QUANTITATIVE STUDIES ON THE CRYOPRESERVATION OF PIROPLASMS WITH PARTICULAR REFERENCE TO *BABESIA RODHAINI*

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This thesis has been composed by me, and it is a record of my own work.

BRIAN J. TAYLOR.

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SUMMARY

Aspects of the cryopreservation of piroplasms have been studied. The majority of the experimental work was carried out on Babesia rodhaini-infected rat blood. The use of three cryoprotectants, glycerol, dimethylsulphoxide (DMSO) and polyvinylpyrrolidone (PVP), was studied. Greatest success was achieved using DMSO at a final concentration of 10%. Three cooling rates, slow, rapid and ultra-rapid, were studied. These cooling rates were also measured and are presented in tabular and graphical form. The best results were achieved using the rapid cooling rate. Two containers, glass ampoules and pp60 polythene capillary tubing, were studied, the best results being achieved using pp60.

Two methods for the estimation of the degree of damage suffered by Babesia rodhaini-infected rat blood during cryopreservation, percentage haemolysis estimation of host red cells and infectivity measurement, were used. Crystals, thought to be associated with haemoglobin, were often found in frozen and thawed blood samples. It was suggested that these crystals affected percentage haemolysis estimations, rendering this estimation technique invalid, for rat blood. The infectivity results obtained in the penultimate experiment in this study cast some doubt also on the validity of the infectivity titration method used in this study. This is discussed at length in the discussion section of that experiment.

Stage of infection in the host, level of parasitaemia in the host and nature of diluent used were all shown to have little effect on the ability of Babesia rodhaini-infected rat blood to withstand

the rigours of cryopreservation. In the last experiment in this study, similar results to those obtained for the cryopreservation of Babesia rodhaini-infected rat blood were obtained for the cryopreservation of Babesia microti- and Nuttallia musculi-infected mouse bloods. From the results obtained for the three piroplasms studied, and from the evidence in the literature, it is suggested that the best method tested, involving the rapid cooling of DMSO-protected blood in pp60 or in glass ampoules could be applicable for the cryopreservation of piroplasms in general.

Introduction

The value of the technique for low temperature preservation (or cryopreservation) of biological material is now well recognised. Many and varied cell types are stored at cryogenic temperatures (usually about -80°C, in solid carbon dioxide or in electromechanical refrigerators, or -196°C, in liquid nitrogen) in experimental or reference banks from which they can be recovered at any future moment for further experiment or reference. These banks of frozen material obviate the need for repeated passage in animals or in culture which is expensive in time and materials and which might subject the particular organism or cell type to selection pressures resulting in possible serious consequences for any comparative study over an extended period.

Workers studying protozoa in one form or another have not been slow to recognise the potential of the technique of cryopreservation, judging from the number of reports of species so far frozen down successfully. (Reviewed by Diamond (1964) and later by Dalgliesh (1972)b.)

Few of these reports, however, have contributed to our knowledge of the basic principles of cryopreservation, about which we still know little. Rather, they have merely been applications of previously successful methods to other species or cell types. This important point was stressed by Meryman (1966) in the foreword to the comprehensive review of the subject, 'Cryobiology', which he edited. Meryman recognised that: "Cryobiology had, by virtue of its many invaluable applications, been propelled headlong far beyond the limits of current basic understanding." He stated the necessity for more basic research in cryobiology in general, a view obviously

shared by the many leading workers in this science who contributed to the symposium on 'The frozen cell' (Wolstenholme and O'Connor (1970)), the stated aim of which was: "... to try to relate the physical to the biological properties necessary for cell viability and to learn more about the basic mechanism or mechanisms of freezing injury and protection."

Such a necessity for basic research into the low temperature storage of protozoa in particular - involving investigations into the mechanisms of injury acquired during the processes of cooling to, storage at, and thawing from low temperatures and its prevention - is discussed by Lumsden (1972). Lumsden observed that there was a lack of quantitative data: "... on the effects of low temperature storage on the organismal potential of all but a few protozoan parasites." It is hoped that this study will afford some such data.

The broad objectives of the work described in this thesis, therefore, were as follows:

(a) To find a successful, preferably convenient, method for the cryopreservation of parasitic protozoa, particularly intraerythrocytic parasites. The parasite to be used for the initial experimental work was the rodent babesia, Babesia rodhaini.

(b) Although it was not the aim of this work to try to find one particular successful method for all protozoan parasites, it was hoped that the results obtained would afford some guide to the probability of success and therefore allow some degree of prediction vis à vis the best and most convenient method.

(c) The only method of estimating the success of any one technique for the cryopreservation of a parasitic protozoan with any degree of accuracy and universal acceptability is a test of infectivity.

(For example, the ID_{63} method of Lumsden, Cunningham, Webber, van Hoeve and Walker (1963) used for trypanosomes) or the log. dose against prepatent period or survival time titration of Overdulve and Antonisse (1970a and 1970b) for babesias. These methods, however, are costly in time, materials and experimental animals and are ruled out in domestic animals for economic reasons. It was hoped that some simpler and less expensive alternative indicator of degree of success might be found.

CHAPTER 1

Review of the literature

Progress in the development of cryopreservation was, at first, slow, but the description by Polge, Smith and Parkes (1949) of the cryoprotective properties of glycerol heralded the beginning of a period of rapid development of the technique of cryopreservation for many and varied types of biological material. It was in this period that the addition of other cryoprotectants to our limited range took place and much about the mechanisms by which cells are protected, damaged, or lethally impaired, was added to our knowledge.

It is important that some details of the necessarily empirical research which afforded these additions to our knowledge be given in order to justify the subsequent experimental design in this study. For example, with regard to the results obtained by other workers in the past, initial studies may be limited to the 'best' cryoprotectants, to the cooling rates likely to give good results, and so on, in order to achieve a situation in which experiments of a reasonable size might produce the maximum information. The justification for such limitations lies in the history of the development of cryopreservation.

History of cryopreservation (a) General

The science, or sub-science, of cryobiology has its origins deep in the history of science itself. In a classic review volume entitled 'Life and Death at Low Temperatures', Luyet and Gehenio (1940) cited references to observations noted as early as 1737. Duhamel and Buffon (1737), dealing with the problem of frozen animals and limbs, suggested that a frozen animal or limb be thawed slowly by bathing

it successively in snow, then water, and then dung.

The importance of the Luyet and Gehenic review can be summed up adequately using the words of A.S. Parkes in the foreword to Audrey Smith's 'Biological Effects of Freezing and Supercooling' (1959), itself a classic review.

"The biological effects of cold have been observed one way or another for centuries and many distinguished scientists have been interested in this field. Apart from the writings of Spallanzani, however, the literature until some twenty years ago was meagre and largely anecdotal. This phase was brought to an end by the work of Luyet in St. Louis, Missouri and the publication in 1940 of his 'Life and Death at Low Temperatures'."

It is however, true to say that Luyet and Gehenic did not mention the first report on the freezing of protozoa, which appeared sixty years before the 1737 paper of Duhamel and Buffon. The author of this first report on the effect of freezing on protozoa, made in 1677, was Antony van Leeuwenhoek, described by Dobell (1932) as "the Father of Protozoology and Bacteriology". Dobell's translation of the relevant passage in a letter from Leeuwenhoek to The Royal Society, dated 23rd. March, 1677, reads as follows:

"Since sending off my letter concerning the little animals in water, I've not remained idle; but I have continued to examine divers sorts of water, examining even that which was distilled or boiled. During the last sharp spell of cold, when all the little animals had perished, I let the water thaw by the fire; and when it had stood a whole day in my bedchamber, with the fire kindled all the time, I saw, after the lapse of 24 hours (and at another time after 17 hours) that living creatures had

come again in the water. Upon this subject I might, indeed, say something further; but I note that my former letter is still under your consideration, so I will spare you more."

Unfortunately, Leeuwenhoek did not pursue this matter further and hence we have no indications as to whether or not the surviving organisms were protozoa. For the next two centuries or so, this sort of simple, anecdotal, observation continued to appear in the literature, but the organisms involved were rarely positively identified. Kuhn (1864), however, reported that amoebae were uninjured at 0°C but killed when the medium froze. Fol (1884) succeeded in culturing amoebae and ciliates from earth which had been frozen to -100°C. There was, however, doubt cast on these findings subsequently.

The first significant report on the cryopreservation of parasitic protozoa was made by Laveran and Mesnil (1904) who showed that trypanosomes survived and retained virulence after exposure for 15 minutes to -191°C in liquid air. In one case, Trypanosoma lewisi produced an infection in a rat after storage at -191°C for 75 minutes. The viability and infectivity were lost, however, after longer periods of storage. Gaylord (1908) and de Jong (1922) also had varying degrees of success with Trypanosoma gambiense and T. equiperdum respectively.

Coggeshall (1939) showed that rapidly cooled and rapidly thawed Plasmodium-infected monkey blood was infective after at least 70 days storage. There was evidence that the infectivity of the frozen and thawed blood was not very different from that of fresh blood. This was a critical observation. It was, at this time, realised that cryopreservation could obviate the need for repeated passage through animals. During the next eight years, several workers adopted and

adapted Coggeshall's technique for other Plasmodium spp., including the human pathogen P. vivax.

Weinman and McAllister (1947) then showed that, for four species of Trypanosoma, two of Leishmania and two of Trichomonas, a two-step slow cooling method was much better than the rapid cooling method of Coggeshall. This was the first indication that slow cooling might be less destructive for extraerythrocytic parasites whereas rapid cooling might be preferable for intraerythrocytic parasites. This was in agreement with the results obtained by Manwell and Edgett (1943) who found that increasing the rate of cooling and thawing of Plasmodium lophurae-infected duck blood increased the proportion of intact normal and parasitised red cells recovered.

The discovery by Polge, Smith and Parkes (1949) of the protective action of glycerol for avian spermatozoa has been, perhaps, the most significant advance in cryobiology yet made. This discovery, and the subsequent discoveries of the protective capacity of other additives, i.e. the macromolecules polyvinylpyrrolidone (PVP) and dextran, shown by Bricka and Bessis (1955) to protect mammalian red cells, and dimethylsulphoxide (DMSO) shown by Lovelock and Bishop (1959) to protect erythrocytes and spermatozoa, during freezing and thawing, made the routine cryopreservation of a large range of materials, including parasitic protozoa, possible.

Protozoologists concerned with parasitic protozoa soon realised the value of the technique of cryopreservation and a large enough number of species had, by 1964, been frozen down successfully to justify an extensive review (Diamond, 1964) in the first issue of the new journal "Cryobiology" exclusively devoted to this subject.

Two papers which appeared within a year of each other are worthy

of particular note. These were by Reusse (1956) and Polge and Soltys (1957). In his paper, Reusse reported the successful freezing and storage, for varying periods of time, of Trypanosoma brucei, T. congolense, T. evansi, Babesia canis, Toxoplasma gondii and Trichomonas foetus. All were protected with glycerol at 7.5% final concentration as cryoprotectant. The material, in glass ampoules, was cooled from +5 to -20°C at c. 1°/min.,* after which it was either cooled rapidly to -55°C (by plunging into an alcohol/solid carbon dioxide mixture at that temperature) or placed directly in a CO_2 cabinet at -76°C. The material was thawed by agitation in a water bath at +37°C. Using these methods, Reusse succeeded in storing Trypanosoma brucei, T. congolense and T. evansi for six months; Babesia canis for some weeks; Toxoplasma gondii for one week and Trichomonas foetus for twenty weeks. These results gave an indication of the potential and wide applicability of such methods, successful as they were for both intra- and extraerythrocytic parasites.

The work of Polge and Soltys (1957) examined the importance of such aspects as concentration of glycerol used as cryoprotectant, composition of medium, and rate of cooling in the cryopreservation of Trypanosoma brucei, T. congolense and T. rhodesiense. Polge and Soltys tested 5%, 10% and 20% final concentration glycerol, eight different media, and the following three cooling methods:

(a) Quick:- from room temperature to -79°C directly.

(b) Two-step:- two minutes at -15°C and then directly to -79°C (After the method of Weinman and McAllister (1947)).

(c) Slow:- 2°/min. to -20°C and then 5°/min. to -79°C.

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* All cooling and thawing rates quoted in this thesis are in Centigrade degrees per minute. In the example above, 1°/min. is used as a convenient shorthand method of writing "a rate of change of temperature of one Centigrade degree per minute". This standard format for expressing cooling and thawing rates will be used throughout the remainder of this thesis.

They showed that up to 50% of the organisms survived when small quantities of infected blood diluted with simple media were frozen by the rapid method. The proportion surviving was increased, however, if glycerol was added to the medium and if the rate of cooling was slower. It was also shown that the surviving organisms showed no change in pathogenicity, drug resistance or antibody tolerance. This illustration of the stability of biological characteristics was of considerable importance, and justified, for trypanosomes at least, further research into, and the use of, cryopreservation techniques.

Thus the success of slow-cooling methods was illustrated independently by Reusse for Trypanosoma, Babesia, Toxoplasma and Trichomonas, and by Polge and Soltys for Trypanosoma. Methods developed since then have usually employed similar slow cooling techniques, particularly since the publication by Cunningham, Lumsden and Webber (1963) and Lumsden, Robertson and McNeillage (1966) of extremely useful methods suitable for small volumes of material.

In these methods, small (25-40 microlitre) volumes of trypanosome-infected blood were preserved in capillary lymph tubes cooled at c. 1°/min. using glycerol at a final concentration of 7.5% as cryoprotectant. The particular usefulness of these methods lay in their applicability to parasitic protozoa other than trypanosomes, and subsequently they were adopted for the storage of a wide variety of protozoan genera including: Trypanosoma, Trichomonas, Plasmodium, Babesia, Leishmania and Theileria. The use of capillary tubes also made possible the storage of large numbers of samples of any one frozen material in a small space in either liquid nitrogen or solid carbon dioxide 'banks'. This, in turn, facilitated the storage of large numbers of different populations or 'strains' of material, often required for immunological studies.

With the development of successful techniques for the cryopreservation of parasitic protozoa, there also developed a specific terminology for those organisms so stored. Lumsden and Hardy (1965) pointed out that material stored in this manner could not be referred to as 'strain' material since that term suggested the possible exposure of the material to selection pressures and resultant changes which would occur during passage. Since the prevention of such changes was one of the main reasons for the development of the technique of cryopreservation, it was necessary that a new, unambiguous, term be introduced for material stored in this manner. The term 'stabilate' was proposed, defined (for protozoans) as a population of an organism preserved in a viable condition on a unique occasion. It followed that there were only as many examples of a stabilate as there were samples of it frozen on that unique occasion. Further discussion on this matter, (Lumsden, 1972) led to the term 'stabilation' being proposed for the process of preparing a stabilate. This term was preferred to 'stabilisation' which, according to Lumsden, is more likely to be misinterpreted. These terms will be used exclusively in this thesis to refer to the materials frozen and to the preparation techniques.

History of cryopreservation (b) Cryopreservation of Babesia spp.

There are, in the literature, several reports of successful cryopreservation of Babesia spp. Of the methods described, no two are the same. There are differences in the cryoprotectant type, its final concentration, the cooling and thawing rates and the container used. (The container being the vessel into which each individual sample is dispensed, e.g. capillary tube, ampoule, bijou bottle, etc.) The methods are described in chronological order.

Reusse (1956) successfully preserved Babesia canis, by the method described above, for some weeks. Waddell (1963) also used glycerol as cryoprotectant in his successful storage for 30 days of Babesia bigemina. The final concentration used was 11.6%. Waddell showed that uncontrolled cooling was successful whereas a rate of cooling of 0.5°/min. was not successful. He stored his material in 4ml. glass ampoules at -79°C. When thawed, the temperature of the material was raised to +38°C in not more than two minutes.

Barnett (1964) described methods for the cryopreservation of Babesia bigemina and Babesia trautmanni. He tested three different slow-cooling methods:

(a) From -5 to -13°C at 1° every two mins.
From -13 to -16°C at 1°/min.
From -16 to -40°C at 4°/min.

(b) From -5 to -13°C at 1°/min.
From -13 to -18°C at 50°/min.
From -18 to -70°C rapidly (Total time for this method, 25 mins.)

(c) On one occasion, Barnett compared method (b) with the method described by Cunningham (1961) which had proved very successful in preserving strains of trypanosomes. Cunningham had enclosed the blood in capillary tubes, but Barnett used tubes containing 1ml. of blood. These tubes were sealed, cooled to +5°C in a refrigerator, and then transferred to a boiling tube containing alcohol at 0°C. The boiling tube was wrapped in cotton wool one inch thick and placed in a 2-pint vacuum flask packed with solid carbon dioxide. The vacuum flask was rotated constantly for two hours after which the boiling tube was removed and instantly placed in a storage cabinet at -79°C.

Barnett used glycerol at a final concentration of 7% as cryo-

protectant. Material thawed (a) rapidly in a water bath at +37°C and (b) slowly to room temperature was tested. Samples (Babesia bigemina) thawed by both methods infected steers successfully. All three cooling rates were successful. Babesia bigemina-infected bovine blood survived for up to 720 days and B. trautmanni-infected pig blood survived for 35 days, the longest period tested.

Pipano and Senft (1966) reported the successful cryopreservation of both Babesia bigemina and Babesiella berbera. Two different cryoprotectants were investigated, glycerol and glucose, both at a final concentration of 15%. The material was dispensed into 10-50ml. bottles, incubated for 30 minutes at +4°C and then placed directly into storage at -70°C in an electromechanical refrigerator. Samples were thawed rapidly by agitation in a water bath at +37°C. Glycerol-protected blood containing B. bigemina was infective for calves for up to 440 days storage. Glycerol-protected B. berbera blood was infective for up to 215 days. Glucose-protected B. bigemina blood was preserved successfully for 240 days (the longest period tested); B. berbera blood was preserved for only 15 days. No change in 'virulence' of frozen parasites was noted. The term 'virulence' was, however, not defined.

Mieth (1966) successfully preserved both Babesia canis and B. rodhaini at -196°C in liquid nitrogen. Glycerol at a final concentration of 10% was used as cryoprotectant. The material, dispensed into plastic vials, was kept at +4°C for 30 minutes and then cooled. Mieth found two rates of cooling which were successful for these parasites. They were:

(a) Slow:- From +4 to -10°C at 0.5°/min.
From -10 to -30°C at 2°/min.
From -30 to -60°C at 5°/min.
then directly into liquid nitrogen.

(b) Rapid:- From +4°C to -196°C directly.

Babesia canis was infective after 178 days storage, B. rodhaini after 185 days.

Frerichs, Johnson and Holbrook (1968) reported the successful cryopreservation of Babesia caballi and B. equi. Glycerol at a final concentration of 11.6% was used as cryoprotectant. The material was maintained at +5°C for two hours, dispensed into glass vials, cooled to -75°C at 1°/min., held at -75°C for 24 hours and then placed directly into liquid nitrogen. In this study, B. caballi survived for 1040 days and B. equi for 603 days. The antigenic characteristics of both parasites were unchanged by this treatment.

Two of these authors, Frerichs and Holbrook (1970), reported the successful cryopreservation of several Babesia spp. These species were:

B. caballi and B. equi in equine blood,

B. canis in canine blood,

B. rodhaini in rat blood,

B. microti in hamster blood,

B. procyoni in raccoon blood,

B. mephitis in skunk blood,

and a then still unclassified Babesia in aardwolf blood.

In this paper, the same cooling procedures were followed and, in addition, the method of thawing was described. This consisted of a process in which the vial was taken from the liquid nitrogen refrigerator and placed in a dry-ice cabinet overnight before allowing it to thaw slowly to room temperature.

Frerichs and Holbrook also stated that they had been successful with simpler procedures in which flame-sealed glass ampoules were cooled from room temperature to -196°C by placing them in liquid

nitrogen directly. The rat and dog erythrocytes appeared, from the results obtained, to be more resistant to lysis under these conditions than the erythrocytes of the other species. B. canis survived for at least 381 days, B. rodhaini 328 days and B. microti for at least 159 days. Due to lack of suitable hosts, it was not possible for Frerichs and Holbrook to test the survival of the other Babesia spp. stored.

Overdulve and Antonisse (1970a, 1970b) published two papers on the measurement of the effect of low temperature on protozoa by titration. An adaptation of their infectivity titration technique was used in this present study and will be discussed later. The aspects of the cryopreservation technique used by Overdulve and Antonisse are considered at this juncture. These workers used, as an illustrative model, the rodent parasite Babesia rodhaini. The method used was a variant of the method described by Cunningham, Lumsden and Webber (1963) and is the first involving the use of dimethylsulphoxide (DMSO) as cryoprotectant. Two concentrations of DMSO were used, 5% and 10%, and both were equally successful. It was recommended, therefore, that the lower final concentration be used. The containers were glass capillary tubes and the cooling rate approximated to 1°/min. The material, which was stored at -76°C, was thawed quickly to room temperature.

The statistical analysis on blood stored in this way for 42 days showed that only 0.16 to 0.65% of the parasites, counted before freezing, remained infective. The virulence of the surviving infective parasites was not altered by deep freezing.

Joyner (1971) in a review article on the laboratory maintenance of piroplasms, described the methods used at his laboratory for the cryopreservation of Babesia spp. The cryoprotectant used was glycerol.

The final concentration used was not stated exactly, but was between 7 and 11.6%, the concentrations recognised by Joyner as having been used by previous workers. The blood/glycerol mixture was allowed to equilibrate for 30 minutes at room temperature. The cooling rate used was c. 1°/min., to -70°C, after which the ampoules were placed directly in liquid nitrogen at -196°C. The ampoules were thawed rapidly by immersion in a large volume of warm water. Joyner also described an alternative method in which 2-2½ inch polythene tubing lengths were used as container, thereby allowing larger numbers of samples to be stored.

A recent series of publications (Dalgliesh, 1971, 1972a, 1972b) has dealt extensively with the cryopreservation of Babesia spp. In the first of these publications, Dalgliesh (1971) described a successful method for the cryopreservation of Babesia bigemina using DMSO, at various final concentrations, as cryoprotectant. Infected calf blood was diluted 1:1 dropwise with one of four different diluents:

(a) Phosphate buffered saline (PBS),

(b) PBS containing 2M DMSO,

(c) PBS containing 3M DMSO,

(d) PBS containing 4M DMSO.

The diluted bloods were then held for 45 minutes at +5°C. Four millilitre samples in 9ml. screw-cap glass vials were then cooled directly to -79°C by immersion in an alcohol/solid carbon dioxide mixture at that temperature. Infectivity of the treated blood was tested before and after freezing by intravenous inoculation into splenectomised calves. The blood was thawed by agitation in a water bath at +40°C. Dalgliesh found that DMSO caused a slight, but not statistically significant, loss of infectivity before freezing.

The loss of infectivity was significant in blood frozen with (a), (b) and (c) above but not with (d). Dalgliesh also measured the post-storage degree of haemolysis and found that the presence of DMSO reduced the degree of haemolysis from 82.8% to as low as 6.7%.

Dalgliesh (1972a) studied the effects of route of inoculation and low temperature preservation on Babesia bigemina-infected bovine blood diluted with glycerol. He compared infected bovine blood diluted with either:

(a) PBS alone, or

(b) PBS containing 4M. glycerol.

The diluted blood was maintained at +38°C for 20 minutes after dilution to ensure that the glycerol had equilibrated properly with the erythrocytes. This had been shown to be necessary by Dalgliesh (1969). The diluted, equilibrated, blood was then cooled directly to -79°C by immersion in an alcohol/solid carbon dioxide bath at that temperature. The containers used were 9ml. glass vials. The samples were thawed by agitation in a water bath at +40°C. Dalgliesh claimed that the cooling rate in the range 0 to -40°C was c. 60°/min. and that the thawing rate in the same range was c. 120°/min. Dalgliesh showed that blood frozen with glycerol was highly infective to non-splenectomised cattle (yearlings) when administered subcutaneously after thawing, whereas blood which had been frozen without glycerol was not infective by this route. The reverse occurred when the same bloods were given intravenously, the protected blood being non-infective and the non-protected blood being highly infective. Dalgliesh also showed that the presence of glycerol reduced the degree of haemolysis from 86% to as low as 4% after freezing and thawing.

Dalgliesh (1972b) published an extensive review article on the

theoretical and practical aspects of freezing parasitic protozoa in which he examined various factors affecting the success of freezing techniques, including the use of chemical cryoprotectants, the cooling rate, the thawing rate, the temperature and duration of storage and other factors of less general importance or applicability. A list of all the methods for Babesia spp. described up to that time was included. All of these methods have been described above.

Dalgliesh made five general recommendations for the cryopreservation of parasitic protozoa:

"(1) A cryoprotectant should be used. Dimethylsulphoxide (DMSO) at a concentration of about 10% is recommended because it is an effective protectant well-tolerated by most protozoa. DMSO rapidly passes through the cell membrane, thereby eliminating the need for a lengthy equilibration period, and reducing possible osmotic stress.

(2) Most parasites survive cooling rates of approx. 1°/min. when a cryoprotectant is used. Parasites of the blood tolerate a wide range of cooling, and many can be frozen successfully by simply transferring samples directly to the final storage temperature.

(3) The storage temperature should be as low as is practicable, and no higher than -60°C. Storage in liquid nitrogen (-196°C) is recommended.

(4) Thawing should be rapid, performed by agitating the sample in a water bath at about +40°C.

(5) There is evidence to suggest that parasites thawed after cryopreservation require a period for structural reconstitution before inoculation. (Here Dalgliesh is referring to the work of Lumsden, Gitatha and Lutz (1968) who showed that a thawed Trypanosoma brucei suspension inoculated into a series of mice during the period of

12 to 197 minutes after thawing had a peak of infectivity at between 30 and 80 minutes after thawing. It was suggested that the initial rise was associated with a structural reconstitution.) Nevertheless (continues Dalgliesh), when DMSO is used, the period between thawing and inoculation should be kept to a minimum, because toxicity of DMSO for some species has been demonstrated."

Dalgliesh and Mellors (1974) studied the survival of Babesia bigemina in blood cooled to -196°C at different cooling rates.

The blood was either stored overnight at +4°C before freezing or was frozen on the day of collection. It was diluted, prior to freezing, with an equal volume of 4M DMSO in phosphate buffered saline and kept cold in an ice bath. The blood was dispensed into 0.5ml. Cassau A.I. plastic straws, sealed, and then placed in an ice bath for 30 minutes.

Eight different methods, producing eight different cooling rates, were described. The rates were measured between -16 and -56°C. The cooling rates produced were:

(1) 3070 ± 48°/min.
(2) 737 ± 67°/min.
(3) 212 ± 11°/min.
(4) 82 ± 3°/min.
(5) 39 ± 1°/min.
(6) 29 ± 0.6°/min.
(7) 3.7 ± 0.4°/min.
(8) c. 0.73°/min.

The temperature of the bloods cooled by methods 3-8 was controlled to below -60°C and then the straws were immersed in liquid nitrogen. The straws were thawed after 2 to 18 hours in liquid nitrogen,

by agitation in a water bath at +40°C, and infectivity was tested in 99 cattle. Blood cooled at each rate was infective, 95 of the 99 cattle becoming infected. The four cattle which did not become infected were among those inoculated with blood cooled at 3070°/min.

The infectivities of blood cooled at 39, 82 and 212°/min. were statistically higher (judged by comparison of mean prepatent periods) than higher or lower rates. The least depression of infectivity occurred with blood cooled at the rate of 82°/min.

Raether and Seidenath (1972) reported the infectivity of several species of parasitic protozoa, including Babesia canis and B. rodhaini, after extended storage in liquid nitrogen. The method used was essentially that described by Mieth (1966).

The material was protected with glycerol at 10% final concentration. It was dispensed into plastic vials and cooled as follows:

(1) The vials were kept at +4°C for 30 minutes.

(2) They were then cooled,

From +4 to -10°C at 0.4°/min.,

From -10 to -30°C at 2°/min.,

From -30 to -60°C at 5°/min.

(3) The vials were then placed directly in liquid nitrogen at -196°C.

The material was thawed by agitation in a water bath at +37°C and tested for infectivity. Babesia canis proved infective after 2621 days storage, B. rodhaini after 2627 days. Tests of infectivity had been made at least once a year during this period.

Bishop, Adams, Thompson and Corrier (1973) used an adaptation of the method described by Pipano and Senft (1966) in making a stabilate of Babesia bigemina separated from contaminating organisms.

Glycerol at a final concentration of 11.6% was used as cryoprotectant. Fifty millilitre samples of material were dispensed into 60ml. plastic, narrow mouth, screw-cap bottles, held for 30 minutes at +4°C and placed in a dry-ice cabinet at -79°C. No mention was made of the infectivity of this material after storage.

CHAPTER 2

Materials and Methods

Biological (1) Parasites.

Babesia rodhaini. This parasite was obtained from Mrs. K.M.G. Adam, Department of Zoology, University of Edinburgh, in 1969. Prior to its original stabilation in the Centre for Tropical Veterinary Medicine, it was passaged in mice 23 times. It was then frozen and stored as TREU 812 in glass capillary lymph tubes, using glycerol at 7.5% final concentration as cryoprotectant, and a cooling rate of c. 1°/min.

In October, 1971, mice were again infected with thawed TREU 812. After one passage in mice, young, 150g., MRC Wistar female rats were successfully infected with fresh whole blood. After nine passages in rats, the parasite was then frozen and stored as TREU 1127 in glass ampoules, using glycerol at 7.5% final concentration as cryoprotectant and a cooling rate of c. 1°/min. The subsequent history of the parasite is quite complicated and is best illustrated by a flow diagram, shown in Fig. 1.

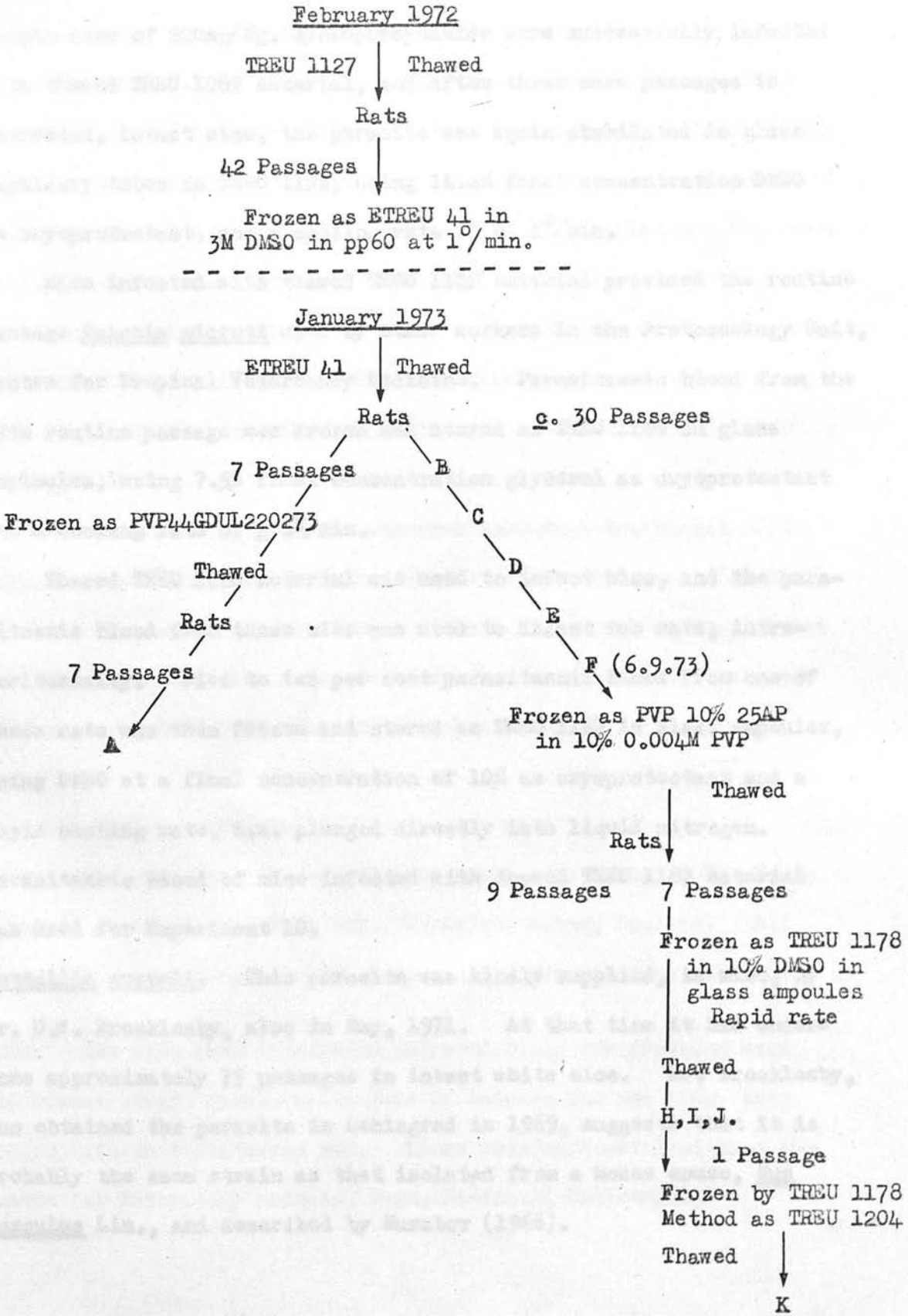
Babesia microti. This parasite was the King's strain, originally isolated from the short-tailed field vole Microtus agrestis and adapted to the laboratory mouse (Cox and Young, 1969).

About fifty weekly passages after its adaptation, the parasite was obtained by Dr. D.W. Brocklesby, Agricultural Research Council, Institute for Research on Animal Diseases, Compton, Nr. Newbury, Berks., England. Dr. Brocklesby, in turn, kindly supplied the parasite, in infected mice, to the Protozoology Unit, Centre for Tropical Veterinary Medicine, in May, 1971, after approximately 65 further passages in intact white mice.

FIG. 1

The passage and stabilation history of the strain of Babesia rodhaini used in the study described in this thesis.

FIG. 1


February 1972
TREU 1127
Thawed
Rats
42 Passages
Frozen as ETREU 41 in
3M DMSO in pp60 at 1°/min.
January 1973
ETREU 41
Thawed
Rats
c. 30 Passages
7 Passages
B
C
D
E
F (6.9.73)
Frozen as PVP44GDUL220273
Thawed
Rats
7 Passages
A
Frozen as PVP 10% 25AP
in 10% 0.004M PVP
Thawed
Rats
9 Passages
7 Passages
Frozen as TREU 1178
in 10% DMSO in
glass ampoules
Rapid rate
Thawed
H,I,J.
1 Passage
Frozen by TREU 1178
Method as TREU 1204
Thawed
K

The parasite was immediately frozen and stored, in glass capillary tubes, as TREU 1082, using 7.5% final concentration glycerol as cryoprotectant and a cooling rate of c. 1°/min. Mice treated with a single dose of 200mg/Kg. cyclophosphamide were successfully infected with thawed TREU 1082 material, and after three more passages in untreated, intact mice, the parasite was again stabilated in glass capillary tubes as TREU 1122, using 14.4% final concentration DMSO as cryoprotectant, and a cooling rate of c. 1°/min.

Mice infected with thawed TREU 1122 material provided the routine passage Babesia microti used by other workers in the Protozoology Unit, Centre for Tropical Veterinary Medicine. Parasitaemic blood from the 69th routine passage was frozen and stored as TREU 1180 in glass amploules, using 7.5% final concentration glycerol as cryoprotectant and a cooling rate of c 1°/min.

Thawed TREU 1180 material was used to infect mice, and the parasitaemic blood from these mice was used to infect two rats, intraperitoneally. Five to ten per cent parasitaemic blood from one of these rats was then frozen and stored as TREU 1187 in glass ampoules, using DMSO at a final concentration of 10% as cryoprotectant and a rapid cooling rate, i.e. plunged directly into liquid nitrogen. Parasitaemic blood of mice infected with thawed TREU 1187 material was used for Experiment 10.

Nuttallia musculi. This parasite was kindly supplied, in mice, by Dr. D.W. Brocklesby, also in May, 1971. At that time it had undergone approximately 75 passages in intact white mice. Dr. Brocklesby, who obtained the parasite in Leningrad in 1969, suggests that it is probably the same strain as that isolated from a house mouse, Mus musculus Lin., and described by Muratov (1966).

This Nuttallia musculi was immediately stabilated in glass capillary tubes as TREU 1081, using glycerol at 7.5% final concentration as cryoprotectant and a cooling rate of c. 1°/min. In January, 1974, a splenectomised CDTO mouse was infected with thawed TREU 1081 material. This mouse showed parasites on day 21 after inoculation. Two intact CDTO mice were then injected intracardially (I.C.) with fresh parasitaemic blood from this mouse. One mouse showed c. 5% infected red cells on day 8 after inoculation, the other on day 19 after inoculation. From the former, two more passages by the intraperitoneal route, were made. The parasite was again stabilated as TREU 1189 in one-third filled glass capillary tubes, using DMSO at 10% final concentration as cryoprotectant and the rapid cooling rate.

Thawed TREU 1189 material was used to infect ten intact CDTO mice, intraperitoneally. By day 6 after inoculation, nine of the ten mice were showing parasites, and fresh blood from these mice was used for Experiment 10. The value of the cryopreservation method described for TREU 1189 will be discussed later.

