

MYCOBACTERIUM LEPRAE MURIUM: PRESERVATION BY FREEZE-DRYING

ROY GOULDING, M. B., B. S., B. Sc.
Department of Pharmacology
Guy's Hospital Medical School
London, England

Despite the various claims advanced from time to time, *Mycobacterium leprae* has never been cultivated *in vitro*, nor has it been convincingly transmitted to any laboratory animal. Similarly, *M. leprae murium* has defied attempts to grow it on laboratory media, although it can be transferred satisfactorily to rats, mice and hamsters (9). This problem of cultivation has become more acute now that antibiotics and chemotherapeutic agents are available which might prove effective against human leprosy infections. A reliable screening test would be an advantage.

Although there is no immediate prospect of using *M. leprae* for this purpose, a number of workers have published the results of therapeutic tests carried out with animals artificially infected with *M. leprae murium* (1-9). Carpenter, in fact, declares "that experimental murine leprosy remains as the most valuable tool with which to investigate the unsolved problems in the human infection." The technical difficulty nevertheless remains that the bacterial strains to be used in this way must be maintained by repeated passage through living hosts, and that is time-consuming. As Hanks (10) has emphasized, murine leprosy bacilli deteriorate rapidly under every *in vitro* condition which has been studied. On the other hand, Wilson and Miles (11) have stated that this microorganism "cannot withstand drying, but it remains viable for 2 years or more in infected organs preserved in 40% glycerol at 0.6°C."

Freeze-drying suggests itself as a means by which such organisms might be preserved in a viable state for use whenever needed.

METHODS

Infesting material.—The strain of organisms used in these experiments was obtained from a mouse kindly provided by Dr. S. R. M. Bushby of the Wellcome Research Laboratories, Beckenham, Kent. Because Grunberg and Schnitzer (9) had encountered difficulty with intravenous inoculations, and because we had sometimes been troubled with immediate, convulsive (probably embolic) deaths when employing the technique described by Barnett and Bushby (1), the attempt was made to obtain a clean sample of organisms in a simple suspending fluid.

A mouse infected five months previously was killed. The liver and spleen were both enlarged, pale and friable, and direct smears made from these organs showed, after Ziehl-Neelsen staining, dense masses of acid-fast organisms. With aseptic precautions these tissues were chopped finely in a petri dish and ground to a smooth

paste in a glass tissue grinder¹ with a small volume of 0.05 per cent Tween 80 in 0.9 per cent saline. The resulting crude suspension was then centrifuged at 1,500 rpm for 10 minutes to remove the gross, particulate matter. Microscopic examination of the supernatant fluid showed it to be still rich in acid-fast organisms. This was separated and recentrifuged at 5,500 rpm for a further 20 minutes. The supernatant fluid was then found to contain a little detritus but no organisms. It was therefore withdrawn and the sediment, amounting to about 0.5 cc., was shaken up with approximately 3 cc. of the Tween-saline suspension fluid. Further centrifuging at 5,500 rpm followed, the supernatant fluid being pipetted off again and the sediment being finally resuspended in Tween-saline. A smear of this suspension showed a dense concentration of well-dispersed organisms and negligible extraneous matter.

When this suspension was diluted 20 times with physiological saline and, in volumes of 0.5 cc., introduced intravenously into adult albino mice each weighing about 25 mgm., there were no early complications and progressive, generalized murine leprosy developed. Most of the animals succumbed to this disease in 3 to 6 months. Autopsy showed the liver and spleen to be chiefly involved, and the lungs and kidneys were also infected. These animals were the control of viability of the bacilli in suspensions prepared in this way.

Suspensions for the experiment.—Two suspensions were prepared for freeze-drying and the subsequent test inoculations.

(1) Horse serum suspension: A mouse was killed four months after being infected in the manner described, when it was in a grossly cachectic state. From the liver and spleen a clean, centrifuged sediment of organisms about 0.5 cc. in volume was prepared by the technique described. This was resuspended in 3.0 cc. of sterile horse serum² and dispersed by shaking.

(2) Glucose-broth suspension: A second, similar specimen of organisms from another mouse was resuspended in 3 cc. of sterile 20 per cent glucose-digest broth, prepared in the laboratory. The broth sample used in this experiment had been kept in stock, protected from light, for about a year.

