The total amount of carbon dioxide produced by the variously treated samples from the Canyon and Dixon Strip mine areas was as follows: nonvegetated < vegetated < undisturbed. The order of production for the Fairmont spoil area was: vegetated < nonvegetated < undisturbed.

REFERENCES

CROXTON, W. C. 1928 Revegetation of Illinois coal stripped lands. Ecology, **9**, 155–175.

HEADLEE, A. J. W. 1955 Characteristics of minable coals of West Virginia. West Virginia Geological Survey, Pt. VI, The structure of bituminous coals, **13**(A) 123–149.

KOHNKE, H. 1950 The reclamation of coal mine spoils. Advances in Agron., **2**, 317–349.

PATERSON, D. D. 1939 *Statistical technique in agricultural research.* McGraw-Hill Book Co., Inc., New York.

STOKLASA, J. AND ERNEST, A. 1905 Ueber den Ursprung, die Menge und die Bedeutung des Kohlendioxyds im Boden. Centr. Bakteriol., Parasitenk., **14**(2), 723–736.

TYNER, E. H. AND SMITH, R. M. 1945 The reclamation of the strip-mined coal lands of West Virginia with forage species. Soil Sci. Soc. Amer. Proc., **10**, 429–436.

TYNER, E. H., SMITH, R. M., AND GALPIN, S. L. 1948 Reclamation of strip-mined areas of West Virginia. J. Am. Soc. Agron., **40**, 313–323.

WAKSMAN, S. A. 1952 *Soil microbiology.* John Wiley & Sons, Inc., New York.

WILSON, H. A. AND STEWART, G. 1955 Ammonification and nitrification in a strip mine spoil. Bull. 379T. West Virginia Agricultural Experiment Station, Morgantown, West Virginia.

WILSON, H. A. AND STEWART, G. 1956 The number of bacteria, fungi and actinomycetes in some strip-mine spoils. Bull. 388T. West Virginia Agricultural Experiment Station, Morgantown, West Virginia.

Rapid Destruction of Bacteria in Commonly Used Diluents and Its Elimination

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The wholesomeness of foods is judged, to a large extent, by the numbers of microorganisms present in them. There has been a tendency recently to establish an allowable upper limit of 100,000 viable bacteria per g of precooked and other frozen foods (Quartermaster Food and Container Institute, specifications 1955; Fitzgerald, 1947). If such specific regulations concerning bacterial numbers are to have proper significance, the methods by which bacterial numbers are determined must be accurate and reliable. There is, however, considerable question concerning the reliability of dilution fluids such as water, saline, and phosphate buffer, commonly used in making bacterial counts.

Sterile water is used frequently to dilute food samples prior to plating. Water, however, and especially distilled water, has been shown repeatedly to kill various bacteria rapidly and extensively. Winslow and Brooke (1927) found that cells of *Bacillus cereus*, *Bacillus megaterium*, and *Serratia marcescens*, even when initially present in concentrations of millions per ml, decreased to less than 1 per cent of the original population within 1 hr when placed in distilled water or physiologic salt solution. Likewise, Butterfield (1932) found that the number of bacteria in natural waters may decrease as much as 60 per cent in 30 min when diluted with distilled water. More recently, it has been reported that *Brucella abortus* is rapidly destroyed in distilled water, Ringer's solution, and in unbuffered and buffered saline (de Mello *et al.*, 1951). Also other bacteria such as *Pseudomonas fluorescens*, a common inhabitant of foods, and *Pseudomonas aeruginosa* rapidly die in water and in saline and phosphate buffer solutions (Gunter, 1954; Stokes and Osborne, 1956).

A random survey by the present authors has disclosed the great extent to which tap and distilled water are employed as diluents. This survey included 44 papers published during the past 20 years, which involved determination of bacterial counts in various foods. Water was used as the diluent in 67 per cent of the investigations, physiologic saline in 18 per cent, phosphate-buffered distilled water in 9 per cent, and the diluent was not mentioned in the remaining 6 per cent of the papers. Of the 67 per cent using water, 21 per cent employed distilled water, 13 per cent tap water, and the remaining 33 per cent did not indicate the type of water used.

Several ways have been suggested to protect bacteria against the destructive action of water and saline diluents. Butterfield (1932) recommended use of distilled water buffered with small amounts of phosphate and this has been adopted to some extent for the assay

of dairy products (American Public Health Association, 1953) and frozen foods (Quartermaster Food and Container Institute, 1955). Phosphate-buffered water, however, does not always provide full protection for bacteria (Butterfield, 1932; Stokes and Osborne, 1956). Also, addition of small amounts of peptone or meat extract to water will protect pure cultures of several bacterial species (Winslow and Brooke, 1927; de Mello *et al.*, 1951), but apparently has not been tested with natural mixed populations.