(2) Laboratory animals.

Mice. The mice used throughout this study were 'Tuck' CDTO strain females, obtained from Tuck Ltd., Rayleigh, Essex, England. All mice were in the range 18-20g. body weight, four to five weeks old.

Rats. The rats used to provide infected blood for freezing were MRC Wistar strain female white rats of between 140 and 180g. body weight, six to seven weeks old. These rats were obtained from the Centre for Laboratory Animals, Bush, Penicuik, Midlothian.

Reagents. The glycerol, dimethylsulphoxide and 44000 molecular weight polyvinylpyrrolidone used as cryoprotectants in this study were obtained from B.D.H. Chemicals Ltd.[1] The 11000 and 24500 molecular weight polyvinylpyrrolidones used as cryoprotectants in this study were obtained from Koch-Light Laboratories Ltd.[2]

The talc used to produce ultrarapid cooling rates in part of this study was obtained from BD.H. Chemicals Ltd. The 'Vaseline' petroleum jelly also used in the production of ultrarapid cooling rates was obtained from Cheeseborough-Ponds Ltd.[3]

Diluents. (1) ABP8.0. The diluent used in the earlier experiments in this study was a phosphate-buffered balanced salt solution 'ABP8.0' as described by Lumsden et al (1965). This diluent was prepared by mixing 9 ml. of a metallic chlorides solution (Sol. A) with 1 ml. of a phosphate buffer, pH 8.0 (Sol. BP8.0). The exact constituents of these solutions were as follows: (Tables from Lumsden et al, 1965)

Solution A — Metallic chlorides	Moles	g./litre	Vol.
NaCl	0.154	9.00	100
KCl	0.154	11.48	4
$MgCl_2 \cdot 6H_2O$	0.110	22.37	3
$CaCl_2 \cdot 6H_2O$	0.110	24.10	1

1. B.D.H. Chemicals Ltd., Poole, England.
2. Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.
3. Cheeseborough-Ponds Ltd., London and New York.

Solution BP8.0 Phosphate buffer, pH 8.0

	Moles	g./litre	Vol.
$NaH_2PO_4 . 2H_2O$	0.154	24.03	0.36
$Na_2HPO_4 . 2H_2O$	0.103	18.33	9.64

This diluent was made up immediately prior to use.

(2) Modified Alsevers Solution. In later experiments, after some doubt about the stability of ABP8.0, a modified Alsevers solution, based on that used by Nowell (1968), was used. The exact constituents of this solution were as follows:

Disodium hydrogen citrate, 0.3M solution	2ml.
Trisodium citrate, 0.3M solution	98ml.
Glucose	2.33g.
NaCl.	0.525g.

The pH of this solution was 7.5.

Anticoagulants. (1) Heparin. In early experiments, the anticoagulant used was heparin. It was used at a concentration of 50 iu/ml. in ABP8.0. One ml. of this anticoagulant solution was used for approximately every 9 ml. of blood, making the final heparin concentration 5 iu/ml. The heparin, in 5 ml. bottles of 5000 iu/ml., was obtained from The Boots Company Ltd.*

(2) EDTA. The use of heparin was discontinued early in this study for two reasons:

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* The Boots Company Ltd., Nottingham, England.

(a) It had been noted that the use of heparin as anticoagulant usually resulted in Giemsa-stained blood films of poor quality due to the 'background' produced by its presence.

(b) There was a suggestion that heparin was deleterious to Babesia rodhaini (T.T. Dolan, personal communication). The anticoagulant then chosen was ethylenediamine tetraacetic acid (EDTA). This was used in dried form in 5 ml. sequestrene bottles obtained from Becton Dickenson Ltd.[1]

(3) Modified Alsevers Solution. When modified Alsevers solution was used as diluent, no additional anticoagulant was required.

Drugs. Cyclophosphamide. The immunosuppressant drug cyclophosphamide was obtained from Ward-Blenkinsop & Co. Ltd.[2]

Freezing equipment. (1) Dry-ice cooling cabinets. The dry-ice cooling cabinet used in the earlier part of this study, was constructed as follows: The compartment in which material to be cooled was placed, was a simple, lidded, aluminium box, 22" long, 4½" wide and 8" deep. This box was built into the centre of an insulated box with internal dimensions of 26" long, 8½" wide and 10" deep, made of two layers of two-inch Purlboard,[3] and fitted with a two-layer, two-inch Purlboard lid. The four-inch space all round the metal cooling box was filled with solid carbon dioxide.

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1. Becton Dickenson Ltd., York House, Empire Way, Wembley, Middlesex, England.
2. Ward-Blenkinsop & Co. Ltd., London & Widnes.
3. Imperial Chemical Industries Ltd., Grangemouth, Scotland.

In later experiments, in order that more material could be simultaneously slow-cooled, a larger cabinet was used. It was made of the same materials, Purlboard and aluminium. The insulation was of three layers of two-inch Purlboard. The cooling box had the dimensions of 10 inches square, 11 inches deep (Fig. 2).

(2) Liquid nitrogen storage containers. When material was to be cooled to, and stored at, liquid nitrogen temperatures, a standard Union Carbide LR33-10 Liquid Nitrogen Refrigerator was used[1] (Fig. 2).

(3) Cooling jackets. The cooling jackets used were essentially the same as those described by Lumsden *et al* (1966) and were made from Onazote[2]. The walls of the jackets were 25 mm. thick and, when placed in a dry-ice cabinet, allowed the contents to cool at a rate of about 1°/min.

In later experiments, when a large number of samples had to be cooled simultaneously, a larger cooling jacket was designed. This jacket, cubic in shape, was made from two layers of half-inch Purlboard, and had internal dimensions of 6 x 6 x 6 inches. This jacket gave a cooling rate for 2 ml. ampoules of g. 1.09°/min. between 0 and -40°C when placed from room temperature into the redesigned dry-ice cooling cabinet mentioned above (Fig. 2).

(4) Polythene capillary tubing. The polythene capillary tubing used as container in this study was low-density polythene capillary tubing (outside diam. 1.22mm; inside diam. 0.76mm., Portex pp60).[3]

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1. Union Carbide, U.K., Ltd., Engineering Products Division, Redworth Way, Aycliffe Industrial Estate, Nr. Darlington, Co. Durham, England.

2. Expanded Rubber and Plastics Ltd., Croydon, England.

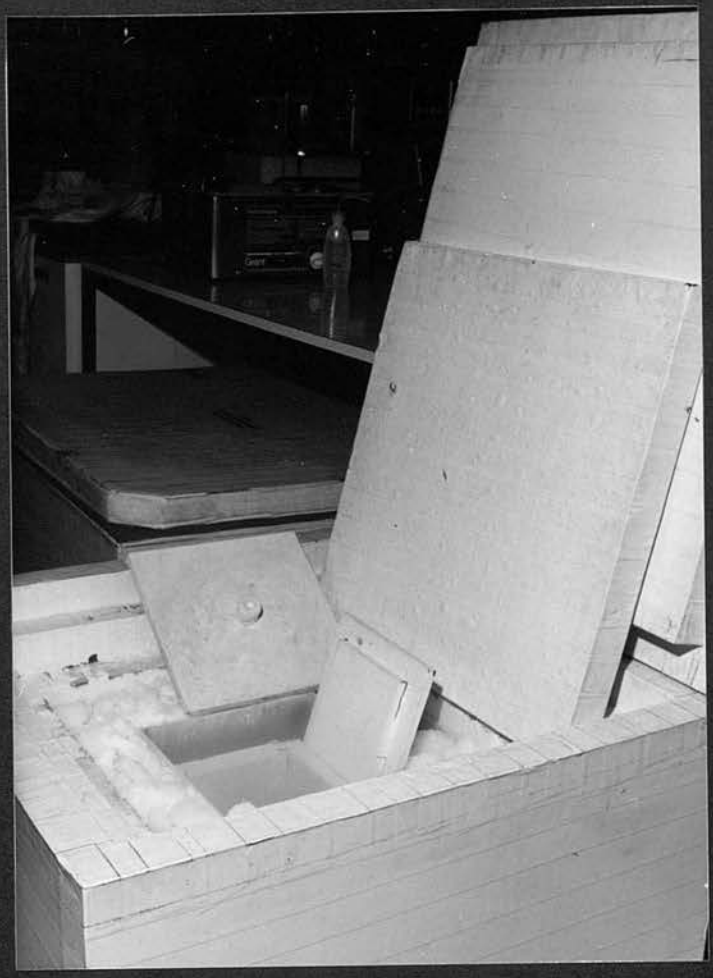
3. Portland Plastics Ltd., Hythe, Kent, England.

FIG. 2(a)

The solid carbon dioxide cooling cabinet used to produce slow, or conventional, cooling rates. Clearly visible are the cabinet lids, the central metal cooling box, the cooling box lid and the lid and part of the large cooling jacket.

FIG. 2(b)

The liquid nitrogen refrigerator used for rapidly cooling of material to, and storage at, -196°C. Clearly visible are the top of one of the ten, ten-inch deep, storage cans, ampoules mounted on an ampoule cane, and a length of pp60 polythene tubing mounted on a metal template.





Lengths of this tubing were mounted for cooling and storage on aluminium templates as shown in Fig. 3. Material being studied was introduced into, and expelled from, this tubing using a syringe fitted with a 21-gauge needle, also shown in Fig. 3.

(5) Glass ampoules. The glass ampoules used for the early experiments in this study were either 1 or 2 ml. clear, neutral-glass vaccine ampoules supplied by Johnsen and Jorgensen.[1] In later experiments, when this stock of ampoules had been used up, 2 ml. borosilicate glass ampoules 11.5 x 69 mm., obtained from Union Carbide Ltd., were used. All ampoules were heat-sealed and, when cooled rapidly to liquid nitrogen temperatures, were mounted on Union Carbide ampoule canes.

(6) Sealing of containers. All containers, whether ampoules or polythene tubing lengths, were heat-sealed. Neutral-glass ampoules were sealed over a standard natural gas bench micro-burner. Borosilicate ampoules required a hotter, stronger flame. This could be obtained using a 'Ronson' Butane hand blowtorch.[2] Even so, it was found desirable and necessary to test the seal on these ampoules by placing the still-warm ampoule into a bath of cold, coloured water. Any ampoule which drew in water was rejected.

Polythene tubing lengths were sealed by melting the ends in a gentle bunsen flame and then pinching between finger and thumb as the polythene hardened again.

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1. Johnsen and Jorgensen, London, EC4.

2. Ronson Products Ltd., Leatherhead, Surrey, England.

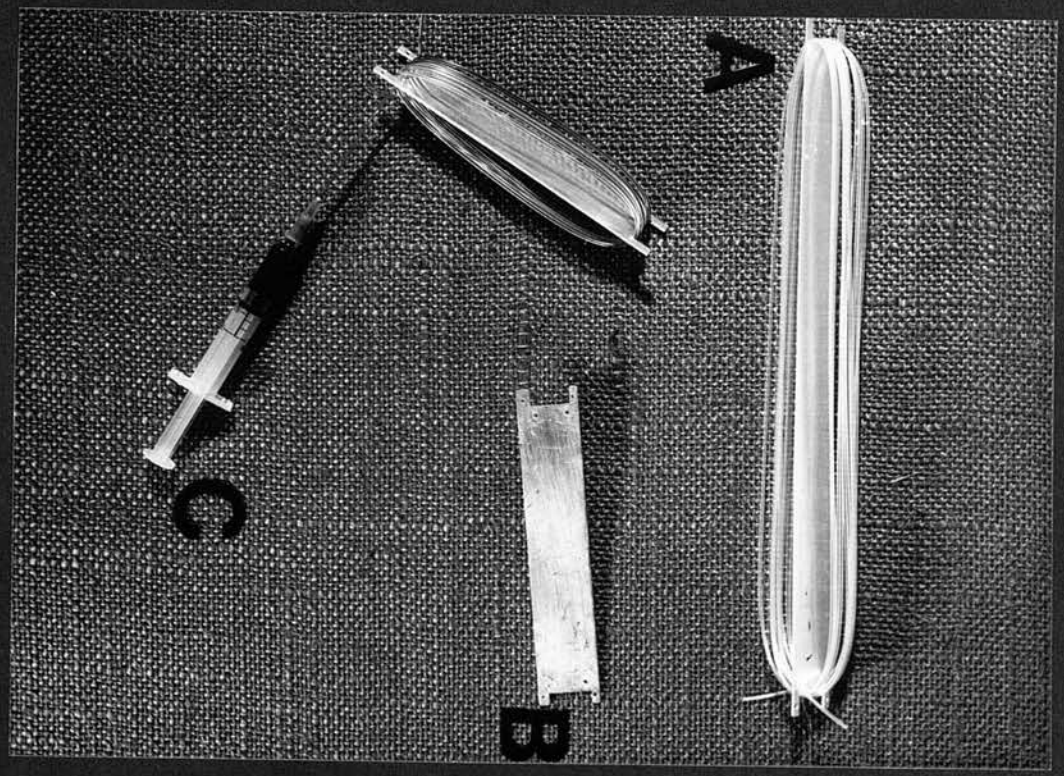
FIG. 3

The method of mounting of, and dispensing of material into, pp60 polythene tubing.

A - shows the method of mounting of pp60 for storage at -196°C in liquid nitrogen. The metal template is c. 10" long.

B - shows the short form of the metal template used for mounting of pp60 tubing when it was to be slowly cooled to, and stored at, -79°C.

C - the method of dispensing infected blood into pp60, shown mounted on a short template.


A
B
C

Treatments of materials before and after freezing

(1) Estimation of red cell count and parasitaemia. Total red cell counts were made using a Model Fn Coulter Counter.[1] This machine was calibrated regularly using a standard cell suspension supplied by courtesy of the Department of Haematology, Edinburgh Royal Infirmary. For prefreeze and post-thaw tests of infectivity of all three parasites studied, 'parasitaemia' was calculated as the percentage of red cells parasitised, irrespective of the number of parasites in each cell, as judged by examination of at least 500 red cells in a Giemsa-stained blood smear. Parasitaemias were calculated to the nearest whole number. Since statistical comparisons were being made within experiments only, any errors were unimportant. (Prior to inoculation, blood samples were always thoroughly mixed. It was then assumed that red cells containing more than one parasite were evenly distributed in the sample and that, therefore, all inocula would contain effectively the same number of parasites.)

(2) Percentage haemolysis estimations. Percentage haemolysis of blood samples was calculated according to the method of Huggins (1963). The formula proposed by Huggins was:

$$\%H = \frac{(100-Hct).Hbs}{Hbt}$$

where %H = Percentage haemolysis,

Hct = Haematocrit,

Hbt = Total haemoglobin,

Hbs = Supernatant haemoglobin.

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1. Coulter Electronics Ltd., High Street South, Dunstable, Bedfordshire, England.

Haematocrit was determined using a Hawkesly Micro-haematocrit Bench Centrifuge. All haematocrits were spun for three minutes. Total and supernatant haemoglobin was estimated by the cyanmethaemoglobin technique in which the haemoglobin is treated with a reagent solution containing potassium ferricyanide, potassium cyanide and potassium bicarbonate.[1] This reagent solution in the first instance causes the lysis of the erythrocytes, if any, the haemoglobin, or any derivatives present, is then oxidised by the ferricyanide to methaemoglobin which is then converted to cyanmethaemoglobin by the cyanide.

The extinction of the cyanmethaemoglobin was read at 546 nm. in 1.0 cm. glass cells using a Pye Unicam SP 1800 Spectrophotometer. No standard was required since;

(a) Only the ratio of supernatant to total haemoglobin was needed,
(b) There is a linear relationship between extinction at 546 nm. and haemoglobin concentration,
(c) The spectrophotometer and cells were 'zeroed' using untreated Drabkins' solution prior to each series of estimations.

(3) Addition of cryoprotectants. In all cases, cryoprotectants were initially made up in diluent to twice the required concentration. Equal volumes of these cryoprotectant solutions and the material being studied were then thoroughly mixed, resulting in the required final concentration of cryoprotectant.

(4) Working temperature. All infected materials, diluents and cryoprotectant solutions were used at room temperatures.

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1. Drabkins' Reagent, obtained from B.D.H. Chemicals Ltd.

(5) Vaseline and talc method for producing ultrarapid cooling rates. Sealed containers (ampoules or polythene tubing lengths) which contained material to be cooled at ultrarapid rates were treated in the following way: The sealed container was lightly smeared with Vaseline petroleum jelly and then dipped in a jar of talc. The excess talc was shaken off and the container was ready for cooling. It was not possible to control the amounts of Vaseline or talc being applied. When containers treated in this way were then plunged directly into liquid nitrogen, the infected material within was cooled at ultrarapid cooling rates.

(6) Thawing of frozen material. Frozen material was always thawed as rapidly as possible by agitation in a bench heated water bath at +37°C.

Cooling curve estimation equipment

(1) Bead thermistors. For the monitoring of slow cooling rates, bead thermistors were used.[1] The output from these thermistors was rendered approximately linear in the range +5 to -45°C by the introduction of a thermistor bridge into the circuit. The circuit diagram for this bridge, which was made in the electronic workshop at the Centre for Tropical Veterinary Medicine, is shown in Fig. 4. Although the readings obtained from such a thermistor set-up were extremely accurate, it was not possible to use thermistors for monitoring of rapid cooling rates or for material in pp60 due to their fragility, cost, and large size.

(2) Thermocouples. For rapid cooling and thawing rates, and for cooling rates of material stored in pp60 polythene tubing, copper/

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1. Model F22, ITT Ltd., Edinburgh Way, Harlow, Essex, England.

FIG. 4

The circuit diagram of the thermistor bridge used to render linear the output of the thermistors used in cooling curve estimations. The following components were necessary:

Resistors

R1 = 1K Ohm R3 = 10K Ohm R5 = 500 Ohm

R2 = 10K Ohm R4 = 10K Ohm R6 = 1K Ohm

(All 10% ½ Watt Carbon)

Thermistor

X1 = F22 (ITT Electronic Services)

Potentiometers

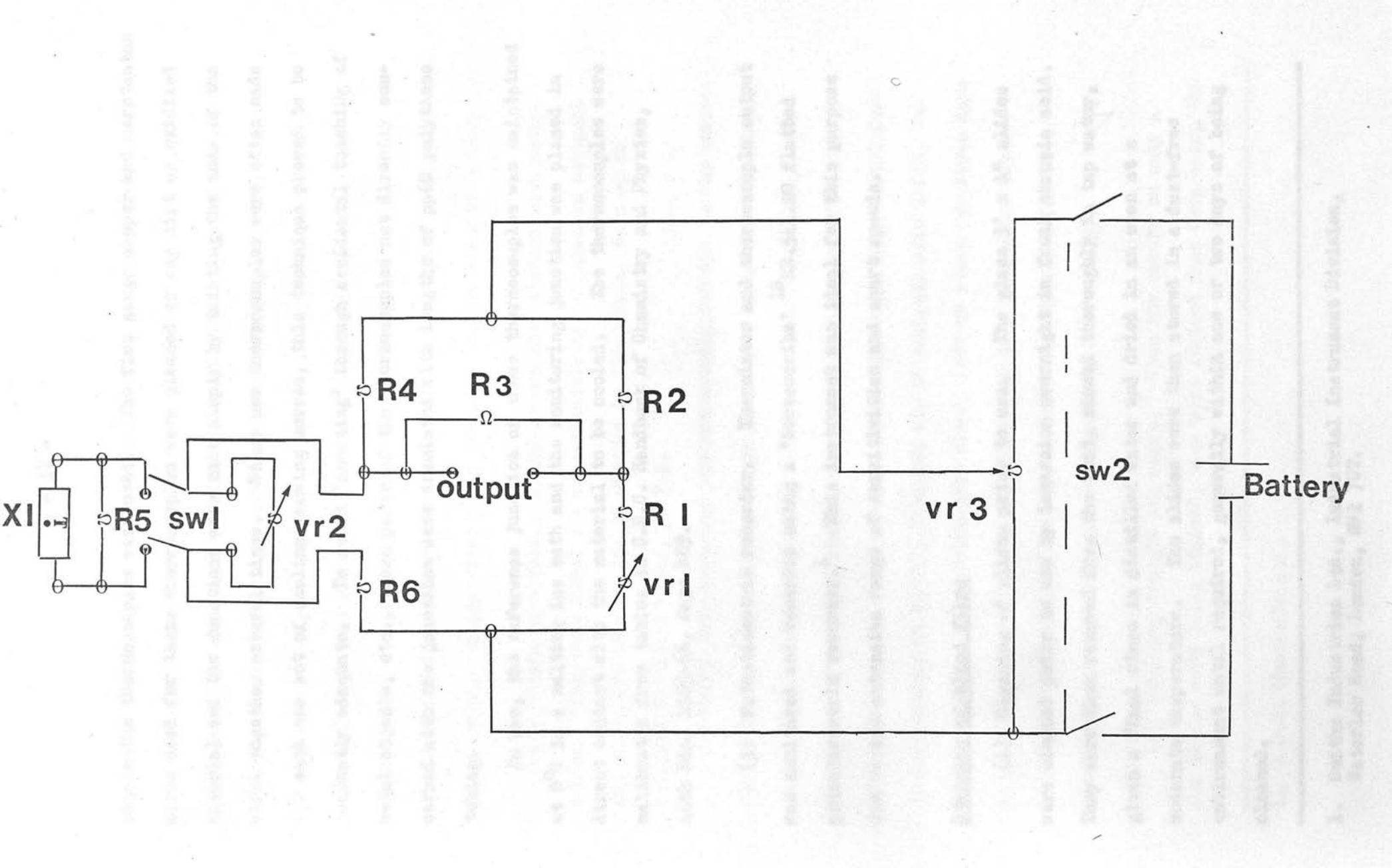
vr 1 1K Ohm vr 2 500 Ohm

vr 3 1.5K Ohm

Switches

SW1 = D.P.D.T.

SW2 = D.P.S.T.


R4
R3
R2
output
XI
R5
swI
vr2
R I
vrI
R6
vr 3
sw2
Battery

constantan thermocouples were used. The fine gauge copper and constantan wires used for these thermocouples were cleaned of any dirt or oxidised material and the junctions were made simply by twisting the ends of the wires together several times. Since new thermocouples were often made for each new set of cooling/thawing curves, this technique proved to be perfectly adequate. To avoid 'shorting' through accidental touching of metal objects, etc., those parts of the thermocouples not directly concerned with the junctions were insulated with lengths of pp60 polythene tubing.

In use, the reference junction of these thermocouples was maintained at 0°C in a melting ice bath and the monitoring junction was placed in direct contact with the material to be cooled. The thermocouples were calibrated from tables in C.R.C. Handbook of Chemistry and Physics, 46th Edn. 1965-66, Page E89.

(3) Potentiometric recorder. Thermistor and thermocouple output was monitored and recorded using a 'Servoscribe' 1S RE.541.20 flatbed potentiometric recorder.[1] This instrument was ideal for this purpose due to its extensive range of sensitivities and chart speeds.

Staining of blood films

(1) Cleaning of slides prior to use. The glass 3" x 1" slides were cleaned prior to use by immersion overnight in fresh chromic acid. They were then removed from the acid, rinsed thoroughly in tap water, given a final rinse in distilled water and dried in an oven at a moderate temperature. The slides were then stored in a dust-free environment until required, generally within one or two days of being cleaned.

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1. Smiths Industries Ltd., Industrial Instrument Division, Waterloo Road, London, NW2 7UT.

(2) Blood smears. Blood smears taken from free-flowing tail blood obtained by cutting the end, or scab, from the tail with very sharp scissors were made on 3" x 1" chromic clean glass slides. Since experimental groups were usually of ten animals, five mice in each of two cages, all five smears from animals in any one cage were put on to one slide. This was accomplished by making the smears (c. 1 cm. wide) across rather than along the slide. Both the scissors and the spreader were washed in alcohol after each individual blood smear. Slides were fully labelled using a diamond marking pen.

(3) Staining method. Blood smears were stained with Giemsa's stain. They were fixed for approximately two minutes in methanol and then were stained for 30 minutes in 15% Giemsa, made up in buffered distilled water, pH 7.2.

Initially the stain used was commercial, but due to the inconsistencies in this product, it was later made up by the following method (Dacie and Lewis, 1970):
Four grams of powdered stain[1] were placed in a conical flask and 264 ml. of glycerol were added. This mixture was then heated for 90-120 minutes at 56°C. Then 264 ml. of methanol were added, the contents of the flask thoroughly mixed, left to stand for seven days at room temperature, filtered, and stored in a dark glass bottle.

After staining, slides were washed in phosphate-buffered distilled water, pH 7.2, as recommended by Baker (1969).

(4) Staining troughs and racks. When the smears on only a few slides were being stained, they were stained face down using the

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1. B.D.H. Chemicals Ltd.

staining troughs shown in Fig. 5(a). This method, as recommended by Baker (1969) prevented deposition of scum and/or particulate material. It was impractical to use this method when large numbers of slides were used. In that event, standard staining racks, as shown in Fig. 5(b) were used.

(5) Acid alcohol. The acid alcohol used in this study to clean staining troughs and racks after use, to prevent build-up of particulate material, was made up as follows: To 970 ml. of 70% methanol was added, dropwise, 30 ml. of concentrated hydrochloric acid, specific gravity 1.18. This mixture was stored in clear glass Winchester bottles ready for immediate use.

Infectivity tests

(1) Dilution technique. Prefreeze, cryoprotectant-added control, or post-storage experimental material, being diluted to give infective doses of fixed size, were always diluted in diluent containing the appropriate cryoprotectant at the same concentration. For example, material stored with 10% DMSO as cryoprotectant was always diluted with diluent containing 10% DMSO. This obviated the possibility of osmotic damage to cells containing high concentrations of cryoprotectant suddenly exposed to cryoprotectant-free diluent.

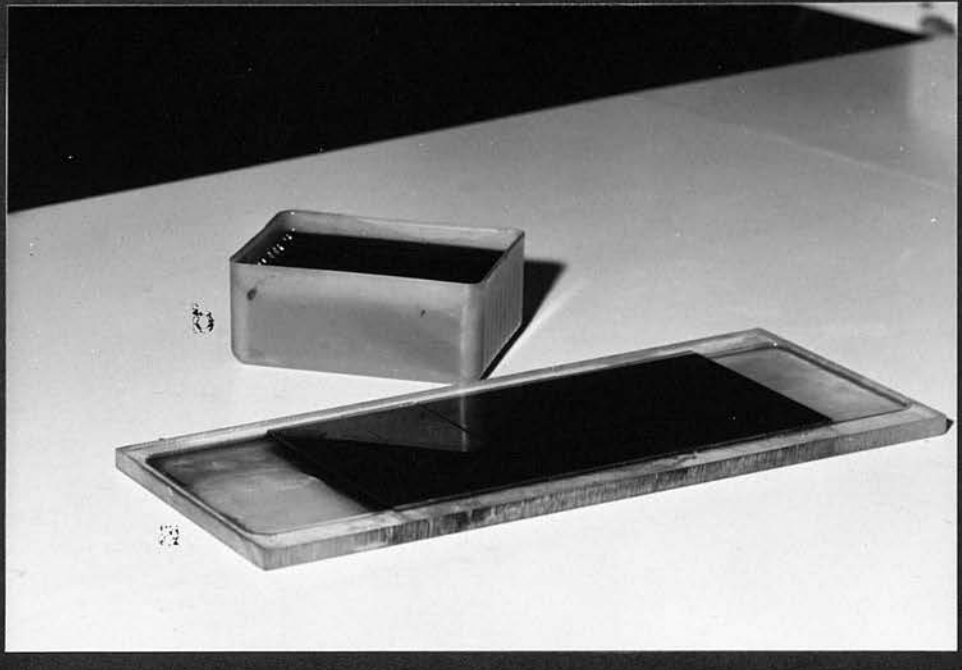
For infectivity tests, tenfold dilutions were made as follows: Using prefreeze red cell counts and percentage parasitised red cells, the first dilution to 2 ml. was prepared in cryoprotectant-added diluent. From this first dilution, 0.2 ml. was transferred to 1.8 ml. cryoprotectant-added diluent and from this 0.2 ml. was transferred to a further 1.8 ml., and so on.

FIG. 5

The apparatus used in staining blood smears

(a) staining troughs

(b) staining racks.



(2) Screening for parasites. In any full-scale infectivity test, all ten animals in the first dilution group were screened for parasites by microscopical examination of a Giemsa-stained blood smear under the oil immersion objective for one minute. During this time, about 40 fields, each containing approximately 100 red cells, were scanned. If any animal in this group was found to be positive, i.e. one or more parasites in c. 4000 red cells, the next dilution group was screened, on that day and on all subsequent days. The mice were examined daily, generally early in the morning. Any animal found positive was examined daily until death. In single-dilution infectivity tests, all ten animals were screened daily for either the presence of parasites or death.

(3) Infectivity titrations. The infectivity titration technique used was based on that described for Babesia rodhaini by Overdulve and Antonisse (1970a and b), who showed that a linear relationship existed between infecting dose and prepatent period or survival time. One difference in this study was that ten animals were used for each dilution group instead of their six. The parasite was titrated for at least three, usually four, dilution groups.

From the equations of the regression lines produced, the mean doubling time, the theoretical number of parasites present at onset of parasitaemia or at death, and the effective number of infective organisms injected could be calculated.

(4) Single-dilution infectivity tests. It can be seen from the above that each full-scale infectivity titration required at least 30, usually 40, animals, in three or four groups of ten. Although it would have been desirable, it was practically and economically impossible

to make full-scale infectivity tests on each experimentally frozen material. For this reason, a smaller, single-dilution, test was used, in which each experimental method material was tested for infectivity in one group of ten animals, usually infected with 10^7 (or a theoretical 10^7) parasitised red cells. Theoretical doses were calculated from prefreeze red cell, and percentage parasitised red cell, counts. Thus, after thawing, the material was diluted to the same extent as it had been prior to cooling, irrespective of the degree of damage incurred.

For these groups of ten animals, mean prepatent period and mean day of death (or survival time) was calculated. The figures obtained for such mean values could then be statistically compared <u>within experiments</u>. In this way, successful and unsuccessful techniques could be separated simply - the unsuccessful techniques were rejected and successful techniques were investigated further.

Statistical tests. (1) On single-dilution infectivity test means. Prepatent period and survival time means of single-dilution infectivity tests were analysed using the Duncan Multiple Range Test (Duncan, 1955). On the advice of the Department of Statistics, University of Edinburgh, some 'rules' were laid down for the treatment of the groups which gave these means, prior to analysis.

<u>Prepatent period means</u>. Quite often, one or possibly more than one animal would appear positive some days after its fellows in that group. This could be accounted for as (a) low infectivity for that particular dose of parasites; (b) variation in host response and (c) misinoculation. It was impossible to say which situation applied and the problem arose as to the treatment of these 'aberrant' individual observations.

In their full-scale titration test, Overdulve and Antonisse (1970b) encountered a similar problem:

"(3) The dispersion in each group of mice both for survival time and for prepatent period, is much higher in Experiment 3, especially in the groups infected with the higher doses; the mice which are clearly outliers are neglected in the calculation of the regression lines of Experiment 3, although it is obvious that the resulting regression lines become more doubtful by this selection."

Rather than simply neglect all 'obvious outliers' in the analysis of prepatent period means, it was decided to trim the data according to the guidelines laid down by Andrews, Bickel, Hampel, Huber, Rogers and Tukey (1972). In this case, the highest individual observation in each group was eliminated from the calculation of each group mean and standard deviation. This effectively removed a large proportion of the 'aberrant results' or outliers obtained. Animals which did not become infected during the screening period were ignored in the calculation of group means and standard deviations.

Survival time means. As with prepatent period calculations, the highest day of death observation in each group was eliminated from the calculation of the mean and standard deviation. Animals which did become infected but did not die were ignored in group mean and standard deviation calculations.

(ii) Full-scale titration tests. A similar data trimming procedure to that described above was used in the calculation of log. dose against prepatent period or survival time linear correlation coefficients and linear regressions; i.e. the highest prepatent period or day of death observation in each dilution group was

eliminated from the linear correlation or linear regression calculations. Non-infected animals and infected animals which did not die were, in addition, ignored in these calculations. Comparisons of linear regression were calculated by the method described by Dawkins (1968).

Because of the degree of data trimming which was used, it was not possible to be completely categorical about borderline differences. In the single dilution tests, since only trends were being looked for, this was of less importance. In titration tests, however, this made conclusions rather more difficult to achieve. For completeness and comparison, therefore, all calculations and tests were repeated in which highest observations were included. In many cases, little or no difference could be found between the results of analyses of trimmed and untrimmed data.

Computer analysis of results. Initially, the task of analysis in both the Duncan Multiple Range Test and the Comparison of Linear regression was carried out 'longhand'. Even, however, with the aid of a programmed calculator, both tests involved many time-consuming arithmetical steps, each of which was a potential source of error. As the amount of data, and the likelihood of errors, increased, it was decided that, for accuracy and economy of labour, computer analysis of results be used.

(1) Duncan Multiple Range Test. These tests were run on the Edinburgh Regional Computing Centre, 'IBM' 370/180 computer, using the BMD07V programme (Dixon, 1971). Data was transferred on to punched cards according to the instructions given in the BMD manual cited above (Dixon, 1971).

(2) Comparison of Linear Regression. At the time that the results in this study were to be analysed, no suitable programme was available for comparison of linear regression as such. It was decided that a programme be written for this test, based on the Dawkins (1968) method, and using the simple FOCAL-12 language which can be used with the small 'DIGITAL' PDP-12 computer. Using the University of Edinburgh Veterinary Faculty, PDP-12 computer, and Restricted FOCAL-12 language the author was able to write a successful programme. (Restricted FOCAL-12 is FOCAL-12 with a few of its functions restricted in order to increase the effective space available for programming.) A copy of the successful programme (&COMLREG) is shown in Appendix Table 94.

An additional attraction of computer analysis of results is that the BMD Duncan Multiple Range Test programme printout is in ideal tabular form for thesis presentation after photo-reduction. The &COMLREG printout format was adjusted to suit this purpose. In many of the Duncan Multiple Range Test tables, however, the list of computed shortest significant ranges has been left out in order to make the table more compact.

CHAPTER 3

Cooling and Thawing Rate Estimation

Theoretically, there are many cooling and thawing rates which might be studied when investigating the cryopreservation of any cell type. These rates could range from the very slow (Meryman, 1967 showed that human erythrocytes, protected with 1.5M glycerol were less damaged when cooled at 0.3°/min. than they were when cooled at higher rates) to the extremely rapid (Luyet and Gehenio, 1955 reduced the degree of haemolysis in unprotected ox blood to 8% by cooling it at an estimated 7800°/min.). Combinations of cooling rates, used for example by Weinman and McAllister (1947) and Polge and Soltys (1957) in the two-step cooling technique mentioned above, further increase the potential number of cooling methods which might be employed. In addition, the combination of variable cooling rates with variable thawing rates brings the number of possibilities virtually to infinity.

It would, of course, be impractical to test every possible combination of cooling and thawing rates, and, therefore, a limit must be set to the number of cooling and thawing rates studied. Cryobiologists recognise the arbitrary separation of cooling rates into the following categories. The limits of these categories are also arbitrary (discussed by Luyet and Rapatz, 1970), each worker setting his own.

(a) Slow or conventional:- usually on or around the historically significant 1°/min.

(b) Rapid:- ranging from a few tens to several hundreds of degrees/min.

(c) Ultrarapid:- in excess of about 2000°/min.

In this study, cooling rates in the above three categories were obtained by the following methods:

Methods

(1) Slow or conventional cooling rates. These cooling rates were achieved by placing glass ampoules or pp60 polythene tubing lengths containing the material to be cooled inside cooling jackets, which have been described above, in a solid carbon dioxide cooling cabinet. The cooling rates were measured using either bead thermistors when the material was stored in ampoules or thermocouples when the material was stored in pp60 polythene tubing. In both cases, the thermistor or thermocouple monitor junction was placed directly in the container and therefore into direct contact with the material being cooled.

(2) Rapid cooling rates. These cooling rates were achieved by placing the ampoules or pp60 lengths containing the material to be cooled directly in liquid nitrogen. The cooling rates were measured using thermocouples placed in the containers and therefore in direct contact with the material being cooled.

(3) Ultrarapid cooling rates. These cooling rates were achieved by placing the material to be cooled, stored in containers coated with Vaseline and talc, directly in liquid nitrogen. Cowley, Timson and Sawdye (1961) outlined the principles of the ultrarapid cooling technique. When, for example, a glass tube is cooled by placing it directly in a boiling liquid, such as liquid nitrogen, three types of boiling, i.e., heat transfer, can occur.

(i) As the temperature of the surface is greater than the boiling point of the liquid, vapour bubbles are formed at specific sites on the surface. These bubbles grow to a critical size and then detach themselves from the surface and rise through the liquid. The greater the difference between the surface temperature and that of the boiling liquid, the greater will be (a) the number of nucleation sites and (b) the frequency of bubble formation at these sites. The turbulence caused by these bubbles causes an increase in the amount of heat transfer from the surface up to a distinct limit.