Freeze-drying.—Freeze-drying was carried out in the Department of Bacteriology, Guy's Hospital Medical School, by means of a modified centrifugal freeze-drier. In this apparatus it was possible to use as the desiccating agent either phosphorus pentoxide in the trays provided in the standard model or solid carbon dioxide in a special condensing unit projecting into the main vacuum chamber.³

Aliquots of 0.15 cc. of the prepared suspension of organisms in horse serum were introduced into 20 tubes for the processing. Ten of the tubes (Group A) were dried by means of solid carbon dioxide for 6 hours and then briefly over phosphorus pentoxide before sealing. The other ten tubes (Group B) were kept for about 15 hours over phosphorus pentoxide after the initial drying with carbon dioxide. The same procedure was applied to the suspension of bacilli in glucose broth, making Groups A and B as before.

¹ Gallenkamp, 1951 - a7623.

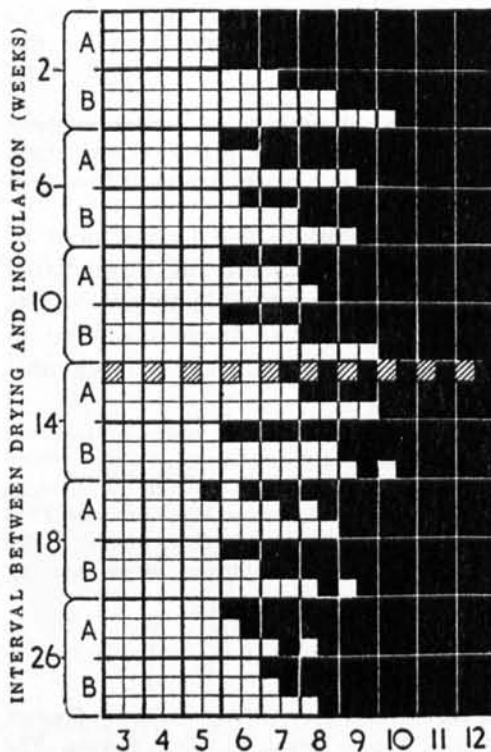
² M. R. C. Serum Research Institute, Carshalton, Surrey.

³ The apparatus used was the Model 2 centrifugal freeze-drier made by Messrs. Edwards (High Vacuum) Ltd., Allendale Works, Manor Royal, Crawley, Sussex. The modification was designed, in consultation with the manufacturers, by Prof. R. Knox, to whom I am indebted for this description. It has been used for several years in the routine freeze-drying of bacterial cultures, sera and virus preparations.

Testing for viability.—As survival of the preserved organisms could not be detected *in vitro*, viability was estimated by their virulence when introduced again into susceptible mice. Each freeze-dried specimen was therefore reconstituted with 4.5 cc. of the Tween-saline, and 0.5 cc. of this inoculum was injected intravenously into each of 6 albino mice weighing about 25 gm. (with one pair of specimens dried in glucose broth only 5 mice in each group were subsequently inoculated.) This represented a dilution equivalent to 30 times that of the original suspension before drying. Groups of mice were inoculated in this way at different intervals following the time of drying, up to a maximum of six months, 12 mice on each occasion. Of each 12 mice, 6 were given the Group A inoculum, subjected to drying with solid carbon dioxide for six hours, and the other 6 were given the Group B inoculum, dried for a further fifteen hours over phosphorus pentoxide. In addition, 6 mice were each given 0.5 cc. of the undried glucose-broth suspension, diluted 30 times with Tween-saline. These animals were all housed and fed in a normal manner.

