serum albumin. This suggests that the binding of one 1-anilinonaphthalene-8-sulfonate molecule to human serum albumin discourages other 1-anilinonaphthalene-8-sulfonate molecules from combining in a greater extent than the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.

Equation 1 was suggested by Scatchard (3) for the special case when the intrinsic association constants are all equal. He also pointed out that, if the plot of $\bar{V}/(D)$ *versus* $\bar{V}$ does not give a straight line, it may be inferred that the intrinsic constants are not equal and/or there is interaction among the bound ions. The former effect is well demonstrated by the results of this study. For comparison, the plots of $\bar{V}/(D)$ *versus* $\bar{V}$ for both protein systems are shown in Fig. 3; these plots are not quite linear. If one were to discount the low values of $\bar{V}$, one could plot a straight line with a high significance factor. The n values for the human and bovine serum albumin systems obtained from the straight-line portion of the Scatchard plots are 2.5 and 2.8, respectively. When using Eq. 3, the results seem to be much more satisfactory. It is possible to detect the number of sites with greatest affinity as well as to detect dramatically the total number of binding sites over a range of small molecule concentrations.

Equation 2 is an empirical equation in which a parameter, m, is introduced into Eq. 1 to obtain a straight-line plot. The fact that m is varied by the protein concentration, buffer agent, and temperature (7) may indicate the effect of these factors on the binding. However, the physicochemical meaning of m is still not clear.

The determination of the binding parameters may also depend on the experimental methods used. The results obtained from the fluorescence technique are different from those of other methods, probably in the manner that only the primary (strong) binding sites are detected, whereas the results obtained from other methods such as dialysis (15, 16) often show larger numbers of binding sites. The curvature of the Scatchard plot has been interpreted (4) as the binding of the small molecule to more than one class of sites on the protein. However, it is possible that the intrinsic association constants of the binding of these sites are not equal even in the same class.

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Low Temperature Maintenance of Test Organism Suspensions for Antibiotic Assays

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Abstract □ The survival rates of 14 test organisms that had been suspended in saline, distilled water, peptone water, or nutrient broth were compared after storage at −70°. Viable cell counts and dose–responses to various antibiotics were determined prior to freezing and at 3-month intervals for 2 years after freezing. Most test organisms were successfully maintained in this manner using water, peptone water, or broth with little or insignificant losses in viable cells or change in dose–response in antibiotic assay systems.

Keyphrases □ Microorganisms—low temperature maintenance for use in antibiotic assays □ Antibiotic assays—low temperature maintenance of test organism suspensions □ Test organisms—low temperature maintenance for use in antibiotic assays

This laboratory maintains a number of test organism suspensions for daily use in turbidimetric and agar diffusion assays of antibiotics (1). Detailed procedures for the preparation of these suspensions are described in the *Code of Federal Regulations* (2). Because of the variety of antibiotics tested and the volume of samples to be assayed, preparing fresh suspensions on a weekly or biweekly basis is a time-consuming task attended by a number of laboratory problems. Ideally, the growth characteristics and specific antibiotic dose–response of any given suspension are reproducible if the suspension has been prepared according to the official method and stored under refrigeration at temperatures just above freezing. In actual practice, even the most exacting

