FACTORS DETERMINING THE VIABILITY OF SELECTED MICROORGANISMS IN INORGANIC MEDIA

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The viability of five selected species of microorganisms in simple, inorganic media was investigated in order to find a basal medium suitable for a comparative series of irradiation experiments. The nature of the studies required that the medium maintain a constant viable count for at least three hours at 20 to 25 C, induce only minimal metabolic activity, be of a simple, reproducible composition, be suitable for a variety of species, and involve only solutes that are practically inert radiochemically.

It seemed likely that phosphate buffer would be satisfactory. However, little is known about the survival of microorganisms in this medium, perhaps as a result of the attitude of early investigators of microbial metabolism. For example, Quastel (1928) pointed out that respiration persisted long after the ability to reproduce was lost and suggested that viability was of no consequence in most metabolic studies.

The suitability of phosphate buffer as a diluent for water analysis was investigated by Butterfield (1933), using river water samples with their natural, mixed populations as the test material. Phosphate buffer, with or without the addition of $CaCl_2$, $MgSO_4$, and $FeCl_3$, gave much better survival and more consistent results than distilled water or autoclaved bicarbonate solution.

The survival of *Escherichia coli* in solutions containing the cations Na^+, K^+, Ca^{++}, or Mg^{++} and the anions Cl^-, $PO_4^{\equiv}$, NO_3^-, or $SO_4^{=}$ was studied by Boissevain and Webb (1928). Greatest viability occurred in solutions of sodium or potassium phosphate buffer, in sodium nitrate, and in magnesium phosphate ($MgHPO_4$). Strains of *Brucella* survived slightly better in phosphate or carbonate buffer than in sodium chloride solution according to ZoBell and ZoBell (1932), who also noted that traces of Ca^{++} and Mg^{++} had a stabilizing effect. However, de Mello, Danielson, and Kiser (1951) reported that *Brucella abortus* could not survive well in saline, with or without the addition of phosphate buffer. Peptone or gelatin was required to maintain viability.

The effect of other inorganic salts such as the chlorides of Na^+, K^+, Ca^{++}, and Mg^{++} on the survival and growth of bacteria has been studied more extensively, and the literature is summarized in reviews by Falk (1923) and Winslow (1934). Some general conclusions may be drawn from these studies which are applicable to the present problem. Viability depends upon salt concentration; survival is usually best in a middle range of concentrations, higher and lower ones being deleterious. Although divalent cations are more toxic than univalent ones, suitable mixtures of the two may improve survival over that found with either cation.

It was felt that sufficient data for the formulation of a medium suitable for the five species selected (*Escherichia coli*, *Saccharomyces cerevisiae*, *Azotobacter agile*, *Pseudomonas fluorescens*, and *Rhodopseudomonas spheroides*) were not available. Consequently, their survival in solutions of potassium chloride and sodium or potassium phosphate buffer, with or without added magnesium sulfate or glucose, was studied. In addition, the effects of buffer concentration, cell concentration, sample volume, and type of sample container were investigated.

MATERIALS AND METHODS

Cultures. The microorganisms employed included *S. cerevisiae* (diploid strain SC-6 and haploid strain SC-7), *E. coli* (strain K-12), *P. fluorescens* (strain A.3.12), *A. agile* (strain M.B. 4.4), and *R. spheroides* (strain ATH 2.4.1).

Preliminary tests were conducted also with the bacteria, *Mycobacterium hyalinum*, *Hydrogenomonas facilis*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides*, and with the colorless alga,

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Prototheca zopfii. These species proved to be unsuitable because of the excessive variation in the results for species of *Mycobacterium* and *Hydrogenomonas* and because unicellular suspensions of the other species could not be obtained.

Culture media. *E. coli*, *P. fluorescens*, and *R. spheroides* were maintained routinely and plated on yeast agar containing NH_4Cl, 0.1 per cent; K_2HPO_4, 0.1 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; yeast extract (Difco), 0.5 per cent; agar, 1.5 per cent. This medium was supplemented with 1 per cent glucose for the culture of *S. cerevisiae* and *A. agile*. All media were adjusted to pH 7 before autoclaving.