RESULTS

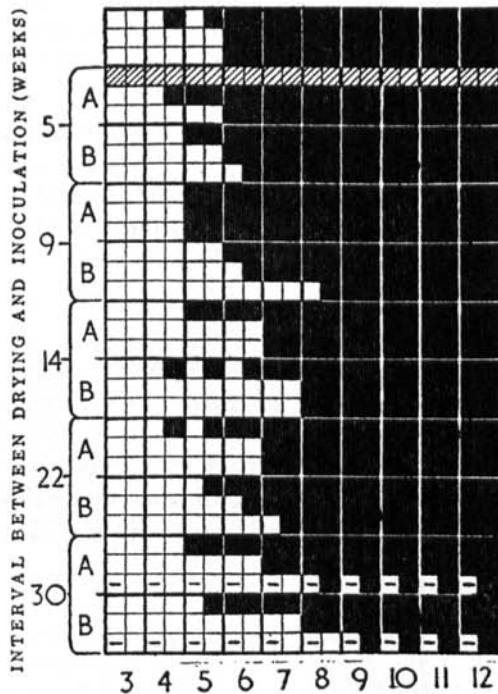
A few of the mice died from intercurrent infection. The rest died from generalized murine leprosy at intervals ranging from 4 to 10 months.



TEXT-FIG. 1. Results of inoculation of groups of 6 mice inoculated with freeze-dried suspensions of *M. leprae murium* in horse serum, by months. A = the material dried over CO₂ and briefly over P₂O₅ before sealing. B = the material dried over CO₂ and for 15 hours over P₂O₅ before sealing. Open squares, infected mice; cross-hatched squares, mouse dying incidentally; solid black squares, mice dying from generalized leprosy.

The results are shown in Text-figs. 1 and 2, the former concerning the suspension made in horse serum and the latter the one made in glucose

broth. All mice were inspected postmortem, and sections were made of the livers, spleens and lungs and stained by the Ziehl-Neelsen technique. The density of acid-fast organisms in the first two of these organs was consistently high in all the animals.



TEXT-FIG. 2. Results of inoculation of groups of 6 mice inoculated with freeze-dried suspensions of *M. leprae murium* in glucose broth, by months. Letters A and B and the symbols as in Text-fig. 1. The 30-weeks groups consisted of 5 mice each.

Messrs. Edwards (High Vacuum) Ltd. kindly assisted by estimating moisture content of a few of the freeze-dried specimens by their vapor pressure method. These are quoted, in percentages, as follows:

Specimen	Suspending fluid	Short drying	Prolonged drying
1	Serum	4.0%	—
2	Serum	3.8%	—
3	Serum	—	0.36%
4	Serum	—	0.70%
5	Glucose broth	9.8%	—
6	Glucose broth	10.9%	—
7	Glucose broth	—	3.41%

DISCUSSION

It is evident that, by freeze-drying in this way, viable organisms are retained and preserved for at least six months. To interpret the data quantitatively, however, may be hazardous.

No figures are yet available (although experiments are meanwhile proceeding) to show the relation between the size of the infecting dose of organisms and the subsequent survival time of the mice. But since the density of acid-fast bacteria found postmortem in all the animals suc-

cumbing to the disease was virtually uniform, the quantitative viability of the reconstituted suspensions of organisms might well be related inversely to the time interval between the original infection and death in each case. If this index be provisionally accepted, then in the case of glucose-broth suspensions (Text-fig. 2) the dried specimens appeared to have suffered little impairment by comparison with the undried material. Moreover, only slight progressive deterioration seems to have occurred with keeping, for the specimens reconstituted after six months were almost as virulent as those used within a few weeks of drying. Finally, there is a suspicion that prolonged drying over phosphorus pentoxide brought about a greater loss of viability than the shorter period of drying over this agent. Significantly, perhaps, the moisture contents of the specimens prepared by the two techniques were markedly different.

SUMMARY AND CONCLUSIONS

A method is described for freeze-drying clean suspensions of *Mycobacterium leprae murium* in horse serum and in glucose-digest broth. Judging by the virulence of the reconstituted specimens when injected into susceptible mice, the viability of the organisms was preserved.

It appears from these experiments that viable bacilli can be satisfactorily preserved by freeze-drying, at least for periods up to six months.

ACKNOWLEDGMENTS

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RESUMEN Y CONCLUSIONES

Se describe un método para congelar-desechar suspensiones limpias del *Mycobacterium leprae murium* en suero de caballo y caldo de glucosa-digestante. A juzgar por la virulencia de los ejemplares reconstituídos, al ser inyectados en ratones susceptibles, se conservó la virulencia de los microbios.

Por estos experimentos parece que, con la congelado-desección, pueden conservarse satisfactoriamente bacilos viables, por períodos a lo menos de seis meses.

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