Table I—Plate Counts before and after Storage at −70°

Test Organism	Vehicle	Initial Count, 10^{10}/ml.	Final Count, 10^{10}/ml.	Percent Loss	Months
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Saline	1.20	1.20	0	18
	Water	1.40	2.40	0	18
	Peptone water	1.40	2.00	0	18
Saccharomyces cerevisiae (ATCC 9763)	Saline	0.049	0.035	29	24
	Water	0.051	0.038	26	24
	Peptone water	0.041	0.034	17	24
Saccharomyces cerevisiae (ATCC 2601)	Saline	0.052	0.005	90	9
	Water	0.052	0.006	88	9
	Peptone water	0.040	0.009	78	9
Staphylococcus epidermidis (ATCC 12228)	Saline	0.42	0.37	12	24
	Water	0.38	0.36	5	24
	Peptone water	0.41	0.52	0	24
Staphylococcus aureus (ATCC 6538P)	Saline	1.50	1.20	20	15
	Water	1.60	1.30	19	12
	Peptone water	2.30	2.50	0	12
	Broth	1.40	1.00	29	15
Bordetella bronchiseptica (ATCC 4617)	Saline	7.90	0.70	91	9
	Water	7.70	1.80	77	15
	Broth	7.70	1.40	82	18
Sarcina lutea (ATCC 9341)	Saline	2.20	1.20	45	24
	Water	0.96	1.80	0	24
	Peptone water	1.50	1.50	0	24
Sarcina subflava (ATCC 7468)	Saline	0.94	0.98	0	24
	Water	1.00	1.20	0	24
	Peptone water	0.78	0.92	0	24
Pseudomonas pyocyanea (ATCC 23389)	Saline	4.40	0.70	84	3[a]
	Water	4.00	1.30	68	3[a]
	Peptone water	4.80	1.20	75	3[a]
Klebsiella pneumoniae (ATCC 10031)	Saline	2.40	0.28	88	3[a]
	Water	2.90	0.94	68	3[a]
	Peptone water	3.60	1.10	69	3[a]
Escherichia coli (ATCC 10536)	Saline	3.50	<0.001	100	3[a]
	Water	4.80	1.40	71	6[a]
	Peptone water	4.20	<0.001	100	3[a]
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Saline	1.70	2.00	0	24
	Water	1.70	2.10	0	24
	Peptone water	1.80	2.10	0	24
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Saline	0.67	0.56	16	24
	Water	0.73	0.80	0	24
	Peptone water	0.66	0.66	0	24
Streptococcus faecalis (ATCC 10541)	Saline	0.68	0.34	50	9[a]
	Water	0.41	0.31	24	9
	Peptone water	0.43	0.31	28	9

[a] Discontinued after the time indicated because of loss of viable cells.

quality controls do not exclude occasional contamination or prevent batch-to-batch variations in the composition of dehydrated media and related problems. Preparing large quantities of test organism suspension for use over an extended period makes it possible to decrease the occurrence of such aberrations and has the further advantage that once an acceptable suspension has been prepared there is a continuity of reproducible assay response.

Conditions for the preservation and maintenance of test organism suspensions have been reported by numerous investigators. Sokolski *et al.* (3) reported the preparation of frozen suspensions for vitamin B_{12} assays. Complete recovery of viable cells was obtained when the suspensions in basal medium were rapidly frozen by direct immersion into liquid nitrogen and rapidly thawed by agitating in a water bath at 40°. McDaniel and Bailey (4) found that liquid nitrogen storage was the most satisfactory method for supplying standard *Streptomyces viridoflavus* inoculum for laboratory and pilot plant experimentation. There were no detectable changes in viability over 12 months when cotton-plugged ampuls were stored in the gas phase of a liquid nitrogen refrigerator. Tanguay (5) reported on the direct freezing of test organisms either in tryptose–saline containing 15% glycerol or in 0.067 *M* phosphate buffer, pH 7.0, containing 15% glycerol. When frozen and stored at −40°, these organisms maintained a satisfactory growth response from 6 months to 1 year or more. The main advantages claimed for this technique are that assays can be done at any time without the necessity of preparing daily inocula or maintaining the sensitivity of test organisms and that assay responses are standardized. Day-to-day fluctuations in growth responses and invalid assays due to inoculum failure are largely eliminated (5). These qualities are essential to the success of the certification program at the National Center for Antibiotic Analysis. This report describes the results of direct freezing of various test organisms used at the National Center.