(ii) At this limit, or the point of critical heat flux, the number of nucleation sites is so great that the bubbles begin to coalesce and a vapour film is formed over the surface. This vapour film is unstable and is constantly being dissipated and reformed at a very high rate.

(iii) When the temperature difference between the surface and the boiling liquid is increased further, the vapour film becomes stable and completely blankets the surface. This stable vapour film has a low thermal conductivity and the heat transfer rate, as a result, reaches a minimum for the system (for temperature differences greater than or equal to that at which the point of critical heat flux was attained). Further increase in the temperature difference between surface and boiling liquid, increases the amount of heat transfer.

With biological material being cooled from room temperature (say +25°C) to -196°C, the critical range of temperature is thought to be from +25 to about -50°C. Unfortunately this is usually the region of temperature difference, i.e. 221 to 146 degrees, at which stable film boiling occurs. Cowley et al showed that heat transfer rates at the minimal point can fall by a factor of ten compared with those at the critical point. They were able to demonstrate a reduction

in this fall by the use of thermally insulating materials such as Vaseline. A surface coated with such a material cooled much more rapidly than an uncoated one. This increase in the cooling rate was due to the surface of the coating which was in contact with the boiling liquid dropping to the temperature of the liquid much more quickly than an uncoated surface. The resultant smaller temperature difference resulted in unstable or nucleate boiling at which heat flux is greater. Cowley et al also stated that the superimposition of a powder over the Vaseline further increased the cooling rate. They also showed that the thickness of the Vaseline coating had an effect on the cooling rate achieved.

(4) Material was always thawed as rapidly as possible by agitation in a water bath at +37°C.

Results

The cooling rates given by the methods described above are presented below:

(1) Slow or conventional cooling rates. (a) using ampoules

Individual estimations (°/min.)

1.1 1.3 1.3 1.4 1.1 1.3

Number of estimations: 6

Mean: 1.25°/min.

Range: 1.1 - 1.4°/min.

(b) using pp60

Individual estimations (°/min.)

2.0 1.6 1.5 1.7 1.9 2.1 1.7

Number of estimations: 7

Mean: 1.78

Range: 1.5 - 2.1°/min.

Figures 6 and 7 show 'typical' slow cooling curves, i.e. curves in which the rates of cooling are close to the means calculated above.

(2) Rapid cooling rates. (a) using ampoules

Individual estimations (°/min.)

166	115	89	183	140	155
89	150	160	150	240	200
133	126	170	138	114	200

Number of estimations: 18

Mean: 151

Range: 89 - 240

(b) using pp60

Individual estimations (°/min.)

2400	2400	2000	1600	1850	2200
2000	2400	2200	1500	1500	1400
1500	1500	1350	1700	2200	1850
1600					

Number of estimations: 19

Mean: 1850

Range: 1350 - 2400

Figures 8 and 9 show typical rapid cooling curves.

(3) Ultrarapid cooling rates. (a) using ampoules

After the breakage of the first five ampoules tested using this cooling method, further investigation was abandoned.

FIG. 6

A 'typical' cooling curve for material slowly cooled in glass ampoules. The estimated rate of cooling between 0 and -70°C in this instance is c. 1.3°/min.

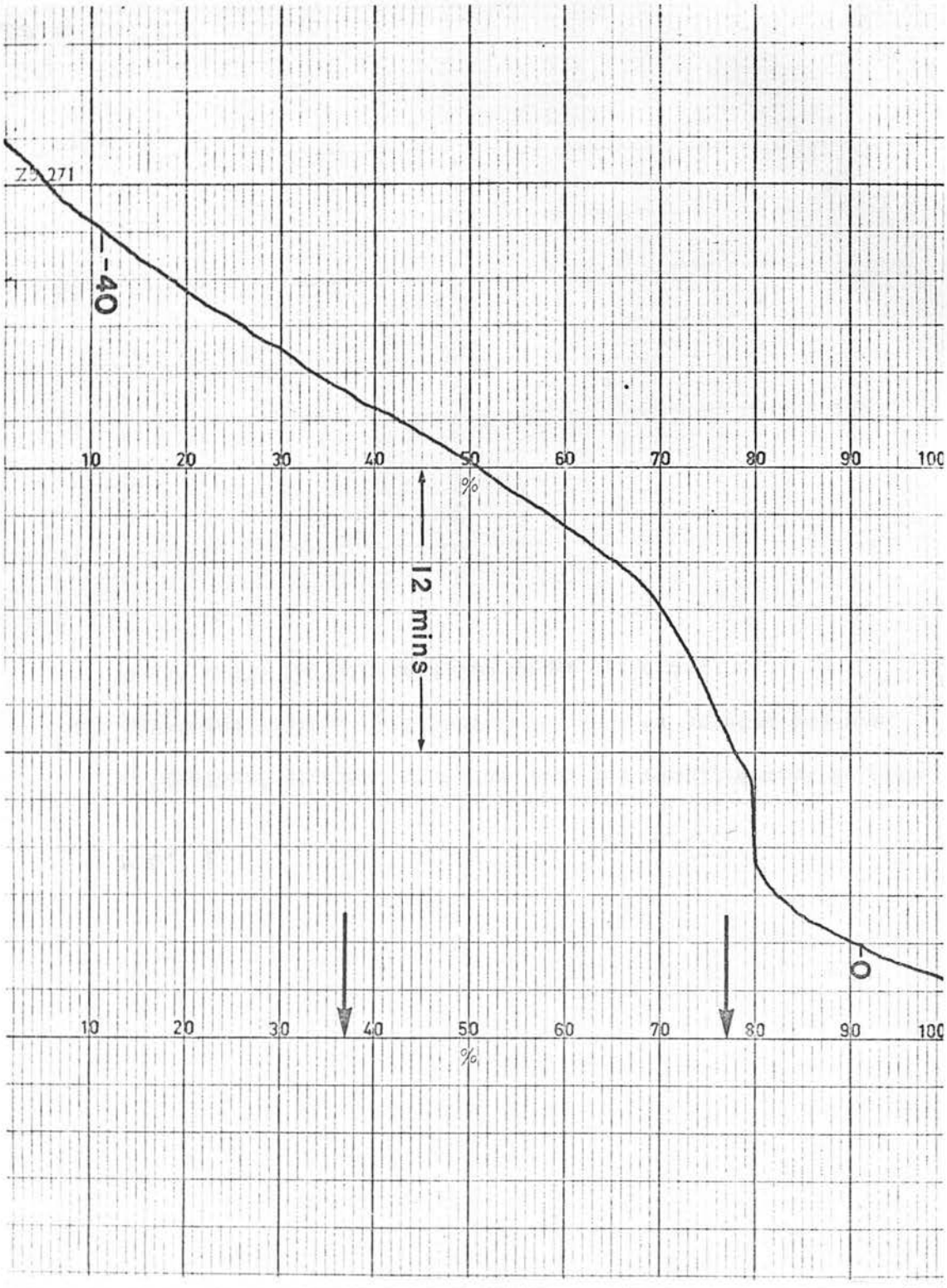

-40
10
20
30
40
50
60
70
80
90
100
%
12 mins
0

FIG. 7

A 'typical' cooling curve for material slowly cooled in pp60 polythene tubing. The estimated rate of cooling between 0 and -40°C in this instance is *c.* 1.7°/min.

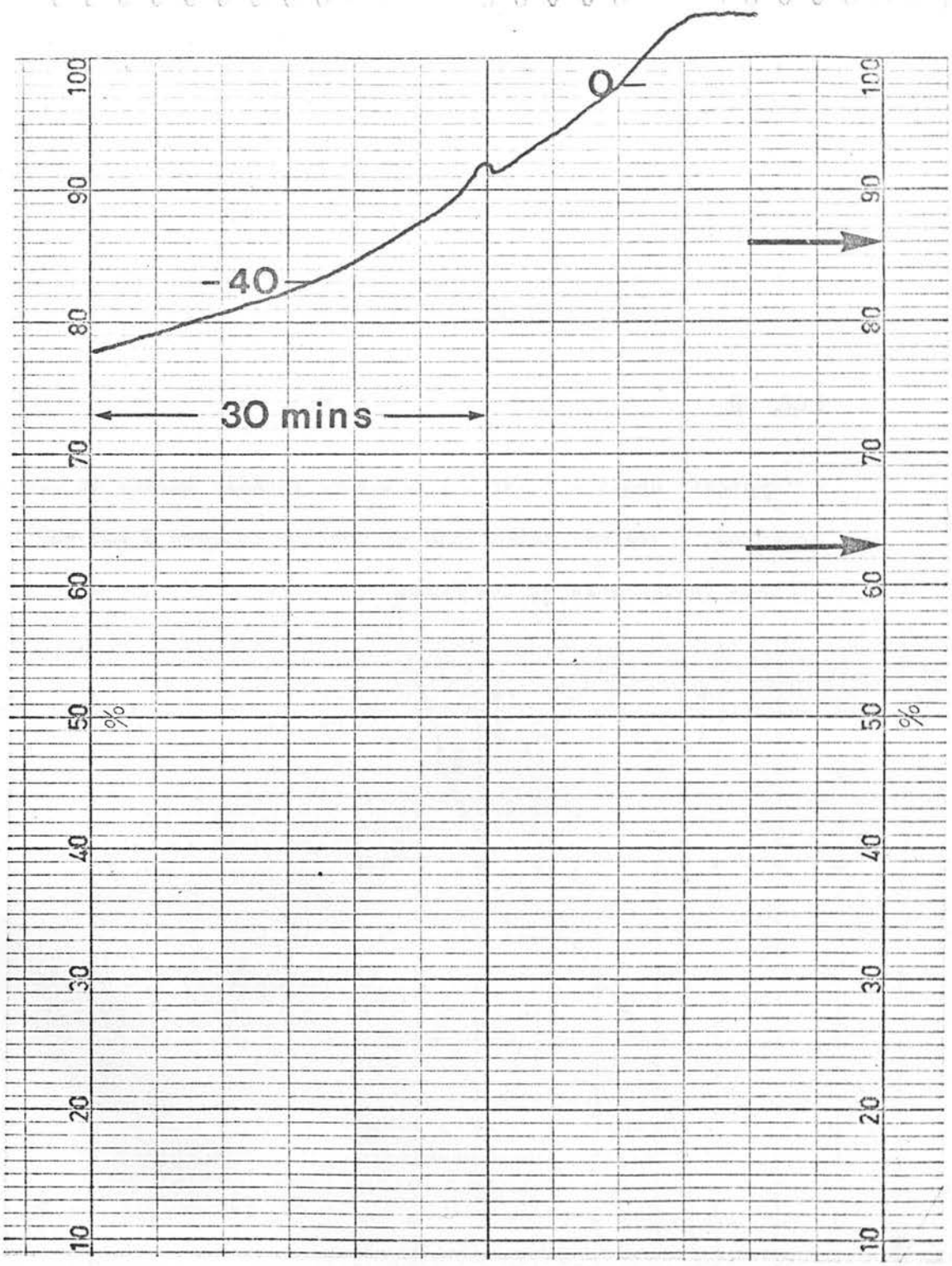

0
-40
30 mins
100
90
80
70
60
50
%
40
30
20
10

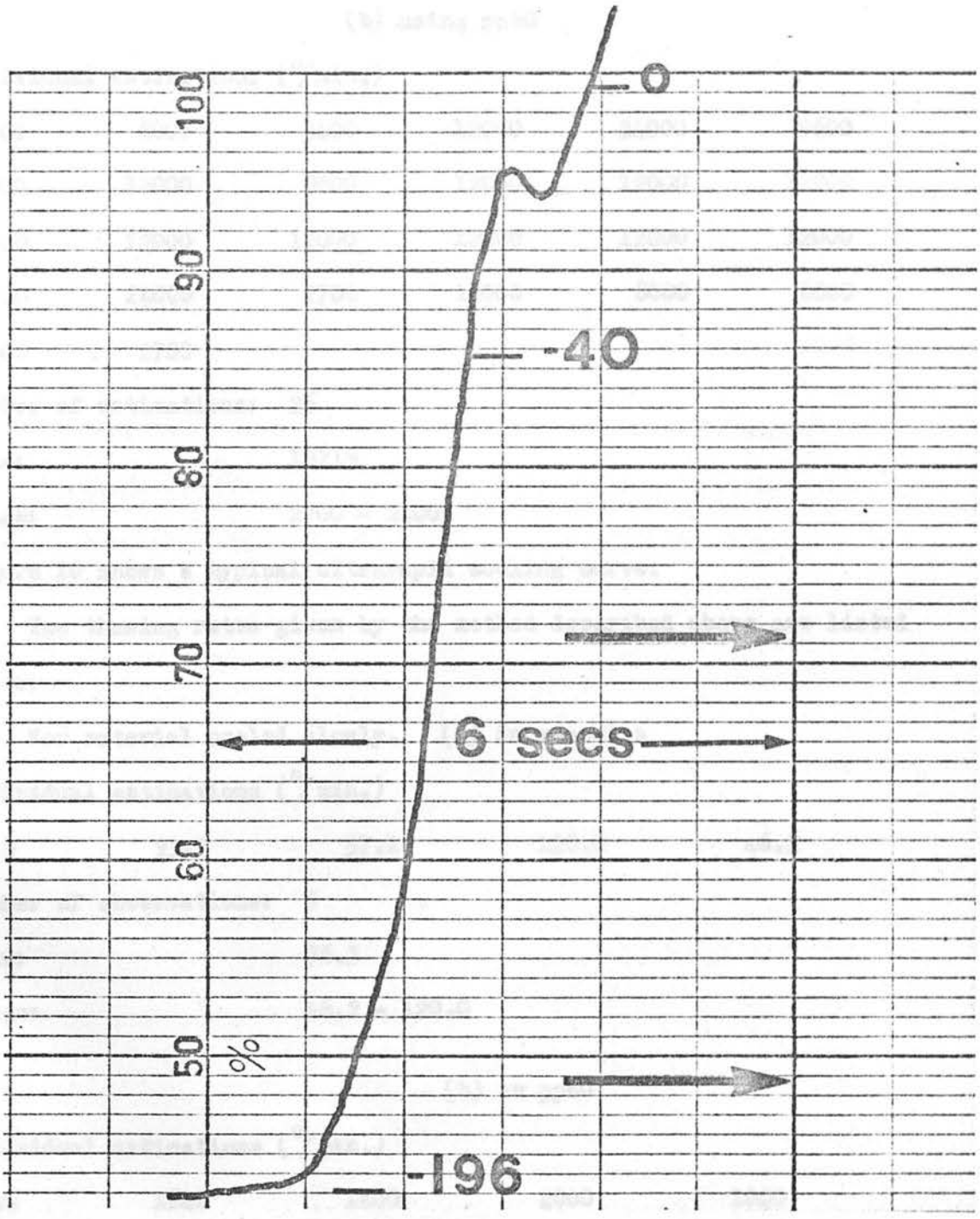
FIG. 8

A 'typical' cooling curve for material rapidly cooled in glass ampoules. The estimated rate of cooling between 0 and -40°C in this instance is c. 150°/min.

0
-40
-80
6 secs

FIG. 9

A 'typical' cooling curve for material rapidly cooled in pp60 polythene tubing. The estimated rate of cooling between 0 and -40°C in this instance is c. 1850°/min.


100
90
80
70
60
50
%
0
-40
-196
6 secs

(b) using pp60

Individual estimations (°/min.)

12000	6000	3400	12000	24000	9600
12000	12000	9600	12000	12000	12000
9600	12000	12000	12000	12000	12000
8000	24000	2700	16000	8000	8000
3000	2700				

Number of estimations: 26

Mean: 10715

Range: 2700 - 24000

Figure 10 shows a typical ultrarapid cooling curve.

The thawing rates given by the method described above are listed below:

(4) For material cooled slowly. (a) in ampoules

Individual estimations (°/min.)

64.9	93.3	57.1	120.0	46.2

Number of observations: 5

Mean: 76.3

Range: 46.2 - 120.0

(b) in pp60

Individual estimations (°/min.)

1846	1846	1600	2000	1000

Number of estimations: 5

Mean: 1658

Range: 1000 - 2000

Figures 11 and 12 show typical thawing curves for material cooled slowly.

FIG. 10

A 'typical' cooling curve for material ultrarapidly cooled in pp60 polythene tubing. The estimated rate of cooling between 0 and -40°C in this instance is c. 9600°/min.

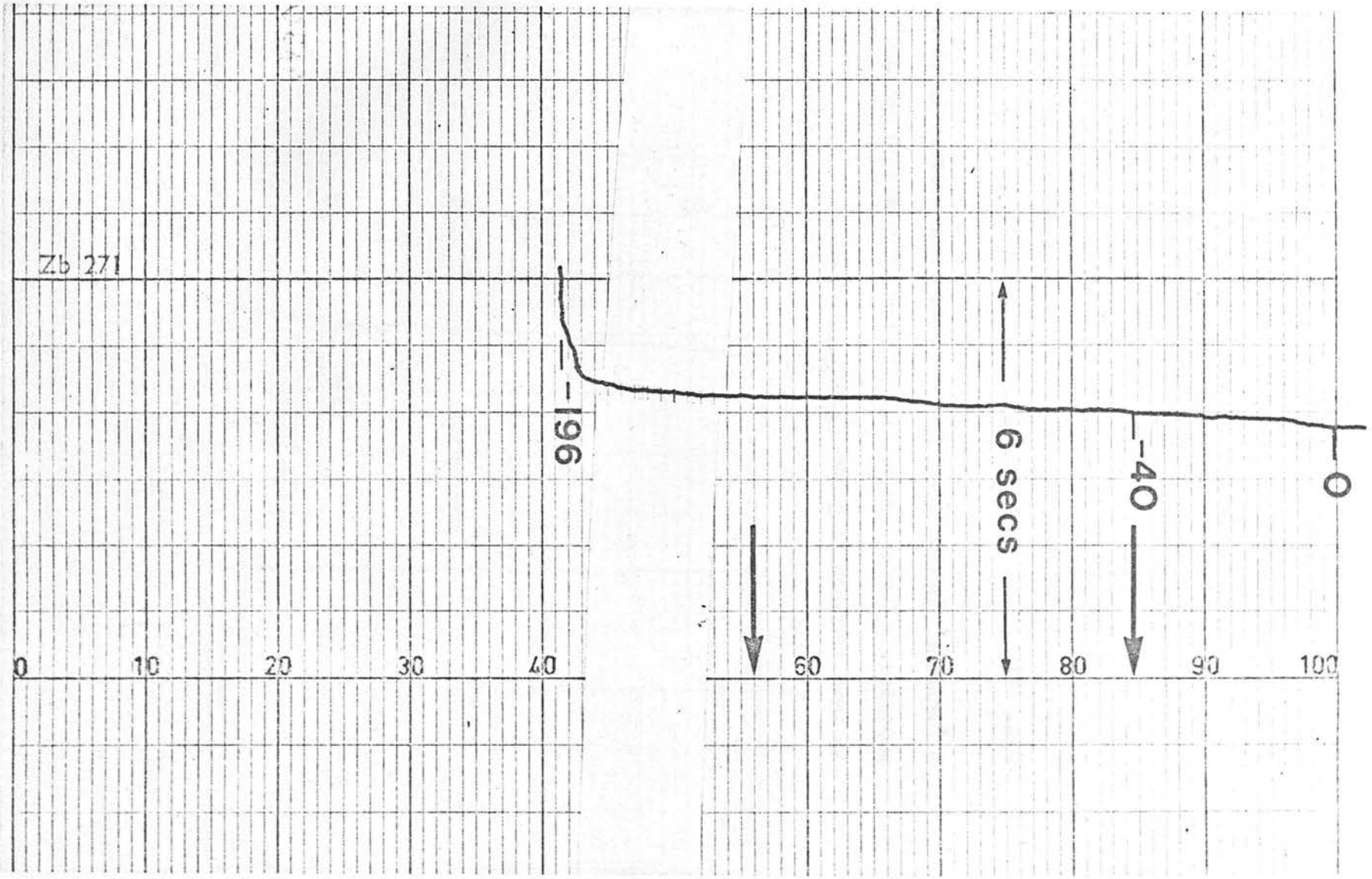

Zb 271
-196
6 secs
-40
0
0
10
20
30
40
60
70
80
90
100

FIG. 11

A 'typical' thawing curve for material cooled slowly in glass ampoules. The estimated rate of thawing between 0 and -40°C in this instance is c. 64.9°/min.

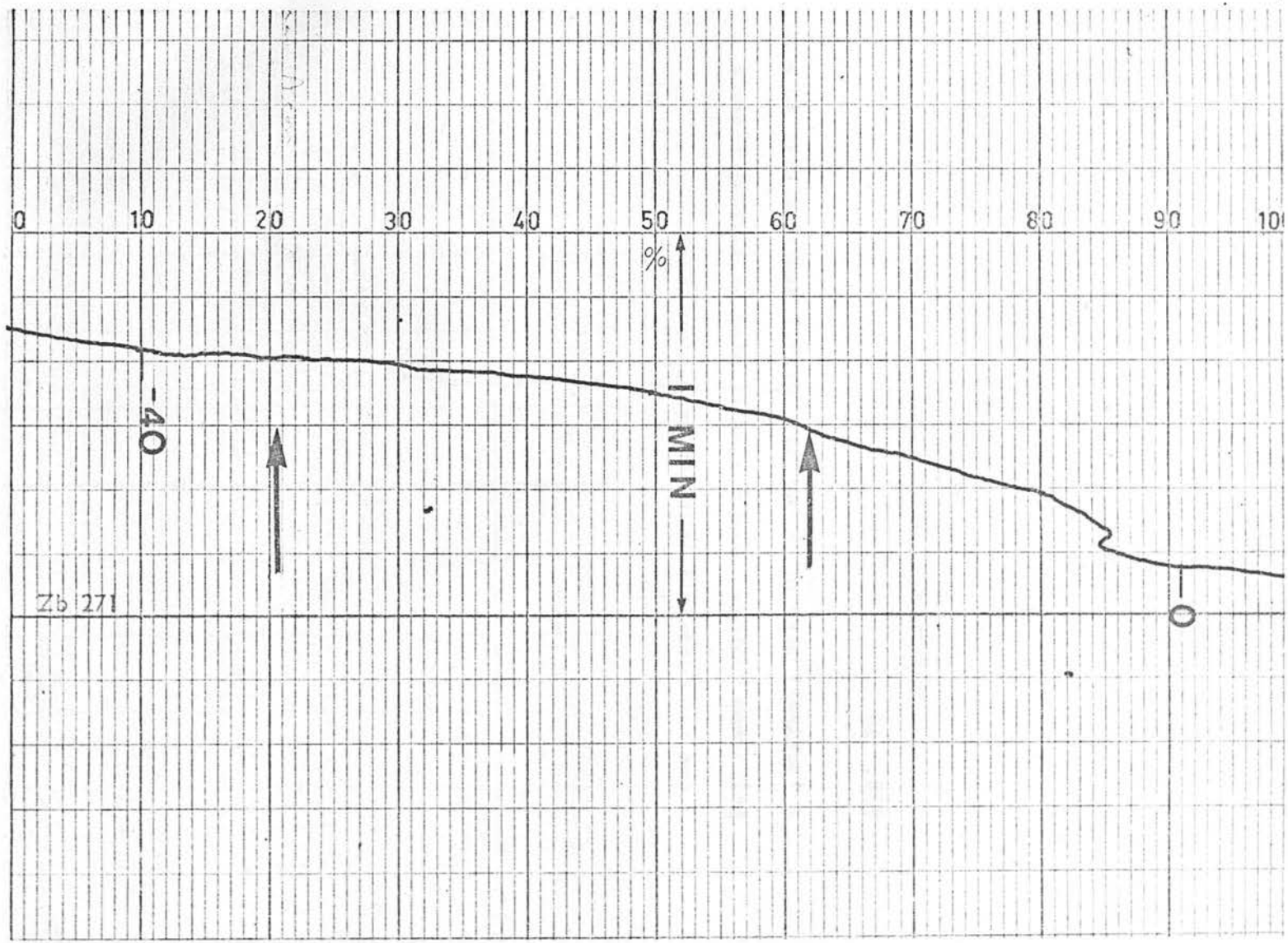
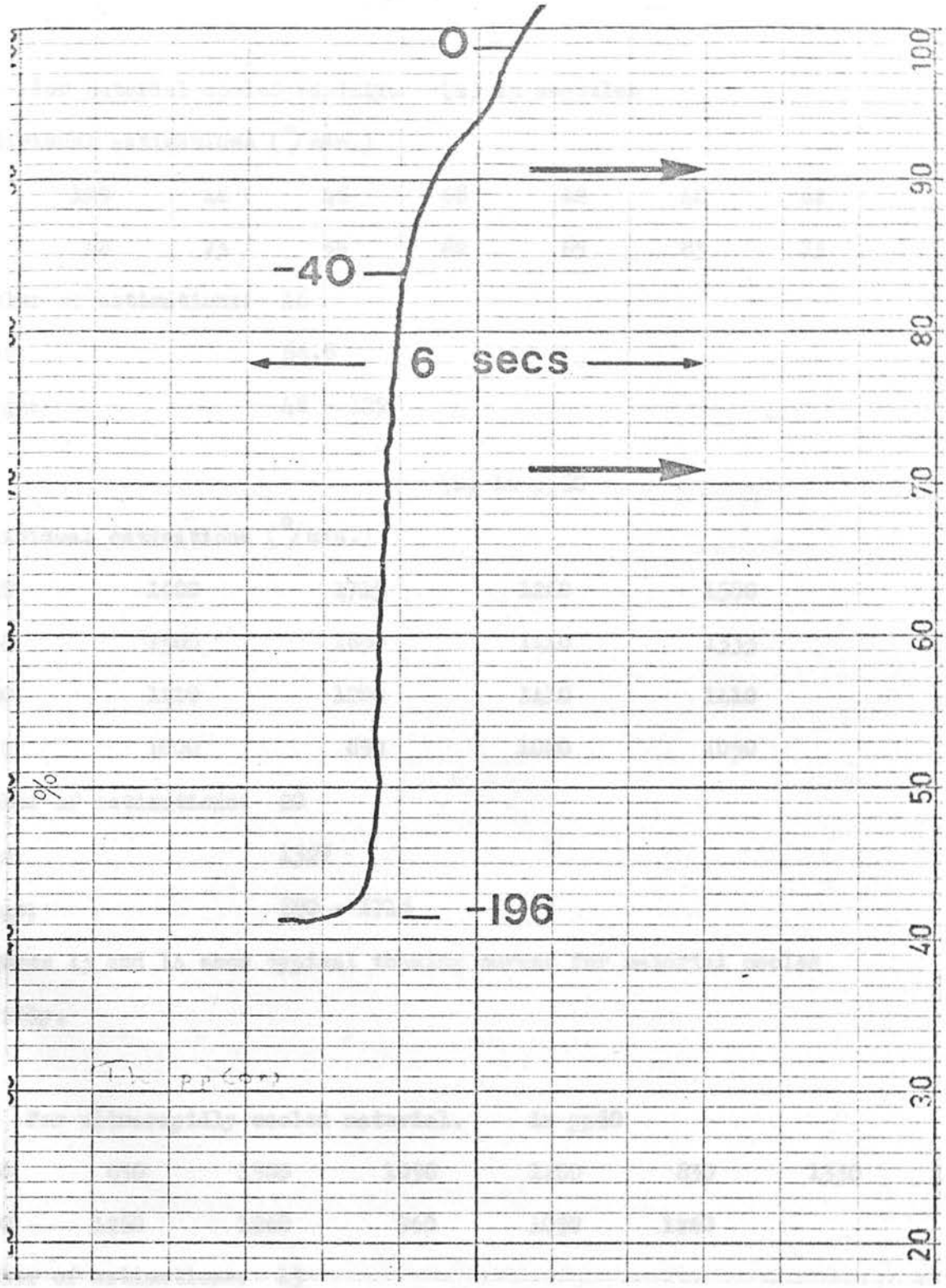

0
10
20
30
40
50
60
70
80
90
%
-40
MIN
0
Zb 271

FIG. 12

A 'typical' thawing curve for material cooled slowly in pp60 polythene tubing. The estimated rate of thawing between 0 and -40°C in this instance is c. 1600°/min.


0
-40
6 secs
-196
%
100
90
80
70
60
50
40
30
20

(5) For material cooled rapidly. (a) in ampoules

Individual estimations (°/min.)

68	129	48	48	68	62	48	62
75	48	73	56	62	65	83	73

Number of estimations: 16

Mean: 66.8

Range: 48 - 129

(b) in pp60

Individual estimations (°/min.)

1600	1600	1715	1260	1500
1330	1500	1600	1410	1335
1200	1410	1200	1410	1410
1000	1000	890	1080	1090

Number of estimations: 20

Mean: 1327

Range: 890 - 1715

Figures 13 and 14 show typical thawing curves for material cooled rapidly.

(6) For ultrarapidly cooled material. in pp60

1090	630	1500	1090	1200	830	1330
960	1260	1260	960	1090	1260	

Number of estimations: 13

Mean: 1112

Range: 630 - 1500

Figure 15 shows a typical thawing curve for material cooled ultrarapidly in pp60.

FIG. 13

A 'typical' thawing curve for material cooled rapidly in glass ampoules. The estimated rate of thawing between 0 and -40°C in this instance is c. 68°/min.

-196
-40
6 secs

FIG. 14

A 'typical' thawing curve for material cooled rapidly in pp60 polytheme tubing. The estimated rate of thawing between 0 and -40°C in this instance is c. 1330°/min.

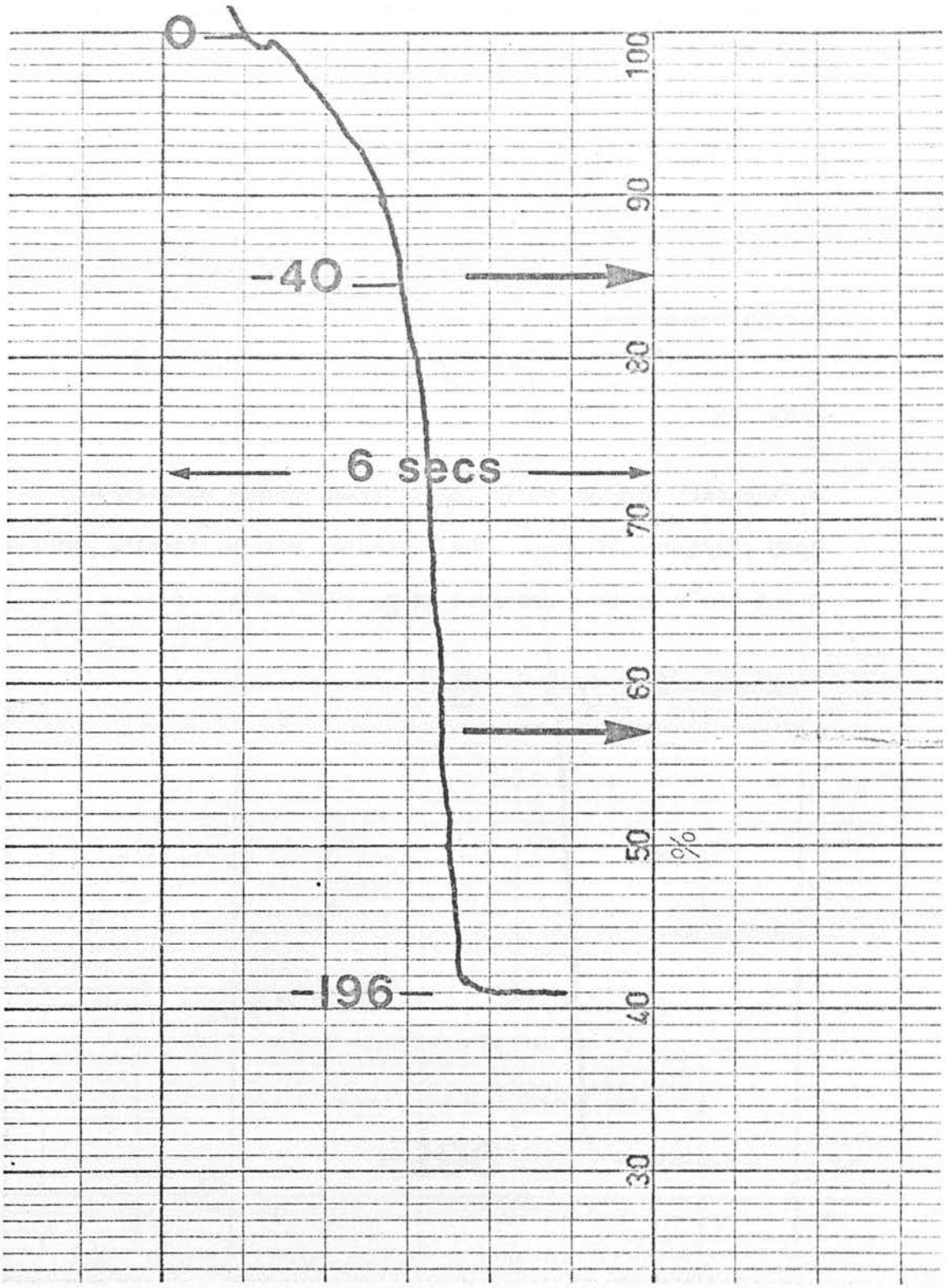
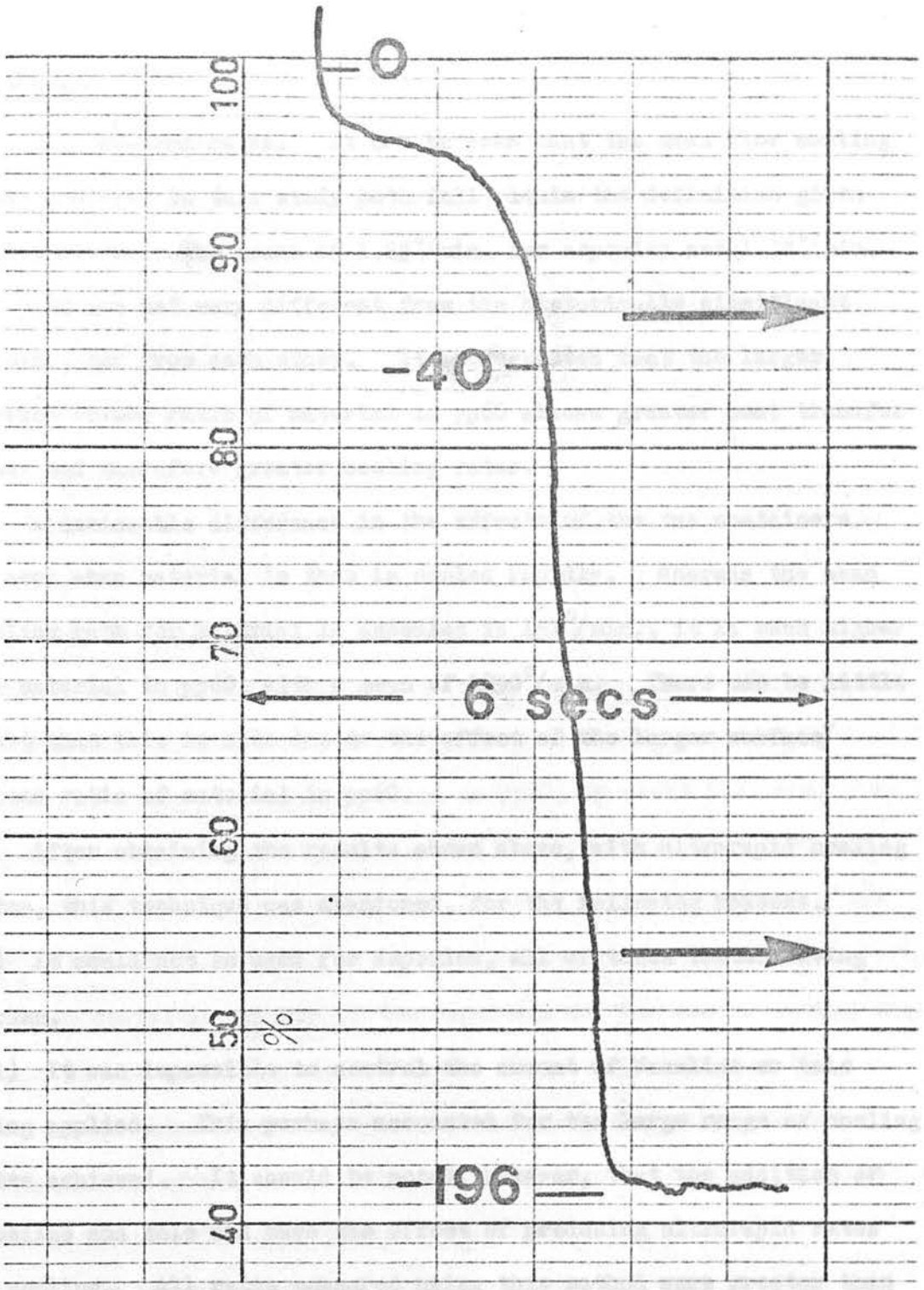

0
-40
6 secs
-196
100
90
80
70
60
50
40
30
%

FIG. 15

A 'typical' thawing curve for material cooled ultrarapidly in pp60 polythene tubing. The estimated rate of thawing between 0 and -40°C in this instance is c. 1090°/min.