The purpose of the present investigation was to examine in detail the effect of various diluents and conditions on the survival of the mixed and diverse bacterial populations found in foods. Several diluents were found that neither appreciably destroy nor stimulate the growth of bacteria for at least an hour. This period should be sufficiently long to permit completion of plating of even a fairly large number of samples.

MATERIALS AND METHODS

Preparation of samples. Most of the experiments were made with precooked frozen chicken and turkey pies purchased from retail stores. The top layer of dough was removed aseptically and discarded. The contents of the pie were transferred to a tared, sterile, screw-cap, aluminum blender jar. A portion of a measured amount of sterile distilled water was added and the mixture blended for 2 min. The remainder of the water, the total of which was sufficient to make a 1:5 dilution of the pie, was then added and the blending continued for an additional minute. Fifty milliliters of the pie suspension were diluted with 50 ml of sterile distilled water. This 1:10 dilution of the pie in distilled water was the starting material for the determination of the effect of various diluents on the microbial population.

Diluents. From the basic 1:10 dilution of sample, further dilutions of 1:100, 1:1000, and 1:10,000 were made in a variety of diluents. For each decimal dilution, 10 ml of sample and 90 ml of diluent were used. These were made in milk-dilution bottles fitted with Escher-type rubber stoppers. The diluents included distilled water, tap water, physiologic saline (0.85 per cent NaCl), 0.0003 M phosphate-buffered distilled water of pH 7.2 (Butterfield, 1932), peptone ranging from 0.5 per cent to 0.0001 per cent, peptone adjusted to pH 5, 6, 7, and 8, with HCl or NaOH and also 0.1 per cent solutions of casein hydrolyzate, yeast extract, L-glutamic acid, glycine, ammonium sulfate, glucose, sucrose, and dextrin.

Test methods. The 1:100 and higher dilutions of the samples in the various diluents were incubated at room temperature, approximately 25 C, to determine the degree of survival of the bacteria contained in them. After 20, 40, 60, and in some cases 120 min, 1-ml aliquots of each dilution, in triplicate, were plated with tryptone glucose extract agar to determine the viable bacterial population. It was considered that, under usual laboratory conditions, there could be a delay of as much as ½ to 1 hr between dilution of a sample and plating when appreciable numbers of samples are being assayed. Colony counts were made after the plates had been incubated for 3 days at 30 C, and the values for the triplicate plates were averaged.

RESULTS

Representative data from a large number of experiments in which distilled, phosphate, and peptone water were compared as diluents are given in table 1. Poultry pies containing from 50,000 to over 10,000,000 bacteria per g are included. The destructive effect of distilled water on the mixed flora of the pies is very clear. As much as 40 to 60 per cent of the bacterial population from some pies was destroyed in the short interval of 20 min. Considerable loss of bacteria occurred with all of the pies. Destruction increased with time so that within 1 hr a minimum of about 40 per cent and a maximum of about 90 per cent of the bacteria could not be recovered. Phosphate dilution water, although less toxic than distilled water, nevertheless permitted a 20 to 30 per cent decrease in bacterial population within 20 min, 30 to 40 per cent in 40 min, and as much as 80 per cent in 1 hr in some instances. It was effective, however, in some cases, in maintaining the bacterial numbers unchanged for 20 min and occasionally for 40 to 60 min.

In sharp contrast to bacterial losses in distilled and phosphate water, the bacteria survived with little or no loss in 0.5 per cent peptone water for at least an hour in all samples. The small decreases in population,

TABLE 1. *Survival of bacteria in various diluents*

Experiment	Source of Bacteria	Diluent	Initial Number of Bacteria per g	Per Cent Change in Number of Bacteria			
				20 min	40 min	60 min	120 min
7	Chicken pie	Water*	5,900,000	−38	−88	−93	−98
		Phosphate†	12,100,000	−34	−56	−79	−96
		Peptone‡	10,500,000	−2	−6	−10	−5
9	Turkey pie	Water	77,000	−26	−40	−47	−62
		Phosphate	66,000	+5	+2	−10	−24
		Peptone	72,000	−1	0	−5	+10
11	Turkey pie	Water	37,000	−60	−62	−70	−86
		Phosphate	43,000	+2	−2	−30	−53
		Peptone	43,000	−10	−14	−12	+23
14	Chicken pie	Water	76,000	−41	−43	−42	−53
		Phosphate	52,000	−10	−31	−25	−37
		Peptone	55,000	−9	−9	−11	+18
17	Pot pie	Water	418,000	−14	−34	−41	−61
		Phosphate	420,000	−21	−25	−40	−50
		Peptone	467,000	−4	−11	−12	

* Distilled water.