EXPERIMENTAL

The 14 official antibiotic assay organisms listed in Table I were tested at 3-month intervals for periods ranging from 6 months to up

Table II—Dose-Response of Test Suspensions after Freezing and Storage at −70°

Test Organism	Vehicle	Antibiotic	Method[a]	Dose Response[b] Initial	Dose Response[b] Final	Months
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Saline	Penicillin	AD	9.07	12.49	18
	Water		AD	9.24	10.30	18
	Peptone water		AD	9.20	11.63	18
Saccharomyces cerevisiae (ATCC 9763)	Saline	Amphotericin	AD	4.95	3.62	24
	Water		AD	4.65	4.05	24
	Peptone water		AD	5.12	4.62	24
Saccharomyces cerevisiae (ATCC 2601)	Saline	Nystatin	AD	6.15	7.97	9[c]
	Water		AD	6.58	7.51	9[c]
	Peptone water		AD	7.11	8.47	9[c]
Staphylococcus epidermidis (ATCC 12228)	Saline	Neomycin	AD	5.51	6.38	24
	Water		AD	3.65	7.57	24
	Peptone water		AD	3.32	5.98	24
Staphylococcus aureus (ATCC 6538P)	Saline	Penicillin	AD	9.53	7.71	15
	Water		AD	9.47	7.71	12
	Water	Tetracycline	T	0.511	0.436	18
	Peptone water		T	0.503	0.212	12[d]
	Broth	Penicillin	AD	8.04	8.47	15
	Broth	Tetracycline	T	0.531	0.402	18
Bordetella bronchiseptica (ATCC 4617)	Saline	Polymyxin	AD	3.72	4.32	6[c]
	Water		AD	3.26	5.22	15
	Broth		AD	3.39	3.82	18
Sarcina lutea (ATCC 9341)	Saline	Erythromycin	AD	6.98	8.24	24
	Water		AD	5.41	6.98	24
	Peptone water		AD	6.15	7.77	24
Sarcina subflava (ATCC 7468)	Saline	Bacitracin	AD	4.88	5.18	24
	Water		AD	5.61	5.68	24
	Peptone water		AD	5.95	5.91	24
Pseudomonas pyocyanea (ATCC 23389)	Saline	Carbenicillin	AD	7.97	9.97	3[c]
	Water		AD	8.37	8.84	3[c]
	Peptone water		AD	8.80	8.97	3[c]
Klebsiella pneumoniae (ATCC 10031)	Saline	Dihydrostreptomycin	T	1.74	No growth	3[c]
	Water		T	1.74	No growth	3[c]
	Peptone water		T	1.41	No growth	3[c]
Escherichia coli (ATCC 10536)	Saline	Chloramphenicol	T	0.949	0.392	3[d]
	Water		T	0.944	0.593	6[d]
	Peptone water		T	0.867	0.614	3[d]
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Saline	Penicillin	AD	14.28	15.02	24
	Water		AD	14.75	14.88	24
	Peptone water		AD	14.75	13.45	24
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Saline	Bacitracin	AD	4.12	5.18	24
	Water		AD	4.92	5.18	24
	Peptone water		AD	4.65	5.45	24
Streptococcus faecalis (ATCC 10541)	Saline	Gramicidin	T	0.423	0.307	9[d]
	Water		T	0.507	0.365	9[d]
	Peptone water		T	0.549	0.236	9[d]

[a] AD = agar diffusion assay, and T = turbidimetric assay. [b] Defined in terms of slope where b = slope = range over 1 log cycle (expressed in millimeters of zone diameter for agar diffusion assay and absorbance values for turbidimetric assay). [c] Discontinued after the time indicated because of loss of viable cells. [d] Discontinued after the time indicated because of significant change in dose-response.

to 2 years. Some tests were terminated earlier than 2 years, *e.g.*, at 6, 12, or 15 months, depending on the number of viable cells present and/or the dose-response at that time. The suspending agents and their compositions were: (*a*) sterile normal saline, (*b*) sterile distilled water, (*c*) sterile 0.5% peptone water, and (*d*) nutrient broth (Medium 3) (2).