0
-40
-196
6 secs
100
90
80
70
60
50
40
%

Discussion

(a) Cooling rates. It can be seen that the mean slow cooling rates achieved in this study both fall within the definition given in (a) above. The means of 1.25°/min. for ampoules and 1.78°/min. for pp60 are not very different from the historically significant 1°/min., nor from each other. It is suggested that the larger surface/volume ratio of material in pp60 allows greater heat transfer rates and therefore greater cooling rates.

A noticeable difference in the effects of the two containers is seen when material in them is cooled rapidly. Whereas the mean cooling rate for material in ampoules is 151°/min., it is much higher for material in pp60, with a mean of 1850°/min. There can be little doubt that this is also due to the effect of the larger surface/volume ratio of material in pp60.

After obtaining the results shown above, with ultrarapid cooling rates, this technique was abandoned, for the following reasons.

(i) It could not be used for ampoules, all of those tested having broken.

(ii) It was impossible to control the amount of Vaseline or talc being applied. This perhaps accounted for the large range of cooling rates achieved. It should be noted, however, that the addition of Vaseline and talc did have the effect of producing ultrarapid rates of cooling. All rates measured using this method were greater than those measured using the simple rapid cooling method, by a factor of up to about 13. However, bearing in mind that one of the objects of this study was to produce a simple repeatable technique, it was decided that with this method repeatability could only be produced with a loss of simplicity, i.e. application of exact amounts of Vaseline and talc would be necessary.

(b) Thawing rates. It can be seen from the results shown above that the mean thawing rates for material cooled either slowly or rapidly in ampoules are similar (76.3 and 66.8°/min. respectively). The range of thawing rates for material cooled by both methods are also similar (46.2 - 120.0 and 48 - 129°/min. respectively). These large ranges probably reflect the degree of agitation of the ampoules in the water bath when thawing is taking place. They are probably also produced as a result of the position of the thermocouple or thermistor in the ampoule, i.e. the nearer it is to the wall of the ampoule, the sooner it will reach 0°C upon thawing.

Mean thawing rates in pp60 are, as can be seen from the results shown above, much more rapid, by a factor of about 20. The results indicate that material cooled slowly in pp60 thaws, on average, faster than material rapidly cooled in pp60, by about 300°/min. The large ranges obtained, 1000 - 2000°/min. for slowly cooled and 890 - 1715°/min. for rapidly cooled material, are indicative of the variability of the thawing method and the means obtained. Therefore, although useful indicators of the magnitude of the thawing rates, they should not be considered definitive. It is suggested that the low mean values obtained for material ultrarapidly cooled may be due to an insulating effect of Vaseline and talc, slowing down the thawing rate of the material they coat.

In the introduction to Chapter 4, which will take the form of a brief review of the principles of cryobiology, mention will be made of the necessity for rapid thawing following rapid cooling. The results shown above indicate that the use of pp60 polythene tubing allows of both rapid cooling and rapid thawing.

CHAPTER 4

Experiments

Introduction. General principles of cryopreservation.

In the discussion of the results of the experiments described in this chapter, reference will inevitably be made to many of the basic principles of cryobiology. It was thought advisable, therefore, that this chapter be preceded by a short introductory section in which these principles are outlined. This introduction will consist of a description of what happens to cells when they are cooled rapidly, slowly, and with a cryoprotectant present.

Rapid cooling. It has long been recognised that red cells could be frozen successfully at rates of up to 3000°/min., provided they were thawed rapidly. Luyet demonstrated this as long ago as 1949 when he successfully froze and thawed red blood cells to and from -196°C between two coverslips.

Briefly, the events which occur when cells in suspension are frozen are as follows:

As the cell suspension is cooled below the freezing point of the extracellular water, extracellular ice is formed. This extracellular ice is prevented by the cell membrane from growing into the now-supercooled interior. The cell begins to equilibrate by losing water, thus concentrating the intracellular solutes. What then happens depends on the rate of cooling and the permeability of the cell membrane to water. Mathematical techniques have been used (Mazur, 1963, 1965a) which enable the theoretical amount of water left in a cell at any given temperature for any particular rate of cooling to be calculated.

FIG. 16

From Mazur (1970).

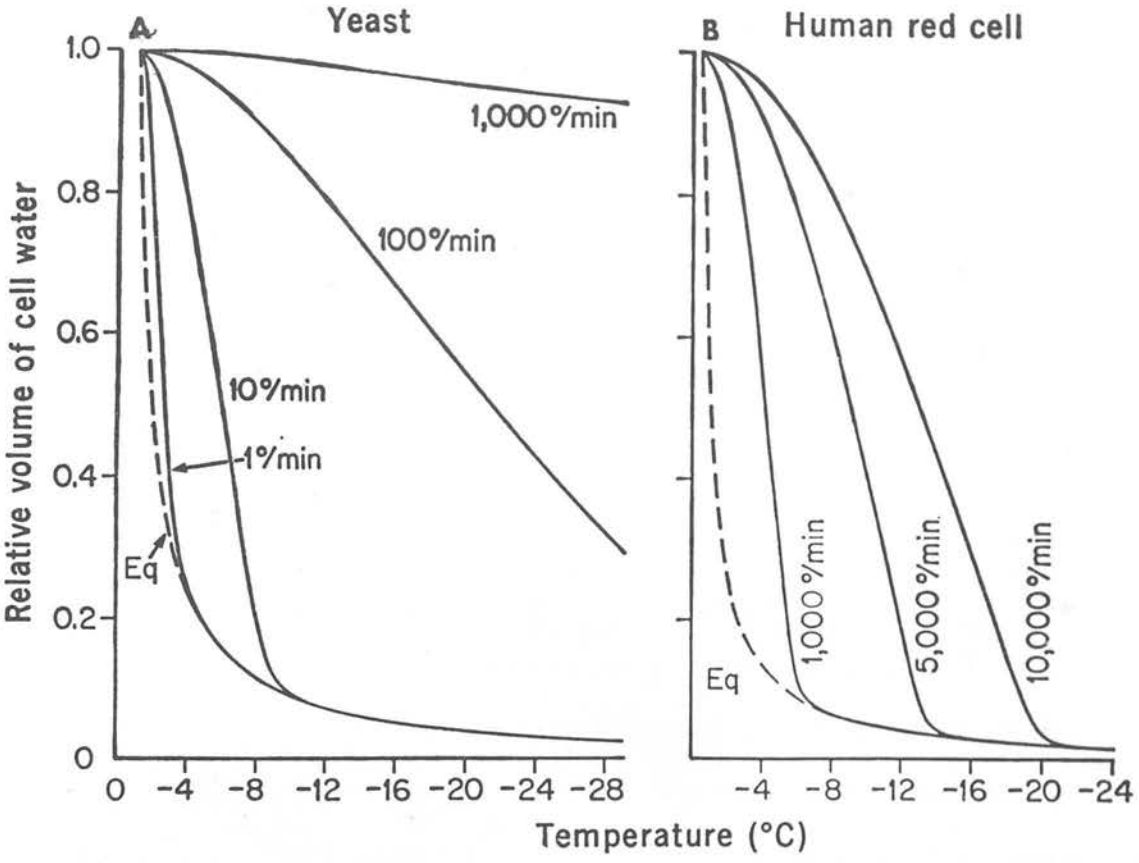


Fig. 16 Calculated fraction of intracellular water remaining in (A) yeast cells and (B) human red cells as they cool to various temperatures at the indicated rates. Curve *Eq* represents the equilibrium water content.

Figure 16, taken from Mazur (1970) shows the calculated fraction of intracellular water remaining in (A) yeast cells and (B) human red cells as they cool to various temperatures at the indicated rates. Curves 'Eq' represent the equilibrium water content of cells cooled infinitely slowly, allowing these cells continuously to maintain vapour pressure equilibrium with the extracellular ice by dehydration. Cells cooled at finite rates, however, will contain more than the equilibrium amount of water and will therefore be supercooled. For example, in 16A, yeast cells cooled at 100°/min. will contain 70% of their original water at -15°C. This water would be supercooled about fourteen degrees, this being the difference between the 70% point on the 100°/min. curve and the corresponding point on the Eq curve.

Such extreme supercooling cannot occur, however, for at temperatures below -10 to -15°C the cell membrane no longer prevents intracellular ice crystal formation and cells that are cooled fast enough to contain supercooled water below these temperatures will then equilibrate by intracellular freezing. The critical cooling rate depends on the surface/volume ratio of the cell and its permeability to water.

One might expect that cells cooled below their freezing point would not be subjected to further stress. This is not necessarily so; reactions can occur in the solid state and one of these is recrystallisation or grain growth. Small convex ice crystals will have higher surface energies than large flat crystals and will tend to reduce their surface energies by growing and fusing with other small crystals. The rate of recrystallisation depends on both the size of crystals formed during cooling and on the subsequent temperatures to which they are exposed, the rate being higher for smaller crystals and at higher temperatures (Mazur, 1966; Kingery, 1960).

Recrystallisation occurs in pure water at temperatures as low as -100°C and it occurs in gels and concentrated aqueous solutions at higher temperatures (Meryman, 1966; Luyet and Rasmussen, 1967; McKenzie and Luyet, 1967).

During rapid cooling the crystals formed will not only be small, they will also tend to recrystallise during warming (thawing), particularly if the warming is slow. It is universally accepted that large intracellular ice crystals are for almost all cell types, and certainly for red cells, lethal. Hence, if we cool rapidly, we should also thaw rapidly.

Slow cooling. With slow cooling, the most likely source of damage to cells comes from what are called 'solution effects'. As the temperature is reduced, the amount of cell water decreases, extracellular and intracellular solutes concentrate, solutes precipitate as their solubilities are exceeded (leading to pH changes) and, finally, all solutes precipitate below the eutectic point of the system. These events are functions of temperature alone in a given cell suspension at constant pressure, provided that the cells remain at a given temperature long enough for them to attain vapour pressure equilibrium. The rate of cooling, therefore, determines the length of time cells are exposed to these solution effects.

The effects of cooling rate on cells - a summary

1. When cooled below zero, cells initially supercool.
2. Subsequent events depend on the rate of cooling and the permeability of the cell membrane to water:

(a) Slow cooling and/or high permeability cause equilibration by dehydration and damage by solution effects.

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(b) Rapid cooling and/or low permeability cause equilibration by intracellular freezing and damage mainly by recrystallisation.

Cryoprotectants. The events summarised above are those which accompany freezing in unprotected cells. However, since the discovery in 1949 of the cryoprotective properties of glycerol, in 1959 of DMSO and of other cryoprotectant materials such as polyvinylpyrrolidone (PVP) and dextran, most workers have tended to use one of these substances in the development of their cryopreservation techniques.

From what has been said above, we could deduce that the theoretical optimum cooling rate for an unprotected cell would be that rate which was fast enough to prevent solution effects - but not so fast as to produce intracellular freezing (Mazur, 1965a). The existence of such optimum cooling rates has been demonstrated for such cells as yeast cells, red cells, sea urchin eggs, nucleated mammalian cells and higher plant cells. These optima have been demonstrated by observations on the survival curves of cells cooled at different rates. Often, however, a cooling rate low enough to prevent intracellular freezing is also low enough to produce lethal exposure to solution effects. Under these conditions there is no optimum rate (Mazur, 1966; Mazur et al, 1970). According to Meryman (1966), this seems to be the case for most nucleated mammalian cells. For example, less than 2% of unprotected mouse marrow cells were shown by Liebo et al (1970) to survive freezing at cooling rates ranging from 0.3 to 600°/min. On the other hand, they will survive if a protective additive such as glycerol is present. Once again, maximum survival occurred at an optimum rate, but the numerical value of this rate depended on the concentration of glycerol used.

Cryoprotective additives appear to protect against solution effects rather than against intracellular freezing. This means that they will tend to increase survival rates in cell suspensions frozen slowly, but will not necessarily do so in suspensions frozen rapidly; survival of rapidly frozen cells may even be decreased (Mazur, 1968; Luyet et al, 1963; Rapatz and Luyet, 1963; Rapatz and Luyet, 1968).

Lovelock (1953a and b) suggested that cryoprotectants act by reducing the concentration of electrolytes in the residual unfrozen solution in and around the cell at any given temperature. If a non-electrolyte is present, then, at any given temperature, the concentration of electrolyte present will be reduced roughly proportionally. He concluded that only low molecular weight hydrophilic solutes with low eutectic points would protect cells because only they could yield solutions of high molar concentrations at sufficiently low temperatures. It seemed a condition also, that these protective additives must be able to penetrate the cell membrane, for otherwise they could not prevent a rise in intracellular electrolyte.

The mechanism of protection by high molecular weight substances has yet to be explained satisfactorily. The ability for these macromolecules to protect at very low molarities (a protein at 10^{-5}M could protect chloroplast membranes, according to Heber, 1970) argues strongly against ascribing protection to colligative effects, but does not absolutely preclude such effects as a factor. Farrant and Woolgar (1970a) were able to show that a 0.008M solution of PVP depressed the calculated mole fraction of electrolyte as extensively as did much higher molar concentrations of glycerol in a partly frozen solution, resulting in a much greater freezing point depression than would have been expected. There is, however, a possible alternative

explanation, i.e. that the macromolecule preferentially binds the free water. Such binding would reduce the amount of free water available for solution of electrolytes and would actually raise their concentration in the residual unbound water.

Whatever the mechanisms of protection, it seems likely that Lovelock's condition that protective additives should penetrate the cell need not apply. There is even some evidence that glycerol need not penetrate the cell in order to protect it. Human red cells (Lovelock, 1953b), bovine red cells (Mazur, 1970b) and unfertilized mouse ova (Sherman, 1963) bathed in glycerol solutions survived freezing even when the glycerol was prevented from penetrating the cell due to lack of time allowed for penetration or to low permeability of the cell membrane. It has been suggested, as a result of these studies, that cell survival may depend rather more on the protection of the cell surface than that of the contents of the cell.

Mechanisms of cryoinjury. There are two major causes of cell death during cryopreservation suggested, i.e. by intracellular freezing and by solution effects. For the latter the picture is complicated inasmuch as 'solution effects' is an all-embracing term for at least four separate events: i.e. water is removed as ice; solutes of high and low molecular weight concentrate; cell volume decreases and solutes precipitate. Each one of these has been the basis of a hypothesis on the mechanism of cryoinjury, but the first three occur simultaneously and hence pinpointing the exact causes of injury becomes very difficult indeed. The theories currently most readily accepted are those of Lovelock, Meryman and Levitt.

Lovelock (1953a and b; 1954; 1959, with Bishop) suggests that the high electrolyte concentrations attained during freezing affect

lipoprotein complexes in cell membranes making them leaky to sodium ions so that the internal concentration of Na^+ rises. Lovelock, however, gave no mechanism to account for a net increase in the amount of solute entering the leaky cell, a condition which would be required for subsequent osmotic shock.

Meryman (1968, 1970), however, has suggested a possible mechanism. He proposes that the damage does not result from the concentration of electrolytes per se, but from the inability of the cell to shrink below a minimum value typical for each cell type. The result of this, he says, is the establishment of a transient pressure gradient across the membrane, which in turn causes the cell to become leaky. The cell then equilibrates by movement of solute into it and it undergoes lysis due to osmotic shock during thawing.

It might be expected therefore, that slow thawing should alleviate this osmotic shock and prevent cell damage, but it does not. It tends to increase the amount of damage, an interesting paradox if the theories of Lovelock and Meryman are to be believed.

Levitt (1962, 1966) suggests that damage is not caused directly by osmotic effects, but rather that macromolecules normally separated in the cell are compacted by dehydration, and abnormal disulphide bonds are formed. There is no evidence for this.

The mechanism of damage by intracellular freezing is even more uncertain. Mazur (1961, 1965a, 1966) has suggested that the growth of intracellular ice crystals exerts sufficient force to rupture plasma membranes or membranes of organelles such as mitochondria. This is based on observations that recrystallising ice crystals can disrupt protein gels and also that cells damaged by intracellular freezing have been shown to suffer membrane damage and have become

leaky. However, similar membrane damage has been shown to occur in cells damaged by extracellular freezing (Heber, 1967).

Experiment 1. Estimation of the degree of damage to Babesia rodhaini - infected rat blood cooled slowly or rapidly in either glass ampoules or pp60 polythene tubing and diluted 1:1 with one of:

(a) phosphate buffered saline (ABP8.0) alone,

(b) 20% final concentration dimethylsulphoxide (DMSO) made up in ABP8.0

(c) 15% final concentration glycerol made up in ABP8.0

(d) 0.008M, 24500 M.Wt. polyvinylpyrrolidone (PVP) made up in ABP8.0.

Percentage haemolysis is used as the method of estimation of degree of damage.

Introduction. In this chapter, individual experiments will be dealt with separately and completely. Experiments will be introduced and described, results and analyses will be presented and discussed, and subsequent experiments will be proposed.

As can be seen from the literature review above, the cooling rates most often investigated in the cryopreservation of Babesia spp. were the 'conventional' slow rate of c. 1°/min. and uncontrolled rates produced by plunging material directly into liquid nitrogen. It was decided to begin this investigation by measuring the effects of such widely differing cooling rates on the degree of damage suffered by Babesia rodhaini-infected rat erythrocytes. Slow cooling rates were obtained by using cooling jackets. Rapid cooling rates were produced by plunging the material to be cooled, in either ampoules or pp60 polythene tubing lengths, directly into liquid nitrogen. The large surface/volume ratio of the polythene tubing (Taylor, 1972) allowed extremely rapid cooling and thawing rates. It was also decided to

test, simultaneously, the effect of presence or absence of cryoprotectant and type of cryoprotectant, using the previously successful cryoprotectants, DMSO and glycerol, and, in addition, an extracellular cryoprotectant, PVP. In order to keep the number of experimental groups to manageable proportions, it was proposed that the various cryoprotectants be used at their most commonly used final concentrations, i.e. 7.5% glycerol, 10% DMSO and 0.004M PVP. As the PVP used in this experiment had a molecular weight of 24500, a concentration of 0.004M was equivalent to approximately 10% w/v.

Although Babesia rodhaini-infected blood was used, infectivity of unfrozen and frozen and thawed material was not measured. It was suggested that degree of damage to the host red cell may be related to the degree of damage to the parasite. There is, in the literature, some evidence to justify this proposal. Rickard (1970) showed that experimentally freed Babesia rodhaini failed to infect normal or splenectomised rats after intraperitoneal injection but did so after intravenous injection. On the other hand, infection developed rapidly after either intraperitoneal or intravenous injection of Babesia rodhaini-infected erythrocytes into normal rats. Similar results were obtained by Bowman, Grant and Kermack (1960) with Plasmodium berghei infections in mice. From these results it could be suggested that an increase in the degree of haemolysis of infected erythrocytes would result in an increase in the number of freed parasites, which would, in turn, result in a loss of infectivity by the intraperitoneal route. All material to be used in infectivity tests in this study would be injected intraperitoneally.

Method. A pool of Babesia rodhaini-infected rat blood was obtained by bleeding passage rats at A in Fig. 1. The number of red cells

present and the proportion of these red cells infected were estimated and found to be 3.6 x $10^6/mm^3$ and 1-2% respectively. Samples of blood were then set aside for prefreeze percentage haemolysis estimations. The remainder of the pooled blood was then divided into four, each part being diluted 1:1 with one of:

(a) Phosphate buffered saline, ABP8.0

(b) Dimethylsulphoxide (DMSO) 20% made up in ABP8.0

(c) Glycerol 15% made up in ABP8.0

(d) Polyvinylpyrrolidone (PVP), 0.008M made up in ABP8.0.

Volumes of c. 1.5 ml of these diluted bloods were then dispensed into either glass ampoules or pp60 polythene tubing lengths. Ten samples were dispensed into each of the two types of container, half of which, after sealing, were then cooled to -79°C at a conventional slow-cooling rate of c. 1-2°/min., the other half being cooled to -196°C at rapid, uncontrolled cooling rates of 1850°/min. for material in pp60 and 136°/min. for material in ampoules.

After storage in solid carbon dioxide and liquid nitrogen for about one week, all samples were then individually thawed as rapidly as possible by agitation in a water bath at +37°C. Each individual thawed sample was then estimated for percentage haemolysis.

Results and Analysis. With so many different treatment groups existing in this and many of the subsequent experiments, persisting with full longhand titles of each treatment group is certain to prove repetitive and bulky and likely to prove confusing. It is proposed, therefore, at the beginning of this, and subsequent, Results and Analysis sections, to list proposed abbreviated treatment group labels and the treatments to which they refer. These labels will be the same as those used in

tables and statistical analyses. For computer programming reasons, the labels will always be six symbols long. The labels for this experiment are:

Label	Treatment
PREFRE	Prefreeze control group
GAMCON	Glycerol/Ampoule/Conventional rate
GPPCON	Glycerol/pp60/Conventional
GAMRAP	Glycerol/Ampoule/Rapid rate
GPPRAP	Glycerol/pp60/Rapid
PAMCON	PVP/Ampoule/Conventional
PPPCON	PVP/pp60/Conventional
PAMRAP	PVP/Ampoule/Rapid
PPPRAP	PVP/pp60/Rapid
DAMCON	DMSO/Ampoule/Conventional
DPPCON	DMSO/pp60/Conventional
DAMRAP	DMSO/Ampoule/Rapid
DPPRAP	DMSO/pp60/Rapid
OAMCON	Zero cryoprotectant (ABP8.0 only)/Ampoule/Conventional
OPPCON	Zero/pp60/Conventional
OAMRAP	Zero/Ampoule/Rapid
OPPRAP	Zero/pp60/Rapid

The results of this experiment are shown in Table 1 which is a copy of computer printout of a Duncan Multiple Range Test which was applied to them.

The Duncan Multiple Range Test compares the difference between two ordered means, which form a range of k' = 2 to k means, adjusted for sample size differences, if any, with a computed shortest significant range of length k'. If the adjusted difference is greater than the computed shortest significant range, then there is a significant difference between the two means, if it is smaller, then the two means are not separable.

It should be noted that this test is a 'conservative' test inasmuch as it requires a greater difference between means to result in significance than would a standard significance test of difference between means, such as the 't-test'. The Duncan Multiple Range Test is particularly useful, however, in indicating trends, this being exactly the type of analysis best suited to the semi-empirical approach necessarily used in this study.

A note of explanation as to what determines the constitution of a homogeneous subset of means would probably be useful at this stage (see Table 1). Basically, the difference between two means, adjusted for sample size difference, is compared with the product of the computed shortest significant range for a subset of that length at Alpha (P) = 0.05 and the within groups (error) root mean square in the overall analysis of variance. This is best explained by a worked example based on the results shown in Table 1.

For the subset (PREFRE, DPPRAP, DAMRAP),

the difference DAMRAP - PREFRE i.e. 7.20 - 3.00 = 4.20

This difference is adjusted for sample size difference using the formula:

$$\sqrt{\frac{2n_1 \cdot n_2}{n_1 + n_2}} \qquad \text{i.e.} \sqrt{\frac{2.4.5}{4 + 5}} = 2.108$$

The adjusted difference is $4.20 \times 2.108 = \underline{8.854}$

This value is then compared with the product of the computed shortest significant range for a subset of size 3 $= 2.9722$

and the within groups root mean square $= \sqrt{9.1152}$

i.e. $2.9722 \times 3.019 = \underline{8.973}$

Therefore, since the adjusted difference is smaller than the shortest significant range for a subset of three, DAMRAP cannot be separated from PREFRE.

It can be seen from Table 1 that the prefreeze haemolysis level was 3.00 + 0.82%. The best post-thaw results were obtained from two of the DMSO-protected groups, DPPRAP (6.00 + 0.71) and DAMRAP (7.20 + 1.79). The statistical analysis of these results shows that DPPRAP and DAMRAP are not separable from the prefreeze control group; i.e. they (PREFRE, DPPRAP and DAMRAP) form a homogeneous subset after a Duncan Multiple Range Test. There is, however, an 'overlap' of this subset with another, namely (DAMRAP, PPPCON and PPPRAP). This subset, in turn, overlaps with a subset of (PPPCON, PPPRAP and PAMCON).

Effectively this means statistically that DPPRAP is no worse than PREFRE but is better than all other groups ranked below it with the exception of DAMRAP. DAMRAP is no worse than PREFRE and DPPRAP but is better than all other groups below it with the exception of PPPCON and PPPRAP. PPPCON is no worse than DAMRAP but is better than all other groups below it with the exception of PPPRAP and PAMCON. PPPRAP and PAMCON are no worse than DAMRAP but are better than all other groups below them. PAMRAP effectively forms a subset on its own, i.e. it is worse than all groups above it, but better than all groups below it.

It has been stated above that the Duncan Multiple Range Test is useful in indicating trends. There is no doubt that, of the sixteen

different treatments tested, the most successful results were obtained with treatments DPPRAP and DAMRAP. Almost as good, however, were treatments PAMCON, PPPCON and PPPRAP. After these three, the next best result was with PAMRAP.

The indicated trends therefore are:

(a) The best results were obtained using the treatments DPPRAP and DAMRAP, both treatments involving DMSO as cryoprotectant and a rapid cooling rate.

(b) The best 'group' results were obtained with the PVP-protected material, all four treatment groups giving very encouraging results.

Also worthy of note are:

(c) OAMCON (unprotected blood cooled slowly in ampoules) gave a surprisingly good result, being better than all other treatments ranked below it.

(d) The treatments GAMCON and DAMCON, which, from the literature review, had been those most frequently applied with success to the storage of Babesia spp. gave the worst results of all in this experiment.

Discussion. For convenience, each of the four points of note above will be discussed in order.

(a) At the time that this experiment was carried out, the only evidence in the literature on the cryopreservation of Babesia spp. to support the finding that a rapid cooling rate combined with protection with DMSO could yield good results was contained in the papers by Dalgliesh (1971, 1972b).

Dalgliesh (1971) rapidly cooled Babesia bigemina-infected bovine blood, protected with various concentrations of DMSO, ranging from zero to 2M, by direct immersion in a cooling bath at -79°C. The

cooling rate he calculated as 60°/min. Dalgliesh showed that the greater the concentration of DMSO used, the greater the post-thaw infectivity. He also showed that the presence of DMSO reduced the degree of haemolysis from 82.2% to as low as 6.7%.

In his review article, Dalgliesh (1972b) gave some details of the work later to be published by Dalgliesh and Mellors (1974). This work involved the use of three widely differing cooling rates of 2.5, 265, and 2785°/min. in the cryopreservation of Babesia rodhaini-infected mouse blood. Without added cryoprotectant, infectivity was achieved using all three rates, but at the lowest rate it was very low. With added DMSO (1.5M) infectivity was increased at least one thousand-fold. The best results were obtained using the cooling rate of 265°/min. Dalgliesh argued that this probably indicated that the optimum cooling rate for Babesia rodhaini was in excess of 1°/min.

The results of Experiment 1, supported by Dalgliesh's evidence in the literature, suggest that further investigation into the DMSO-protected, rapid cooling method is justified, probably to the extent of a full-scale infectivity test. Such a test would also afford some information on the value of percentage haemolysis estimations as an indicator of the degree of success of any cryopreservation method.

(b) There has been, in the literature on the cryopreservation of Babesia spp., no evidence to suggest that PVP might be a useful cryo-protectant. There has been, however, a considerable amount of interest shown in this cryoprotectant by workers engaged in research on the cryopreservation of other cell types. In a study on the molecular weight of PVP and its cryoprotective properties with Pseudomonas, Ashwood-Smith and Warby (1971) showed that dialysed PVP gave good protection against freeze-thaw damage. The cooling

rate used was c. 25°/min. Undialysed PVP (with a pH as low as 3.8 - 4.0), not toxic to Pseudomonas per se, was toxic when the bacterium was frozen in its presence.

Liebo, Farrant, Mazur, Hanna and Smith (1970) showed that marrow stem cells from mice could be successfully cryopreserved using PVP as cryoprotectant. The survival rate was found to be dependant on cooling rate, there being distinct maxima at 7-14°/min. for 15% PVP (M. Wt. 40000) and 11°/min. for 7.5% PVP.

In the light of previously published work on the cryopreservation of erythrocytes of species other than the rat, the apparent success of PVP in this experiment is not surprising. For some years prior to this experiment, PVP has been actively investigated as a promising cryoprotectant for the cryopreservation of human red cells. Both glycerol and DMSO have been used successfully for cryopreservation of human erythrocytes, but the high concentrations required and the subsequent problems of pretransfusion removal of cryoprotectant, have diminished the popularity of both these cryoprotectants for human erythrocyte cryopreservation. Using a rapid cooling rate, post-thaw survivals of up to 97% have been reported with PVP, and 24-hour posttransfusion survivals of 80-92% have been observed. (Bloom, Witebsky, Rinfret, Doebbler and Cowley, 1965)

Polyvinylpyrrolidone, therefore, having given such good 'group' results in this experiment, and being in wide use for many cell types, merits further study as a possible cryoprotectant for Babesia spp. Aspects such as its molecular weight, its concentration and whether it is dialysed or undialysed should be considered as these have all proved to be of some importance in studies on other cell types. Estimation of success by percentage haemolysis alone would be justified for a preliminary study of this nature.

(c) The fact that OAMCON gave such good results is surprising. Not only is this treatment better than all of the treatments using glycerol as cryoprotectant and two of those using DMSO, it is also better than the other unprotected treatments, including those in which rapid cooling was used.

Since the report by Luyet (1949) on the success of rapid cooling of small volumes of unprotected bovine blood between coverslips, it has been universally accepted that better results will be achieved in the freezing of unprotected blood when it is cooled rapidly rather than slowly. There is no shortage of evidence in the literature to support this belief. This evidence is reviewed by Luyet and Rapatz (1970); however, some of the more significant studies merit some mention here.

Meryman and Kafig (1955) developed a technique in which very small droplets (0.45 to 0.9 mm. diameter) of unprotected human blood were sprayed on to the surface of liquid nitrogen where they floated momentarily before sinking. The frozen pellets were then thawed rapidly by sprinkling into plasma or physiological saline at+42°C. Haemolysis was reduced to 10-15% by this method.

Luyet and Gehenio (1955) showed that with unprotected bovine blood, haemolysis decreased with increase in cooling rate, up to about 7800°/min. At more rapid cooling rates, percentage haemolysis increased again.

All of these successful techniques have one important aspect in common. As Pegg (1966) pointed out, for rapid freezing, a rapid heat exchange is necessary, which in turn requires a favourable sample geometry, usually achieved by having a small sample size. Luyet (1949) used thin films of blood, Meryman and Kafig (1955) used

small droplets, and Luyet and Gehenio (1956) used thin-walled metal capillary tubes.

The point being stressed here is best summed up by Doebbler, Rowe and Rinfret (1970), who state "Both slow and rapid rates of cooling can yield frozen (human) red cells suitable for transfusion, but only when cryoprotective solutes are employed. Without one or more such materials being present, unmodified blood will, at best, yield about 80% unhaemolysed red cells with rapid freezing and almost none with slow freezing."

It is clear from examination of the cooling rates achieved, that rapid cooling in pp60 treatments also utilise small sample geometry with some success, but with larger volumes of material. The failure, therefore, of the OPPRAP treatment to give better results than the OAMCON treatment is, to say the least, unexpected and somewhat difficult to explain.

The various reports above, concerning the general decrease in haemolysis of unprotected bloods with increase in cooling rate deal only with bovine and human bloods. Rapatz and Luyet (1971) examined the degree of haemolysis in three species after rapid cooling. Unfortunately, no cooling rates were given - only the temperature of the cooling bath into which the samples were plunged. The material being cooled was placed, in 50 mm^3 volumes, in thin-walled capillary tubes similar to those used by Luyet and Gehenio (1955). Rapatz and Luyet showed that bath temperatures of -40°C, -50°C and -70°C gave minimum lysis (30%, 20% and 20%) with human, bovine and ovine bloods respectively, i.e. the optimum cooling rate for human blood was lower than that for bovine and ovine bloods, and so on. The differences, they suggested, result from differences in average

cell volumes (87, 48 and 30 μ^3 respectively); average water contents (72%, 62% and 59% respectively) and permeabilities (8.35, 3.8 and 1.9 seconds to haemolyse in 0.02M NaCl hypotonic salt solution respectively).

It is possible, though unlikely, that the cell volume, water content and permeability of rat erythrocytes are such that the rapid cooling rates used in this experiment (136°/min. for ampoules and 1850°/min. for pp60) are so far removed from the optimum that the slow cooling rates of 1.25°/min. for ampoules and 1.78°/min. for pp60 are better. Dalgliesh (1972b), however, stated that unprotected Babesia rodhaini-infected mouse blood gave very low infectivity when cooled slowly and greater infectivity when cooled at rates of 265 and 2785°/min. This part of Experiment 1 has been repeated and is reported below.

(d) The very poor results given by the treatments DAMCON and GAMCON are not surprising. Increasingly, in the literature, the 1°/min. or 'conventional' cooling rate has met with criticism. Mazur, Liebo, Farrant, Chu, Hanna and Smith (1970) showed that human red cells protected with 1.25M glycerol cooled to -196°C at different rates, and thawed rapidly, had vastly differing degrees of survival dependant on the cooling rates. Cells cooled at 2-3°/min. showed only 5-10% survival whereas cells cooled at c. 3000°/min. showed up to 70% survival. In their words "Although most cryobiologists freeze cells at around 1°/min., this is not necessarily the cooling rate that yields maximum survival."

Mazur (1970) is more direct. Referring to the Mazur et al (1970) findings quoted above, he states "Another significant feature of the curves is that they raise doubts about the general validity of the widely accepted practice of cooling cells at 1°/min., a practice that has become the sine qua non of cryobiology, partly for historical

reasons (Smith and her colleagues determined that this rate was optimal for bull spermatozoa: Smith and Polge, 1950a and b; Polge and Lovelock, 1952), partly because it yields high survival of some cells, and partly because it yields sufficient cell survival for the purposes of preserving cultures. Sometimes, however, it leads to failure." Similar sentiments have also been expressed by Meryman (1963, 1970) and Meryman and Weinmann (1968).

Most of the reports on the cryopreservation of parasitic protozoa have been based on the pioneering work of Reusse (1956) and Polge and Soltys (1957), who showed that a combination of glycerol as cryoprotectant and a slow cooling rate could give good results when used for the cryopreservation of a variety of parasitic protozoa. Cunningham, Lumsden and Webber (1963) and Lumsden, Robertson and McNeillage (1966) adapted the Reusse and Polge and Soltys methods for other protozoa and standardised on a cooling rate of c. 1°/min., as used by Reusse.

Frerichs and Holbrook (1970) showed that a slow cooling technique was successful for the glycerol-protected bloods of several species infected with various Babesia spp. They went on to describe a successful rapid cooling method, similar to that used for treatment GAMRAP, and noted that the bloods of different species treated in this way showed different degrees of lysis. It was also stressed that Babesia canis-infected dog blood and B. rodhaini-infected rat blood were more resistant than the blood of other species (gerbil, skunk, aardwolf and raccoon) when treated in this way.

The work described by Dalgliesh (1972b) is relevant in this respect. In his paper, Dalgliesh states that he and Mellors had found that DMSO-protected Babesia rodhaini-infected mouse blood was

more infective when cooled at 265°/min. than it was when cooled at 2.5°/min., indicating an optimum cooling rate which was probably in excess of 1°/min.

There is, therefore, an increasing amount of evidence to show that a 'conventional' 1°/min. cooling rate might not be as successful as rapid rates for the cryopreservation of red cells in general and, very probably, for babesia-infected red cells in particular. It is proposed, however, that such 'conventional' treatments be included in future experiments for comparative purposes.

Experiment 2. (1) Further studies on the degree of damage suffered by Babesia rodhaini-infected rat blood slowly or rapidly cooled in either glass ampoules or pp60 polythene tubing lengths using either (a) ABP8.0 alone, or (b) 0.004M PVP as cryoprotectant. Studies on the effect of (2) molecular weight and dialysis and (3) concentration on the cryoprotective properties of PVP.

Introduction. The good group results achieved in Experiment 1 for treatments using PVP as cryoprotectant, indicated that further investigation of this cryoprotectant could yield valuable results. The results obtained by Ashwood-Smith and Warby (1971) who used PVP as a cryoprotectant in the cryopreservation of Pseudomonas, indicate that aspects such as molecular weight and dialysis could have an effect on its cryoprotective properties. Liebo et al (1970) demonstrated a relationship between degree of success achieved and concentration of PVP used as cryoprotectant in the cryopreservation of mouse stem cells.

This experiment was designed to test the effect of the above factors on the cryoprotective capacity of PVP for Babesia rodhaini-infected rat blood.

Method. (1) A Babesia rodhaini-infected rat blood pool was taken from passage rats at B in Fig. 1. Parasitaemia of this blood pool was estimated and was found to be less than 1% infected red cells. Samples of the pooled blood were then set aside for predilution percentage haemolysis estimation. The blood was then divided into two, each half being then diluted 1:1 with

(a) ABP8.0

(b) 0.008M PVP, M.Wt. 24500, made up in ABP8.0.

