of a microorganism. J. Bacteriol., **63,** 147.

CLARKE, S. K. R. 1953 A simplified plate method for detecting gelatin-liquefying bacteria. J. Clin. Pathol., **6,** 246–248.

CLARKE, P. H. AND COWAN, S. T. 1952 Biochemical methods for bacteriology. J. Gen. Microbiol., **6,** 187–197.

FRAZIER, W. C. 1926 A method for the detection of changes in gelatin due to bacteria. J. Infectious Diseases, **39,** 302–309.

GREENE, R. A. AND LARKS, G. G. 1955 A quick method for the detection of gelatin liquefying bacteria. J. Bacteriol., **69,** 224.

KOCHALTY, W., WEIL, L., AND SMITH, L. 1938 Proteinase secretion and growth of *Clostridium histolyticum*. Biochem. J., **32,** 1685–90.

KOHN, J. 1953 A preliminary report of a new gelatin liquefaction method. J. Clin. Pathol., **6,** 249.

LAUTROP, H. 1956*a* A modified Kohn's method for detecting gelatin-liquefying properties of bacterial cultures. Transactions of the Eleventh Scandinavian Pathological Congress, 1955. Acta Pathol. Microbiol. Scand., Suppl. III, 190–191.

LAUTROP, H. 1956*b* A modified Kohn's test for the demonstration of bacterial gelatin liquefaction. Acta Pathol. Microbiol. Scand., **39,** 357–369.

LEVINSON, H. S. AND SEVAG, M. G. 1954 Manganese and the proteolytic activity of *Bacillus megaterium* in relation to germination. J Bacteriol., **67,** 615–616.

OAKLEY, C. L., WARRACK, G. H., AND WARREN, M. E. 1948 The kappa and lambda antigens of *Clostridium welchii*. J. Pathol. Bacteriol., **60,** 495–503.

WEIL, L. AND KOCHALTY, W. 1937 Studies on the proteinase of *Clostridium histolyticum*. Biochem. J., **31,** 1255–1267.