JOURNAL OF CLINICAL MICROBIOLOGY, July 1977, p. 4-9

Vol. 6, No. 1
Printed in U.S.A.

Survival of Chlamydiae After Cooling to −196°C

M. J. PRENTICE[1] AND J. FARRANT*

Divisions of Communicable Diseases and Cryobiology, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, United Kingdom

Received for publication 25 March 1977

Factors influencing the survival of chlamydiae after freezing were reexamined. From the data presented, it is suggested that preservation of laboratory-grown chlamydiae is best achieved through the use of sucrose as the cryoprotective agent, in the presence of 10% serum. Dimethyl sulfoxide and glycerol are more toxic. The period of exposure to sucrose before freezing must be kept as short as possible and be at 4°C rather than at room temperature. The rate of cooling during freezing in sucrose is not important; however, cooling at a rate slower than 1°C/min should be avoided. Since chlamydial survival is increased by rapid thawing, the volume of the sample should be kept to a minimum. Thawed suspensions should be inoculated onto cell monolayers without delay. The application of these methods may increase the proportion of stored clinical specimens in which chlamydiae can be found.

There is increasing evidence that the pathological effects of *Chlamydia trachomatis* extend beyond the concern of the ophthalmologist. Although the pathogenic role of this species of chlamydiae in genital disease of man has not been confirmed by fulfillment of Koch's postulates, recent work strongly supports this association (5, 8, 9). Studies of chlamydial genital infection depend partly on a reliable method of detecting the microorganisms. The true efficiency of the isolation techniques used at present is unknown.

The most sensitive system currently used to detect chlamydiae is growth in tissue culture monolayers. Since these microorganisms are labile, specimens awaiting culture are frequently stored in the frozen state. The damaging effect of freezing on chlamydial organisms was reported by Hanna (4), but this reduction in infectivity must be weighed against the lability of the microorganisms in the unfrozen state. The advantages of storing clinical specimens containing chlamydiae at or below −70°C have been reported by Gordon et al. (3) and Darougar et al. (1), whereas Reeve et al. (10) maintain that freezing specimens reduces the number of chlamydial isolates by 20%. The methods used by these groups were not, however, comparable.

The storage of chlamydiae during transport from the patient to the tissue culture monolayer is an important part of the isolation procedure. The ways in which loss of these microorganisms due to freezing might be diminished have now been investigated by cooling cultures under controlled conditions.

[1] Present address: Department of Virology, The Churchill Hospital, Headington, Oxford OX3 7LJ, United Kingdom.

MATERIALS AND METHODS

Culture of microorganisms. Three strains of chlamydiae were used in these experiments. Lymphogranuloma venereum 404 L (LGV) and *C. trachomatis* T/19 C/TN/IOL-564/OT from a trachoma patient were obtained from the Institute of Ophthalmology, London. The third strain was *C. trachomatis* cultured from the urethra of a patient suffering from a nonspecific urethritis. The organisms used in these experiments had been passed two to six times in McCoy cell cultures. Fresh inoculum was prepared from McCoy cells infected with chlamydiae 3 or, rarely, 4 days previously. Glass beads were added to a flat-bottomed 5-ml vial containing a culture, and the cells were disrupted by a Whirlimixer for 40 s and then centrifuged at 600 × *g* for 4 min. The supernatant suspension was diluted, in some cases, to a concentration suitable for the assay system.

Composition of media. The phosphate-buffered sucrose solutions used to preserve chlamydiae are based on the work of Weiss and Dressler (11), who recommended the use of a 0.4 M sucrose-sodium phosphate medium. A recent symposium (12) suggests the use of 0.2 M sucrose-potassium phosphate as a transport medium for clinical specimens (3) and a sodium-containing solution for storage of harvested chlamydiae.

