10953

Preservation of Viable Malaria Parasites in the Frozen State.

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The study of malaria has often been seriously handicapped by the lack of a suitable method for the maintenance of parasites in a viable state outside a living host. All attempts to cultivate them *in vitro* have been unsatisfactory, as have various freezing and drying procedures which generally give satisfactory results with viruses and bacteria. An important contribution to the problem of preservation of treponematas was made by Turner[1] when he showed that syphilis spirochetes can be kept virulent and active for more than a year in glass containers immersed in a mixture of alcohol and CO_2. Numerous attempts were made, with negative results, in this laboratory to preserve malaria parasites in a viable state by a similar procedure. In these attempts infected blood and tissues from avian, monkey and human sources, as well as infected mosquitoes, were used, and the preserved material, although frozen rapidly, was thawed slowly for the infectivity test. Recently Turner,[2] after his experience with the preservation of treponematas, emphasized the importance of freezing and thawing the material as rapidly as possible and the necessity for low storage temperatures within a very narrow range of fluctuation.

With the thermos jugs used in this laboratory for storage of biologically active material in a mixture of alcohol and solid CO_2, a fairly uniform temperature can be maintained only when there is an excess of CO_2; but as the amount of CO_2 diminishes through evaporation, the temperature of the mixture rises, and under ordinary storage conditions wide variations in the temperature occur. To overcome this difficulty Horsfall[3] recently has devised a dry low temperature storage cabinet in which the total fluctuation in the temperature does not exceed 8°C from the average of −76°C. In investigating once more the possibility of preserving malaria parasites in frozen state, use was made of this cabinet for storage purpose, and the practice of freezing and thawing the material as rapidly as possible was routinely adopted. The results obtained form the basis of this report.

1 Turner, T. B., *J. Exp. Med.*, 1938, **67**, 61.

2 Turner, T. B., personal communication.

3 Horsfall, F. L., Jr., in press.

Materials. The material for freezing was infected whole blood from rhesus monkeys with subacute or chronic malaria infections. The plasmodia used were *Plasmodium knowlesi* and *Plasmodium inui.* The blood to be frozen was either defibrinated, heparinized, or citrated, using one part of a 2% sodium citrate solution to 3 parts of blood.

Freezing and Thawing. The blood was placed in screw-capped celluloid tubes 4⅞ inches in length and ⅞ of an inch in diameter, with a capacity of 17 cc. In order to freeze the blood as rapidly as possible, the amounts were limited usually to 1 cc. With a whirling motion each tube was immersed in a mixture of solid CO_2 and alcohol. As soon as the material was frozen solid it formed a coating on the inside of the tube. It was then rapidly transferred to a low temperature storage cabinet until ready for testing. The cabinet mentioned above kept cold by dry solid CO_2, maintained a temperature between –72°C and –80°C. Considerable precaution was exercised in order to thaw the frozen specimens as rapidly as possible. Immediately upon removal from the cabinet, the tubes were immersed and rapidly rotated in a water bath at 37.5°C placed beside the cabinet. In this way the contents were reduced to a liquid state at body temperature within a few seconds. Before being injected interperitoneally into normal monkeys, samples were examined for dissolved hemoglobin, number of intact red cells, and parasites.

Determination of Viability of Frozen Parasites. The details of the tests for the viability of the malaria parasites maintained at low temperatures are summarized in Table I. Monkeys 1 and 2 were inoculated with 1 cc of infected *P. knowlesi* and *P. inui* blood which had been stored at –76°C for 70 days, and each animal devel-

TABLE I.
Results of Infectivity Test in Normal Rhesus Monkeys, After Injection with Malaria Parasites Stored at —76°C.

No. of Monkey	Species of parasite	Anti-coagulant used	Conc.* of parasites in preserved blood	Days in frozen state	Amt of preserved blood tested, cc	Parasites in blood of monkeys on
1	*P. knowlesi*	Citrate	113	70	4	7th day
2	*P. inui*	"	+	70	1	14th "
3	*P. knowlesi*	Heparin	490	19	1	5th "
4	" "	Defibrinated	490	19	1	5th "
5	" "	Citrate	490	19	1	6th "
6	" "	None†	490	19	1	5th "
7	" "	None‡	490	19	1	5th "
8	" "	Heparin	+	19	1	9th "
9	" "	Citrate	+	19	1	14th "

* Number of parasitized red cells per 10,000 normal cells.
† Placed in icebox before clotting.
‡ Allowed to clot before transferring to icebox.

oped typical infection characteristic of the respective organisms. Monkeys 3 through 9 likewise became infected after receiving infected blood from different sources maintained at the same temperature for 19 days. Of particular interest were Monkeys 2, 8, and 9, which became infected from 1 cc of blood in spite of the fact that the source monkeys had a maximum of less than one parasite per 10,000 red cells. The incubation periods observed in all of the monkeys were approximately the same as those obtained from comparable inoculations of fresh blood, thus indicating that there is relatively little loss of infectivity by freezing in the manner described. The data at hand indicate that the method as described for the preservation of malaria parasites of monkeys offers a more practical method than animal passage for maintaining strains or species. The possibilities whereby this method may facilitate the study of malaria are apparent.

Summary. A method of preserving monkey malaria parasites for as long as 70 days by rapid freezing, storage at –76°C, and rapid thawing without loss of viability is described.

10954 P

Relation of Age to Immune Response of Mice to Formolized Equine Encephalomyelitic Virus.

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This paper presents a study of the relation of age to the ability of animals to be immunized to a viral disease, as studied by injecting mice with non-infective formolized equine encephalomyelitic virus.[1] The immunity induced was measured by resistance to active virus and by development of neutralizing antibodies.

The capacity to form antibodies to non-infective antigens of non-viral nature is known to increase with age. This has been demonstrated with phenolized typhoid bacilli in guinea pigs and rats,[2] formolized typhoid bacilli, sheep red cells, horse serum and egg-albumin in rabbits,[3] and formolized *Trypanosoma lewisi* in rats. In

[1] Cox, H. R., and Olitsky, P. K., *J. Exp. Med.*, 1936, **63**, 745; Olitsky, P. K., and Morgan, I. M., *J. Am. Vet. Med. Assn.*, 1939, **95**, 530.

[2] Kligler, I. J., and Olitzki, L., *Z. Hyg. u. Infektionskrankh.*, 1929, **110**, 459.

[3] Freund, J., *J. Immunol.*, 1930, **18**, 315.