Post-dilution percentage haemolysis estimations were then made. Volumes of c. 1.5 ml of these diluted bloods were then dispensed into ampoules or pp60 lengths, ten samples into each. After heat-sealing, five ampoules or pp60 lengths were then slowly cooled at a rate of c. 1-2°/min. to -79°C. The other five were rapidly cooled to -196°C by plunging them directly into liquid nitrogen.

After three days storage at either -79 or -196°C, each sample was then individually thawed as rapidly as possible by agitating it in a water bath at +37°C, and its percentage haemolysis was estimated.

(2) A Babesia rodhaini-infected rat blood pool was taken from passage rats at C in Fig. 1. This blood pool parasitaemia was c. 3% infected red cells. After predilution percentage haemolysis estimations, the blood pool was divided into six. The six parts were then diluted 1:1 with one of the following:

20% PVP, M.Wt. c. 11000, undialysed, pH6.4
" " " " , dialysed, pH7.3
" " " c. 24500, undialysed, pH5.3
" " " " , dialysed, pH7.15
" " " c. 44000, undialysed, pH4.5
" " " " , dialysed, pH6.9,

and post-dilution percentage haemolysis estimations were made.

One millilitre samples of each of these diluted bloods were then dispensed and cooled as in (1) above, the only difference being that, due to lack of material, only eight samples of each could be frozen. After two days storage, at -79°C or -196°C, the samples were individually thawed and estimated for percentage haemolysis, as described in (1) above.

(3) A Babesia rodhaini-infected blood pool was taken from passage rats at D in Fig. 1. The parasitaemia was less than 2% infected red cells. After predilution percentage haemolysis estimations, the pool was divided into six. These six parts were then diluted 1:1 with one of:

15% PVP, M.Wt. 24500, undialysed, pH5.3
10% " " " " pH5.4
5% " " " " pH5.4
15% " " ", dialysed, pH7.2
10% " " " " pH7.2
5% " " " " pH7.1

and post-dilution percentage haemolysis estimations were made. The dialysed PVP concentrations were calculated after dialysis had been completed.

Five 1 ml samples of each of these diluted bloods were then dispensed into, and rapidly cooled to -196°C in, pp60 polythene tubing lengths. After four days storage at -196°C, the samples were individually thawed as in (1) above and percentage haemolysis estimations were made.

Results and Analysis. (1) The results and statistical analysis of the first part of Experiment 2 are shown in Table 2 which is a copy of the results of a Duncan Multiple Range Test on these results. The computed shortest significant ranges have been omitted as unnecessary, thereby allowing the table to be presented in a more compact form. The labels used are:

Label	Treatment
PREFRO	Prefreeze undiluted control blood
PREFRP	Prefreeze, PVP-diluted control blood
OPPCON	Zero Cryoprotectant (ABP8.0 only)/pp60/ Conventional rate
OAMCON	Zero/Ampoule/Conventional
OPPRAP	Zero/pp60/Rapid rate
OAMRAP	Zero/Ampoule/Rapid
PPPCON	PVP/pp60/Conventional
PAMCON	PVP/Ampoule/Conventional
PPPRAP	PVP/pp60/Rapid
PAMRAP	PVP/Ampoule/Rapid

The prefreeze buffer-diluted blood showed 0.8 ± 0.21 percentage haemolysis, and the prefreeze PVP-diluted blood showed zero percentage haemolysis. As can be seen from the results of the Duncan Multiple Range Test, these two prefreeze treatment results cannot be statistically separated. It can be said, however, that the two prefreeze treatments can be separated from all other treatments ranked below them. The best post-thaw results are obtained from the treatments OPPCON and OAMCON. This subset overlaps with a subset of (OAMCON, OAMRAP and PPPRAP), i.e. OPPCON is better than all those treatments ranked below it with the exception of OAMCON, and OAMCON in turn is better than all treatments ranked below it with the exception of OAMRAP and PPPRAP. The best PVP-protected blood, PPPRAP, is not separable from two of the three treatments ranked above it, OAMCON and OAMRAP, but is separable from all treatments ranked below it. The two slowly cooled PVP-protected samples cannot be separated, but are both worse than PPPRAP and both better than PAMRAP. The poorest results came from two

rapidly cooled bloods PAMRAP and OPPRAP.

The following points are of particular interest when considering these results.

(a) Two slowly cooled, unprotected bloods, OPPCON and OAMCON, gave the best results.

(b) Rapidly cooled, unprotected blood (OPPRAP) gave the least successful results.

(c) The best PVP-protected treatment results came from the rapidly cooled treatment PPPRAP.

(2) The results and statistical analysis of the second part of Experiment 2 are shown in Table 3. The computed shortest significant ranges have been omitted. The labels used are:

Label	Treatment
PREDIL	Prefreeze, undiluted control blood
PRE11N	Prefreeze control blood diluted with undialysed 11000 M.Wt. PVP
PRE11D	Prefreeze control blood diluted with dialysed 11000 M.Wt. PVP
PRE24N	Prefreeze control blood diluted with undialysed 24500 M.Wt. PVP
PRE24D	Prefreeze control blood diluted with dialysed 24500 M.Wt. PVP
PRE44N	Prefreeze control blood diluted with undialysed 44000 M.Wt. PVP
PRE44D	Prefreeze control blood diluted with dialysed 44000 M.Wt. PVP
11NPPC	11000 PVP, Undialysed/pp60/Conventional rate
11NAMC	11000 PVP, Undialysed/Ampoule/Conventional
11NPPR	11000 PVP, Undialysed/pp60/Rapid rate
11NAMR	11000 PVP, Undialysed/Ampoule/Rapid

Label	Treatment
11DPPC	11000 PVP, Dialysed/pp60/Conventional
11DAMC	11000 PVP, Dialysed/Ampoule/Conventional
11DPPR	11000 PVP, Dialysed/pp60/Rapid
11DAMR	11000 PVP, Dialysed/Ampoule/Rapid
24NPPC	24500 PVP, Undialysed/pp60/Conventional
24NAMC	24500 PVP, Undialysed/Ampoule/Conventional
24NPPR	24500 PVP, Undialysed/pp60/Rapid
24NAMR	24500 PVP, Undialysed/Ampoule/Rapid
24DPPC	24500 PVP, Dialysed/pp60/Conventional
24DAMC	24500 PVP, Dialysed/Ampoule/Conventional
24DPPR	24500 PVP, Dialysed/pp60/Rapid
24DAMR	24500 PVP, Dialysed/Ampoule/Rapid
44NPPC	44000 PVP, Undialysed/pp60/Conventional
44NAMC	44000 PVP, Undialysed/Ampoule/Conventional
44NPPR	44000 PVP, Undialysed/pp60/Rapid
44NAMR	44000 PVP, Undialysed/Ampoule/Rapid
44DPPC	44000 PVP, Dialysed/pp60/Conventional
44DAMC	44000 PVP, Dialysed/Ampoule/Conventional
44DPPR	44000 PVP, Dialysed/pp60/Rapid
44DAMR	44000 PVP, Dialysed/Ampoule/Rapid

The results show that, the prefreeze predilution blood, all six PVP-diluted prefreeze bloods and the frozen and thawed bloods 24NPPR, 44NPPR and 24NAMR form one subset and therefore cannot be statistically separated. Of the frozen and thawed bloods the best result came from 24NPPR (2.95 $\pm$ 0.95%), which is separable from all treatments ranked below it with the exception of PRE44N, PRE24N, 44NPPR, 24NAMR and 11NPPR.

It should be noted that adjustment for sample size difference has meant the elimination of treatment 11NAMR from this best subset, although it was ranked above 11NPPR. The next best treatment, 44NPPR, was better than all treatments ranked below it with the exception of 11NAMR, 11NPPR, 44NPPC and 44NAMR. The next best group of treatments, 11NAMR, 11NPPR and 44NPPC could not be separated from each other and all were more successful than all treatments ranked below them, with the exception of 44NAMR, 24NPPC and 44NAMC.

Of the twelve undialysed PVP treatments, only three, 11NPPC, 24NAMC and 11NAMC showed any overlap with the noticeably less successful treatments using dialysed PVP. This overlap involved only the best four dialysed PVP-protected bloods, 44DAMC, 24DPPC, 24DPPR and 44DPPC.

The trends in this part of Experiment 2, therefore, are:

(a) Undialysed PVP's gave, in general, better results than dialysed PVP's, as estimated by percentage haemolysis.

(b) Within these undialysed PVP treatment groups, all three treatments involving rapid cooling in pp60 were found in the best post-thaw subset.

(c) Both undialysed, 24500 M.Wt., PVP-protected, rapidly cooled bloods were in the best subset.

(3) The results and statistical analysis of part 3 of Experiment 2 are shown in Table 4, a copy of the results of a Duncan Multiple Range Test. The computed shortest significant ranges have been omitted. The labels used are:

Label	Treatment
PREDIL	Prefreeze undiluted control blood
PREF5N	Prefreeze control blood diluted with 5% undialysed 24500 M.Wt. PVP
PREF5D	Prefreeze control blood diluted with 5% dialysed 24500 M.Wt. PVP
PRE10N	Prefreeze control blood diluted with 10% undialysed 24500 M.Wt. PVP
PRE10D	Prefreeze control blood diluted with 10% dialysed 24500 M.Wt. PVP
PRE15N	Prefreeze control blood diluted with 15% undialysed 24500 M.Wt. PVP
PRE15D	Prefreeze control blood diluted with 15% dialysed 24500 M.Wt. PVP
THAW5N	Frozen and thawed blood protected with 5% undialysed 24500 M.Wt. PVP
THAW5D	Frozen and thawed blood protected with 5% dialysed 24500 M.Wt. PVP
THA10N	Frozen and thawed blood protected with 10% undialysed 24500 M.Wt. PVP
THA10D	Frozen and thawed blood protected with 10% dialysed 24500 M.Wt. PVP
THA15N	Frozen and thawed blood protected with 15% undialysed 24500 M.Wt. PVP
THA15D	Frozen and thawed blood protected with 15% dialysed 24500 M.Wt. PVP

It can be seen that all prefreeze bloods, diluted or otherwise, showed percentage haemolysis levels which could not be statistically separated. The prefreeze blood, PREF5D, not separable from all other prefreeze bloods, was separable from all frozen and thawed bloods. All other prefreeze bloods were separable from all frozen and thawed bloods, with the exception of THA15N, the best frozen and thawed blood. All other frozen and thawed bloods, with the exception

of THA10N and THAW5D, are separable from each other.

Points of note in this part of Experiment 2 are:

(a) The best results were obtained with blood protected with 15% final concentration undialysed PVP.

(b) The second and third best results were obtained from bloods protected with dialysed PVP at 10 and 15% final concentration respectively.

(c) In general, lower concentrations of PVP were less successful, whether dialysed or not.

Discussion. (1) (a) (b) When comparing the results of part 1 of Experiment 2 with those of Experiment 1, some apparent inconsistencies can be noted. For instance, although in both experiments, the best results from an unprotected blood came from OAMCON, there was a considerable difference between the two experiments for mean percentage haemolysis results, i.e. 30.75 ± 2.63% in Experiment 1 and 18.10 ± 4.43% in Experiment 2. Statistical comparison of these results would be of little value inasmuch as one would be comparing results from two unrelated biological populations. The observation of these differences is useful in that it indicates that each experiment (using different bloods, at different stages of infection and with varying prefreeze percentage haemolysis levels) must be considered as a separate entity and comparisons only of a very general and conservative nature (i.e. of trends) should be made between experiments.

It was intended in part 1 of Experiment 2 to confirm or refute the finding in Experiment 1 that unprotected Babesia rodhaini-infected rat blood was less damaged by freezing and thawing when slowly cooled than when rapidly cooled. Looking at percentage haemolysis results alone, this finding would seem to have been confirmed.

The two best results were obtained from unprotected bloods slowly cooled in either ampoules or pp60, and clearly the worst result came from unprotected blood rapidly cooled at 1850°/min. in pp60.

In the discussion of Experiment 1, it was stressed that such findings were contrary to the usual pattern when freezing unprotected blood. There is, however, a possible explanation for these results.

When samples of thawed unprotected bloods were centrifuged to provide supernatant for percentage haemolysis estimation, it was noted that some of the centrifuged red cell pellets were atypical in appearance and were as pale in colour as pellets found when heavily damaged blood was centrifuged. Microscopical examination of these blood pellets (Figs. 17 and 18) showed that some of these bloods were in fact heavily damaged and there was, in addition, evidence of crystals of various shapes and sizes. Similar crystals had been observed before by the author in trypanosome-infected, glycerol-protected, slow cooled rat blood thawed from the low temperature storage banks at the Centre for Tropical Veterinary Medicine (Fig. 19).

It is suggested that these crystals in some way involve, or 'bind' the haemoglobin molecule. They are then pelleted and therefore effectively remove haemoglobin from the supernatant solution, resulting in erroneous, low, percentage haemolysis results. Post-thaw total haemoglobin readings were, on average, unchanged, suggesting that, if indeed the crystals did involve haemoglobin in some way, then that haemoglobin could still be estimated by the cyanmethaemoglobin method.

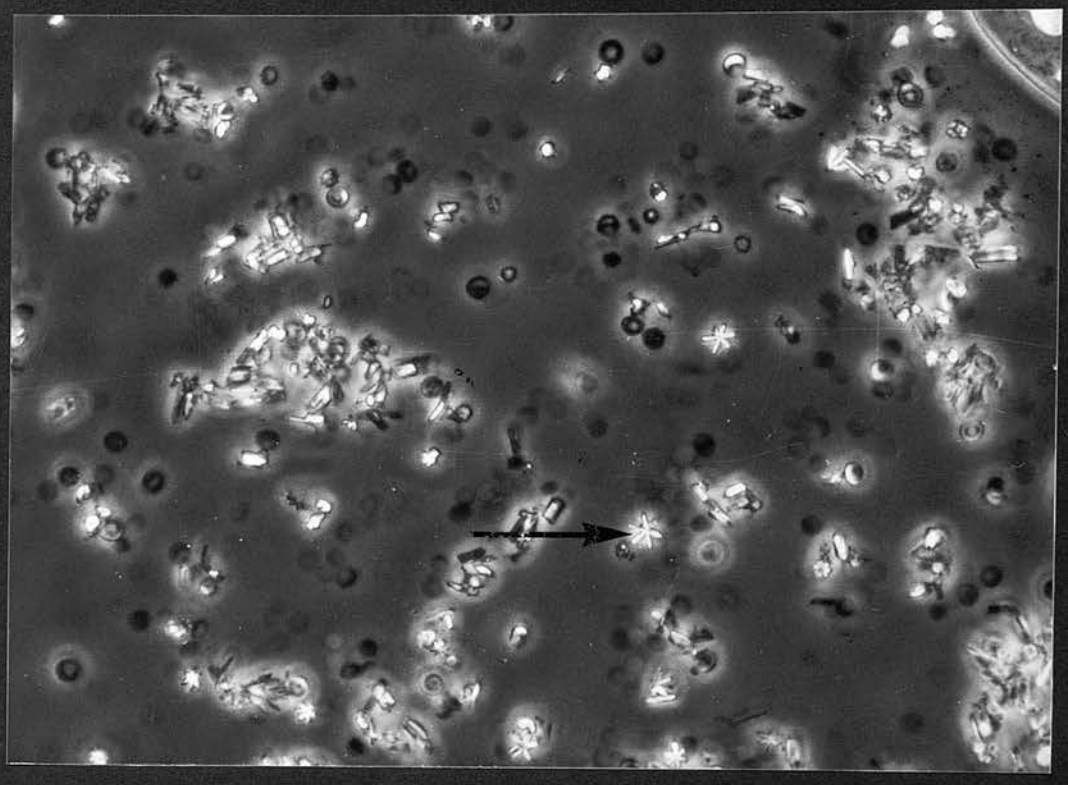
It is further suggested that degree of damage to the red cell population and/or the rate of cooling of these red cells determines

FIG. 17

FIGS. 17 & 18

Phase contrast photomicrographs showing the appearance, after thawing, of unprotected, Babesia rodhaini-infected rat blood which had been slowly cooled to -79°C. The extensive damage suffered is evident. Also evident are the crystals often found in frozen and thawed rat blood (Arrowed).

FIG. 18



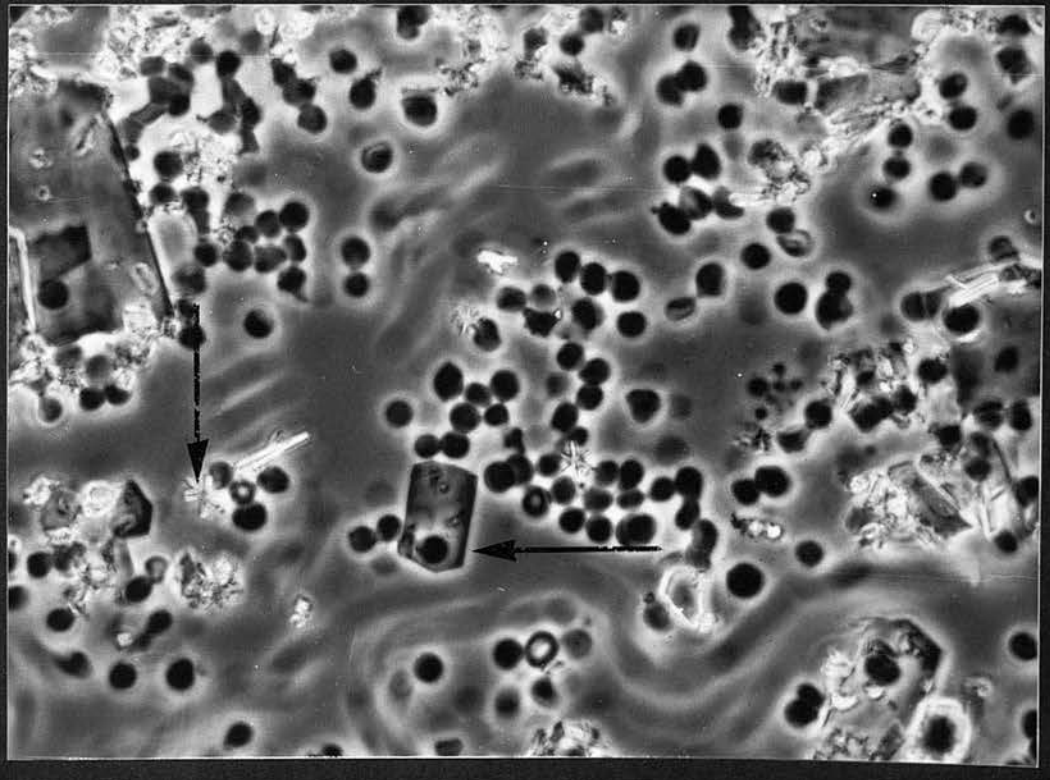
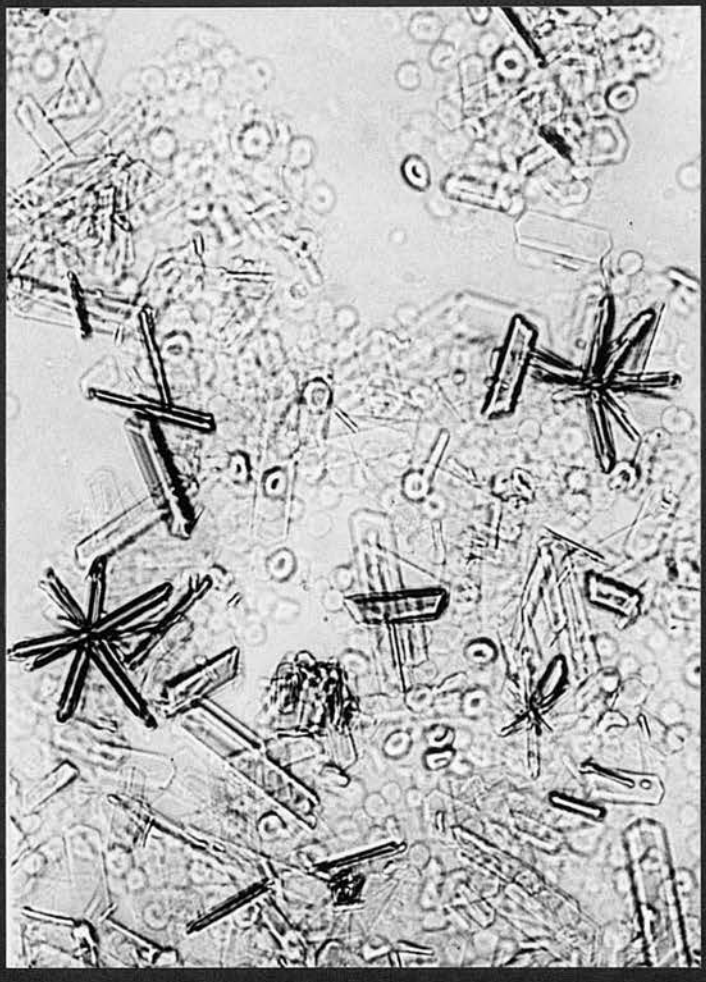


FIG. 19

The appearance after thawing of a stabilate of trypanosome-infected rat blood taken from the Centre for Tropical Veterinary Medicine storage bank. Clearly evident are the crystals often associated with frozen and thawed rat blood.



the extent to which these crystals are formed, i.e. the greater the degree of damage to the red cells (perhaps caused by the rate of cooling), the larger the amount of freed haemoglobin and the more likely that these crystals will be formed. None of the workers cited above in the discussion of Experiment 1, in connection with the usual success of rapid cooling of unprotected blood, working with human, bovine, ovine and mouse bloods, noted the existence of such crystals. Infected mouse blood, also commonly frozen and stored in the Centre for Tropical Veterinary Medicine low temperature storage banks, has not, in the experience of the author, shown the presence of such crystals when thawed.

To summarise, although percentage haemolysis results indicated that unprotected Babesia rodhaini-infected rat blood was less damaged when slowly cooled rather than rapidly cooled, microscopic examination of the thawed bloods indicated that this was probably not the case. It is suggested that crystals found in some thawed bloods remove haemoglobin molecules from solution, resulting in low percentage haemolysis results. Degree of damage to the red cells, which results in an increase in the level of free haemoglobin in solution, may be the trigger for the formation of these crystals. It could, therefore, be the case that a surprisingly low percentage haemolysis indicates a large scale removal of haemoglobin from solution. This would be in agreement with the microscopic evidence in this experiment. Whatever the exact explanation of these results, it is without doubt, evident that there is a considerable degree of damage to unprotected blood whether slowly or rapidly cooled, judged by microscopical and percentage haemolysis evidence respectively.

As a result of this experiment, the importance of checking

microscopically the morphological state of thawed bloods, particularly when percentage haemolysis alone is used as a measure of success, is evident. The use of percentage haemolysis alone must now be in doubt as a potential indicator of degree of success of particular freezing techniques for infected rat blood.

(1) (c) Whereas in Experiment 1 the two treatments PPPCON and PPPRAP were not separable as the best PVP-protected treatments, PPPRAP is clearly best in Experiment 2. This finding is not surprising. Although there has been some variation found in the optimum cooling rate for PVP-protected cells, most workers are agreed that cooling rates of more than 1°/min. are necessary for maximum survival.

Bricka and Bessis (1955), working with human erythrocytes, found that, when using PVP at concentrations of between 25 and 40%, maximum survival (c. 95%) occurred when the cooling rate was c. 60°/min. Doebbler, Buchheit and Rinfret (1961) showed that up to 95% recovery and more than 90% 24 hour posttransfusion viability could be obtained with rabbit blood protected with 7% PVP (M.Wt. 40000) when rapidly cooled to -196°C in aluminium containers. Pert et al (1965) showed that rapidly cooled human red cells protected with between 13 and 25% PVP, could give recoveries of up to 99% when rapidly cooled. Bloom et al (1965) reported that human red cells protected with PVP and rapidly cooled showed up to 97% post-thaw survivals. Rapatz and Luyet (1965) found that ox blood protected with 10% PVP gave results intermediate between the protective agents of the type glycerol and those of the type dextrose. At low cooling velocities, it exerted an injurious action, although less than glycerol or DMSO.

Liebo et al (1970) demonstrated that nucleated mouse marrow stem cells had optimal cooling rates of 7-14°/min. when protected with

15% PVP and 11°/min. with 7.5% PVP (M.Wt. 44000). They also showed that Chinese hamster tissue culture cells, unlike mouse marrow stem cells, had a broader optimum range of between 15 and 100°/min. when protected with 0.004M PVP and when rapidly thawed. In addition, they showed that rapidly cooled cells showed less survival when thawed at slow rates.

It would, therefore, in the light of these reports in the literature, be not surprising to find that Babesia rodhaini-infected rat erythrocytes would show good survival rates when protected with PVP, rapidly cooled and rapidly thawed, i.e. frozen by the PPPRAP method (pp60 allowing rapid thawing).

(2) (a) The finding that dialysis reduced the cryoprotective qualities of the PVP's used is also surprising. It is normal procedure when experimenting with PVP in biological systems to adjust the pH to a figure approaching neutrality. In a study with mouse marrow stem cell suspensions, Liebo et al (1970) adjusted the pH of their PVP-containing solutions to pH7.0 using 0.1N NaOH, not by dialysis. Farrant and Woolgar (1970a) in a study on the effect of cryoprotective additives on the degree of haemolysis suffered by human red cells exposed to hypertonic solutions of sodium chloride, adjusted the pH of their PVP-containing solutions to 6.8 using 1.0N NaOH. At the mole fractions of sodium chloride studied, a 15% PVP (40000 M.Wt.) solution adjusted in this way reduced the degree of haemolysis markedly.

Ashwood-Smith and Warby (1971) made an extensive study on the cryoprotective properties of PVP with Pseudomonas and with rabbit erythrocytes. This study involved such aspects as the molecular

weight of the PVP used, whether or not it was dialysed and the effect of this dialysis on the PVP itself. Many of their findings are relevant to this study. The PVP used had a nominal molecular weight of 40000. They found that unbuffered solutions of 5% or more of this PVP had a pH of between 3.8 and 4.0. They stated that there was nothing associated with the structure of PVP to account for this acidity. This characteristic had been noted, however, by several other workers, including the author. Ashwood-Smith and Warby suggested that traces of catalyst and other small molecular weight contaminants were responsible. When they dialysed the PVP against distilled water, at 4°C for 18 hours, the pH rose to 6.0. When this material was freeze dried, about 12% of the weight of the original sample had been lost. The dialysis membrane used had a 'cut-off' point at about 12000-13000 daltons, indicating that about 12% of the PVP they used probably had a molecular weight of 12-13000 or less. It was found that undialysed PVP was not toxic per se to rabbit erythrocytes or to Pseudomonas. With undialysed PVP, cryoprotective properties were unchanged with respect to rabbit erythrocytes, but with Pseudomonas dialysed PVP was "worse than useless", i.e. worse than the unprotected control by a factor of about seven. It was shown, in addition, that adjustment of undialysed PVP to the pH of dialysed PVP (i.e. 6.0) still led to a considerable (44% to 14%) drop in survival of Pseudomonas thus protected, after cryopreservation. This indicated that pH was not the only detrimental aspect of undialysed PVP, but that the presence of small molecules assumed a toxic effect during or after cryopreservation.

Thus, there is evidence for and against the practice of dialysing PVP in order to raise the pH. It may well be the case, when dealing

with some biological systems, that pH should simply be adjusted with NaOH, for example. This would seem to be indicated by the work on rabbit erythrocytes. It may also be the case, however, that some biological systems are more susceptible to pH and/or the presence of toxic small molecules, for example Pseudomonas. Further investigation into this matter is dealt with in part 3 of this experiment.

(b) The finding in part 2 of this experiment that the overall best cooling and thawing method for PVP-protected blood involved rapid cooling and storage in pp60 tubing confirms a similar finding in part 1 above. It would be reasonable, therefore to base subsequent investigations involving PVP as a cryoprotectant on this rapid cooling in pp60 method.

(c) From the results in part 2 of this experiment, PVP of a molecular weight of 24500 has been shown to be the best of the three tried. None of the workers cited above has used such small molecular weight PVP's as 11000 and 24500; most have used PVP's of the order of 40000 M.Wt. Steinbruch and Quentin (1958) showed that when the concentration of red cells in a 20% PVP-protected sample was reduced to one sixth, the degree of haemolysis suffered was reduced to one third. At one fourth concentration of cells, they found that an increase in the concentration of PVP from 20% to 40% reduced haemolysis to less than a half its original value. They also considered the effect of the agglomeration of the red cells by the PVP and the countereffect of agglomeration-limiting substances. These effects, however, they found to be of little significance. Agglomeration of rat erythrocytes was also noted by the author, in this experiment. This was particularly true when PVP of molecular weight 44000 was used.

There was virtually no agglomeration when PVP's of 11000 and 24500 M.Wt. were used. Since the best results were obtained with 24500 M.Wt. PVP, there is no justification in continuing with the use of 44000 M.Wt. PVP.

The 11000 M.Wt. PVP should probably also be eliminated from further study. In common with the results of Ashwood-Smith and Warby (1971), all of the PVP's used in this experiment probably lost a fraction of their molecules during dialysis. This would not only have had the effect of removing small molecules but also of reducing the effective concentration of the PVP's. According to the suppliers, the Visking tubing used for dialysis in this experiment, had a nominal 'cut-off' point at 20000 daltons. If this figure was correct, then a large proportion of the molecules of the 11000 M.Wt. PVP would be lost during dialysis, effectively reducing its concentration considerably. In all experiments involving dialysis of PVP, the volume of the concentrated PVP solution always increased during dialysis due to the movement of buffer into it. This also had the effect of reducing the effective concentration of PVP, i.e. an initial 20% PVP solution may be reduced to 15% during dialysis.

It was proposed that a further study be made of the effect of reduction in concentration on the cryoprotective properties of say, dialysed and undialysed 24500 M.Wt. PVP, cooled rapidly in pp60. Part 3 of Experiment 2 was designed with this proposal in mind.

(3) (a) As stated in the materials and methods section above, the PVP concentrations in this part of Experiment 2 were calculated after dialysis. This meant that if 25 ml. of 40% PVP increased in volume to 30 ml during dialysis, then it was assumed that the

concentration of that PVP was reduced to $\frac{25 \times 40}{30}$ = 33%. The post-dialysis concentrations obtained by calculation were 15, 10 and 5%. Undialysed PVP's were made up to the same concentrations. At that point, it was noticed that the genuine solutions of 15, 10 and 5% PVP were noticeably more viscous than their calculated counterparts. This was evidence to support the suggestion that, in addition to an increase in buffer volume reducing the PVP concentration, a loss of PVP molecules, probably of less than 20000 daltons molecular weight, further decreased the PVP concentration, in agreement with the findings of Ashwood-Smith and Warby (1971).

Such evidence would also help to explain why THA15N was the most successful treatment. Evidence has already been cited (Liebo et al, 1970; and Steinbruch and Quentin, 1958) that an increase in PVP concentration increases the survival of erythrocytes and nucleated mammallian cells. Undialysed PVP at a concentration of 15% was the most concentrated solution of PVP used and should, therefore, have resulted in the best survival.

(b) (c) The fact that dialysed 10% PVP gave better results than undialysed 10% PVP is less easy to explain. A simple net concentration relationship would not seem to apply here, or the undialysed 10% PVP would have been better. It is suggested that two factors may influence success:

(1) The effect of PVP concentration, i.e. as the PVP concentration increases, so the survival of the red cells increases.

(2) The effect of dialysis and loss of toxic small molecules (Ashwood-Smith and Warby, 1971), i.e. the presence of small toxic molecules decreases the survival of red cells. At sufficiently

high concentrations of PVP, e.g. 15%, factor (1) masks the effect of factor (2). At lower concentrations of PVP, e.g. 10%, the effect of factor (2) is more evident. This hypothesis could explain why undialysed 15% PVP gave the best results and also why dialysed 10% PVP gave better results than undialysed 10% PVP.

The fact that dialysed 10% PVP gave better results than dialysed 15% PVP is inconsistent with this hypothesis. If this hypothesis held true, one would expect that dialysed 15% PVP would be more successful than dialysed 10% PVP. It would be more concentrated and be free of toxic small molecules. One possible explanation for this apparent paradox, and in support of the original hypothesis, is that in the PVP solution which was dialysed to give a concentration of 15%, less small molecules were removed during dialysis and those toxic small molecules which remained exerted their deleterious effect to the full.

It is proposed that, with regard to the results obtained above, the use of PVP as a cryoprotectant in the cryopreservation of Babesia rodhaini-infected rat blood be further investigated, preferably to the extent of a full-scale infectivity titration. It is proposed that the method to be investigated should incorporate the following characteristics:

(a) the blood should be cooled rapidly,

(b) the blood should be stored in pp60, allowing of rapid thawing,

(c) PVP of molecular weight 24500 should be used,

(d) the PVP should be dialysed and be at a concentration of about 10% (0.004M).

Experiment 3. Estimation of the degree of damage in Babesia rodhaini-infected rat blood;

(a) protected with phosphate buffered saline (ABP8.0) alone and slowly cooled in ampoules to -79°C.

(b) protected with 7.5% glycerol and slowly cooled in ampoules to -79°C.

(c) protected with 0.004M (c. 10%) PVP, M.Wt. 24500, and rapidly cooled to -196°C in pp60 polythene tubing lengths.

(d) protected with 10% DMSO and rapidly cooled in pp60 polythene tubing lengths to -196°C.

Estimation by (1) percentage haemolysis measurement, and by

(2) full-scale infectivity titrations.

Introduction. The results of Experiments 1 and 2 showed that good survival of Babesia rodhaini-infected rat blood could be achieved if it was rapidly cooled to -196°C in the presence of either 0.004M PVP or 10% DMSO. It was suggested that these methods, shown to have been successful by the estimation of percentage haemolysis, should be investigated further to the extent of measuring the infectivity of the parasites thus preserved. This experiment was designed to do this. The method often reported previously to have been successful, i.e. slow cooling in the presence of 7.5% glycerol, shown in Experiment 1 not to have been as successful as would have been expected, is included for comparative purposes, as is a method involving unprotected slowly cooled blood which gave surprisingly good results in Experiments 1 and 2.

Method. A Babesia rodhaini-infected pool of rat blood was obtained, by the method described earlier, from passage rats at E in Fig. 1.

The number of red cells present and the proportion of these cells infected were estimated. The values were 1.80×10^6 red cells/mm^3 and 67% respectively. Predilution percentage haemolysis was also estimated and found to be c. 6%.

The blood pool was then divided into four, each part being diluted 1:1 with one of:

Phosphate buffered saline alone (ABP8.0)

15% glycerol made up in ABP8.0

0.008M (c. 20%) PVP, M.Wt. 24500, dialysed, made up in ABP8.0, pH7.1

20% DMSO made up in ABP8.0.

Samples were then set aside for prefreeze percentage haemolysis estimations and infectivity titrations. These samples were diluted in such a way as to give 10^7, 10^6, 10^5 and 10^4 infected red cells per 0.1 ml dose. The diluent used was phosphate buffered saline (ABP8.0) containing the same type and concentration of cryoprotectant as that present in the original cryoprotectant-diluted bloods. Doses of 0.1 ml of each of the four dilutions of the four cryoprotectant-diluted bloods were then injected intraperitoneally into each of ten mice, i.e. using 4 x 4 x 10 = 160 mice.

The mice were screened daily for parasites in Giemsa-stained blood smears for an arbitrary period of twenty days. Animals showing parasites within this twenty-day period were examined daily until they died or until a further arbitrary period of ten days had elapsed.

The remainders of the ABP8.0- and glycerol-protected bloods were then dispensed into glass ampoules and slowly cooled to -79°C. The remainders of the DMSO- and PVP-protected bloods were dispensed into pp60 polythene tubing lengths and rapidly cooled to -196°C. Due to the small amount of infected blood initially available, only

two samples of each of the four diluted bloods could be frozen.

After storage in solid carbon dioxide or liquid nitrogen for about six weeks, both samples of each of the four bloods were thawed rapidly by agitation in a water bath at+37°C. Post-thaw percentage haemolysis estimations were made. The remainders of the bloods were then diluted to give theoretical 10^7, 10^6, 10^5 and 10^4 infected red cells/0.1 ml dose (i.e. dilutions were made based on prefreeze, undamaged, red cell numbers and parasitaemia estimations). Mice were infected, and screened for the presence of parasites and day of death, using the same methods and time limits as those used in the prefreeze infectivity titrations.

Results and Analysis. (1) Percentage haemolysis estimations. The percentage haemolysis results obtained in this experiment are shown below. Since only two readings could be taken, no statistical analysis can usefully be employed.

	PREFREEZE	FROZEN AND THAWED
Predilution	6%	-
Buffer only	5%	76%, 65%
PVP	7%	25%, 34%
Glycerol	9%	63%, 70%
DMSO	5%	42%, 47%

Since the methods used were essentially the same as those labelled OAMCON, GAMCON, PPPRAP and DPPRAP of Experiment 1, these results may be compared with those obtained in Experiment 1. It can be seen

that the order of success in Experiment 3 was; PPPRAP, then DPPRAP, then GAMCON and then OAMCON, whereas in Experiment 1 (Table 1) it was DPPRAP, then PPPRAP, then OAMCON and then GAMCON. In Experiment 3, few if any crystals were observed in any of the thawed bloods.