Incubation. The cultures were incubated at 30 C in the dark. The viability of the photosynthetic bacterium, *R. spheroides*, was not influenced by the presence or absence of light.

Preparation of suspensions. Organisms were obtained from 16 to 24 hour plate cultures which had been inoculated with a suspension prepared from a 24 hour slant culture. The cells were harvested in the selected salt solution, centrifuged, washed, and resuspended in the same type of solution. Then they were shaken for five minutes in a 250 ml Erlenmeyer flask containing glass beads in order to break up clumps. The cell concentration was determined with a Coleman model 6A spectrophotometer and adjusted to a convenient value.

Counting procedure. Viable cell counts were determined by the surface plating technique, using an aliquot of 0.1 ml of suspension per plate. Each count represents the average of eight replicate plates. The plates were incubated at 30 C for 1 to 3 days.

Preparation and maintenance of glassware. Scrupulous cleaning of the glassware by methods which assure removal of all traces of the cleansing agent is necessary in a study of this type. After preliminary experiments with detergents and other chemicals, all glassware with the exception of petri dishes was cleaned with chromic acid solution and rinsed repeatedly in hot tap water and distilled water. Glassware other than petri dishes was replaced every six months to avoid the problem of deterioration due to repeated autoclaving noted by Kohn and Harris (1941).

Purity of chemicals. Chemicals of analytical reagent grade were used without further purification. The distilled water was obtained from a new Barnstead still, model SMQ-5. Redistillation of the water through Pyrex was not feasible for experiments on this scale. Trial determinations made with such redistilled water showed no significant differences from the results obtained otherwise.

Sample containers. In most experiments, the suspensions were incubated in standard, round bottom, Pyrex test tubes (22 by 175 mm). To determine the effect of the composition of the container on survival, cups of aluminum, Pyrex glass, and Teflon (polytetrafluorethylene), a nonreactive, heat resistant plastic, were employed. The cups, similar in shape to test tube covers, were 12 mm in depth and 18 mm in diameter.

RESULTS

Effect of buffer concentration. The dependence of viability upon salt concentration was studied with buffers of potassium phosphate or sodium phosphate, pH 6.8 to 7.2, over a concentration range of 0.5 to 0.001 M. The cells were harvested, washed, and diluted to a density of 2×10^5 cells per ml in 0.05 or 0.01 M buffer. A subsequent hundredfold dilution with buffer or distilled water gave a series of samples varying in buffer concentration, but of fixed cell concentration. A suspension prepared in the buffer used for the washing procedure was plated immediately to obtain the initial cell count. All of the samples were allowed to stand at room temperature, 22 to 25 C, for 1.5 hours, following which the viable count was determined.

The results obtained with potassium phosphate buffer, summarized in figure 1, show that the organisms varied considerably both in the range of concentrations tolerated and in the concentrations that were optimum for survival. *E. coli* and *S. cerevisiae* did not decrease significantly in numbers at any concentration tested, whereas *P. fluorescens*, *R. spheroides*, and *A. agile* survived best over a rather narrow range of molarities. The data for *A. agile* and *R. spheroides* indicate that the ability of certain organisms to survive at a given buffer concentration is dependent upon the concentration of the solution used in harvesting the cells. There were no significant differences in the survival data found with potassium and sodium phosphate buffers.

Viability in potassium chloride solution and phosphate buffers. Cells were washed and sus-

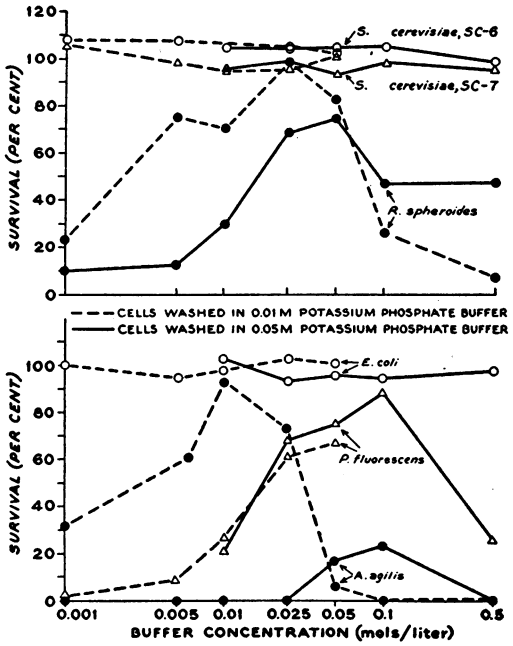

Figure 1. Effect of buffer concentration on the survival of microorganisms in potassium phosphate buffer.