The constituents of the media used in this study are summarized in Table 1. The complete medium with antibiotics (CMA) (3) was only used as a growth medium for McCoy cells. CMA with added glucose (4.5 mg/ml) and double the amount of fetal calf serum (FCS) is denoted CMGA. This was used

TABLE 1. *Composition of media*

Medium	Diluent	FCS (%, vol/vol)	Buffer	Final concn of additional constituents
CMA	MEM[a]	5	4.4% (wt/vol) sodium bicarbonate adjusted to pH 7.4 (unless otherwise stated)[b]	Glutamine, 200 μM Vancomycin, 100 μg/ml Streptomycin, 50 μg/ml Vitamins, 1% (vol/vol) (Flow Labs)
CMGA	MEM	10	As for CMA	As for CMA, with glucose, 4.5 mg/ml = 0.03 M
NaSP	CMGA (1:1) with 0.4 M sucrose in water = 0.2 M final concn (=6.8%, wt/vol)	10	0.02 M Na_2HPO_4	
KSP	0.2 M sucrose (=6.8%, wt/vol) in water	3 or 10	0.02 M K_2HPO_4/KH_2PO_4	
Sucrose freezing solution	MEM	10	As for CMA	Sucrose concn as stated, between 5 and 25% (wt/vol)

[a] MEM, Eagle minimal essential medium.
[b] A 20 mM solution of HEPES was occasionally used instead.

regularly to overlay the monolayers after inoculation with chlamydiae and to dilute inocula; it was also investigated as a cryoprotective agent.

The potassium phosphate-buffered sucrose solution (KSP) used by Darougar et al. (1) as a transport medium for frozen clinical specimens awaiting chlamydial culture contained 3% (vol/vol) FCS. The inoculum used in these experiments necessarily diluted the solution. The usual ratio of KSP to inoculum was 4:1, and occasionally 11.5:1. The sodium phosphate-buffered sucrose solution (NaSP), diluted 1:1 with CMGA, was used as a cryoprotective agent to store harvested chlamydiae.

To study the cryoprotective properties of glycerol, dimethyl sulfoxide (Me_2SO), and sucrose, solutions were prepared in Eagle minimal essential medium and autoclaved before the addition of buffer and 10% FCS. Organisms suspended in CMGA were diluted with an equal volume of double-strength cryoprotective additive, whether glycerol, Me_2SO, or sucrose solution. The final concentrations of protective substances are given for each experiment.

Sodium bicarbonate (4.4%, wt/vol) was used to buffer all solutions to pH 7.4 except those containing phosphate buffer (KSP and NaSP) and where the use of 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) is specifically mentioned.

Method of freezing. Unless otherwise stated, samples of microorganisms in the different cryoprotective media were frozen in 0.2-ml volumes in 2-ml screw-capped polypropylene tubes (NUNC). The method of controlling the rates of cooling was that described by Leibo et al. (6) as modified by Morris and Farrant (7), the tubes being immersed in different vessels cooled by liquid nitrogen. When the temperature of a replicate uninfected sample, as recorded by a calibrated copper constantan thermocouple, reached −55°C, the samples were plunged into liquid nitrogen. Some samples were cooled by being placed in a −70°C electric refrigerator (Revco). When samples of 5% (wt/vol) sucrose freezing solution were cooled in polypropylene tubes by direct plunging into liquid nitrogen, the rate of cooling to −55°C was 175°C/min for 1-ml samples and 367°C/min for 0.2-ml samples.

Small volumes (0.2 ml) were used during freezing to increase the rate of thawing and to dilute the cryoprotective agent by the medium overlying the monolayers, thus avoiding the possible hazards of a centrifugation step. Unless stated otherwise, samples were agitated individually in a water bath at 37°C until they thawed.