† Prepared according to Butterfield (1932): 0.0003 M $PO_4^{=}$, pH 7.2.

‡ Peptone, 0.5 per cent.

TABLE 2. *Influence of peptone concentration on survival of bacteria**

Per Cent Peptone	Experiment A			Experiment B		
	Bacteria per g × 10^4		Per cent reduction	Bacteria per g × 10^4		Per cent reduction
	Initial	After 1 hr		Initial	After 1 hr	
0.5	149	151	1†	124	122	2
0.1	169	161	5	131	124	5
0.01	169	164	3	133	97	27
0.001	145	110	24	114	87	24
0.0001	155	117	25	125	87	30
Distilled water	156	123	21	107	69	35

* The natural mixed population of precooked frozen poultry pies.

† Increase.

approximately 10 per cent, may be more apparent than real, since the plating method used for the quantitative determination of bacterial numbers is also accurate to about 10 per cent.

The results obtained after 120 min are instructive, since this longer period accentuates and therefore makes clearer the destructive action of distilled and phosphate water. They also indicate that some bacterial multiplication may take place in the peptone water if the dilutions are left at room temperature for as long as 2 hr.

Physiologic saline was used in a few experiments. The bacterial losses were approximately the same as those in distilled water.

Influence of peptone concentration. To determine the minimum amount of peptone necessary to afford complete protection of the bacteria for an hour, experiments were made in which the peptone concentration was varied between 0.5 and 0.0001 per cent (table 2). In experiment A, 0.01 per cent peptone was sufficient to completely protect the bacteria, whereas 0.1 per cent peptone was required in experiment B. It appears therefore that 0.01 per cent peptone is a critical concentration which is sometimes but not always adequate. The minimum concentration of peptone required, therefore, in order to obtain consistent and full protection of the bacteria is 0.1 per cent and this concentration was used in subsequent experiments.

Effect of pH. In the previous experiments no attempts were made to control pH of the diluents although values were recorded. The distilled water ranged between pH 5.7 and 6.3, the phosphate water between pH 6.7 and 7.2, and the peptone water between pH 6.6 and 6.9. In the present experiment, the influence of pH on the protective action of 0.1 per cent peptone was determined. The results are shown in table 3. Essentially full protection of the bacteria was obtained for 1 hr in the range of pH 6.0 to 7.3. Considerable bacterial destruction occurred, however, at pH 5.3.

The loss of bacteria previously obtained in distilled water cannot be attributed, except possibly in a minor way, to the lower pH of the water. It is evident from table 3 that 39 per cent destruction of bacteria occurred in distilled water of pH 6.0, whereas only 9 per cent loss occurred in peptone water at exactly the same pH level. Moreover, as has been shown previously, considerable and frequent bacterial destruction takes place in phosphate water which is close to pH 7.

Also, in some of our experiments we have noted as much bacterial loss in tap water at pH 7 as in distilled water at pH 6.

There is very little change in pH of the peptone-water dilution of the food material during the 1 hr of storage.

Effectiveness of additional diluents. In order to characterize more closely the nature of diluents which will protect bacteria, a number of additional compounds were tested. These included yeast extract, which like peptone is a complex proteinaceous material, a mixture of amino acids in the form of hydrolyzed casein, individual amino acids, inorganic nitrogen, and carbo-

TABLE 3. *Effect of pH on the protective action of 0.1 per cent peptone diluent*

Diluent	pH*		Bacteria per g		Per Cent Reduction
	Initial	After 1 hr	Initial	After 1 hr	
Peptone	5.3	5.2	31,000	16,000	48
Peptone	6.0	6.1	33,000	30,000	9
Peptone	6.8	6.7	34,000	33,000	3
Peptone	7.3	7.2	31,000	29,000	6
Distilled water	6.0	6.1	31,000	19,000	39

* Of the 10^3 dilution of the poultry pies from which the bacterial counts were obtained.