Points of note in these results are:

(1) The excellent DPPRAP results obtained in Experiment 1 are not evident in Experiment 3.

(2) The OAMCON results are very poor compared to those in Experiments 1 and 2.

(2) Infectivity titrations. The results of the prefreeze and post-thaw infectivity titrations, with and without data trimming, are shown in Appendix Table 5. In each titration forty mice were infected, ten in each of four dilution groups. In all but one of the titrations, at least one, usually more than one, animal failed to show an infection in the first twenty days, and was ignored in subsequent statistical analyses. In the titrations of the unprotected, i.e. buffer only-diluted and PVP-protected post-thaw bloods, no animals became infected, even at the highest doses.

The first part of the table shows;

r = the computed correlation coefficient, and

n = the number of observations = the number of infected animals, or deaths.

In this case all r values are significant, at p = 0.05 for n-2 degrees of freedom, for prepatent period and survival time against log. dose. The second part of the table shows the computed regression lines for both prepatent period and survival time against log. dose where;

b = slope of the regression line and

m = its constant.

The numbers against 7 and 5 or 4 are the computed Y values for the X values of 7, 5 or 4 on the computed regression line having the equation:

$$Y = \bar{y} + b(X-\bar{x}) \qquad (1)$$

where $\bar{y}$ = mean of all observed y values (prepatent periods or survival times), in days

and $\bar{x}$ = mean of the logarithm$_{10}$ of all x values (log. doses)

Substituting m for $\bar{y}-b\bar{x}$ in (1) gives;

$$Y = bX + m \qquad (2)$$

1. Appendix Tables 6 to 8 are copies of computer printout of comparisons of linear regression between:

Table 6. BPPPWI = Regression of mean prepatent periods on log. dose in prefreeze blood diluted with buffer only, untrimmed data,

and

BPPPWO = Regression of mean prepatent periods on log. dose in prefreeze blood diluted with buffer only, trimmed data.

Table 7. BSTWI and BSTWO, as for Table 6, but for survival time regressions.

Table 8. BPPPWO and BSTWO

The labels used for the regressions in this experiment are:

Label	Regression
	(1) Prefreeze bloods
BPPPWI	Buffer only diluted/prepatent period/untrimmed data.
BPPPWO	Buffer only diluted/prepatent period/trimmed data.
BSTWI	Buffer only diluted/survival time/untrimmed.
BSTWO	Buffer only diluted/survival time/trimmed.
DPPPWI	DMSO-diluted/prepatent period/untrimmed.
DPPPWO	DMSO-diluted/prepatent period/trimmed.
DSTWI	DMSO-diluted/survival time/untrimmed.
DSTWO	DMSO-diluted/survival time/trimmed.
GPPPWI	Glycerol-diluted/prepatent period/untrimmed.
GPPPWO	Glycerol-diluted/prepatent period/trimmed.
GSTWI	Glycerol-diluted/survival time/untrimmed.
GSTWO	Glycerol-diluted/survival time/trimmed.
PPPPWI	PVP-diluted/prepatent period/untrimmed.
PPPPWO	PVP-diluted/prepatent period/trimmed.
PSTWI	PVP-diluted/survival time/untrimmed.
PSTWO	PVP-diluted/survival time/trimmed.
	(2) Post-thaw bloods
G2PPPWI	Glycerol-protected/prepatent period/untrimmed.
G2PPPWO	Glycerol-protected/prepatent period/trimmed.
G2STWI	Glycerol-protected/survival time/untrimmed.
G2STWO	Glycerol-protected/survival time/trimmed.
D2PPPWI	DMSO-protected/prepatent period/untrimmed.
D2PPPWO	DMSO-protected/prepatent period/trimmed.
D2STWI	DMSO-protected/prepatent period/untrimmed.
D2STWO	DMSO-protected/prepatent period/trimmed.

As with the Duncan Multiple Range Test, a note of explanation on the various symbols used and the mechanism of comparison would be appropriate and so is given below.

In Table 6, Groups 1 and 2 are the two regressions being compared, i.e. Group 1 is BPPPWI and Group 2 is BPPPWO. In each group, the X's represent the log. doses and the Y's represent the mean observed prepatent periods for the animals infected with these doses.

Immediately below each set of data, is an analysis of variance which tests the significance of the computed slope, which is, in effect, a comparison of the sloped line with a horizontal line. The total sum of squares of Y's (T SS in the Table) represents the total squared deviations. We can calculate the sum of the squared deviations from the sloped line (E SS in the Table) which is a measure of the inherent error of the data. The difference between the total sum of squared deviations (T SS) and the error squared deviations (E SS) represents the regression sum of squares (R SS in the Table) which results from using a line which is not horizontal. In practice, T SS and R SS are computed and E SS is obtained by subtraction. The total degrees of freedom in the analysis of variance is n-1 (in this case = 3). The regression itself takes only one (the comparison of the sloped line with the horizontal), therefore, by subtraction, the error degrees of freedom are n-1-1 = n-2 (in this case 2). The various degrees of freedom are not listed in the printout because of limitations of computer programming space. The mean squares, or variances (MS) of both regression and error, calculated from SS divided by degrees of freedom, are shown.
The variance ratio F for $\frac{\text{Regression MS}}{\text{Error MS}}$ is shown.

This is compared with tables of F values for $n_1 = 1$ (regression) and $n_2 = 2$ (error) degrees of freedom at $p = 0.05$. (See Appendix Table 93)

At the bottom of Table 6 is the comparison of linear regression analysis of variance. In this, the total sum of squares and total degrees of freedom have been omitted, again to save computer programming space. Each line is read horizontally, i.e. C R DF is the Combined Regression degrees of freedom, the SS on that line the Combined Regression sum of squares, the MS the Combined Regression mean square, or variance, and the F the $\frac{\text{Combined Regression}}{\text{Error}}$ variance ratio. The other lines begin with:

CON DF - Between Constants degrees of freedom

SL DF - Between Slopes degrees of freedom

ERR DF - Error degrees of freedom.

The computed variance ratios are compared with tabulated F values at $p = 0.05$ for the appropriate n_1 and n_2 degrees of freedom.

In Table 6, it can be seen from the BPPPWI and BPPPWO regression analyses, that their computed regression lines, with slopes of -1.9120 and -1.9000 respectively, are significant. (Tabulated F at $p = 0.05$ for 1:2 degrees of freedom = 18.51). Also significant is the resultant comparison of linear regression, having a combined regression F of 6290.11 against the tabulated 7.71 for 1:4 d.f. at $p = 0.05$. It can also be seen that the two regressions do not differ significantly either between slopes or between constants, with F values of 0.0727 and 3.1281 respectively. In this instance, therefore, the procedure of data trimming has made little difference to the regression obtained. Either regression, therefore, could be used in subsequent comparisons. As a rule, when this is the case, the trimmed data regression will be used.

Table 7 shows the comparison of the lines for BSTWI and BSTWO. Again, both regressions are significant, as is their comparison. The computed F values of 0.0010 and 1.0131 are not significant and therefore the lines do not differ in either slope or constant. The BSTWO regression will be used for subsequent comparisons with other prefreeze control and post-thaw survival time regressions.

Appendix Table 8 shows the results of a comparison between BPPPWO and BSTWO using individual items of data. In this table, the items of data have been omitted in order to make it more compact. The results of this comparison indicate:

(a) the comparison is significant,

(b) the two computed regressions do not differ significantly in slope,

(c) the two computed regressions differ significantly in constant.

It can be seen, when comparing the results obtained in the comparisons shown in Tables 6 and 7 with those shown in Table 8, that the results obtained, for example for slope, differ slightly depending on whether group means or individual items of data are used. Although in many cases these differences may be very small, it was considered that, in all future comparisons, individual items of data should be used.

2. All of the following trimmed and non-trimmed data regressions proved not to differ in either slope or constant:

DPPPWO and DPPPWI

DSTWO and DSTWI

D2PPPWO and D2PPPWI

D2STWO and D2STWI

GPPPWO and GPPPWI

GSTWO and GSTWI

G2PPPW0 and G2PPPWI

G2STW0 and G2STWI

PPPPW0 and PPPPWI

PSTW0 and PSTWI

In subsequent comparisons in this experiment, therefore, the use of trimmed data regressions is justified.

Appendix Tables 9-17 show the results of comparisons between:

9 BPPPW0 and GPPPW0

10 BSTW0 and GSTW0

11 GPPPW0 and GSTW0

12 BPPPW0 and DPPPW0

13 BSTW0 and DSTW0

14 DPPPW0 and DSTW0

15 BPPPW0 and PPPPW0

16 BSTW0 and PSTW0

17 PPPPW0 and PSTW0

The results of the comparisons in Tables 9-17 indicate:

(1) The prepatent period regressions of buffer only-diluted prefreeze blood and glycerol-diluted prefreeze blood differ significantly in both slope and constant.

(2) The survival time regressions of these bloods differ significantly in slope only.

(3) The prepatent period and survival time regressions of glycerol-diluted prefreeze blood differ significantly in constant but not in slope.

(4) The prepatent period regressions of buffer only-diluted prefreeze blood and DMSO-diluted prefreeze blood differ significantly in both slope and constant.

(5) The survival time regressions of these bloods differ significantly in constant and in slope.

(6) The prepatent period and survival time regressions of DMSO-diluted prefreeze blood differ significantly in constant and slope.

(7) The prepatent period regressions of buffer only-diluted prefreeze blood and PVP-diluted prefreeze blood differ significantly in both slope and constant.

(8) The survival time regressions of these bloods differ significantly also in both slope and regression.

(9) The prepatent period and survival time regressions of PVP-diluted prefreeze blood differ significantly in constant and in slope.

3. Appendix Tables 18-23 show the results of comparisons between:

18	BPPPW0	and	D2PPPW0
19	BSTW0	and	D2STW0
20	D2PPPW0	and	D2STW0
21	BPPPW0	and	G2PPPW0
22	BSTW0	and	G2STW0
23	G2PPPW0	and	G2STW0

These results indicate:

(1) The prepatent period regressions of buffer only-diluted prefreeze blood and DMSO-protected frozen and thawed blood are significantly different in both slope and constant.

(2) The survival time regressions of these bloods also differ significantly in both slope and constant.

(3) The prepatent period and survival time regressions of the DMSO-protected frozen and thawed blood differ significantly in constant but not in slope.

(4) The prepatent period regressions of buffer only-diluted prefreeze blood and glycerol-protected frozen and thawed blood differ

significantly in both slope and constant.

(5) The survival time regressions of these bloods also differ significantly in both slope and constant.

(6) The prepatent period and survival time regressions of glycerol-protected frozen and thawed blood differ significantly in both slope and constant.

4. Appendix Tables 24-31 show the results of comparisons between:

24 GPPPW0 and DPPPW0

25 GSTW0 and DSTW0

26 GPPPW0 and G2PPPW0

27 GSTW0 and G2STW0

28 DPPPW0 and D2PPPW0

29 DSTW0 and D2STW0

30 D2PPPW0 and G2PPPW0

31 D2STW0 and G2STW0

These results indicate:

(1) The prepatent period regressions of glycerol- and DMSO-diluted prefreeze bloods do not differ in either slope or constant.

(2) The survival time regressions of these bloods also do not differ in either slope or constant.

(3) The prepatent period regressions of glycerol-diluted prefreeze and glycerol-protected frozen and thawed bloods differ significantly in both slope and constant.

(4) The survival time regressions of these bloods also differ significantly in both slope and constant.

(5) The prepatent period regressions of DMSO-diluted prefreeze and DMSO-protected frozen and thawed bloods differ significantly in slope

but not in constant.

(6) The survival time regressions of these bloods also differ significantly in slope but not in constant.

(7) The prepatent period regressions of DMSO- and glycerol-protected frozen and thawed bloods differ significantly in constant and in slope.

(8) The survival time regressions of these bloods differ significantly in both slope and constant.

Discussion. Undoubtedly the most significant findings in Experiment 3 are the completely negative results given by both the buffer only- and PVP-protected bloods. The poor results with the buffer only-protected blood might perhaps have been predicted. The good percentage haemolysis results obtained for blood treated in this way in Experiment 1 were not confirmed in Experiment 2 and a possible explanation was suggested, i.e. that the buffer only-protected blood in Experiment 1 was indeed heavily damaged, but that the damage was not properly measurable due to the removal of haemoglobin from the supernatant fluid used for percentage haemolysis estimations. The percentage haemolysis results in Experiment 3 also indicated a high degree of damage to buffer only-protected frozen and thawed blood. The non-appearance of crystals in the frozen and thawed bloods in this experiment is difficult to explain. One explanation which might apply is that the blood, when taken from the donor rats, had already lost a considerable amount of haemoglobin *via* haemoglobinuria, due to the advanced state of the disease. Total haemoglobin estimations made in the course of percentage haemolysis estimations support this (of the order of 3.7 g. per cent in the undiluted prefreeze blood). The suggested explanation is that the haemoglobin was never at a high enough concentration to allow of the triggering of crystal formation.

The total failure of the buffer only-protected blood to infect any animals, even at the highest dose level, is of considerable interest, particularly when it is noted that percentage haemolysis estimations indicated that more than 25% of the red cells originally present survived cryopreservation. If the true survival of red cells, whether parasitised or not, was only one per cent, i.e. a loss of two log. units, the highest dose injected would then have contained at least 10^5 parasitised red cells. Such a dose, according to the prefreeze infectivity results, should infect mice which would show parasites on average on days 5-6, and be dead on days 10-12, which was obviously not the case. The following explanations for this disagreement between the percentage haemolysis and infectivity results obtained for the buffer only-protected frozen and thawed blood are suggested:

(1) The percentage haemolysis estimations were wrong.

(2) The infected red cells were selectively lysed and the parasites were rendered non-infective by the intraperitoneal route.

(3) The parasites within intact or semidamaged red cells were damaged or in some way rendered non-infective.

The first suggested explanation is unlikely to apply. The visual and microscopical appearance of the frozen and thawed blood indicated the likelihood that at least one per cent of the red cells was intact. This would be supported by the findings of other workers studying bovine, human, ovine and mouse bloods. (See discussion of Experiment 1.)

The second suggested explanation is quite possible, but extremely difficult to prove. The only report in the literature of successful freezing of babesia-infected unprotected rodent blood is that of Dalgliesh (1972) who showed that unprotected mouse blood was infective

when rapidly cooled but gave a very low infectivity when slowly cooled. This might be evidence against selective lysis. However, from the results of Experiment 2 in particular, it could be said that rat blood might be atypical in that it shows the formation of crystal structures and therefore could invalidate comparison with the results obtained from bloods of other species.

The same arguments apply to the third suggested explanation. It is quite possible that the increase in salt concentration within a red cell due to its dehydration as a result of 'solution effects' could seriously affect the parasite within, whilst the red cell itself survives. The Dalgliesh (1972a) results, however, would suggest that, in mouse blood at least, some of the parasites still manage to survive.

The results obtained with buffer-only protected blood are, however, of relatively little importance. This method was intended to be used as a 'control', to demonstrate what happens to unprotected, Babesia rodhaini-infected frozen and thawed rat blood.

Of considerably more importance, however, are the negative infectivity results obtained with the PVP-protected frozen and thawed blood. In view of the extremely encouraging percentage haemolysis results obtained with PVP-protected blood in Experiments 1 and 2 and in this experiment, these infectivity results are very disappointing, and difficult to explain.

It is unlikely that the percentage haemolysis occurring in Experiments 1-3 is falsely low. The workers cited in the discussion of Experiment 2: Bricka and Bessis (1958); Doebbler et al (1961); Pert et al (1965); Bloom et al (1965); and Rapatz and Luyet (1965), all showed that red cells (human, rabbit or bovine) survived well when cooled rapidly in the presence of PVP. The estimations of

success all involved the use of percentage haemolysis estimations, but Doebbler *et al* (1961) also measured posttransfusion viability and found it to be *c.* 90%.

There is little doubt, therefore, that good survival of PVP-protected frozen and thawed blood, as measured by percentage haemolysis estimation, is likely also to mean correspondingly good red cell viability. It appears also that the dialysed PVP used was not toxic to the red cells or the parasite. This can be seen from the excellent prefreeze infectivity titration results (Appendix Tables 15 and 16), in which the significant differences found between both the prepatent period and survival time slopes and constants were differences in which the PVP-diluted blood gave better results (i.e. lower values for slopes) than the buffer only-diluted 'control' blood.

It is difficult to explain, then, why PVP-protected rapidly cooled rat blood infected with *Babesia rodhaini* should prove non-infective after freezing and thawing. It is not because PVP is toxic to the red cell *per se* and probably not toxic during or after freezing and thawing, since about 70% of the red cells would appear to have survived, as measured by percentage haemolysis estimation. This does not mean that the PVP used was not toxic to the parasite within the red cell. It is, however, unlikely that any toxicity of PVP for the parasite is direct, since PVP, especially dialysed PVP with any small molecules removed, does not enter the red cell. Any investigation, however, into this aspect of the PVP/parasite relationship is beyond the scope of this study.

At this stage, before discussing the positive results which came from this experiment, a closer examination of the titration methods for *Babesia rodhaini* described by Overdulve and Antonisse (1970a and b)

and adapted for use in this study, would be valuable.

Since there is a linear relationship between prepatent period and survival time on one hand and log. dose on the other, all regressions computed and shown in Appendix Tables 6-31 being significant, in agreement with the findings of Overdulve and Antonisse, the length of the prepatent period or survival time is dependent upon the number of infective organisms injected into the recipient animal. If, as stated by Overdulve and Antonisse, when comparing the regressions of two identically diluted suspensions of infective organisms, one finds a significant difference in constant but not in slope, then, for prepatent period, this difference is a measure of the difference between the proportions of infective organisms in the initial suspensions, relative to the theoretical number calculated from prefreeze red cell count and parasitaemia estimations. For survival time, such differences can be used as measures of the differences between these proportions only if the virulence of individual parasites is not altered by treatment (e.g. freezing and thawing). According to Overdulve and Antonisse, changes in virulence of the individual parasites are reflected in the durations of the parasitaemias, i.e. the distance between the regression lines for prepatent period and survival time against log. dose.

Two lines which differ in constant, but not in slope, may be regarded, statistically, as parallel. This is an important point when considering the usefulness of this method of estimation of parasite survival, especially if, as Overdulve and Antonisse did, one estimates only survival time in order to minimise the amount of work involved. Overdulve and Antonisse stressed this point in their discussion. They suggested that to be able to generalize the use of such a technique to species other than Babesia rodhaini, one would

require experimental evidence that:

"(a) There should be a linear relationship between log. dose and survival time.

(b) The expectation of the number of infective organisms at death is independent of the number inoculated, i.e. that the lines for prepatent period and survival time are parallel or that the duration of parasitaemia for all infective doses is a constant.

It is evident that the actual cause of death is not merely the presence of a certain number of parasites in the host but a pathological condition initiated by the parasites, e.g. destruction of cells and organs by the parasites and disturbance of their normal physiological activity as in coccidiosis, production of toxins as in many bacterial infections, agglutination of erythrocytes, and obstruction of capillaries as in malaria. If conditions (a) and (b) are proved experimentally, then the finding of pathological changes does not make this technique useless. Especially when the effect of the pathological changes is rapid in comparison to the time necessary for the parasites to multiply to a sufficiently high level, the inaccuracy made by neglecting these actual causes of death will be small."

Overdulve and Antonisse went on to say that, in their experiments, it may have been that most of the parasites no longer multiplied in the latest phase of life in the host, so that their estimations of survival time were increased by a small constant term. In the comparison of two parallel lines, however, the existence of such a constant term does not make any difference so long as a third condition is fulfilled:

(c) That the virulence of the individual parasites is not altered by treatment.

If the 'pathological changes' term is not constant for all doses in the same titration, it may be expected that the error mean square based on prepatent period should be smaller than that based on survival time. Alterations of the virulence of the individual parasites must be reflected in differences in the duration of parasitaemia before and after treatment. Neither the former or the latter (with one exception) were found by Overdulve and Antonisse in their experiments. Therefore, they argued, they were able to use the terms 'virulence' and 'rate of multiplication' as synonyms.

In the present experiment, the effect of freezing and thawing on the parasite is estimated by the comparison for both slope and constant of the prepatent period and survival time regressions of the prefreeze cryoprotectant-diluted and frozen and thawed bloods. The prepatent period comparisons should be particularly useful insofar as they are less likely to be affected by a 'pathological changes' term. If the slopes are not different, then any difference in constant indicates a difference in the proportion of infective organisms in the injected suspension. If the slopes are different, this indicates a change in the rate of multiplication, or virulence, of the parasite, assuming that the conditions listed above are met.

Such indications are very useful, but can, at times, be difficult to interpret. It is possible, however, to derive from the results obtained, values for the rate of multiplication or doubling time and for the effective number of infective organisms injected.

It is possible to calculate the doubling time, $\mathcal{Q}$, of a parasite using the expression:

$$\mathcal{Q} = b. \log 2, \qquad (3)$$

where b is the numerical value of the slope of the regression line

of prepatent period or survival time against log. dose for that parasite. Obviously, the lower the value of b, the lower will be the value of Q.

According to Overdulve and Antonisse, the number of organisms present at death should, then, be

$$N = n.2^{t/Q} \qquad (4)$$

where n = the initial number of organisms injected,

t = the survival time,

and Q = the computed doubling time of the parasite = b. log2.

Knowing t and Q, and assuming that N does not change with treatment and that, for the sake of calculation, one infected prefreeze red cell represents one infective organism, it is possible to calculate for a treated blood, the effective number of infective organisms at injection from the expression:

$$n = \frac{N}{2^{t/Q}} \qquad (5)$$

this number can then be expressed as a percentage of the theoretical number of infective organisms injected.

In this experiment, the prefreeze results and those from the glycerol- and DMSO-protected frozen and thawed bloods confirm the finding by Overdulve and Antonisse for Babesia rodhaini-infected mouse blood, in that there also exists, for Babesia rodhaini-infected rat blood, a linear relationship between prepatent period and survival time on one hand and log. dose on the other. With reference to the points of note listed above, it can be seen that the addition of penetrating cryoprotectant, i.e. glycerol or DMSO, caused an increase in the numerical value for the slopes of the prepatent period and

survival time regressions, which is equivalent to an increase in doubling time, or a reduction in virulence (points 2: 1, 2, 4 and 5). The addition of the non-penetrating cryoprotectant, PVP, resulted in a shortening of the doubling time of the parasite and hence an increase in its virulence (points 2: 7 and 8).

It can also be seen from the prefreeze results, that in three of the four bloods, the regression lines for prepatent period and survival time differ in constant but not in slope (points 1: b and c; 2: 3 and 9). Thus for these bloods, the survival time regression line may be regarded as parallel to the prepatent period line. This relationship was also found, in their experiments, by Overdulve and Antonisse. Such a situation would be expected if, as pointed out by Overdulve and Antonisse, all infective organisms injected multiply continuously with a constant rate until the moment that the infection becomes apparent and/or death occurs.

Points 3: 1, 2, 4 and 5 indicate that, although infective parasites did survive the dilution, cooling, storage and thawing procedures, these parasites were reduced in number, relative to prefreeze numbers. This is evident from the significant increases in constant with at the same time significant decreases in the numerical value of slope. This applies to both the prepatent period and survival time regression comparisons of prefreeze diluted and frozen and thawed bloods diluted with either glycerol or DMSO.

It will be recalled that in the derivation of equation (2) above, $Y = bX+m$, 'm', the constant, had been substituted for the expression $\bar{y}-b\bar{x}$ in equation (1). It can therefore be seen that decreasing the numerical value of 'b' in $\bar{y}-b\bar{x}$ (b is algebraically always negative in such titrations) can only serve to decrease the value of the '$b\bar{x}$'

part of the expression, and therefore decrease the value of 'm'. Therefore if 'm' is shown to be significantly greater when, at the same time, 'b' is significantly smaller, the increase in 'm' must to a large extent be due to an increase in the value of '$\bar{y}$' (the mean of all observed prepatent period or survival time values). It is possible that an increase in the value of '$\bar{x}$', the mean of all doses administered to animals showing parasites or dying, as the case may be, might occur when individual items of data are used, especially if fewer animals become infected or die in the lower dilution groups, but this is also likely to result in a corresponding increase in '$\bar{y}$'.

It is in a situation such as this, where comparisons of linear regression indicate a loss of parasite numbers, that estimation of the effective number of infective organisms injected is particularly useful. In these experiments, such estimations can be based on both the prepatent period and survival time results obtained. Since, as we have seen above, the addition of penetrating cryoprotectant causes a reduction in virulence, then these estimations should be made comparing the numbers of infective organisms before dilution, after dilution and after treatment, i.e. freezing and thawing. The results of these estimations are shown below:

(a) From prefreeze, buffer only-diluted blood regressions:

ESTIMATE OF NUMBER OF ORGANISMS PRESENT AT:

(1) Onset of parasitaemia	(2) Death
4.4×10^7	2.25×10^{10}

(b) From treated blood regressions:

COMPUTED NUMBER OF INFECTIVE ORGANISMS INJECTED

(Expressed as a percentage of 10^7; computed post-thaw numbers shown in brackets)

	(1) From prepatent period regressions	(2) From survival time regressions
PREG	57%	88%
PRED	46%	240%
PREP	37%	10.9%
POSTG	0.016% (1625)	0.00052% (52)
POSTD	3.55% (355398)	0.35% (35175)

The results shown above are of considerable interest. From part (a) of the table, it can be seen that onset of parasitaemia occurred when there were a calculated 4.4×10^7 parasites present in the host. Death occurred when a calculated 2.25×10^{10} parasites were present, i.e. when parasite numbers had increased by a factor of 2250 from 10^7.

It can be seen from part (b) of the table that the computed numbers of infective organisms injected calculated from prepatent period data regressions are all credible, i.e. prefreeze dilution reduces the number of infective organisms from 100% down to 37 to 57%. The number of parasites surviving freezing are further reduced to 0.016% for glycerol-protected blood and 3.55% for DMSO-protected blood. These last estimations could also be expressed as percentages of the corresponding prefreeze diluted blood values. The percentage survivals of parasites in diluted bloods then become:

$0.016 \times \frac{100}{57} = 0.028\%$ for glycerol-protected blood and

$3.55 \times \frac{100}{46} = 7.72\%$ for DMSO-protected blood. For the latter, a

loss of only 93% of parasites during the processes of cryopreservation is very encouraging indeed.

When the computed numbers of infective organisms injected are calculated from survival time regressions, they are less credible. For instance, using this data, prefreeze dilution with DMSO apparently increased the number of infective organisms at injection by a factor of 2.4, which is obviously wrong. This paradox can perhaps be explained, at least in part, by the abnormally high value obtained for the slope of the PREDST regression, which, as can be seen from the comparison shown in Appendix Table 14, is significantly greater than the PREDPPP slope. As a matter of interest, it may be noted that, if the survival time regression slope had been the same as the prepatent period slope, a figure of 66% survival would have been obtained.

Another notable point from the survival time-based calculations, is the extremely low figure obtained for the survival of glycerol-protected frozen and thawed blood, i.e. the top dilution group was calculated to contain only the equivalent of 52 infective organisms. It is likely that this estimation is too low. It can be seen that, in this titration, this top dilution suspension was diluted twice further, i.e. by 100 times and still could infect animals. Since all ten doses in this latter dilution together would theoretically contain only five infective organisms, and since three animals became infected, this would mean that these three animals were being infected with only one, two or three infective organisms. The evidence, however, shown above does not support this suggestion. For example, with DMSO-protected frozen and thawed blood, which from survival time data had theoretically about one thousand times more infective organisms

at injection in the theoretical 10^7 dilution (35175), no animals infected with doses from the theoretical 10^4 dilution showed parasites. This suggests that a minimum number of infective organisms in the region of 10^2-10^3 is required for an infection to develop. This agrees with the results obtained for Babesia rodhaini-infected mouse blood by Overdulve and Antonisse, who found an 'end point' at about 10^3-10^4 parasites injected and with observations made by the author and his colleagues (Dolan, personal communication; Trees, personal communication) in the course of routine passage of Babesia rodhaini.

It might, of course, be possible that glycerol-protected, slowly cooled, frozen and thawed Babesia rodhaini becomes atypical in being able to produce a patent infection and death at very low levels of parasites injected. Investigation and discussion of this, is however, not within the scope of this study. It is, however, important to note that calculations of effective numbers of infective organisms injected may, in the light of these results, be regarded as, at best, approximations.

However, when considering the differences between the various prefreeze and frozen and thawed bloods, listed in points 3: 1-6 and 4: 1-8, the computed numbers of infective organisms injected shown above are useful. For instance, comparison of linear regression has shown that the prefreeze glycerol- and DMSO-diluted blood regressions are not separable in either slope or constant, when either prepatent period or survival time data are used (points 4: 1 and 2). The numerical values shown for the computed number of infective organisms injected are in agreement with the corresponding prepatent period comparison of linear regression results and act as a

useful addition to the survival time comparison results, i.e. in the prepatent period comparison (Appendix Table 24) the computed F values were very small (0.1641 and 0.0665 for constant and slope respectively) whereas in the survival time comparison (Appendix Table 25) they were quite large, though not significant (1.0802 and 2.5860). The important point to be illustrated here is that because a difference is not shown to be significant does not necessarily mean that it does not exist.

The dramatic loss of parasite numbers in glycerol-protected frozen and thawed blood indicated above is also indicated by the prepatent period and survival time comparison of linear regressions of these bloods (points 4: 3 and 4). Similarly, the results of the prefreeze and frozen and thawed DMSO-diluted blood regressions (points 4: 5 and 6) are quantified (in approximate terms).

The overall results of this experiment have been disappointing. In view of the encouraging results obtained from percentage haemolysis estimations in Experiments 1 and 2, better results might have been expected. It would appear, from the results of this experiment, that the best treatment of those so far studied for the cryopreservation of Babesia rodhaini-infected rat blood is a rapid cooling method, using 10% final concentration DMSO as cryoprotectant. It is suggested, however, that the results obtained in Experiment 1 be confirmed, possibly with the addition of infectivity studies to safeguard against possible misleading percentage haemolysis results.

Experiment 4. Estimation of the degree of damage in Babesia rodhaini-infected rat blood cooled either slowly to -79°C or rapidly to -196°C in either pp60 polythene tubing lengths or glass ampoules and protected with one of:

(a) Phosphate buffered saline alone (ABP8.0)

(b) 7.5% final concentration glycerol made up in ABP8.0

(c) 10% final concentration DMSO made up in ABP8.0

(d) 0.004M final concentration PVP, M.wt. 24500, dialysed, made up in ABP8.0

(e) 0.7M final concentration sucrose, made up in ABP8.0.

Damage is estimated by:

(a) Percentage haemolysis measurement.

(b) Single-dilution infectivity tests.

Introduction. Because of the rather poor results obtained in Experiment 3, it was decided that, before further investigation, the results obtained in Experiments 1 and 2, upon which the design of Experiment 3 was based, be confirmed. In order to obtain more information, it was suggested that, in addition to the percentage haemolysis estimations made in Experiments 1 and 2, infectivity tests should also be carried out on the prefreeze and frozen and thawed bloods. Since the numbers of groups being tested was large, single-dilution infectivity tests were used. To elucidate the problem of highly damaged bloods giving possibly misleading low percentage haemolysis results, it was also proposed that a blood be included treated in such a manner that considerable damage was inevitable. This would indicate whether or not percentage haemolysis estimations could be accepted when the amount of haemolysis was very high. It was known, from previous observations,

that rat blood, diluted 1:1 with 1.4M (47.88% w/v) sucrose was heavily damaged by freezing and thawing. It was suggested, therefore, that a portion of the blood pool be diluted in this manner.

Woolgar and Morris (1973) showed that human red cells cooled from +25°C to 0°C in the presence of a hypertonic solution of sucrose (40% w/v) were damaged upon resuspension in an isotonic solution. This damage caused by lowering the temperature of cells suspended in hypertonic solutions is called thermal shock. Lovelock (1954, 1955), who first described thermal shock haemolysis, used hypertonic sodium chloride solutions. Morris and Farrant (1973) were also able to produce thermal shock haemolysis using 35, 40 or 45% sucrose solutions, even without the added stress of resuspension in isotonic solutions. The thermal shock damage increased gradually with time of exposure at low temperatures, and was decreased by increasing the cooling rate.

Method. A Babesia rodhaini-infected rat blood pool was obtained by the methods described above, from rats at F in Fig. 1. The number of red cells present and the proportion of these cells parasitised were estimated and found to be $3.0 \times 10^6/mm^3$ and 38% respectively. Predilution percentage haemolysis was also estimated and was found to be negligible.

The blood pool was then divided into five, each part then being diluted 1:1 with one of the following:

(a) Phosphate buffered saline alone (ABP8.0)

(b) 15% glycerol made up in ABP8.0

(c) 20% DMSO made up in ABP8.0

(d) 0.008M, 24500 M.Wt., dialysed PVP made up in ABP8.0

(e) 1.4M sucrose made up in ABP8.0.

Samples of the diluted bloods were then set aside for prefreeze percentage haemolysis estimations (all of which were negligible except for the sucrose-diluted blood which showed 6%) and single-dilution infectivity tests. For the infectivity tests, each sample was diluted in such a manner as to give 10^7 infected red cells in each 0.1 ml dose, the diluents used being ABP8.0 containing the same type and concentration of cryoprotectant present in the original cryoprotectant-diluted blood. Doses of 0.1 ml of each of these dilutions were then injected intraperitoneally into each of ten mice. These mice were screened daily for the presence of parasites in the blood by the methods described above and deaths were recorded.

The remainders of the diluted bloods were then dispensed into either glass ampoules or pp60 polythene tubing lengths and slowly cooled to -79°C or rapidly cooled to -196°C. After storage at these temperatures for ten days, all the samples of the frozen bloods were thawed rapidly in a water bath at +37°C and individually estimated for percentage haemolysis. The pooled remainders of each blood were then diluted in the same manner, and to exactly the same extent, as the prefreeze diluted bloods to give a theoretical 10^7 parasitised red cells in each 0.1 ml dose. Single-dilution infectivity tests were made, using these dilutions, by the methods described above.

Results and Analysis. Percentage haemolysis results.

Appendix Table 32 shows the results of a Duncan Multiple Range Test made on the percentage haemolysis results obtained in this experiment. The labels used in this experiment are:

Label	Treatment
OPPCON	Zero cryoprotectant (ABP8.0 only)/pp60/Conventional rate.
OAMCON	Zero/Ampoule/Conventional
OPPRAP	Zero/pp60/Rapid rate
OAMRAP	Zero/Ampoule/Rapid
PPPCON	PVP/pp60/Conventional
PAMCON	PVP/Ampoule/Conventional
PPPRAP	PVP/pp60/Rapid
PAMRAP	PVP/Ampoule/Rapid
DPPCON	DMSO/pp60/Conventional
DAMCON	DMSO/Ampoule/Conventional
DPPRAP	DMSO/pp60/Rapid
DAMRAP	DMSO/Ampoule/Rapid
GPPCON	Glycerol/pp60/Conventional
GAMCON	Glycerol/Ampoule/Conventional
GPPRAP	Glycerol/pp60/Rapid
GAMRAP	Glycerol/Ampoule/Rapid
SPPCON	Sucrose/pp60/Conventional
SAMCON	Sucrose/Ampoule/Conventional
SPPRAP	Sucrose/pp60/Rapid
SAMRAP	Sucrose/Ampoule/Rapid

It can be seen from the results of this analysis, that treatment PPPRAP was statistically better than all other treatments, effectively forming a subset on its own. The next best results were obtained from the treatments in the subset (GPPRAP, DPPRAP and PAMRAP), all of which were statistically better than all those treatments ranked below

them. The two next best treatments, DAMRAP and GAMRAP are separable from each other and from all those treatments ranked below them.

It is also evident from these results that:

(a) The sucrose-'protected' bloods all, as expected, having been heavily damaged, did indeed give realistic percentage haemolysis results.

(b) All rapidly cooled bloods, with the exception of those which were diluted with sucrose, gave significantly better results than all slowly cooled bloods, regardless of the cryoprotectant used.

(c) Unprotected, slowly cooled blood gave surprisingly good results once again. The results of microscopic examination of the frozen and thawed bloods are shown below. This table has been included because, notably, no crystals were seen in blood treated by the OAMCON method. This observation rather confounds the theory of haemoglobin removal by crystal formation proposed earlier and will be discussed later.

Treatment	Mean % H	Crystals seen
OPPCON	86.8	0
OAMCON	65.4	0
OPPRAP	88.2	+
OAMRAP	87.7	+
PPPCON	77.2	++
PPPRAP	7.5	0
PAMCON	42.4	++
PAMRAP	17.8	0
DPPCON	55.2	0
DAMCON	67.8	0
DPPRAP	15.8	0
DAMRAP	23.0	0
GPPCON	77.4	0
GAMCON	69.2	0
GPPRAP	15.2	0
GAMRAP	32.8	0
SPPCON	90.2	0
SAMCON	93.2	0
SAMRAP	82.7	0
SPPRAP	86.2	0

0 - no crystals seen.
+ - few crystals seen.
++ - many crystals seen.