Method and evaluation of assay. The quantity of chlamydiae remaining after any treatment was estimated by inoculating duplicate samples, diluted if necessary in CMGA at room temperature, onto irradiated McCoy cell monolayers, as described by Darougar et al. (2). Occasionally, treatment of the monolayers with 25 μg of 5-iodo-2'-deoxyuridine per ml for 3 days was used instead of irradiation. Three days after inoculation, the cultures were fixed in methanol and stained with Giemsa (10%, vol/vol), and the number of inclusion-forming units was estimated by dark-ground microscopy. In some experiments with a heavy chlamydial inoculum, the monolayers were fixed after 2 days of incubation, before the inclusions coalesced. The usual counting technique was to observe at least 10 microscope fields on each cover slip at ×120 magnification. If, however, the count was low, consistent results were obtained by examining the whole surface of the cover slip; alternatively, if a large number of inclusions were crowding the monolayer, the magnification was increased to ×240 and 10 fields were counted.

The accuracy of this counting procedure was in-

vestigated by inoculating 12 serial twofold dilutions of a suspension of genital chlamydiae and counting the resulting inclusions by the most convenient method. To compensate for the different magnifications used, the total number of inclusions on the cover slip was calculated in each instance. The linear relationship between the concentration of organisms and the estimated number of inclusion-forming units is demonstrated in Fig. 1.

Analysis of significant differences. The significance of differences between treatment means, for example between survival at two different rates of cooling, was assessed by a one-way analysis of variance of all the data in each experiment. At least two samples of each treatment were processed identically, and from each sample at least 10 field counts were made. Significant differences were taken at the $P = 0.05$ level.

RESULTS

Effect of concentration of chlamydiae. The survival of chlamydiae after thawing might be affected by the concentration of microorganisms during freezing and by whether inocula were diluted immediately after thawing. To study these possibilities, LGV was prepared in five dilutions and buffered with HEPES. After freezing in 20% sucrose at 2°C/min to −196°C and rapidly thawing, the concentrated suspensions were diluted to equal the most dilute sample, and the numbers of inclusion-forming units were assessed. The survival of organisms was not apparently affected either by their concentration during freezing or by dilution immediately after thawing.

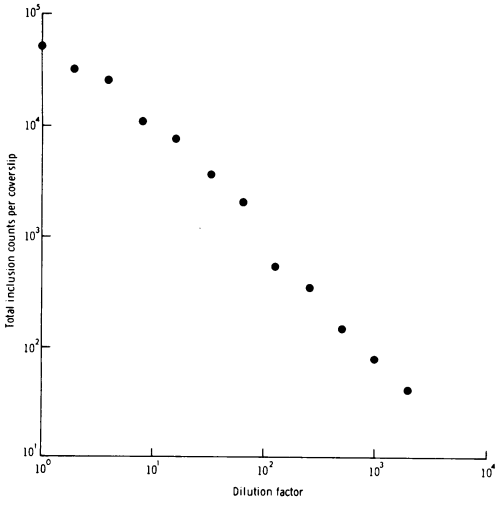

FIG. 1. *Inclusion counts obtained over 12 serial twofold dilutions of a suspension of genital chlamydiae. To compensate for the different magnifications used, the total number of inclusions on the cover slip was calculated. The linear relationship between the concentration of chlamydiae and the estimated number of inclusion-forming units can be seen.*

Effect of cryoprotective substances on chlamydiae at room temperature. Preliminary investigations into the effect of cryoprotective agents on chlamydial survival at room temperature for 1 h showed that glycerol (10%, vol/vol) reduced the inclusion count to less than 10% of the original. The survival in Me_2SO (10%, vol/vol) varied between 10 and 45%. For this reason, further investigation was confined to sucrose- and FCS-containing solutions.

To study the toxicity of sucrose and serum, equal concentrations of chlamydiae of genital origin were prepared in a variety of sucrose-, sucrose–phosphate-, and FCS-containing media. These were left at room temperature (23°C) for 2.5 h before inoculation onto McCoy cells (Table 2).

(i) Sucrose. The inclusion counts of chlamydial suspensions that had been standing in 5, 10, and 15% sucrose freezing solutions were not significantly different. However, in 25% sucrose there was a significant loss of infectivity.

(ii) Fetal calf serum. The greatest survival was obtained using 10% FCS; increasing the concentration to 20, 30, or 40% resulted in lower counts.