pended in potassium phosphate buffer, Sorenson's buffer (Na_2HPO_4-KH_2PO_4), potassium chloride, and potassium chloride supplemented with potassium phosphate buffer at a concentration of 0.005 M. All four solutions were of the molar concentration found optimum for the given species. The exposure time was 1.5 hours.

The survival of *S. cerevisiae*, *E. coli*, and *P. fluorescens* was 100 per cent in all cases. *R. spheroides* exhibited 100 per cent survival in potassium phosphate and Sorenson's buffer, 75 per cent viability in buffered potassium chloride, and no survival in potassium chloride alone. The lethality of the latter solution was due to the low pH of the medium, although the decreased viability in the buffered solution suggests that the chloride may be somewhat less satisfactory than the phosphate.

Effect of magnesium sulfate and glucose on survival in potassium phosphate buffer. The effect of these compounds was studied when the viability of three species in unfortified phosphate buffer was found to fall sharply below 100 per cent after intervals greater than 1.5 hours.

The cultures were grown in the usual manner and harvested in each of four test solutions. The suspensions were diluted to a concentration of about 2×10^4 cells per ml, and one ml samples were dispensed into tubes. The viable count was determined immediately and at intervals of 2, 3, 4, 5, and 6 hours. Before plating, the necessary tenfold dilution was made by adding nine ml of diluent to each tube. The data obtained at intervals of 3 and 6 hours give a representative picture and are summarized in table 1.

S. cerevisiae and *E. coli* exhibited approximately 100 per cent survival in all of these media throughout the 6 hour period.

In phosphate buffer alone, the viable counts of *A. agile* and *R. spheroides* decreased with time at rates characteristic of each, while that of *P. fluorescens* was somewhat variable. Addition of magnesium sulfate led to a constant count for *R. spheroides* and to an increase in count of approximately 25 per cent which began during the fourth to sixth hours for *A. agile* and *P. fluorescens*. Addition of glucose improved the viability of *R. spheroides* but decreased that of *A. agile* to 25 per cent of the survival found in unsupplemented buffer.

The survival of all species in buffer containing both glucose and magnesium sulfate was at least as good as that in buffer fortified with magnesium sulfate alone. The addition of both compounds caused a greater increase in count of *A. agile* and *P. fluorescens* than did magnesium sulfate alone.

The magnesium sulfate concentration was selected on the basis of its frequent use in synthetic media. Subsequent tests showed that it may be decreased to 0.0005 M before becoming limiting for any of the sensitive strains. Addition of a sterile magnesium sulfate solution to the buffer following autoclaving is recommended to prevent formation of a precipitate.

Effect of cell concentration and sample volume. Various investigators, including Ballantyne (1930), Winslow and Brooke (1927), de Mello, Danielson, and Kiser (1951), Spangler and Winslow (1943), have reported that an increase in the cell concentration favors survival. The last two reports indicated that concentrations of 10^7 cells per ml or greater were required for this effect. Reports dealing with the effect of sample volume on survival under comparable conditions were not found.