TABLE 4. *Extent of protection of bacteria* by various diluents*

Diluent†	pH‡	Bacteria per g × 10^4		Per Cent Change
		Initial	After 1 hr	
Peptone	6.4	132	125	−5
Yeast extract	6.1	132	125	−5
Casein hydrolyzate	6.6	114	118	+3
Glutamic acid (neutralized)	7.4	102	114	+12
Glycine	7.0	140	140	0
Ammonium sulfate	5.8	124	104	−16
Glucose	5.6	118	30	−75
Sucrose	6.4	112	99	−12
Dextrin	6.1	117	83	−29
Distilled water	6.3	114	76	−33

* The natural mixed population of precooked frozen poultry pies.

† All compounds were present in 0.1 per cent concentration.

‡ Of the 10^4 dilution of the poultry pies from which the bacterial counts were obtained.

hydrates. As usual, peptone and distilled water served as controls. Representative results are presented in table 4.

Yeast extract protects bacteria fully as well as peptone. This is true also of casein hydrolyzate, glutamic acid, and glycine. Ammonium sulfate may afford some protection. Among the carbohydrates, glucose and dextrin are ineffective although sucrose appears to offer considerable protection to the bacteria. Autoclaved glucose solutions tend to be acid, and this fact may account for the greater bacterial losses in glucose diluent than in distilled water. On the basis of the limited number of compounds tested, it appears that, in general, bacteria are protected best by diluents which contain complex proteinaceous substances such as peptone and yeast extract and by mixtures of amino acids or even single amino acids.

DISCUSSION

Not all types of bacteria die rapidly when placed in water or saline. The coli-aerogenes group and the morphologically and biochemically related *Salmonella* and *Proteus* groups can survive, in undiminished numbers, for many hours and even days in water (Cohen, 1922; Stokes and Osborne, 1955, 1956). But as has been indicated, many bacteria, including aerobic sporeformers, pseudomonads, and other common inhabitants of foods die very rapidly, within minutes, when suspended in water or saline. Unfortunately, this marked bacterial destruction has not received adequate consideration, since apparently most investigators continue to use water as a diluent for quantitative bacterial counts. Our data indicate that this practice can lead to gross quantitative errors in the assay of foods. The degree of error will vary with the number of sensitive bacterial cells present, the length of time they remain in contact with water, and the size of the initial population. In connection with the latter, it has been our experience that frequently foods with low numbers of bacteria show smaller losses than foods with high bacterial populations when the foods are diluted in water. This is due probably to the protective action of the food material itself. The protection is greater in dilutions of food with relatively few bacteria simply because such foods are diluted to a smaller extent prior to plating than the foods with a great many bacteria per gram.

Destruction of bacteria during dilution can be avoided by the use of 0.1 per cent peptone water as the diluent or the other organic nitrogenous substances described. The peptone water is easy to prepare and is relatively inexpensive because of the low concentration of peptone required for full bacterial protection. At first glance, use of such a nutrient solution as a diluent would seem to offer the danger of bacterial multiplication as contrasted to bacterial destruction in water. Experimentally, however, growth did not occur in peptone water within 1 hr and was absent or only small after 2 hr. This result is understandable, since bacteria placed in nutrient media normally do not begin to multiply for 1 to 2 hr or longer.

The peptone water affords adequate protection for bacteria, provided it is not brought below about pH 6 by the food material. In the case of highly acid foods such as fruits and fruit juices, it may be necessary to neutralize the food with alkaline phosphates or bicarbonate prior to assay or to add such neutralizing agents to the peptone water.

As previously indicated, distilled water was used in all of our experiments to prepare the initial 1:5 and 1:10 dilutions of the food materials. Some bacterial destruction may occur at this stage. The extent would probably be small because of the shortness of time of exposure of the bacteria to the water, about 5 min in our experiments, and the protective effect of the large amount of food material present in these low dilutions. However, it may be best to make all dilutions in 0.1 per cent peptone water. We have adopted this practice as a routine procedure. For other types of materials it may be advisable to redetermine the optimum peptone concentration.

The reason for the marked sensitivity of some bacteria and the resistance of others to destruction in water and saline is not known. Nor is it known how peptone and the related organic nitrogenous substances protect the sensitive bacteria. These are intriguing problems which merit investigation.

SUMMARY

Rapid and extensive destruction of bacteria (the natural mixed population of poultry pies) occurs in the commonly used diluting fluids, namely distilled, tap, and phosphate water, and saline. As much as 40 to 60 per cent of the bacterial population may die in distilled water within 20 min and over 90 per cent in 1 hr. Phosphate water, although less toxic than distilled water, nevertheless permitted, in some instances, a 20 to 30 per cent decrease in bacterial numbers within 20 min and as much as 80 per cent in 1 hr. It is apparent that use of these diluents can lead to large errors in the quantitative determination of bacterial numbers by plating methods.