(iii) Sucrose-phosphate. KSP (diluted 4:1 by inoculum) gave similar results whether 3% or 10% FCS was included. However, the inclusion count after exposure of the chlamydial suspension to NaSP was significantly higher than it was when the inoculum was diluted in either of the KSP solutions.

TABLE 2. *Effect of cryoprotective agents on genital chlamydiae at room temperature for 2.5 h*

Medium	Mean inclusion counts/field[a]
Sucrose	
5% (wt/vol)	33.8
10% (wt/vol)	34.2
15% (wt/vol)	31.5
25% (wt/vol)	23.7
FCS	
10% (vol/vol)	39.8
20% (vol/vol)	27.6
30% (vol/vol)	25.6
40% (vol/vol)	26.1
KSP[b]	
3% (vol/vol) FCS	26.4
10% (vol/vol) FCS	28.3
NaSP,	
10% (vol/vol) FCS	35.7

[a] Pairs of means differing by 5.1 or more are significantly different at the $P = 0.05$ level.

[b] Diluted 4:1 by inoculum.

TABLE 3. *Stability of LGV at 23 and 4°C in CMGA and 20% sucrose*

Medium	Holding temp (°C)	Inclusion counts/field (n = 20) (mean ± standard error)			
		0 h	2 h	4 h	23 h
CMGA	23	24.7 ± 0.7	25.4 ± 0.7	23.3 ± 0.5	23.6 ± 0.8
	4		22.4 ± 0.8	26.2 ± 0.7	24.6 ± 1.1
Sucrose (20%, wt/vol)	23	24.7 ± 1.3	21.6 ± 0.9	22.5 ± 0.9	12.7 ± 0.7
	4		24.6 ± 1.1	24.2 ± 0.9	28.7 ± 1.1

The highest concentrations of cryoprotective agents that did not significantly affect survival at room temperature were used in subsequent freezing experiments.

Stability of LGV at 23 and 4°C. A freshly grown culture of LGV was suspended in either CMGA or 20% sucrose solution and held at either 23 or 4°C. The infectivity of samples was assayed after standing for 0, 2, 4, and 23 h. There was a significant reduction in infectivity in the sucrose solution held at 23°C for 23 h in contrast to that of the CMGA suspensions at both temperatures and the sucrose solution at 4°C (Table 3).

Effect of rate of cooling on survival of chlamydiae during freezing. In the presence of 20% sucrose, the survival of LGV was little affected by varying the rate of cooling between 0.1 and 367°C/min (Fig. 2). In one of the experiments, a fresh culture of LGV was frozen in 20% sucrose buffered with 20 mM HEPES. After thawing, an assay in cultures also buffered with HEPES resulted in an average inclusion count of 90% of the original unfrozen sample. This control sample was exposed to sucrose for less than 30 min at room temperature and had an average of 24.2 inclusions per field. The survival of LGV at the two cooling rates slower than 1°C/min was significantly lower than that at rates between 1 and 10°C/min. In the other experiment shown in Fig. 2, recently thawed LGV was used, buffered by bicarbonate throughout. The resulting overall survival was lower, averaging about 70%. The unfrozen control had a mean of 21.2 inclusions per field. In this instance the rate of cooling had no significant effect on LGV survival after freezing.

Similarly, the rate of cooling had a negligible effect on survival of the genital strain of chlamydia when sucrose was used as the cryoprotective additive (Fig. 3). Using either 15% sucrose freezing solution or KSP, containing 6.8% sucrose, diluted by inoculum 4:1, there was good survival at all the cooling rates examined. At only one cooling rate, 7.4°C/min, was the survival in KSP significantly better than in 15% sucrose. None of the results was significantly different from 100% survival.