The organisms were washed in potassium phosphate buffer of the optimum concentration, without and with magnesium sulfate, and diluted

TABLE 1

Effect of magnesium sulfate and glucose on survival in potassium phosphate buffer

ORGANISM	BUFFER CONCENTRATION	PER CENT SURVIVAL*							
		Diluent†							
		A		B		C		D	
		Incubation time (hr)							
		3	6	3	6	3	6	3	6
	moles/L								
Saccharomyces cerevisiae, strain SC-6	0.05	100	98	98	98	112	107	95	98
Saccharomyces cerevisiae, strain SC-7	0.05	96	98	93	96	104	111	101	113
Escherichia coli	0.05	100	94	97	84	95	87	96	95
Azotobacter agile	0.01	68	56	100	128	25	14	103	164
Rhodopseudomonas spheroides	0.025	87	62	96	89	88	83	92	96
Pseudomonas fluorescens	0.1	86	112	97	126	91	102	133	213

* The values represent the average result of from 2 to 5 determinations in each case.

† Composition of diluents:

A—Potassium phosphate buffer of the concentration specified, pH 6.9 to 7.1.

B—Diluent A supplemented with magnesium sulfate at a final concentration of 0.002 M.

C—Diluent A supplemented with glucose at a final concentration of 0.01 M.

D—Diluent A supplemented with magnesium sulfate and glucose at final concentrations of 0.002 M and 0.01 M, respectively.

TABLE 2

Effect of cell concentration and sample volume on viability

ORGANISM	SAMPLE VOLUME	BUFFER CONCENTRATION	PER CENT SURVIVAL*					
			Potassium phosphate buffer			Potassium phosphate buffer + $MgSO_4 \cdot 7H_2O$		
			Cell concentration in organisms/ml					
			2×10^8	2×10^6	2×10^4	2×10^8	2×10^6	2×10^4
	ml	*moles/L*						
Saccharomyces cerevisiae, strain SC-6	1.0	0.05	102	97	98	102	99	89
	0.2		99	98	98	104	103	95
Saccharomyces cerevisiae, strain SC-7	1.0	0.05	100	97	100	99	99	100
	0.2		96	96	98	96	98	83
Escherichia coli	1.0	0.05	98	88	92	95	96	92
	0.2		94	92	89	94	91	91
Azotobacter agile	1.0	0.01	94	63	34	98	94	155
	0.2		91	75	45	92	120	180
Rhodopseudomonas spheroides	1.0	0.025	75	68	49	113	108	101
	0.2		74	59	49	112	109	102
Pseudomonas fluorescens	1.0	0.1	89	102	117	91	112	162
	0.2		78	112	83	86	137	137

* Determined at 6 hours.

to give suspensions containing 2×10^8 to 2×10^6 cells per ml. The most dilute sample was plated immediately, and the viable counts of the other suspensions were determined after incubation of 1.0 and 0.2 ml samples for 6 hours at room temperature. The results are presented in table 2.

The viable counts of *S. cerevisiae* and *E. coli* remained practically unchanged under all conditions. The results for the other species depended upon the presence of magnesium sulfate.

In unsupplemented phosphate buffer, the survival of *A. agile* and *R. spheroides* decreased as the cell concentration decreased. No significant trend was found with *P. fluorescens*. In buffer fortified with magnesium sulfate, growth of *A. agile* and *P. fluorescens* occurred only at 2×10^4 and 2×10^6 cells per ml. The count of *R. spheroides* remained at the original value at all cell concentrations.

The sample volume had no effect upon the survival of *E. coli*, *S. cerevisiae*, and *R. spheroides* at any cell concentration, nor upon that of *A. agile* and *P. fluorescens* at a concentration of 2×10^8 cells per ml. At lower concentrations the response of the latter two species varied from experiment to experiment. When significant differences occurred, the viability of *A. agile* was greater in the 0.2 ml samples than in the 1.0 ml samples, and the reverse was found with *P. fluorescens*.

Effect of composition of incubation cups. These tests were carried out in conjunction with the experiments of the two preceding sections. Suspensions containing 2×10^4 cells per ml in magnesium sulfate-fortified and unfortified buffer were incubated in cups of aluminum, Pyrex glass, and Teflon, and in Pyrex glass tubes, 1.0 ml per container. The survival after 6 hours was determined.

E. coli and *S. cerevisiae* showed no change in viability in any type of container or buffer solution. In unsupplemented buffer, the survival of *A. agile* and *R. spheroides* in all cups was the same or less than that found in glass test tubes. *P. fluorescens* exhibited 100 per cent survival in glass and Teflon cups and apparent growth in aluminum cups.

