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Experiment IX. Effect of freezing on the viability of spores of A. flavus NOT suspended in any vehicle during freezing. 5/31/52.

Procedure: Culture 32 (Age 14 days) and Culture (33) Age 12 days were utilized.

Spores were tapped and brushed onto sterile weighing paper and then two small amounts tapped from these into the bottom of the freezing tubes. Some spores undoubtedly stuck to the upper sides of the tube; but they probably were not among those removed during inoculation.

After freezing, approximately 0.1 ml double sterile distilled was pipetted into each freezing tubes; the contents well stirred, and approx 0.03 ml. introduced onto each agar disk. Exact amount were not quantities were not used here as there was beleived to be a very large difference in the number of spores introduced in the various freezing tubes.

One slight mistake was made: Tubes # 6 (3 tubes) were treated in resuspension as follows: 0.1 ml. H_2O added to 1st tube, and stirred; then same pipette used to draw up ## double distilled water for second tube. This undoubtedly introduced some spores into the test tube containing appox 50 ml. of double distilled water. The error thus introduced into later tubes (in which in a given set (for which one pipette was utilized) all ~~tod~~ 3 tubes were filled with 0.1 ml. D_2O, then stirring was done) was

probably very slight because 1. Spores became greatly diluted in 50 ml - only 0.1 ml. withdrawn for a sample
2. From experience, many spores tend to remain on surface layer of water, and resuspending water was always removed with the mouth of the pipette well below the surface.
The error must have been well under 1%.

Otherwise Procedure was as outlined before.

Experimental: 5 sets of tubes utilized.

3 tubes	A/	No Vehicle;	Quick freezing;	Quick warming; Quick Thawing
3 "	B/	"	"	Slow warming; " "
3	C/	"	"	slow warming Slow thawing
1	D/	Double distilled water (vehicle) *	"	Quick warming; Quick thawing
1	E/	" *	"	Slow warming; Slow Thawing

Each tube was inoculated in duplicate.

* In D and E the procedure was as in previous exp. except "dry" spores added to tubes first, then 0.1 ml. water added. ~~Two~~

~~Duplicate~~ E tubes also served as check for preceding experiment i.e. Frozen at -70°, then put in bath at -15° and allowed to warm then thawing (~~DE~~).

In C, tubes were removed when temperature reached -2° C and then thawed for 35 sec in water at +35°

Since no ~~liquid~~ water vehicle was present, thawing could not actually be observed; but 30-40 sec. has been the usual amount of time.

D. was quick thawed in usual way.

Initial freezing

Temperature = 67° (at beginning) and -69 to -70° during most of freezing.

Duration of freezing = 18 minutes.

Warming

A) Quick warming; quick thawing. ~~Tubes~~ Dry spores; Tubes # 6-II, 3-I and an unmarked tube

Tubes in water at + ~~37°C~~ (+34.5 °C) - immediately (or within 30 sec after removing from initial freezing bath.

B) "Dry" spores; slow warming; quick thawing

Time (after removal from freezing bath	Temperature Therm. # 2, # 3		Time	Temp. (#2	#3 ~~#3~~	#4
6 min		-49°	22 min.	-31.5	-30° - ~~31~~	
9		-45°	27 min	-27.5	-27°	
12.5	-41	-39°C	33 min.	-22	-21	
18	-36	-34°	43.5	-13	-8.5	-13°
			48	-10	-10.5	-11°

B) Continued

Time	Temp.	Therm. # 2	3	4
~~#8~~ 54 min		-6.2	-7	-7.0
58 min		-4.0	-4.5	-5.0
61		-2.5	-2.5	-3.0
62.5 minutes		-1.5	-2°	-2.0

Then removed and thawed for 35 sec. in water at +35°C. Warming in 600 ml. beaker containing 390 ml. of methyl alkasolve.

Total measured time = 56.5 minutes (ie 62.5 − 6)

Total # °C = 49 − 2 = 47°

Avg. warming Rate = 47°/56.5 min = 0.83°/min.

C) "Dry" spores - slow warming slow thawing

Time (after initial freezing)	Temperatures Therm. # 2	#3
7 min		-46.5°C
9.5 min		-42°
13	-36	-34°
19	-25.5	-27°
21	-25.5	-25
24.5	-21.5	-20.8°
28	-17	-16
33.5	-13.5	-12
42	-3.5	-3.5

Time	Therm # 2	#3
45 min	-1.6	-2°
47 min	0°	0° (Thawing began)
57 min	+3.4°	+4°
59 +5 min	+5.5°	+5.5°

Tubes removed

Warming was done in a 600 ml. beaker containing 150 ml. of methyl cellosolve.

Past experience has shown that thawing of ice in tube occurs when outside bath is approx + 5°C. Hence this temp was gained before tubes were removed. Time for bath to go from 0° to +5.5° = 12.5 minutes.

Measured time elapsed = 59.5 - 7 = 52.5 minutes.
°C warmed = 46.5 + 5.5 = 52.0

Avg. Rate = $52°/52.5$ mins = 0.99°/min.

D. See p. 13.

E. Spores in Double distilled water. Warmed and thawed in bath starting at −15°C.

Time (after initial freezing)	Temperature			
5 mins	−8.4°C	Therm. #2	20 ~~#~~ mins	−0.1°C
8.5	−6°		20.5	+0.1° — Thawing begins
12	−4.6°	Therm. #4 ↓	23.5	+ 1.7°
14	−3.3°		26.5°	+ 3°
16	−1.5°		29°	+ 4°
			30.5	+ 4.7
			~~50~~ 32	+ 5.6° — Thawing complete

Warming vessel was 1000 ml. Beaker containing 280 ml. methyl cellosolve.