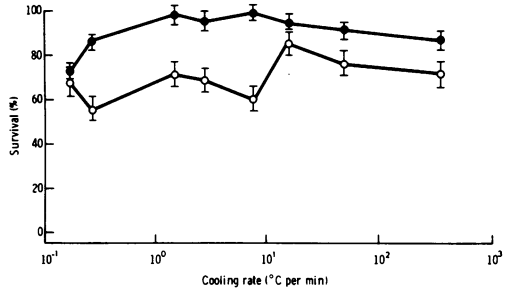

FIG. 2. *Effect of rate of cooling on survival of LGV after freezing to −196°C in two different solutions of sucrose (20%, wt/vol): fresh culture buffered with HEPES (●); and thawed culture buffered with bicarbonate (○). Both experiments indicate that survival is little affected by the rate of cooling.*

Effect of the cryoprotective agent on the survival of chlamydiae. A comparison between the cryoprotective abilities of four different media is shown in Table 4. Genital chlamydiae were suspended in either CMGA, 15% sucrose solution, NaSP, or KSP with 10% FCS (diluted 11.5:1 by the inoculum). Volumes of 0.2 ml of each suspension were prepared in eight tubes; two were assayed immediately, and the rest were frozen. The three cooling rates used were: 2°C/min using the standard cooling rate equipment; approximately 10°C/min produced by placing the tubes in a −70°C refrigerator; and a direct plunge into liquid nitrogen, which for a 0.2-ml sample is equivalent to a cooling rate of 367°C/min. Results showed that the infectivity of the unfrozen controls was similar except that that of the unfrozen control CMGA suspension was significantly below that of the KSP control. In this experiment all treatments resulted in significantly less than 100% survival. Organisms frozen in CMGA were best recovered at the intermediate rate of cooling, whereas, in general, the addition of sucrose resulted in similar survival at each cooling rate. Chlamydiae plunged into liquid nitrogen in the KSP, which contained a smaller amount of CMGA (1:11.5) than in the experiments shown in Fig. 3, were damaged as much as the organisms frozen in CMGA alone, thus showing the inherent variability of the system, especially after rapid cooling.

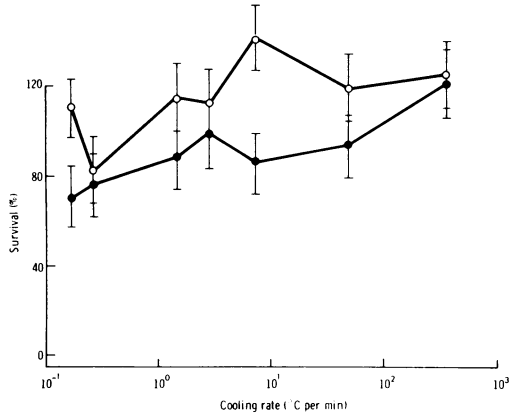

FIG. 3. *Effect of rate of cooling on survival from −196°C of a genital strain of chlamydia using sucrose as the cryoprotective agent. Using both KSP* (○) *and sucrose freezing solution (15%, wt/vol)* (●), *there is good survival at all the cooling rates examined.*

TABLE 4. *Survival of genital chlamydiae after freezing in four different solutions by three different methods*

Solution	Inclusion counts/field[a] (mean; n = 20)			
	Unfrozen control	2°C/min[b]	10°C/min[c]	367°C/min[d]
CMGA	19.3	9.7	14.4	10.3
Sucrose (15%, wt/ vol)	20.3	14.0	12.4	15.0
NaSP	19.8	15.7	14.7	14.6
KSP	22.1	16.5	15.6	11.6

[a] Pairs of means are significantly different if they differ by 2.2 or more.
[b] As in Materials and Methods.
[c] −70°C refrigerator.
[d] Directly into liquid nitrogen.

Effect of thawing rate on the survival of chlamydiae. The genital strain of *C. trachomatis* suspended in a 15% sucrose solution frozen by plunging into liquid nitrogen was thawed at three different rates. Survival was significantly greater when the samples were thawed rapidly by immersion of the whole vial in a turbulent 37°C water bath, whether the volume of suspension was 0.25 or 1.0 ml (Fig. 4). There was no significant loss of titer after freezing the smaller volume if the thawing was done rapidly.