In buffer containing magnesium sulfate, the viable count of *R. spheroides* decreased 30 per cent in aluminum cups but showed no change in the glass and Teflon containers. The counts of *A. agile* and *P. fluorescens* remained at the initial level or increased in all containers.

DISCUSSION

The five strains studied can be divided into two groups on the basis of their viability in the test solutions. The survival of *E. coli* and *S. cerevisiae* was unaffected by any of the factors tested, in marked contrast to the survival of *A. agile*, *R. spheroides*, and *P. fluorescens*. It is apparent that the two groups differ in some fundamental way, perhaps in the mechanism controlling the exchange of materials between cell and medium.

In unsupplemented phosphate buffer, pH 7, the viability of *P. fluorescens*, *A. agile*, and *R. spheroides* was dependent upon the buffer concentration and was maximal in the middle of the concentration range tested (0.001 to 0.5 M), falling sharply at both ends. In the case of *A. agile* and *R. spheroides* the optimum buffer concentration also depended upon that of the buffer employed in washing the cells. Cells harvested in 0.05 M buffer required a higher optimum concentration than those washed in 0.01 M buffer. When both types of washed cells were incubated in the same concentration of buffer, they differed in survival by as much as 90 per cent. The change in salt tolerance of growing cultures, usually due to selection, is well known, but the modification of salt tolerance in nondividing cells appears to have been overlooked. This process may be comparable to the adaptation of resting cells to substrate utilization although the results also could be due to selection.

The stabilizing effect of mixtures of di- and univalent cations has been reported by Flexner (1907) and Shearer (1917) for the meningococci and by Duthoit (1923) and Winslow and Falk (1918, 1923) for the enteric organisms. The present findings with magnesium sulfate demonstrate the phenomenon in other types of bacteria. Following studies with the lactic acid bacteria, MacLeod and Snell (1950) suggested that "ion antagonisms" may result from nutritional requirements and involve competition of ions for enzyme surfaces. The profound stabilizing effect of magnesium sulfate upon resting cells in the present work might be explained on this basis.

Microscopic examination of dense suspensions showed them to be free of clumped cells, indicat-

ing that the increase in viable count of *A. agile* and *P. fluorescens* during the fourth to sixth hours of incubation represented cell division and not dispersion of clumps. Since cell division occurred only in the most dilute suspension, it appeared that growth was supported by a limited supply of nutrients, present as contaminants of the chemicals or glassware. If insufficient washing of the cells had been the cause, growth should have occurred in the concentrated suspensions also.

On the basis of the above results, potassium phosphate buffer, pH 7, containing 0.002 M magnesium sulfate has been selected for the irradiation experiments. The five species undergo no changes in viable count when suspended in this medium for 3 hours. The composition is varied only in respect to buffer concentration, which is adjusted to suit the requirements of the individual strains. Teflon cups are recommended.

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SUMMARY

The viability of five selected species was investigated in simple inorganic media of pH 7 at 20 to 25 C. *Escherichia coli* and *Saccharomyces cerevisiae* (haploid and diploid strains) were maintained without change in viable count for 1.5 hours in potassium phosphate buffer of concentrations varying from 0.001 to 0.5 M and for 6 hours in 0.05 M phosphate buffer, with or without the addition of magnesium sulfate (0.002 M) or glucose (0.01 M). The survival of *Azotobacter agile*, *Rhodopseudomonas spheroides*, and *Pseudomonas fluorescens* depended upon the phosphate buffer concentration, cell concentration, composition of the sample container (Pyrex, aluminum, Teflon), duration of the experiment, and, in the case of *A. agile* and *R. spheroides*, upon the concentration of the medium used in washing the cells. The addition of magnesium sulfate decreased or eliminated the deleterious effects of these factors. In conjunction with a suitable buffer concentration, it sustained a constant viable count for at least 6 hours in the case of *R. spheroides* and for 3 hours in that of *A. agile* and *P. fluorescens*, with an increase in count occurring during the ensuing 3 hours.

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