The test organisms were maintained on agar slants containing 10 ml. of appropriate medium, and the suspensions were prepared as described in the *Code of Federal Regulations* (2). Growth was washed from the agar surface of the Roux bottles with 50 ml. of the appropriate suspending agent. Washings from three Roux bottles were pooled for each diluent to give a total of 150 ml. of suspension. From each pooled suspension, 12-ml. portions were placed in 30-ml. polycarbonate centrifuge tubes [1.27-cm. (0.5-in.) mouth] and closed with polypropylene screw caps. The tubes of suspension were quick frozen by a mixture of equal parts of dry ice and 95% ethanol (6) and then placed in a low temperature freezer (one capable of temperature to −90°) at −70°.

Viable cell counts and dose-responses (7) to the appropriate antibiotics were determined prior to freezing and at 3-month intervals up to 2 years. At each interval, a tube of frozen suspension was thawed by placing in a refrigerator for 2 hr. Each thawed suspension was tested initially upon thawing and then was stored for 2 weeks under refrigeration and tested each week for viable cell count and dose-response. The percent inoculum varied with the organism being tested; the incubation time also varied, depending on whether the agar diffusion or turbidimetric assay method was used to determine the dose-response to a particular antibiotic.

RESULTS AND DISCUSSION

As shown in Table I, most test organisms can be maintained satisfactorily for up to 2 years without any significant loss in viability and, as shown in Table II, such a procedure maintains an acceptable dose-response to the various antibiotics. Outstanding success was obtained with *Staphylococcus aureus* (resistant to novobiocin), *Sarcina subflava*, and *Sarcina lutea* (resistant to erythromycin) with no loss of viable cells in saline, water, or peptone water (Table I). In these instances, peptone water was selected as the vehicle of choice because of the stability of most organisms under refrigeration after they are thawed. Table I also shows that, of the organisms tested, the least success was obtained with *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas pyocyanea;* tests were discontinued early in the study because of losses in viable cells ranging from 70 to 100% after only 3 months.

Table III—Recommended Procedures for Storage at −70°

Test Organism	Suspending Vehicle	Maximum Storage Time, Months Frozen	Stability (Weeks Unfrozen) under Refrigeration
Staphylococcus aureus, resistant to novobiocin (ATCC 12692)	Peptone water	12	1
Saccharomyces cerevisiae (ATCC 9763)	Peptone water	12	2
Saccharomyces cerevisiae (ATCC 2601)	—	Not recommended	4
Staphylococcus epidermidis (ATCC 12228)	Peptone water	12	1
Staphylococcus aureus (ATCC 6538P)	Broth	12–18 (Agar diffusion assay) 6 (Turbidimetric assay)	1 1
Bordetella bronchiseptica (ATCC 4617)	Water	6	1
Sarcina lutea (ATCC 9341)	Peptone water	12	2
Sarcina subflava (ATCC 7468)	Peptone water	12	2
Pseudomonas pyocyanea (ATCC 23389)	—	Not recommended	2
Klebsiella pneumoniae (ATCC 10031)	—	Not recommended	1
Escherichia coli (ATCC 10536)	—	Not recommended	2
Sarcina lutea, resistant to erythromycin (ATCC 15957)	Peptone water	12	2
Sarcina subflava, resistant to dihydrostreptomycin (ATCC 7468/d)	Peptone water	12	2
Streptococcus faecalis (ATCC 10541)	—	Not recommended	24 hr.

Table III indicates that the recommended maximum storage time for most tested organisms is 1 year in peptone water. The 1-year time limit was selected to allow for some margin of error, even though excellent results were obtained for up to 2 years for most organisms.

In conclusion, most tested organisms can be frozen for 6 months to 2 years in water, peptone water, or broth with little or no loss in viable cells or change in dose-response. The method is simple and requires no elaborate equipment other than a low temperature freezer capable of maintaining temperatures of −70°. Frozen suspensions, although not specifically mentioned in the *Code of Federal Regulations*, are implicit in the directions to maintain the suspensions under "refrigeration." The findings show that subfreezing refrigeration temperatures offer a suitable and reliable method for maintaining suspensions of antibiotic test organisms.

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