Interrupting the thaw at −20°C. Small volumes (0.25 ml) were warmed from −196°C by placing in a refrigerator at −20°C for 20 min before immersion in a 37°C water bath. This

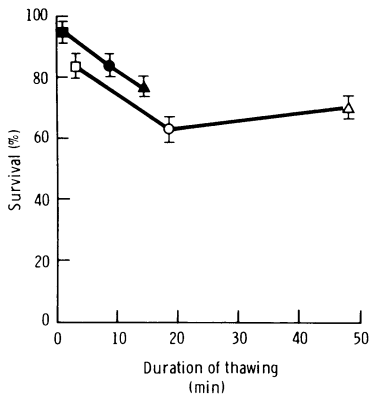

FIG. 4. *Effect of duration of thawing on the percent survival of a genital strain of chlamydia frozen in sucrose freezing solution (15%, wt/vol) by direct immersion of the samples into liquid nitrogen. The sample volumes were 0.25 ml (filled symbols) or 1.0 ml (open symbols). The thawing procedures were agitation in 37°C water bath (squares), standing at 23°C room temperature (circles), and in a 4°C refrigerator (triangles). Survival was best if thawing was rapid; even using the water bath, this required the use of a small sample volume.*

procedure resulted in a significantly lower survival (77.8%) than when samples were transferred from −196°C directly to the water bath (94.8% survival).

DISCUSSION

Cryoprotective agent. The results of this work show that chlamydiae can be recovered completely after freezing to −196°C with sucrose as the cryoprotective agent. In contrast, Reeve et al. (10) reported an 80% loss of infectivity after a yolk sac suspension had been frozen to −70°C. For many cells and microorganisms, the incorporation of a cryoprotective agent is essential to obtain significant survival after freezing. These agents are highly water-soluble compounds that are either of low molecular weight, like glycerol, Me_2SO, and sucrose, or polymers, like serum albumin. There is evidence that they protect by reducing the amount of ice formed during freezing and thus indirectly reduce the rise in the concentration of damaging solutes, for example, electrolytes. The choice of cryoprotective agent in any system is determined initially by its toxicity in that system. The results of the experiments described show that either the chlamydial preparations or the McCoy cells used in the assay system are injured by a toxic effect of the conventional cryoprotective agents glycerol and Me_2SO, but are less affected by sucrose or serum. It is therefore reasonable to use the latter

agents at the highest concentration that is relatively nontoxic, since cryoprotection is improved as the concentration of the agent is increased.

Rate of cooling. Most of our experiments showed that, in the presence of sucrose, the survival of chlamydiae after thawing from −196°C is little affected by the rate of cooling. This agrees with the report of Leibo et al. (6) that in other systems an effective concentration of cryoprotective agent, particularly sucrose, tends to improve survival over a wide range of cooling rates. Variations in the composition of the sucrose-containing solutions used in the present study did not seem to have any marked effect on the post-thaw survival over a range of cooling rates; the damaging effects of the components of the solutions were far more apparent at room temperature before freezing. The recovery of microorganisms before freezing from CMGA alone was less variable than that from the other solutions, but the cryoprotective properties of CMGA were less marked than those of the sucrose-containing solutions, in that survival was low at both faster and slow cooling rates. It is not possible, however, from these data to say whether the variability in survival of chlamydiae at room temperature was due primarily to the predominant action of the solution, to the proportion of CMGA present, or to the concentration of sucrose itself.

Estimation of survival. Differences between the survival after freezing chlamydiae in different experiments may be due to the inadequate method of liberating organisms from cells while preparing the inocula. Intact inclusions may still be present in the unfrozen control, whereas the membrane damage occurring during freezing may separate the infectious particles and so increase the number of inclusion-forming units. The very high survival rates seen in some experiments may thus be erroneous.