7 tins for thawing = 11.5 minutes.

Total measured elapsed time = 32-5 = 27 min.

0°C = 8.4 + 5.6 = 14 0°

Avg. Rate = 14°/27 min = 0.52°/min.

Inoculations & killing

Designation	Description	Time Inocc.	Time killed	Length of Incub.
A	Dry. Quick warm & Thaw	11:45 PM.	3:45	16 hrs.
B	Dry, Slow " quick thaw	11:45 PM.	"	16 hrs.
C	" " Slow thaw	11:45 PM.	"	16 hrs.
D	Water, GW. QT	11:30	"	15 hrs 45 min
E.	Water SW. Slow thaw	11:30	"	" "
Controls	A *	11:45	3:45	16 hrs.

* Prepared by stirring spores in bottle distilled water at 11.45 - Pipetting .03 ml. of suspension into each of two Syar dishes.

Slide #	Tube #	Description	# Germ.	# Not Germ.	% Germin.	Comments
A. 1-A	3-I	~~Spores~~ Spores Dry	422	47	422/469 = 89.9	
1-B	3-I	Fast Warm	642	46	642/688 = 93.4	
2-A	6-II	Fast thaw	789	69	789/858 = 92.0%	one Large Clump*
2-B			622	83	622/705 = 88.3%	A few small clumps.
3-A	Blank		422	99	422/521 = 80.9%	
3-B			No Spores on slide.			
B - 1-A	2-I	Spores Dry	217	48	217/265 = 81.8%	Spores scarce - in clumps - clumps same # of unsperm spores?
1-B		Slow Warm	154	30	154/184 = 83.7	
2-A	2-II	Fast thaw	343	104	343/447 = 76.8%	
2-B			279	72	279/351 = 79.4%	Spores Scarce crystals on agar.
3-A	~~5-I~~ ~~2-III~~		277	144	277/421 = 65.7%	Spores Scarce - crystals on Agar
3-B						
C 1-A	1-I	Spores "Dry"	486	258	486/744 = 65.3%	Some Broken spore particles - Not many.
1-B		Slow Warm	359	176	359/535 = 67.2%	
2-A	1-II	Slow Thaw	342	47	342/389 = 87.9%	Middle too thick to count. :: by eye
2-B			330	40	330/370 = 89.3%	- But not low % in center
~~3-B~~ 3-C	~~5-II~~		573	87	573/660 = 86.8%	Agars v. crowded in central regions -
3-C			575	191	575/766 = 75.0%	count.

* This clump contained a good number of ungerminated spores - but most of these spores had not wetted. Prob. the % (ie not including clump of several to many thousand spores) is probably accurate -

Slide	Tube	Description	# Germ.	# Not	% Germ.
D	1-A	9-II Spores in H_2O.	416	181	416/597 = 69.7%
	1-B	" Fast warm, Fast thaw	459	159	459/618 = 74.2%
E	1-A	9-II-B spores in water - Slow warm and thaw	43	573	43/616 = ~~7.0%~~ 6.97% *
	1-B		42	702	42/744 = ~~5.7%~~ 5.65% *

Control A-1 See bottom p. 16. - Around 65% germ. ~~not~~ Uncon - Must be
A-2 due to killing spores in transfer or to some dead spores remaining in tube

* There were numerous & large clumps of spores which had not wet; these had not germinated, but were not counted.

from previous times.

Conclusions.

Although the variation is quite great in some cases, it is quite clear that spores ~~frozen~~ cooled in air to −70°C are not harmed by the process. This would seem to be best explained by no freezing occuring. ie ~~outside~~ an aqueous vehicle is necessary for freezing to occur.

~~A~~ Statistics

	m	x	m−x	$(m-x)^2$
A. 89.9	88.9	89.9	+1.0	1
93.4	88.9	93.4	+4.5	20.25
92.0	88.9	92.0	+3.1	9.61
88.3	88.9	88.3	−0.6	0.36
80.9	88.9	80.9	−8.0	64.00
Σ = 444.5				95.22

Mean = 88.9

$\sigma = \sqrt{\frac{95.22}{5}} = \sqrt{19.05} = 4.36$

Mean = 88.9 ± 4.36 %

	m	x	m−x	$(m-x)^2$
B. 81.8	77.4	81.8	+4.4	19.36
83.7	77.4	83.7	+6.3	39.69
76.7	77.4	76.7	−0.7	0.49
79.4	77.4	79.4	+2.0	4.0
65.7	77.4	65.7	−11.7	136.9
Σ = 387.3				Σ = 200.44

$\sigma = \sqrt{\frac{200.44}{5}} = \sqrt{40.2} = 6.34$

Mean = 77.4 ± 6.34 %

	m	x	m−x	$(m-x)^2$
C. 65.3	79.1	65.3	−13.8	190.44
67.2	79.1	87.9	+8.8	77.44
87.9	79.1	89.3	+10.2	104.04
89.3	79.1	67.2	−11.9	191.61
86.8	79.1	86.8	+7.7	59.29
78.0	79.1	78.0	−1.1	1.21
474.5				Σ = 574.03

$\sigma = \sqrt{\frac{574.03}{6}} = \sqrt{95.7} = 9.76$

Mean = 79.1 ± 9.76 %

D. 69.7
74.2
143.9 ÷ 72.0 %

Mean = 72.0 ± 2.2 %

E. 6.97
5.65
12.62 Mean = 6.31 %
5.65
.66

Mean = 6.31 ± 0.66 %