Bacterial losses during dilution can be avoided by the use of peptone water as the diluent. As little as 0.1 per cent peptone is sufficient to provide essentially full protection of bacteria for at least 1 hr. Other compounds which will also protect bacteria, although they have not been tested as extensively as peptone, include yeast extract, casein hydrolyzate, glutamic acid, and glycine. Carbohydrates are generally ineffective.

REFERENCES

American Public Health Association. 1953 *Standard methods for the examination of dairy products*, 10th ed. New York.

BUTTERFIELD, C. T. 1932 The selection of a dilution water for bacteriological examinations. J. Bacteriol., **23**, 355–368.

COHEN, B. 1922 Disinfection studies. The effects of temperature and hydrogen ion concentration upon the viability of *Bact. coli* and *Bact. typhosum* in water. J. Bacteriol., **7**, 183–230.

DE MELLO, G. C., DANIELSON, I. S., AND KISER, J. S. 1951 The toxic effect of buffered saline solutions on the viability of *Brucella abortus*. J. Lab. Clin. Med., **37**, 579–583.

FITZGERALD, G. A. 1947 Are frozen foods a public health problem? Am. J. Public Health, **37**, 695–701.

GUNTER, S. E. 1954 Factors determining the viability of selected microorganisms in inorganic media. J. Bacteriol., **67**, 628–634.

Quartermaster Food and Container Institute 1955 Military Specification Meal, Precooked, Frozen, MIL-M-13966 (QMC).

STOKES, J. L. AND OSBORNE, W. W. 1955 A selenite brilliant green medium for the isolation of *Salmonella*. Appl. Microbiol., **3**, 217–220.

STOKES, J. L. AND OSBORNE, W. W. 1956 Effect of the egg shell membrane on bacteria. Food Research, **21**, 264–269.

WINSLOW, C. E. A. AND BROOKE, O. R. 1927 The viability of various species of bacteria in aqueous suspensions. J. Bacteriol., **13**, 235–243.

Microbiological Process Report

Analytical Microbiology

II. The Diffusion Methods

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I. PRINCIPLE. In these methods the substance to be assayed is allowed to diffuse through solid, inoculated culture medium. If the substance being assayed is a bacteriostatic or bactericidal agent, a zone of inhibition results. If the substance is a growth factor, a zone of growth [zone of exhibition (Bacharach and Cuthbertson, 1948)] develops. The size of the zone, either of inhibition or growth, is a function of the concentration or in certain circumstances, the amount (Heatley, 1948) of the substance being assayed. This function can be expressed as a linear relationship between the size of the zone and the logarithm of the concentration of the substance (Bliss, 1944; Davies, 1945–1946; Bacharach and Cuthbertson, 1948). By measuring the distance the substance diffuses, as evidenced by growth or lack of growth of the test organism, and comparing it with that of a known standard preparation, the potency of the sample may be calculated.

II. TYPES OF DIFFUSION. There are two types of diffusion, vertical (linear diffusion) and horizontal (radial diffusion).

A. Vertical (linear) diffusion. In vertical diffusion methods, a given volume of the solution to be assayed is placed on top of a column of inoculated agar and allowed to diffuse down the column. The length of the column, in which the inhibition or exhibition takes place, is measured and the concentration of substance corresponding to this distance is read from a standard curve. The standard curve is prepared from a similar series of columns of known concentrations of the substance being assayed. This method is seldom used. Some Japanese workers (Torii *et al.*, 1947) use it routinely, and several other investigators have developed workable methods (Florey *et al.*, 1949; Davis and Parke, 1950: Davis *et al.*, 1950). Its main disadvantages are:

(1) Must necessarily use a facultative anaerobe.
(2) Test solutions must be sterile.
(3) Difficulty in observing the end point.
(4) There is a relatively narrow spread of readings of the zone lengths.
(5) The method is cumbersome. There is a lengthy time period involved in cleaning and setting up the apparatus.

One major advantage of this method (Davis and Parke, 1950) is the ability to better define and control the geometry of the diffusion system. To gain this one advantage, it is necessary to sacrifice others such as time, ease of handling, and so forth. From a practical standpoint, the increase in accuracy by better control of diffusion is not worth the sacrifice. The literature does not indicate the use of this method for the assay of substances other than antibiotics.

B. Horizontal (radial) diffusion. There are several