Rate of thawing. Survival of chlamydiae was best when thawing was rapid, which agrees with results of tests on many other cells and microorganisms. Indeed, there is now considerable evidence that a large proportion of freezing injury occurs during the rewarming phase and that this can be minimized by rapid thawing.

Application of the findings to clinical specimens. The results described may not necessarily apply to clinical specimens that possibly contain a variety of toxic substances, especially if taken from a genital region. It cannot be assumed that, as shown for experimental suspensions, there would be a negligible loss of organisms kept in CMGA or sucrose solutions at 4°C for 23 h. The unknown toxic factors may be more damaging than the effects of freezing or the toxicity of the cryoprotective agent. However, the detrimental effect of freezing was noticed by Reeve et al. (10), who cultured identical clinical specimens before and after a cycle of freezing and thawing. They found chlamydiae in 20% fewer specimens after this procedure. Using methods recommended in this study, this loss may be reduced so that the technique of using frozen clinical specimens for the detection of chlamydiae would be not only very convenient but also more reliable than in the past.

ACKNOWLEDGMENTS

We are grateful to S. Darougar for supplying strains of chlamydiae and to D. Taylor-Robinson for helpful advice and discussion.

LITERATURE CITED

1. **Darougar, S., B. R. Jones, J. R. Kinnison, J. D. Vaughan-Jackson, and E. M. C. Dunlop.** 1972. Advances in the diagnostic isolation of *Chlamydia* including TRIC agent, from the eye, genital tract and rectum. Br. J. Vener. Dis. 48:416–420.
2. **Darougar, S., J. R. Kinnison, and B. R. Jones.** 1971. Simplified irradiated McCoy cell-culture for the isolation of Chlamydiae, p. 63–70. *In* R. L. Nichols (ed.), Trachoma and related disorders. Excerpta Medica, Amsterdam and New York.
3. **Gordon, F. B., I. A. Harper, A. L. Quan, J. D. Treharne, R. St. C. Dwyer, and J. A. Garland.** 1969. Detection of chlamydia (Bedsonia) in certain infections of man. 1. Laboratory procedures: comparison of yolk sac and cell culture for detection and isolation. J. Infect. Dis. 120:451–461.
4. **Hanna, L.** 1962. Isolation of trachoma and inclusion conjunctivitis viruses in the United States. Ann. N.Y. Acad. Sci. 98:24–30.
5. **Holmes, K. K., H. H. Handsfield, S. P. Wang, B. B. Wentworth, M. Turck, J. B. Anderson, and E. R. Alexander.** 1975. Etiology of nongonococcal urethritis. N. Engl. J. Med. 292:1199–1205.
6. **Leibo, S. P., J. Farrant, P. Mazur, M. G. Hanna, and L. H. Smith.** 1970. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose or glycerol. Cryobiology 6:315–332.
7. **Morris, G. J., and J. Farrant.** 1972. Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to −196°C. Cryobiology 9:173–181.
8. **Oriel, J. D., P. Reeve, B. J. Thomas, and C. S. Nichol.** 1975. Infection with *Chlamydia* group A in men with urethritis due to *Neisseria gonorrhoeae*. J. Infect. Dis. 131:376–382.
9. **Prentice, M. J., D. Taylor-Robinson, and G. W. Csonka.** 1976. Non-specific urethritis: a placebo-controlled trial of minocycline in conjunction with laboratory investigations. Br. J. Vener. Dis. 52:269–275.
10. **Reeve, P., J. Owen, and J. D. Oriel.** 1975. Laboratory procedures for the isolation of *Chlamydia trachomatis* from the human genital tract. J. Clin. Pathol. 28:910–914.
11. **Weiss, E., and H. R. Dressler.** 1962. Investigation of the stability of the trachoma agent. Ann N.Y. Acad. Sci. 98:250–258.
12. **World Health Organization.** 1975. Guide to the laboratory diagnosis of trachoma, p. 25 and 28. World Health Organization, Geneva.