Single-dilution infectivity tests. Appendix Tables 33-36 show the results and analysis of the single-dilution infectivity tests for both prepatent period and survival time using both trimmed and untrimmed data. It can be seen from Table 33 that the three best prepatent period infectivity test results from frozen and thawed bloods using untrimmed data were obtained from the two DMSO-protected, rapidly cooled bloods DPPRAP and DAMRAP, together with the glycerol-protected, rapidly cooled blood GPPRAP. These three bloods are included in the best subset which also contains four of the prefreeze bloods PREFRO, PREFRP, PREFRD and PREFRG. From Table 34 it can be seen that, using trimmed prepatent period results, the best subset again contains DPPRAP and DAMRAP and contains only two of the prefreeze bloods, PREFRO and PREFRP. From Table 35, it can be seen that, using untrimmed survival time results, the best subset contains the frozen and thawed bloods DAMRAP, DPPRAP, GPPRAP and GAMRAP, with the prefreeze bloods PREFRP, PREFRD, PREFRO and PREFRG. In Table 36, which shows the analysis of the trimmed survival time results, the best subset contains the frozen and thawed blood DPPRAP and the prefreeze bloods PREFRO, PREFRG and PREFRD.

Returning to Table 33, it can be seen that the best subset is overlapped by the next best subset which contains the frozen and thawed bloods DPPRAP, DAMRAP, GPPRAP and GAMRAP. It can be seen therefore, that the four rapidly cooled glycerol- and DMSO-protected bloods give overall better results than the corresponding slowly cooled bloods.

The prepatent period trimmed data results (Table 34) show also that the four best frozen and thawed bloods were those protected by glycerol and DMSO and rapidly cooled in either pp60 or ampoules.

Similar results are obtained from the survival time untrimmed data (Table 35) and from the survival time trimmed data (Table 36).

It should also be noted that three of the sucrose-protected, three of the buffer only-protected and one of the PVP-protected bloods showed only one animal or none at all infected and, therefore, did not appear in the prepatent period untrimmed data test analysis (Table 33). Data trimming further removes those treatments in which only two animals were infected, i.e. the only remaining buffer only- and sucrose-protected bloods and one of the PVP-protected bloods, PPPCON. Of the six animals infected with the GPPCON blood, only two died and therefore this treatment was removed from the analysis of the survival time trimmed data results.

Discussion. Percentage haemolysis results.

From the point of view of least damage measured, the best results in this experiment, as judged by percentage haemolysis estimation, were given by blood frozen and thawed by treatment PPPRAP. The next best results were obtained from GPPRAP, DPPRAP and PAMRAP. These results agree quite closely with those obtained in Experiment 1 in-asmuch as PPPRAP and DPPRAP are ranked high and are statistically better than all but two of the slowly cooled bloods in Experiment 1 and all of the slowly cooled bloods in this experiment. It is interesting to note, however, that, in contrast to the results of Experiment 1, GPPRAP gave much better results, being statistically better than all bloods ranked below it, with the exception of DPPRAP and PAMRAP. In general, however, percentage haemolysis estimations in this experiment have confirmed those of Experiment 1 in that:

(a) Rapidly cooled, glycerol-, DMSO- or PVP-protected bloods are

less damaged by cryopreservation than their slowly cooled counterparts. (b) Rapidly cooled blood contained in pp60 polythene tubing is less damaged than that contained in glass ampoules.

This second point, as seen from the cooling and thawing rate data above, can be explained by the fact that material rapidly cooled in ampoules is cooled and thawed less rapidly than that in pp60. The survival of red cells has been shown to be related to the cooling rate (discussed above), as indeed has the survival of Babesia rodhaini-infected mouse blood (Dalgliehs, 1972b). Most workers are agreed that thawing as rapidly as possible is advisable when recovering rapidly cooled material from storage (Liebo et al, 1970, for marrow stem cells; Farrant and Morris, 1973, for red cells). Theoretically, this would be advisable, in order to limit the extent of intracellular ice crystal growth due to slow warming. Material stored in ampoules is thawed at an overall rate much below that in pp60. An increase in the amount of damage in blood stored in ampoules after rapid cooling would therefore be expected.

As predicted, the bloods suspended in 0.7M sucrose were heavily damaged by freezing and thawing, indeed, they exhibited some damage even before cooling (prefreeze percentage haemolysis being 6%). It was expected that, if haemoglobin was being removed from heavily damaged blood supernates, this might be shown in these sucrose-suspended bloods. Microscopic examination of these thawed bloods revealed no crystals. Unprotected, slowly cooled blood frozen in ampoules showed only 65.4% ± 7.57% haemolysis and at the same time, no crystals. In Experiments 1 and 2, the presence of crystals in thawed blood treated thus (OAMCON) was used to explain the unexpectedly low percentage haemolysis results obtained. This explanation

is not applicable in this instance and the conclusion that may be reached is that, against all the 'rules' of cryobiology, a slowly cooled, unprotected blood was less damaged than one which was rapidly cooled.

The author, however, familiar with the appearance of blood treated in this way, does not support this view. Macroscopically, the pellet is a very pale pink, usually indicative of severe damage. Microscopically, pellet material consists of little but amorphous debris. There is little or no evidence of the presence of intact erythrocytes, certainly not to the extent of 30-40% that the percentage haemolysis results would indicate. What has caused the apparent reduction in concentration of the supernatant haemoglobin is not clear.

It is clear, however, that the percentage haemolysis results in this experiment, in conjunction with the corresponding infectivity test results, indicate strongly that percentage haemolysis estimation is very unreliable:

(1) as an indicator of red cell damage

(2) as an indicator of probable success or failure of a given cryopreservation technique.

This second point is emphasised by the completely negative infectivity test results obtained from blood preserved by the PPPRAP method, which gave the best percentage haemolysis results.

The percentage haemolysis method for estimation of damage to infected blood is, nevertheless, not without its merits. In its favour could be argued that the rapidly cooled DPPRAP, GPPRAP, DAMRAP and GAMRAP which rank high in percentage haemolysis estimations also rank high in infectivity test results. It is obvious, however, that if percentage haemolysis estimations are to be of any use in estimating

damage to parasitised blood, they can be so only if used in conjunction with tests of infectivity.

Single dilution infectivity tests. The results of the infectivity tests in this experiment confirm those obtained in Experiment 3, inasmuch as the best results from frozen and thawed bloods were obtained from bloods rapidly cooled in either pp60 polythene tubing or in glass ampoules. From the four separate Duncan Multiple Range Tests on the prepatent period and survival time trimmed and untrimmed data, the indicated trends are that the best results are obtained from rapidly cooled DMSO-protected and rapidly cooled, glycerol-protected bloods, in that order.

As stated above, the Duncan Multiple Range Test is particularly useful in indicating trends. There can be little doubt that the indications in this experiment are that rapid cooling is preferable to slow cooling for the cryopreservation of Babesia rodhaini-infected rat blood. Such trends have already been indicated by percentage haemolysis results in this and earlier experiments and by the infectivity test results in Experiment 3. The indications are also, that when rapid cooling is employed, results are best when 10% DMSO is used as cryoprotectant and when the material is frozen and stored in pp60 polythene tubing.

It has been shown, therefore, that in Experiment 3 and in this experiment, DMSO-protected, rapidly cooled blood can give very good results. In this experiment, the results from blood treated in this way could not be statistically separated from prefreeze control bloods. Such a finding is indeed significant and should be confirmed if possible. If an overall trend has been indicated in the

results of the experiments so far, it must be that, within the limits of the experimental design, Babesia rodhaini-infected rat blood gives better results when rapidly cooled than when slowly cooled, in the presence of penetrating cryoprotectants. Although these results are not entirely at variance with results obtained by previous workers (Dalgliesh, 1972; Frerichs and Holbrook, 1970), it is interesting to note that in general, penetrating cryoprotectants such as glycerol and DMSO are reputedly most effective at slow cooling rates (Rapatz and Luyet, 1965). The evidence from this study so far supports the view, in common with the findings of Dalgliesh (1972), that although slow cooling in the presence of penetrating cryoprotectants would probably result in some success, it might, in many cases, be true to say that better results would be obtained if higher cooling rates were used.

Experiment 5. Estimation of the degree of damage in Babesia rodhaini-infected rat blood cooled either slowly to -79°C or rapidly to -196°C in either pp60 polythene tubing or in glass ampoules and protected by either:

(a) 7.5% final concentration glycerol, or

(b) 10% final concentration DMSO.

Estimation by:

(1) percentage haemolysis, and by

(2) single-dilution infectivity tests.

Introduction. This experiment was designed to confirm the two main indicated trends which have appeared in the first four experiments in this study, viz.

(a) That rapid cooling gives better results in the cryopreservation of Babesia rodhaini-infected rat blood than slow cooling.

(b) That the overall best method was a method in which DMSO-protected blood in pp60 polythene tubing was rapidly cooled.

Method. A pool of Babesia rodhaini-infected rat blood was obtained from rats at G in Fig. 1 by the methods described above. The number of red cells present and the proportion of these red cells infected were estimated and found to be $4.8 \times 10^6/mm^3$ and 10% respectively. Predilution percentage haemolysis was also estimated and was found to be 0.1%.

The blood pool was then divided into two, each part being diluted 1:1 with either:

(a) 15% glycerol made up in ABP8.0

(b) 20% DMSO made up in ABP8.0

Samples of these diluted bloods were set aside for prefreeze percentage haemolysis estimations and single-dilution infectivity tests. The diluted bloods were prepared for these infectivity tests by the method described above (Experiment 4). The mice were screened for the presence of parasites and the day of death was recorded as described above.

The remainders of the two diluted bloods were then dispensed into either pp60 polythene tubing lengths or glass ampoules (six of each) and cooled either slowly to -79°C or rapidly to -196°C. After one week storage at these temperatures, all three samples of each of the frozen bloods were rapidly thawed in a water bath at +37°C and then percentage haemolysis was estimated individually. The remainders of the frozen and thawed bloods were then prepared for single-dilution infectivity tests as described above. Animals were screened for the presence of parasites and the day of death was recorded as described above.

Results and Analysis. Percentage haemolysis.

The post-thaw percentage haemolysis results and analysis are shown in Appendix Table 37. The labels used in this experiment are:

Label	Treatment
PREFRD	Prefreeze DMSO-diluted control blood
PREFRG	Prefreeze glycerol-diluted control blood
DAMRAP	DMSO/Ampoule/Rapid rate
DAMCON	DMSO/Ampoule/Conventional rate
DPPRAP	DMSO/pp60/Rapid
DPPCON	DMSO/pp60/Conventional

Label	Treatment
GAMRAP	Glycerol/Ampoule/Rapid
GAMCON	Glycerol/Ampoule/Conventional
GPPRAP	Glycerol/pp60/Rapid
GPPCON	Glycerol/pp60/Conventional

The interpretation of these results is quite simple. It can be seen that the two treatments, DPPRAP and GPPRAP form the best subset, and are statistically separable from all other treatments ranked below them, including all four slowly cooled treatments. The second best subset consisted of the two other rapidly cooled bloods, GAMRAP and DAMRAP, also separable from all four of those slowly cooled.

Single-dilution infectivity tests. Appendix Tables 38-41 show the results and analyses of the single-dilution infectivity tests for both prepatent period and survival time trimmed and untrimmed data. The analyses were made by the Duncan Multiple Range Test.

The results of the test on the untrimmed prepatent period data is shown in Table 38. It can be seen that the results obtained with all treated bloods are clearly separable from those with the prefreeze bloods. The best results from treated bloods came from the rapidly cooled (DPPRAP, GPPRAP and DAMRAP) which form the best post-thaw subset. This subset, however, overlaps with a second subset of (GPPRAP, DAMRAP, GAMRAP, DPPCON and DAMCON) which in turn overlaps with the subset (DAMRAP, GAMRAP, DPPCON, DAMCON and GAMCON). This shows that, although the best results were undoubtedly obtained with the rapidly cooled bloods, these were not statistically separable from all the slowly cooled bloods, with the exception of those obtained with DPPRAP which was separable from all those ranked below it, with the exception of

GPPRAP and DAMRAP.

Appendix Table 39 shows the results of a test on the trimmed prepatent period data. In this test, the best post-thaw results were also obtained from (DPPRAP and DAMRAP) which are contained in the best post-thaw subset. This subset overlaps with a subset containing the treatments (DAMRAP and GPPRAP), which in turn overlaps with a subset containing (GPPRAP, GAMRAP and DPPCON). This analysis indicates that the best results were obtained from the rapidly cooled bloods. In this case, the results obtained with two of the rapidly cooled bloods, DPPRAP and DAMRAP, were statistically separable from those obtained with all of the slowly cooled bloods.

Appendix Table 40 shows the results of a test on the untrimmed survival time data. It can be seen that the rapidly cooled bloods are all found in the best post-thaw subset. However, three of the slowly cooled bloods are also in this subset. It can be seen, therefore, that although the rapidly cooled bloods gave, in general, better results inasmuch as they were ranked higher, they were not statistically separable from three of the slowly cooled bloods.

Appendix Table 41 shows the results of a test on the trimmed survival time data. It can be seen that all four rapidly cooled bloods are found in the same subset of (DPPRAP, DAMRAP, GPPRAP, DPPCON and GAMRAP), which, however, overlaps with a subset containing (DAMRAP, GPPRAP, DPPCON, GAMRAP, DAMCON and GPPCON). This subset in turn overlaps with a subset of (GPPRAP, DPPCON, GAMRAP, DAMCON, GPPCON and GAMCON). These results agree with those shown in Table 38 inasmuch as the only rapidly cooled bloods which are statistically separable from any slowly cooled bloods are DPPRAP and DAMRAP, although the rapidly cooled bloods are, in general, ranked above

the slowly cooled bloods.

Discussion. Percentage haemolysis results.

The percentage haemolysis results obtained in this experiment agree closely with those obtained in previous experiments. They indicate that rapidly cooled bloods were less damaged by cryopreservation than slowly cooled bloods and that rapidly cooled bloods in pp60 suffered less damage than rapidly cooled bloods in ampoules. Discussion of results such as these has been quite extensive in various sections above and need not be repeated here.

Single dilution infectivity test results. The results obtained in infectivity tests in this experiment illustrate the value of confirming 'significant' findings by repeating the experiment which afforded those findings. In experiments where the results obtained are not unexpected or controversial, or in which no significant differences between any of the methods or treatments tested are found, a second, repeat, experiment might not be justified. Where a significant result or difference is seen (e.g. rapid cooling being significantly better than slow cooling, or DPPRAP results not being separable from prefreeze control results) then confirmatory experiments should be carried out.

In view of the results obtained in Experiment 4, it might have been expected that rapidly cooled bloods would be statistically better than slowly cooled bloods and that rapidly cooled bloods protected with DMSO and in pp60 polythene tubing would be statistically inseparable from the prefreeze control. Neither situation applied in this experiment. The indications were that rapidly cooled bloods gave better results than slowly cooled bloods, but this difference

was not as statistically 'clear-cut' as in Experiment 4. The indication was also that treatment DPPRAP was the overall best treatment, but it was, in all tests, always statistically separable from the prefreeze control.

If we look again at the results obtained in Experiment 3, they might help to explain the results of this experiment. It was shown in Experiment 3 that a proportion of between 0.35% and 3.55% of infected red cells survived the processes of cryopreservation. It can be seen from Appendix Table 5 that the computed values (using trimmed data) for prepatent period and survival time for undiluted prefreeze, DMSO-diluted prefreeze and DMSO-protected frozen and thawed bloods, at the theoretical dose of 10^7, were:

Treatment	Prepatent period	Survival time
Undiluted	1.23	6.97
DMSO-diluted	2.13	7.55
Frozen and thawed	3.62	9.68

Had such results been analysed by a Duncan Multiple Range Test, the likelihood would have been that the frozen and thawed blood would have been shown to be statistically separable from the prefreeze controls. Such statistical differences are indicated for these bloods using the full titration results (Appendix Tables 18, 19, 28 and 29).

It might, therefore, be said that the results obtained in this experiment confirm those obtained in Experiment 3 in that blood frozen and thawed by the DPPRAP method can be statistically separated from the prefreeze control. It is suggested that the contrary results obtained in Experiment 4 may be more a consequence of the

prefreeze controls giving unexpectedly poor results than a consequence of the DMSO-protected, rapidly cooled frozen and thawed bloods giving unexpectedly good results. Examination of the raw data in Experiment 4 supports this suggestion.

Returning to Appendix Tables 33-36, it can be seen that, even when using trimmed prepatent period or survival time data, the standard deviations for the prefreeze bloods PREFRO and PREFRD are considerably larger than those for DPPRAP. This can be indicative of at least two, if not more, 'outliers' (Overdulve and Antonisse, 1970b). These outliers would usually be a result of animals becoming infected, or dying, late in the observation period and therefore would tend to increase the values of the mean for these bloods, thus bringing them closer to the DPPRAP mean. PREFRO had for prepatent period two outliers and for survival time three. PREFRD had two outliers for prepatent period and two for survival time. The frozen and thawed DPPRAP had no obvious outliers.

Another reason is also suggested to explain the differences between the results obtained in Experiments 3, 4 and 5. It had been noted in this and in previous experiments, that, after the preparation of material for infectivity tests, the diluent, ABP8.0 tended to become cloudy after a period of 1-2 hours at room temperature, particularly when DMSO had been added. It is thought that this cloudiness might be due to the precipitation of calcium or possibly magnesium phosphate. The DMSO-water reaction is exothermic (Huggins, 1963) and this would tend to exacerbate the effect of calcium or magnesium precipitation at temperatures around and above room temperature. Usually this precipitation was noticed long after the various dilutions had been made and the material had been cooled, or had begun its cooling, and

was therefore considered to be of little significance. It is now being suggested, however, that damage might occur as a result of these changes in the composition of the diluent at any time after dilution, after thawing and before injection, resulting in a possible loss of infectivity. If such damage occurred, its extent could be variable, affecting some bloods in some experiments more than others, depending upon, for instance, room temperature, mode of addition of DMSO, and length of time for preparation of dilutions for infectivity tests. It is doubtful if the use of ABP8.0 as a diluent can be regarded as acceptable. It could be, to a greater extent, an uncontrollable variable which should if possible be eliminated. Another more suitable diluent should be investigated.

Experiment 6. Estimation of the effect of diluent on the survival of Babesia rodhaini-infected rat blood protected with either:

(a) 7.5% final concentration glycerol

(b) 10% final concentration DMSO

and cooled either slowly to -79°C or rapidly to -196°C in either pp60 polythene tubing lengths or in glass ampoules. The diluents compared are ABP8.0 and modified Alsever's solution. Estimation of degree of damage is by:

(1) percentage haemolysis estimations

(2) single-dilution infectivity tests.

Introduction. This experiment was designed to test the suggestion that the diluent used in previous experiments, a phosphate buffered balanced salts solution, ABP8.0, could in itself be deleterious to Babesia rodhaini-infected rat erythrocytes before or after cryopreservation. Nowell (1968) showed that Alsever's solution was a suitable medium for the suspension and dilution of bloods infected with Babesia spp. It was decided therefore to compare the results achieved when Babesia rodhaini-infected rat blood was prepared for cryopreservation in one or other of these diluents. In case cooling rate, cryoprotectant type, or container also had some bearing on the possible damage caused by using ABP8.0, it was suggested that rapid and slow cooling, glycerol and DMSO, and pp60 and ampoules also be tested simultaneously.

Method. A pool of Babesia rodhaini-infected rat blood was obtained by the methods described previously from rats, at H in Fig. 1, infected with material from the stabilate TREU 1178. EDTA was used as anti-coagulant. The number of red cells present and the proportion of these red cells infected were estimated and found to be $4.3 \times 10^6/mm^3$

and c. 1% respectively. Predilution percentage haemolysis was also estimated and found to be c. 1-2%. The blood pool was then divided into four. These four parts were diluted 1:1 with one of:

(a) 15% glycerol made up in ABP8.0

(b) 15% glycerol made up in modified Alsever's solution (MAS)

(c) 20% DMSO made up in ABP8.0

(d) 20% DMSO made up in MAS.

Samples of these diluted bloods were then set aside for prefreeze percentage haemolysis estimations, all of which were shown to be c. 4-5%, and for prefreeze single-dilution infectivity tests. The infective doses for these infectivity tests were prepared by the methods described above, using the appropriate cryoprotectant-added diluent. The mice infected with these doses were screened for the presence of parasites and day of death was recorded, also by the methods described above.

The remainders of the four diluted bloods were then dispensed into either pp60 polythene tubing lengths or into glass ampoules and cooled either slowly to -79°C or rapidly to -196°C, four samples by each method. After 14 days storage at these temperatures, each sample was individually thawed as rapidly as possible in a water bath at +37°C, and individual percentage haemolysis estimations were made. From the remainders of the thawed samples, dilutions were made, as described above for prefreeze bloods, for post-thaw single-dilution infectivity tests. The mice used in these tests were screened for presence of parasites and day of death by the methods described above.

Results and Analysis. Percentage haemolysis.

Appendix Table 42 shows the results and analysis of the per-

centage haemolysis obtained in this experiment for the frozen and thawed bloods. The labels used in this experiment are:

Label	Treatment
	(i) Prefreeze bloods
PREALD	Prefreeze, DMSO-diluted blood/Diluent MAS
PREALG	Prefreeze, glycerol-diluted blood/Diluent MAS
PREABD	Prefreeze, DMSO-diluted blood/Diluent ABP8.0
PREABG	Prefreeze, glycerol-diluted blood/Diluent ABP8.0
	(ii) Frozen and thawed bloods
ALDPPC	DMSO/MAS/pp60/Conventional rate
ALDPPR	DMSO/MAS/pp60/Rapid rate
ALDAMC	DMSO/MAS/Ampoule/Conventional
ALDAMR	DMSO/MAS/Ampoule/Rapid
ALGPPC	Glycerol/MAS/pp60/Conventional
ALGPPR	Glycerol/MAS/pp60/Rapid
ALGAMC	Glycerol/MAS/Ampoule/Conventional
ALGAMR	Glycerol/MAS/Ampoule/Rapid
ABDPPC	DMSO/ABP8.0/pp60/Conventional
ABDPPR	DMSO/ABP8.0/pp60/Rapid
ABDAMC	DMSO/ABP8.0/Ampoule/Conventional
ABDAMR	DMSO/ABP8.0/Ampoule/Rapid
ABGPPC	Glycerol/ABP8.0/pp60/Conventional
ABGPPR	Glycerol/ABP8.0/pp60/Rapid
ABGAMC	Glycerol/ABP8.0/Ampoule/Conventional
ABGAMR	Glycerol/ABP8.0/Ampoule/Rapid

The reason why there are less than four observations for ABDAMR and ALGAMR is due to the breakage of some of the glass ampoules during thawing, probably due to faulty sealing.

It can be seen from these results that all of the rapidly cooled bloods gave statistically better results than all of the slowly cooled bloods, in agreement with the findings of previous experiments. It can be seen also that the best subset of these post-thaw results contains the treatments ABGPPR, ALDPPR, ABDPPR, ABDAMR and ALDAMR. This subset contains all four DMSO-protected, rapidly cooled bloods whether diluted with ABP8.0 or MAS. It also contains three of the four PPRAP treated bloods. This subset is overlapped by a subset containing (ABDAMR, ALDAMR, ABGAMR, ALGAMR and ALGPPR). It can be seen therefore, that three of the PPRAP-treated bloods are separable from all those ranked below them with the exception of ABDAMR and ALDAMR, suggesting, in support of previous findings above, that a PPRAP treatment is likely to give the best results, whether protected by glycerol or DMSO and whether diluted with ABP8.0 or MAS. There does not appear to be any evidence from these percentage haemolysis results that MAS is a better diluent than ABP8.0, or <u>vice versa</u>.

Single-dilution infectivity tests

Appendix Table 43 shows the results of a Duncan Multiple Range Test on the untrimmed prepatent period data obtained in infectivity tests in this experiment. It can be seen that the best subset, (PREALG, PREABD, ALDPPR, PREALD, ABDAMR, PREABG, ABDPPR and ALDAMR), contains all of the prefreeze diluted bloods plus all four DMSO-protected frozen and thawed bloods. This indicates, therefore, that the best post-thaw results are given by DMSO-protected, rapidly

cooled bloods, regardless of diluent and container used. This subset overlaps with two other subsets of (PREALD, ABDAMR, PREABG, ABDPPR, ALDAMR and ABGAMR) and (PREABG, ABDPPR, ALDAMR, ABGAMR and ABGPPR), which encompass two more rapidly cooled bloods ABGAMR and ABGPPR. Thus the trend towards better results being given by rapid cooling persists. There might also be evidence to suggest that ABP8.0 is a better overall diluent than MAS since all four ABP8.0-diluted, glycerol- and DMSO-protected, rapidly cooled bloods appear in the best three subsets, whereas only two of the corresponding MAS-protected bloods appear therein. Statistically, however, there is no evidence that any of the glycerol-protected, rapidly cooled bloods can be separated from any other, since they all appear in the same subset (ABGAMR, ABGPPR, ALGAMR, ALDPPC, ALGPPR, ALDAMC, ABDAMC and ALGAMC).

Table 44 shows the results of a test on trimmed prepatent period data. On this occasion, data trimming has made some impact on the subsequent analysis. In this test, the best subset contains three of the prefreeze bloods, PREALG, PREABD and PREABG. This subset overlaps with two others;

(PREABD, PREABG, PREALD and ALDPPR) and

(PREABG, PREALD, ALDPPR and ABDAMR).

It can be seen therefore that three of the prefreeze bloods PREALG, PREABD and PREABG are statistically separable from all bloods ranked below them with the exception of PREALD, ALDPPR and ABDAMR. The best post-thaw results came from the bloods in the two subsets PREALD, ALDPPR, ABDAMR and ABDPPR, and ALDPPR, ABDAMR, ABDPPR and ALDAMR. These results agree with those above inasmuch as the best post-thaw results are obtained from bloods protected with DMSO,

rapidly cooled, and diluted with either ABP8.0 or MAS. Also, as above, all glycerol-protected, rapidly cooled bloods are found in the subset, (ABGAMR, ABGPPR, ALDPPC, ALGPPR, ALGAMR, ALDAMC and ABDAMC), and therefore cannot be statistically separated.

Table 45 shows the results of a test on the untrimmed survival time data. In agreement with the prepatent period results above, the best post-thaw results were obtained from rapidly cooled bloods, including all four DMSO-protected bloods. The best subset contains these bloods plus all four prefreeze bloods (PREALG, ALDPPR, ABDAMR, PREABD, ABDPPR, PREABG, PREALD, ALDAMR and ABGPPR). There would again appear to be no evidence of either ABP8.0 or MAS being more satisfactory than the other as diluent. Also, all four glycerol-protected, rapidly cooled bloods were again found in the same subset and therefore could not be statistically separated.

Table 46 shows the results of a test on the trimmed survival time data. In this test, the best subset, (PREALG, ABDPPR, PREABD, ALDPPR, PREALD, PREABD and ABDAMR), contains all four prefreeze bloods and three of the four DMSO-protected, rapidly cooled bloods. This subset overlaps with a subset containing (PREABD, ALDPPR, PREALD, PREABG, ABDAMR and LADAMR). These results agree with those found above inasmuch as the best post-thaw results are obtained with DMSO-protected, rapidly cooled blood whether diluted with ABP8.0 or MAS. It can also be seen that, in agreement with previous findings, all four glycerol-protected, rapidly cooled bloods are found in one subset and therefore cannot be statistically separated.

The trends in this experiment are, therefore:

(1) Rapidly cooled bloods are more successfully cryopreserved than slowly cooled bloods.

(2) DMSO-protected, rapidly cooled bloods give better results than glycerol-protected, rapidly cooled bloods.

(3) The best overall results were obtained from rapidly cooled, DMSO-protected blood in pp60 polythene tubing.

(4) There is no evidence that either ABP8.0 or MAS is better than the other when used as a diluent in the cryopreservation of Babesia rodhaini-infected rat blood.

Discussion. Percentage haemolysis results.

The percentage haemolysis results obtained in this experiment are in agreement with those of previous experiments in that:

(1) Rapidly cooled bloods showed less damage than slowly cooled bloods,

(2) Rapidly cooled bloods in pp60 gave, in general, better results than rapidly cooled bloods in glass ampoules,

(3) Some evidence is shown to suggest that DMSO-protected, rapidly cooled bloods are less damaged than glycerol-protected, rapidly cooled bloods.

The only other point of note is that, with regard to the primary object of this experiment, no difference could be shown between the results obtained when using the two diluents ABP8.0 and MAS. Further discussion of these results is unnecessary.

Single-dilution infectivity tests.

The first point which should be made concerning the analysis of the infectivity test in this experiment is that, in common with the indicated trend in the percentage haemolysis results, neither ABP8.0 nor MAS can be shown to be better than the other as diluent in the cryopreservation of Babesia rodhaini-infected rat blood. This might suggest that the erratic results obtained in Experiments 3, 4 and 5

were probably not due to the damaging effect of ABP8.0. This does not, however, eliminate the possibility of an unpredictably variable damaging effect of ABP8.0. In some experiments, as in this one, ABP8.0 may prove to be harmless whereas in others, in response to any one of the factors mentioned above in the discussion of Experiment 5 it may prove to be very harmful. A study of the factors, if any, affecting the possible unpredictability of the affect of ABP8.0 would involve a considerable amount of work and would hardly be justifiable. A simpler device would be to discontinue the use of ABP8.0 in favour of a diluent such as MAS, which has been shown in this experiment to be no worse than ABP8.0 and which, because of its simpler chemical constitution, is unlikely to undergo potentially harmful changes. MAS also has the additional benefit of being in itself an anti-coagulant solution and therefore may be used from the outset of each experiment, i.e. at the withdrawal of blood from the donor rats.

The other indicated trends from the analysis of the infectivity test results in this experiment all confirm previous results. These are listed above in the results section and need not be repeated here. It is, however, interesting to note that, in all four tests in this experiment, in common with the results of Experiment 4, some of the frozen and thawed bloods could not be statistically separated from their prefreeze controls. With untrimmed data, this applied to both DMSO- and glycerol-protected rapidly cooled bloods, but using trimmed data, this applied only to DMSO-protected rapidly cooled bloods.

Examination of the data listed in Appendix Tables 43-46, shows that, as in Experiment 4, the standard deviations of the prefreeze bloods are quite large when compared with those of frozen and thawed bloods. Examination of the raw data confirmed this. The prefreeze

blood PREALD had three outliers in its prepatent period results and two in its survival time results. PREALG had one outlier in both prepatent period and survival time results, PREABD had one outlier in prepatent period results and two in survival time results, and PREABG had one in prepatent period results and two in survival time results. These outliers probably indicate why PREALD was ranked so low in all four analyses including those in which trimmed data were used.

With PREALG, the removal by data trimming of the one outlier in both prepatent period and survival time data reduces the mean and standard deviation in both cases, but to a greater degree with prepatent period data. This is sufficient with prepatent period results to make PREALG separable from all frozen and thawed bloods. This trimming is not sufficient, however, to separate PREALG from three of the four DMSO-protected rapidly cooled frozen and thawed bloods (ALDPPR, ABDAMR and ALDAMR) in the survival time trimmed data analysis.

Removal of the one outlier in the PREABD prepatent period results reduces its mean and standard deviation considerably. This is not sufficient, however, to separate it from ALDPPR. Removal, by data trimming, of one outlier in the PREABD survival time results does not reduce the mean sufficiently to make this blood separable from three of the four DMSO-protected, rapidly cooled frozen and thawed bloods, ALDPPR, ABDAMR and ALDAMR. The standard deviation of PREABD, it will be noted, remains relatively large at 2.15.

Removal, by data trimming, of the one outlier in the PREABG prepatent period results reduces its mean and standard deviation considerably. This is not sufficient, however, for this blood to be separated from ALDPPR and ABDAMR. Similarly, removal of one

of the two outliers in the survival time results is not sufficient to separate this blood from three of the DMSO-protected, rapidly cooled frozen and thawed bloods, ALDPPR, ABDAMR and ALDAMR.

It would appear, therefore, that the removal of one outlier in the best prefreeze blood PREALG has enabled it to be separable from all frozen and thawed bloods, but only when prepatent period results are considered. Removal of the single outlier in the survival time results given by this blood still does not bring about its separation from three frozen and thawed bloods. This indicates that the results with these three frozen and thawed bloods, ALDPPR, ABDAMR and ALDAMR, are genuinely good, and not the consequence of one or two outliers rendering the results given by their prefreeze counterparts unrealistically poor.

Experiment 7. Estimation of the effect of:

(a) Different stages of infection in the host

(b) Different levels of parasitaemia artificially produced by dilution with normal rat blood,

on the degree of damage suffered by Babesia rodhaini-infected rat blood protected with 10% DMSO and rapidly cooled to -196°C in pp60 polythene tubing. Estimation of degree of damage is by:

(1) percentage haemolysis estimation

(2) single-dilution infectivity tests.

Introduction. It had been suggested that the stage of infection in the host might influence the ability of Babesia rodhaini-infected rat blood to withstand the rigours of cryopreservation. For trypanosomes, certainly, it has been shown that best results are obtained when the parasite is frozen at the stage at which the parasitaemia is rising (Cunningham, van Hoeve and Lumsden, 1963). It has often been suggested, and in many cases required, that the parasites be stabilated at a point at which the parasitaemia is approaching its peak. This would be a simple device for ensuring that, even with very low proportions of infective organisms surviving cryopreservation, sufficient would survive to be useful for subsequent experimental procedures. Most of the workers mentioned above in the literature review, who have reported the successful cryopreservation of Babesia spp., made no comment about any stage of infection being better for cryopreservation than another. A few, Joyner (1971), Frerichs et al (1968), Barnett (1964), Mieth (1966), Pipano and Senft (1966) and Overdulve and Antonisse (1970b) indicated that they had either withdrawn the blood 'at peak parasitaemia' or 'during the acute stage of the disease'.

In consideration of the results of rapidly cooled bloods in

Experiments 4 and 5 only, it was suggested that actual level of parasitaemia might also have a bearing on the measured ability to withstand cryopreservation. If infected blood is frozen with low levels of parasitaemia such as that of 10% in Experiment 5, and if, as suggested, parasitised cells are selectively lysed by cryopreservation, then, under such circumstances, very few infected red cells would survive the cryopreservation processes, resulting in lengthened prepatent periods and survival times in infectivity tests. With high levels of parasitaemia, even if all cells lysed were parasitised, there would be little effect on the infectivity, relative to pre-freeze values.

It is unlikely that such a situation does apply, especially in the light of the results obtained in Experiments 3 and 6. In Experiment 3 poor results were obtained when the parasitaemia of the blood used was high (67%) and in Experiment 6 good results were obtained when the parasitaemia of the blood used was low (1%). It was, however, considered that an investigation into the effect of level of parasitaemia could profitably be carried out at the same time as an investigation into the effect of stage of infection. The following experiment was, therefore, designed.

Method. Babesia rodhaini-infected rat blood was obtained by the usual methods from rats at I in Fig. 1. Several rats were infected on the same day with doses of Babesia rodhaini designed to produce a range of parasitaemias at any one time. When these rats showed parasitaemias ranging from 5-50% infected red cells, they were bled out, using MAS as anticoagulant. Four of these bloods, with parasitaemias of 45, 16, 10 and 5% were diluted 1:1 with 20% DMSO made

up in MAS, dispensed into pp60 polythene tubing and rapidly cooled to -196°C. Prefreeze percentage haemolysis estimations and single-dilution infectivity tests were made. None of these four prefreeze bloods showed any haemolysis.

At the same time, similar levels of parasitaemia were artificially produced by dilution of a 50% parasitaemic blood with normal rat blood. These diluted bloods were also diluted 1:1 with 20% DMSO made up in MAS, dispensed into pp60 polythene tubing and rapidly cooled to -196°C. Samples had been previously taken for prefreeze percentage haemolysis estimation and single-dilution infectivity tests. No haemolysis was found in these. The dilutions were prepared for, and the animals screened in, infectivity tests by the methods described above. After storage at -196°C for ten days, the samples of all eight frozen bloods were thawed rapidly in a +37°C water bath and individual percentage haemolysis estimations were made. From the diluted pooled remainders of these samples, mice were infected for, and screened in, single-dilution infectivity tests by the methods described above.

Results and Analysis. Percentage haemolysis

The results and analysis of the post-thaw percentage haemolysis estimations in this experiment are shown in Appendix Table 47. The labels used in this experiment are:

Label	Treatment
	(i) Prefreeze bloods
NATU45	Prefreeze blood with a naturally occurring parasitaemia of 45%
NATU16	Prefreeze blood with a naturally occurring parasitaemia of 16%
NATU10	Prefreeze blood with a naturally occurring parasitaemia of 10%
NATUR5	Prefreeze blood with a naturally occurring parasitaemia of 5%
DILU45	Prefreeze blood with an artificially produced parasitaemia of 45%
DILU16	Prefreeze blood with an artificially produced parasitaemia of 16%
DILU10	Prefreeze blood with an artificially produced parasitaemia of 10%
DILUT5	Prefreeze blood with an artificially produced parasitaemia of 5%
	(ii) Frozen and thawed bloods
NA2T45	Frozen and thawed blood/Natural parasitaemia of 45%
NA2T16	Frozen and thawed blood/Natural parasitaemia of 16%
NA2T10	Frozen and thawed blood/Natural parasitaemia of 10%
NA2TU5	Frozen and thawed blood/Natural parasitaemia of 5%
DI2L45	Frozen and thawed blood/Artificial parasitaemia of 45%
DI2L16	Frozen and thawed blood/Artificial parasitaemia of 16%
DI2L10	Frozen and thawed blood/Artificial parasitaemia of 10%
DI2LU5	Frozen and thawed blood/Artificial parasitaemia of 5%

Six of the eight frozen and thawed bloods are in the best subset, (DI2LU5, DI2L10, NA2TU5, NA2T10, DI2L16 and NA2T16). This subset overlaps with a second subset of (DI2L10, DI2L16, NA2T16 and NA2T45). The effect of adjustment for sample size difference can be seen in the results of this test inasmuch as, although DI2L10 and NA2TU5 have the same arithmetic mean, NA2TU5 can be separated in the second subset from DI2L16, NA2U16 and NA2T45 whereas DI2L10 can not. The indications are, therefore, that when the parasitaemia is lower, whether naturally or by dilution, percentage haemolysis is lower. This difference is not statistically significant, except when the extremes are compared, i.e. DI2LU5 is statistically separable from NA2T45 and DI2L45.

Single-dilution infectivity tests

The results of the Duncan Multiple Range Test on the single-dilution infectivity test data in this experiment are shown in Appendix Tables 48-51. Table 48 shows the results of a test on the prepatent period untrimmed data. The interpretation of these results is simple. The frozen and thawed bloods were all separable from the prefreeze bloods but not separable from each other. Table 49 shows the results of a test on the prepatent period trimmed data. The results of this test are the same as those for the untrimmed data, in that all frozen and thawed bloods were separable from the prefreeze bloods but were not separable from each other.

In Table 50, the results of a test on the untrimmed survival time data are shown. It can be seen from these results that, in agreement with the prepatent period test results, the frozen and thawed bloods cannot be separated from each other. There is, however, a small degree of overlap between the prefreeze and the

frozen and thawed blood results, i.e. the two subsets (NATU10, NATU45 and NA2TU5) and (NATU45, NA2TU5, DI2LU5 and DI2L16) contain prefreeze and frozen and thawed bloods.

Table 51 shows the results of a test on the trimmed survival time data. Once again, it can be seen that all of the frozen and thawed blood results are contained in the same subset and, therefore, cannot be statistically separated.

Discussion. Percentage haemolysis results

As stated above, the only indication from the percentage haemolysis results obtained in this experiment is that there might possibly be a relationship between parasitaemia, whether natural or artificial, and degree of damage suffered as a result of cryopreservation. The blood DI2LU5 has been shown to be separable from (i.e. better than) NA2T45 and DI2L45. DI2L45 has also been shown to be statistically separable from (i.e. worse than) all other bloods ranked above it. There does not, however, appear to be any difference in the degree of damage suffered by bloods with either naturally occurring or artificially produced levels of parasitaemia, i.e. stage of infection in the host prior to cryopreservation does not appear, when judged by percentage haemolysis estimation, to have any effect on the ability of Babesia rodhaini-infected rat blood to withstand the rigours of cryopreservation.

Single-dilution infectivity tests

There is very little evidence from the results of the infectivity tests carried out in this experiment to indicate any relationship between parasitaemia, whether natural or artificial, and loss of infectivity due to cryopreservation. This is indicated in all four

statistical tests made on these results and is in contrast with the indications from the analysis of the percentage haemolysis results.

It is also interesting to note that, with trimmed data in particular, there is little or no overlap between prefreeze and frozen and thawed blood results. This is in contrast with the results of Experiments 4 and 6 and in agreement with the results of Experiments 3 and 5. All of the bloods frozen in this experiment were treated by the method DPPRAP, previously shown to be the best method studied. In Experiments 4 and 6, blood treated by this method gave very good results and in several statistical tests was shown not to be separable from prefreeze controls. In Experiments 3 and 5, however, although DPPRAP was still shown to be the best treatment tested, it was nevertheless shown to be statistically separable from prefreeze controls.

Why such differences in results between experiments should exist is not clear. Suggestions that type of diluent, stage of infection in the host, and level of parasitaemia might have some effect on the ability of Babesia rodhaini-infected rat blood to withstand the rigours of cryopreservation, have all proved to be unfounded. Considering the results of Experiments 3-7 as a whole, it is the opinion of the author that:

(1) there is little doubt that, of the methods tested for the cryopreservation of Babesia rodhaini-infected rat blood, the treatment DPPRAP has been shown to be most successful,

(2) the results of Experiments 3 and 7 in particular indicate that although DPPRAP-treated blood is the most successful, it is nevertheless separable from, or less successful than, prefreeze controls. The contrary results shown in Experiment 4 may possibly be explained

by insufficient data trimming of the prefreeze results. The same explanation cannot be used to explain similar contrary results obtained in Experiment 6.

It is suggested that this matter be given a final investigation in a subsequent experiment. This experiment, it is suggested, should take the form of a comparison, by full scale infectivity titration methods, of the most successful treatments studied, DPPRAP and DAMRAP, and their prefreeze controls. Such full-scale infectivity titrations are probably the most sensitive indicators of degree of success, and should, therefore, confirm the opinions of the author that, although it is the best treatment of those tested, DPPRAP is not successful to the extent that it cannot be statistically separated from prefreeze controls.

Experiment 8. Estimation of the degree of damage in DMSO-protected Babesia rodhaini-infected rat blood:

(i) slowly cooled to -79°C and stored at (a) -79°C or (b) -196°C,

(ii) rapidly cooled to, and stored at, -196°C, in either pp60 polythene tubing or in glass ampoules.

Estimation of degree of damage is by:

(1) percentage haemolysis estimation

(2) single-dilution infectivity tests.

Introduction. Evidence from several of the experiments described above indicated that DMSO-protected Babesia rodhaini-infected rat blood was less damaged by cryopreservation when rapidly cooled rather than slowly cooled. It has been suggested that this difference might not be due solely to the difference in cooling and thawing rates, but might also be due to some extent to the difference in final storage temperature. (Slowly cooled material was stored at -79°C and rapidly cooled material was stored at -196°C.) This experiment was designed to investigate if any differences existed between slowly cooled, DMSO-protected bloods stored at these temperatures.

Method. A pool of Babesia rodhaini-infected rat blood was obtained by the methods described above from rats at J in Fig. 1. These rats had previously been infected with the stabilate TREU 1178. MAS was used as both anticoagulant solution and diluent. The number of red cells present, and the proportion of these red cells infected were estimated and found to be $4 \times 10^6/mm^3$ and 4%, respectively. Predilution percentage haemolysis was estimated and was found to be c. 2%. A sample of this blood was set aside for a predilution

single-dilution infectivity test. The remainder of the pooled blood was then diluted 1:1 with 20% DMSO made up in MAS. Samples of this diluted blood were set aside for prefreeze percentage haemolysis estimation (c. 4%) and a single dilution infectivity test. The remainder of the diluted blood was dispensed into either pp60 polythene tubing or into glass ampoules. Eight of each of these samples were then slowly cooled to -79°C. When they had equilibrated at -79°C, after about two hours, four of each of the pp60 lengths and ampoules were transferred directly to -196°C for storage. Another four of each of the samples in pp60 and in ampoules were rapidly cooled to -196°C.

After storage at -196°C or -79°C for one week, each sample was individually thawed in a +37°C water bath and examined for percentage haemolysis. From the pooled remainders of each of the six different frozen and thawed bloods, samples were taken for post-thaw single-dilution infectivity tests. Material was prepared for, and animals were screened in, these tests by the methods described above.

Results and Analysis. Percentage haemolysis estimations

Appendix Table 52 shows the results and analysis of the post-thaw percentage haemolysis estimations. The labels used in this experiment are:

Label	Treatment
PREFRO	Undiluted prefreeze control blood
PREFRD	DMSO-diluted prefreeze control blood
PP6-79	Slowly cooled/pp60/Stored at -79°C
PP-196	Slowly cooled/pp60/Stored at -196°C
AMP-79	Slowly cooled/Ampoule/Stored at -79°C
AM-196	Slowly cooled/Ampoule/Stored at -196°C
DPPRAP	Rapidly cooled/pp60/Stored at -196°C
DAMRAP	Rapidly cooled/Ampoule/Stored at -196°C

It can be seen that the two rapidly cooled bloods, DPPRAP and DAMRAP, are statistically separable from each other and from all other frozen and thawed bloods. The best slowly cooled blood result was obtained from PP6-79 which was separable from all those bloods ranked below it. The next best results were obtained from the bloods which formed the subset (AMP-79 and AM-196) which can be statistically separated from the blood giving the worst result, PP-196. The indications from these results are, therefore, that storage of slowly cooled blood at -79°C results in less damage than storage at -196°C. Evidence, however, of crystal formation in all four slowly cooled bloods casts some doubt on the validity of these results.

Single-dilution infectivity tests

Appendix Tables 53-56 show the results of Duncan Multiple Range Tests on the trimmed and untrimmed prepatent period and survival time data. The shortest significant ranges have been omitted.

Table 53 shows the results of a test on the untrimmed prepatent

period data. The two prefreeze bloods, not separable from each other, are separable from all of the frozen and thawed bloods. The two rapidly cooled bloods, not separable from each other, are separable from all four slowly cooled bloods, which are ranked below them. One of the slowly cooled bloods, AM-196, is separable from all other slowly cooled bloods. The other slowly cooled bloods form a single subset and therefore cannot be statistically separated. The analysis of trimmed prepatent period results is shown in Table 54. The results of this analysis are exactly the same as those for the untrimmed prepatent period data.

Table 55 shows the results of a test on the untrimmed survival time data. In agreement with the prepatent period test results, the two prefreeze bloods, not separable from each other, are separable from all of the frozen and thawed bloods, with the exception of DPPRAP which forms a subset with PREFRD. The two rapidly cooled bloods, not separable from each other, are separable from all of the slowly cooled bloods with the exception of AM-196 which forms a subset with DAMRAP. In agreement with the prepatent period test results, the slowly cooled blood AM-196 is separable from all three other slowly cooled bloods ranked below it, which cannot be separated from each other.

Table 56 shows the results of a test on the trimmed survival time data. These results are in agreement with the prepatent period test results, inasmuch as the two prefreeze bloods are separable from all of the frozen and thawed bloods, the two rapidly cooled bloods are separable from all of the slowly cooled bloods and the slowly cooled blood AM-196 is separable from all three other slowly cooled bloods, which cannot be statistically separated from each other.

Discussion. Percentage haemolysis results

The percentage haemolysis results obtained in this experiment are in general agreement with those obtained in earlier experiments in this study. The best results were achieved when the DMSO-protected Babesia rodhaini-infected rat blood was rapidly cooled to -196°C in pp60, the next best when it was rapidly cooled to -196°C in ampoules. The slowly cooled bloods all showed a markedly higher degree of damage, whether or not they were stored at -79°C or -196°C. Although these results are of doubtful validity (due to the presence of crystals in some of the frozen and thawed bloods) it is interesting to note that the two best results from slowly cooled bloods were obtained from those stored at -79°C, the blood stored at -79°C in pp60 tubing being statistically better than all three other slowly cooled bloods.

Single-dilution infectivity tests

The results of the analyses of the single-dilution infectivity tests data obtained in this experiment are in agreement with those obtained with the percentage haemolysis data, with one major difference. This major difference is that, in contrast with the indications from the analysis of the percentage haemolysis data, the best result from a slowly cooled blood is obtained from the blood AM-196. Since there must be some doubt as to the validity of the percentage haemolysis data, the indications from the infectivity test analysis should probably be regarded as more accurate.

It is not surprising that slowly cooled Babesia rodhaini-infected rat blood gives better results when stored at -196°C than it does when stored at -79°C. Mamwell and Edgett (1943), working with Plasmodium lophurae; Polge and Soltys (1957), working with

various Trypanosoma spp.; and Diamond (1964), working with Pentatrichomonas hominis all showed that survival was better, and for a longer period of time, when the storage temperature was lower. Dalgliesh (1972b), in reviewing this topic, recommends that: "The storage temperature should be as low as practicable, and no higher than -60°C."

Lovelock (1953) demonstrated an increasing rate of storage injury as the temperature of storage was reduced (with glycerol-protected human erythrocytes). Meryman (1966) suggested that this increase in injury rate was related to a net increase in concentration of biochemically active substances due to the removal of more and more free water by freezing, resulting in higher rates of harmful reactions. At temperatures below -35°C, where no further concentration occurs, Lovelock found that further decrease in storage temperature resulted in less storage damage. It is suggested by Lovelock, and Meryman, that this is in general agreement with the Arrhenius expression which predicts that the rate of a reaction will bear an exponential relationship to temperature, i.e. the lower the temperature, the lower will be the rate of reaction. Meryman (1966), reviewing this topic, discusses the evidence that a temperature of about -130°C appears to be the point below which little or no biochemical activity takes place. He recommends storage at liquid nitrogen temperatures, but points out, however, that there is evidence that some cell types and some enzyme activities are adversely affected by long term storage, even at the temperature of liquid nitrogen. Meryman and Weinman (1968) state that storage temperatures are generally considered stabilising below -100°C.

Much of the discussion above is of academic interest only. The indications from this experiment are that Babesia rodhaini-infected rat blood slowly cooled to -79°C should be stored at -196°C. Such a recommendation, however, applies only when the only refrigerant available for cooling is solid carbon dioxide. If liquid nitrogen is available for cooling as well as storage, the material should be rapidly cooled to -196°C, the results obtained with this method being consistently better for Babesia rodhaini-infected rat blood than those obtained with a slow cooling method.

Experiment 9. Estimation of the degree of damage suffered by Babesia rodhaini-infected rat blood protected with 10% final concentration DMSO and cooled rapidly to -196°C in either pp60 polythene tubing or glass ampoules. Estimation of degree of damage is by:

(1) percentage haemolysis estimation, and

(2) infectivity titrations.

Introduction. The results of Experiment 3 indicated that rapidly cooled, DMSO-protected blood frozen in pp60, demonstrated the best infectivity of the four treated bloods tested. In spite of this, the post-thaw survival of infective organisms was estimated to be no more than 0.35% to 3.55%. Subsequent experiments have confirmed that a DPPRAP method gives the best post-thaw results, but the degree of success, estimated by single-dilution infectivity tests, has been variable, from very good to quite poor (Experiment 7). These experiments have also indicated that stage of infection, level of parasitaemia, and nature of diluent have no significant effect on the results obtained. This experiment was designed to confirm the degree of survival of infective organisms after cryopreservation when using the two best methods of the limited number tested, i.e. DPPRAP and DAMRAP.

Method. A pool of Babesia rodhaini-infected rat blood was obtained by the methods described above, using MAS as anticoagulant, from rats at K in Fig. 1, previously infected with the stabilate TREU 1204. The number of red cells present and the proportion of these red cells infected were estimated and found to be $4.3 \times 10^6/mm^3$ and 2% respectively. Samples were set aside for a predilution percentage

haemolysis estimation (found to be c. 3%) and infectivity titration.

The remainder of the blood was diluted 1:1 with 20% DMSO made up in MAS. Samples of this diluted blood were set aside for a prefreeze percentage haemolysis estimation (found to be c. 8%) and infectivity titration. The two prefreeze blood samples were prepared for, and the animals infected were screened in, infectivity titrations as described above (Experiment 3).

The remainder of the diluted blood was dispensed into either pp60 polythene tubing or glass ampoules and rapidly cooled to -196°C. After storage for eight days at -196°C, the samples were individually thawed in a water bath at +37°C and estimated for percentage haemolysis. The pooled remainders were then prepared for, and the animals infected were screened in, infectivity titrations as described above.

Results and Analysis. Percentage haemolysis estimations

The post-thaw percentage haemolysis results are shown below.

DAMRAP	16.00 ± 3.39	5 observations
DPPRAP	14.67 ± 1.86	6 observations

A 't-test' on these two means gave a computed value for t of 0.74, which for $n_1+n_2-2 = 9$ degrees of freedom, is not significant at $p = 0.05$. Therefore it can be seen that the percentage haemolysis results given by these two treated bloods are not statistically separable.

Infectivity titrations

The results and analysis of the prefreeze and post-thaw infectivity titrations are shown in Appendix Tables 58 to 76. The results of the

prefreeze and post-thaw infectivity titrations, with and without data trimming, are shown in Appendix Table 58. In each titration, forty mice were injected, ten in each of four dilution groups. In all titrations, several animals did not show an infection on the first twenty days and were ignored in the subsequent statistical analyses. It is interesting to note that a surprising number (8 out of 40) of the animals in the prefreeze undiluted blood titration did not become infected. The importance of this observation will be discussed later. The labels used in this experiment are:

Label	Regression
	(1) Prefreeze bloods
OPPPWI	Buffer only-diluted/Prepatent period/Untrimmed data
OPPPWO	Buffer only-diluted/Prepatent period/Trimmed data
OSTWI	Buffer only-diluted/Survival time/Untrimmed
OSTWO	Buffer only-diluted/Survival time/Trimmed
DPPPWI	DMSO-diluted/Prepatent period/Untrimmed
DPPPWO	DMSO-diluted/Prepatent period/Trimmed
DSTWI	DMSO-diluted/Survival time/Untrimmed
DSTWO	DMSO-diluted/Survival time/Trimmed
	(2) Post-thaw bloods
ppPPPWI	Frozen in pp60/Prepatent period/Untrimmed
ppPPPWO	Frozen in pp60/Prepatent period/Trimmed
ppSTWI	Frozen in pp60/Survival time/Untrimmed
ppSTWO	Frozen in pp60/Survival time/Trimmed
APPPWI	Frozen in ampoules/Prepatent period/Untrimmed
APPPWO	Frozen in ampoules/Prepatent period/Trimmed
ASTWI	Frozen in ampoules/Survival time/Untrimmed
ASTWO	Frozen in ampoules/Survival time/Trimmed

(1) Tables 59 to 61 show the results of comparisons of linear regression between:

59 OPPPWI and OPPPWO

60 OSTWI and OSTWO

61 OPPPWO and OSTWO

These comparisons indicate that:

(a) The trimmed and untrimmed data prepatent period regressions of the undiluted prefreeze blood do not differ significantly in either slope or constant.

(b) The trimmed and untrimmed survival time regressions of this blood also do not differ significantly in either slope or constant.

(c) The prepatent period and survival time trimmed data regressions of this blood are significantly different in both slope and constant.

The following trimmed and untrimmed data regressions proved not to differ significantly in either slope or constant:

DPPPWI and DPPPWO

DSTWI and DSTWO

ppPPPWI and ppPPPWO

ppSTWI and ppSTWO

APPPWI and APPPWO

ASTWI and ASTWO

In view of these results, it was decided that trimmed data regressions be used exclusively in all subsequent comparisons in this experiment.

(2) Tables 62 to 64 show the results of comparisons between:

62 OPPPWO and DPPPWO

63 OSTWO and DSTWO

64 DPPPWO and DSTWO

These comparisons indicate that:

(a) The prepatent period regressions of the undiluted prefreeze and the DMSO-diluted prefreeze bloods do not differ significantly in either slope or constant.

(b) The survival time regressions of these bloods also do not differ significantly in either slope or constant.

(c) The prepatent period and survival time regressions of the DMSO-diluted prefreeze blood differ significantly in constant but not in slope.

(3) Tables 65 to 70 show the results of comparisons between:

65 OPPPWO and ppPPPWO

66 OSTWO and ppSTWO

67 ppPPPWO and ppSTWO

68 OPPPWO and APPPWO

69 OSTWO and ASTWO

70 APPPWO and ASTWO

These comparisons indicate that:

(a) The prepatent period regressions of undiluted prefreeze blood and DMSO-protected blood frozen and thawed in pp60 polythene tubing do not differ significantly in either slope or constant.

(b) The survival time regressions of these bloods also do not differ significantly in either slope or constant.

(c) The prepatent period and survival time regressions of DMSO-protected blood frozen and thawed in pp60 polythene tubing differ significantly in both slope and constant.

(d) The prepatent period regressions of undiluted prefreeze blood and DMSO-protected blood frozen and thawed in glass ampoules do not differ significantly in either slope or constant.

(e) The survival time regressions of these bloods also do not differ significantly in either slope or constant.

(f) The prepatent period and survival time regressions of DMSO-protected blood frozen and thawed in glass ampoules differ significantly in both slope and constant.

(4) Tables 71 to 76 show the results of comparisons between:

71 DPPPW0 and ppPPPW0

72 DPPPW0 and APPPW0

73 ppPPPW0 and APPPW0

74 DSTW0 and ppSTW0

75 DSTW0 and ASTW0

76 ppSTW0 and ASTW0

These comparisons indicate that:

(a) The prepatent period regressions of DMSO-diluted prefreeze blood and DMSO-protected blood frozen and thawed in pp60 polythene tubing do not differ significantly in either slope or constant.

(b) The prepatent period regressions of DMSO-diluted prefreeze blood and DMSO-protected blood frozen and thawed in glass ampoules do not differ significantly in either slope or constant.

(c) The prepatent period regressions of DMSO-protected blood frozen and thawed in pp60 polythene tubing and glass ampoules do not differ significantly in either slope or constant.

(d) The survival time regressions of DMSO-diluted prefreeze blood and DMSO-protected blood, frozen and thawed in pp60 polythene tubing, do not differ significantly in either slope or constant.

(e) The survival time regressions of DMSO-diluted and DMSO-protected blood, frozen and thawed in ampoules, do not differ significantly in either slope or constant.

(f) The survival time regressions of DMSO-protected bloods, frozen and thawed in pp60 polythene tubing or glass ampoules, do not differ significantly in either slope or constant.

Discussion. Percentage haemolysis estimations

All that need be said concerning the percentage haemolysis results obtained in this experiment is that they are in general agreement with those obtained in earlier experiments. The results with the DPPRAP method are better, but not statistically so, than those obtained with the DAMRAP method.

Infectivity titrations

Of considerable interest in this experiment are the results of the various comparisons of linear regressions which are outlined above. The only comparisons in which significant differences could be shown were those between the various prepatent period and survival time results. Differences in constant would be expected in such comparisons. If, however, as discussed above (Experiment 3), the only factor of importance in determining the time of death is the attainment of a lethal number of infective organisms, then if the multiplication of these infective organisms is constant from the time of injection till death, the regression lines for prepatent period and survival time should be parallel, i.e. the time between onset of parasitaemia and death should be a constant, regardless of the number of infective organisms injected. If the prepatent period and survival time regressions are parallel, then their computed slopes should not be statistically different. In the prefreeze undiluted and both post-thaw blood titrations, however, the prepatent period

and survival time regressions differ significantly in slope.

Since no significant differences could be shown between constants or slopes in any of the prepatent period or survival time regressions of all four bloods tested, the indications are that none of the bloods has been shown to be any more or less infective than any of the others. This in turn means that, using this particular method of statistical analysis, no significant difference could be shown between any of the bloods whether prefreeze or frozen and thawed, i.e. the number of organisms rendered non-infective during dilution, and during cryo-preservation in ampoules and pp60 was not sufficient to increase prepatent periods or survival times significantly, regardless of dose. If this last statement were to be believed, it would indeed be a significant finding. It would suggest that blood frozen and stored by the DPPRAP and DAMRAP methods do not differ significantly in infectivity from prefreeze control bloods.

Such a finding, however, is not in agreement with the results achieved in previous experiments in this study, and, therefore, examination of the figures calculated for the numbers of infective organisms present at onset of parasitaemia and death and effective numbers of infective organisms injected should prove interesting. These computed figures are shown below:

(a) From the prefreeze buffer only-diluted blood:

Estimate of number of organisms present at:

(1) Onset of parasitaemia (2) Death

5.786×10^{12} 8.385×10^{20}

(b) From treated bloods:

Estimate of number of infective organisms injected:

(Expressed as a percentage of 10^7)

	(1) From prepatent period data	(2) From survival time data
PRED	1.151%	279.3%
DPPRAP	6125%	2.87%
DAMRAP	2456%	490700%

(d) It can be seen that these figures do little to clarify the results of the comparisons of linear regression in this experiment, indeed, if anything they further confuse matters. Three of the four derived figures, from post-thaw data, for the effective numbers of infective organisms injected indicate that more infective organisms were present in each dose after, than were present before cryopreservation. The derived figures for the DMSO-diluted prefreeze blood are equally confusing. The figure derived from prepatent period data is only 1.151% whereas the figure derived from survival time data is 240 times larger at 279.3%.

Such figures are clearly unacceptable, and cast considerable doubt on the validity of certain assumptions made in their derivation. The following assumptions were made by Overdulve and Antonisse (1970a and b), and by the author in the analysis of the results obtained in Experiment 3, with respect to the relationship between log. dose and prepatent period and survival time:

(a) There existed (or could be shown to exist) a linear relationship between log. dose on one hand and prepatent period or survival time on the other.

(b) The expectation of the number of infective organisms present at onset of parasitaemia or at death was independent of the number injected.

(c) The inoculated infective organisms, to quote Overdulve and Antonisse, "multiply continuously with a constant rate until the

moment that the infection becomes apparent (onset of parasitaemia or death)". It followed, according to Overdulve and Antonisse, that there was, therefore, a direct relationship between time and number of infective organisms present.

(d) If the effect of any pathological changes is rapid in comparison with the time necessary for the infective organisms to multiply to a sufficiently high level, any inaccuracy made by neglecting these actual causes of death will be small.

It is suggested that the anomalous results obtained in this experiment are due to the invalidity of one or more of these assumptions. Considering the figures derived from the buffer only-diluted blood, which acts as the 'control', it can be seen that the calculated numbers of infective organisms present at onset of parasitaemia and at death are much too high. Schermer (1967) quoting the figures of several workers, indicates that the total red cell count in the white mouse is only c. 10×10^9 red cells/ml. He also lists several estimates of the total blood volume of the white mouse and the maximum estimate was one of 8.3% of body weight, i.e. about 1.66 ml in a 20g mouse. It can be seen, therefore, from these estimates that the derived figure of 5.786×10^{12} infective organisms present at onset of parasitaemia means that there were in the mouse

$$\text{c. } \frac{5.786 \times 10^{12}}{1.66 \times 10^{10}} = 350 \text{ infective organisms for every red cell present.}$$

The derived figure of c. 10^{21} infective organisms present at death need hardly be stated as impossible.

It is suggested that the assumption that the infective organisms injected multiply continuously at a constant rate from the moment of

injection is invalid and that this accounts, in part, for the erroneous derived figures. In contrast to the parasite used in Experiment 3, the Babesia rodhaini used in this experiment was taken from rats which had been infected with stabilated material, TREU 1204. It had often been noted by the author that stabilated material when injected initially into rats, or mice, took considerably longer than passage material to show a parasitaemia and to kill the host. After several passages, however, the parasite returned to its highly adapted state, i.e. the state it was in when used for Experiment 3. It is suggested that this 'slowing down' of the onset of parasitaemia and death is not a result of a lengthening of the doubling time of the parasite, but rather a result of the existence of a 'lag phase' during which little or no multiplication of the parasite occurs. (Assumption 1) This is supported to a certain extent by the value obtained for slope in the prefreeze buffer only-diluted blood prepatent period regression in this experiment which is considerably lower than that obtained for the corresponding regression in Experiment 3.

The calculation used above for the effective number of infective organisms injected involves extrapolation of the linear relationship shown to exist in the period of observations (from the time at which parasites were seen in the 10^7 dilution group to the time at which animals died in the 10^4 dilution group) back to the moment of injection. When a highly-adapted parasite is involved (in Experiment 3 only 2.1 doublings had taken place according to the derived onset of parasitaemia figure) the existence of a lag phase, if any, would be unlikely seriously to affect the calculation of the effective number of infective organisms injected.

Examination of the derived figures for the number of infective organisms present at onset of parasitaemia and at death in Experiment 3 show that they are, at least, credible. The indications were that 4.4×10^7 parasites were present at onset of parasitaemia. If, from the estimates quoted by Schermer, the total number of red cells present in a 20g mouse is taken to be 2×10^{10}, then 4.4×10^7 parasites present would indicate one parasite for approximately every 500 red cells. The derived figure of 2.25×10^{10} infective organisms present at death would indicate the presence of approximately one parasite for every red cell.

If these figures are used as close approximations of the number of infective organisms which should be present at the onset of parasitaemia and death, (Assumption 2) then, using the former, an estimation of the length of the lag phase in this experiment may be made. The method of calculation is as follows:

Using the prefreeze buffer only-diluted 'control' blood prepatent period regression data:

It is assumed, for simplicity, that 4×10^7 infective organisms are present at the onset of parasitaemia, (Assumption 3) i.e. that two doublings have taken place.

The time required for two doublings would be $2 \times Q$, where $Q = b\log 2$, and b = the numerical value of slope in the prepatent period regression. Unlike the situation which applied in Experiment 3, and in Overdulve and Antonisse's experiments, the linear relationship which has been shown to exist in the observed period is being extrapolated only as far back as the end of the lag phase. The only justification for doing this is that the observed 'b' is the

only estimate which can be used for measuring the extent of the doubling time of the parasite before the onset of parasitaemia. (Assumption 4)

In this experiment:

$Q = 0.875 \times 0.3010 = 0.2634$ days (6.32 hours)

and $2 \times Q = 0.527$ days.

The lag phase is taken as the computed prepatent period at a dose level of 10^7, minus the computed 'true' prepatent period

$= 5.000 - 0.527$

$= 4.473$ days.

It is very probable that this lag phase, if it exists, is variable, depending on the subsequent treatment of the infective organisms, e.g. dilution or cryopreservation. Since there is no way in which the extent of this variation, if any, can be measured, the simplest situation which could apply, i.e. that the lag phase is a constant length, is assumed. (Assumption 5)

If the lag phase calculated above is simply subtracted from all the computed prepatent periods for a dose level of 10^7 in the treated blood regressions, and the effective numbers of parasites injected are then estimated by the usual methods, the following figures are obtained:

Treated blood	Estimate of number of infective organisms injected (Expressed as percentages of 10^7)
PRED	14.3%
DPPRAP	195%
DAMRAP	100%

The fact that these figures now approach credibility suggest that Assumptions 1-5 above may not be without some degree of justification.

The mere subtraction of the length of the hypothetical lag phase from the computed survival time of animals infected with prefreeze buffer only-diluted blood at the 10^7 level would result in a new estimate of the number of infective organisms present at death of 2.68×10^{15}. This estimate is obviously still much too high.

It is now further suggested that the assumption made by Overdulve and Antonisse, and by the author in Experiment 3, that the 'pathological changes' term could, with little loss of accuracy be ignored, is invalid in this instance, in which the parasite is not highly adapted. It is suggested that the large figures obtained for the derived number of infective organisms present at death indicate that, in this experiment, the constant term referred to by Overdulve and Antonisse as the pathological changes term is not small relative to the time required by the parasite to attain a 'sufficiently high level', and therefore cannot be ignored.

An estimate may be made of the extent of this pathological changes term. Using the 'control' prefreeze buffer only-diluted blood, it is first of all assumed that the 'sufficiently high level' of parasites required is, as indicated in Experiment 3, about 2.25×10^{10}. (Assumption 6)

Overdulve and Antonisse suggested that the length of the pathological changes phase would be a constant. There is no evidence why this should be the case, especially after treatment. However, it is not possible to estimate the extent of any variation, and the simplest situation which could apply, i.e. that it is indeed a constant, is assumed. (Assumption 7)

In the calculation of the extent of this phase, the length of time required for the parasite to double to 2.25×10^{10} from 10^7 parasites in the buffer only-diluted prefreeze blood is estimated and subtracted from the observed computed survival time for a dose level of 10^7

i.e. $\frac{2.25 \times 10^{10}}{10^7} = 2.25 \times 10^3$ = the factor by which the number of infective organisms has to increase to reach a "sufficiently high level"

$= 2250$

$= 2^{11.7}$

Therefore 11.7 doublings must take place in order that the "sufficiently high level" be attained. In the buffer only-diluted prefreeze blood the length of time required for these doublings would be:

11.7 x Q, where Q = blog2 and 'b' is the numerical value of the slope of the survival time regression of the buffer only-diluted blood. (Assumption 8)

This length of time is therefore, $= 11.7 \times 0.3010 \times 0.8091$

$= 2.849$ days.

This is then subtracted from the computed 10^7 level survival time $= 11.263 - 2.849$

$= 8.414$

This figure of 8.414 days is an estimate of the sum of the lag phase and the pathological changes phase. The length of the pathological changes phase would be: $= 8.414 - 4.473$

$= 3.941$ days.

When the total of 8.414 days is subtracted from all of the computed survival times at the 10^7 level in the treated bloods and the effective numbers of infective organisms injected are then estimated in the normal way, the following results are obtained:

Treated blood	Effective number of infective organisms injected (Expressed as percentages of 10^7)
PRED	31%
DPPRAP	10%
DAMRAP	546%

Since two of these three estimated figures are quite credible, and the third is rather more acceptable than the corresponding results before Assumptions 1-8 were made, although still obviously too high, it can be suggested that Assumptions 1-8 above may be justified to some degree.

It was assumed above that the lag and pathological changes phases were of constant length. It is unlikely that this would be true, especially after the various treatments undergone by the bloods involved. Yet another assumption made by Overdulve and Antonisse and by the author in Experiment 3, may not be valid, i.e. that assumption which states that the number of infective organisms expected to be present at the onset of parasitaemia or at death is independent of the number injected. Since, as shown above, the lines for prepatent period and survival time are not parallel in the titrations of three of the four bloods, this could be evidence that the assumption immediately above is not valid in this experiment. The invalidity of this assumption would make even more complex the

relationship existing between log. dose and prepatent period and survival time in this experiment.

All that has been discussed above is purely theoretical, supported by some circumstantial evidence. It was originally expected that this experiment would clarify the results obtained earlier in this study, thereby bringing the studies on Babesia rodhaini-infected rat blood to a suitable conclusion. As it happened, this experiment created more problems than it cleared up. The experiment has been valuable, however, in demonstrating that relationships shown to exist for a blood infected with a particular parasite do not necessarily exist for bloods infected with that same parasite if that parasite has been treated in such a manner as to change its biological characteristics.

Experiment 10. Estimation of the degree of damage in

(i) Babesia microti-infected mouse blood, and

(ii) Nuttallia musculi-infected mouse blood

cooled either slowly to -79°C in glass ampoules or rapidly to -196°C in pp60 polythene tubing and protected with 10% final concentration DMSO as cryoprotectant.

Estimation by (a) percentage haemolysis measurement

(b) full-scale infectivity titration.

Introduction. As a result of the experiments described above, it has been shown that, of the treatments investigated for the cryopreservation of Babesia rodhaini-infected rat blood, the best results were obtained when the infected blood, protected with 10% final concentration DMSO as cryoprotectant, was rapidly cooled to -196°C in pp60 polythene tubing. In contrast, Babesia rodhaini-infected blood protected with 10% final concentration DMSO and slowly cooled to -79°C in glass ampoules gave very poor results, although techniques similar to this had previously been commonly reported as being successful, by other workers. This experiment was designed to test the effectiveness of these two methods for another two piroplasms, Babesia microti and Nuttallia musculi.

Method. For both parasites, pools of infected mouse blood were obtained from groups of ten mice infected with the stabilates TREU 1187 (Babesia microti) and TREU 1189 (Nuttallia musculi). These mice were bled out, using the methods described above and with MAS as anticoagulant. The numbers of red cells present and the proportions of these red cells infected were estimated.

These were found to be $5.7 \times 10^6/mm^3$ and 6% (Babesia microti) and 2.8×10^6 and 25% (Nuttallia musculi). Samples of the predilution blood pools were set aside for percentage haemolysis estimations (c. 1% for B. microti and c. 3% for N. musculi). The remainders of the blood pools were then divided into two and diluted 1:1 with either:

(a) MAS alone

(b) 20% DMSO made up in MAS.

Samples of the diluted bloods were set aside for prefreeze percentage haemolysis estimation (c. 2% for B. microti and 6% for N. musculi) and infectivity titrations. The remainders of the diluted bloods were then dispensed into either pp60 polythene tubing and rapidly cooled to -196°C (label DPPRAP), or into glass ampoules and slowly cooled to -79°C (label DAMCON).

After storage at -196°C or -79°C for one week, the individual samples were thawed as rapidly as possible in a water bath at +37°C and estimations of post-thaw percentage haemolysis were made. From the pooled remainders of the thawed bloods samples were taken for post-thaw infectivity titrations. The dilutions for the prefreeze and post-thaw infectivity titrations were made as described above, giving 10^7, 10^6, 10^5 and 10^4 infected red cells in every 0.1ml dose. The mice injected with these doses were screened for the presence of parasites only by the methods described above. Neither Babesia microti nor Nuttallia musculi kill mice, even at the highest doses injected.

Results and Analysis. Percentage haemolysis estimations

(a) The post-thaw percentage haemolysis results obtained from Babesia microti-infected mouse blood are shown below.

Treatment	Mean	S.D.	No. Observations
DPPRAP	19.8	± 1.09	5
DAMCON	74.4	± 4.34	5

The variance ratio, $\frac{4.34^2}{1.09^2}$ is equal to 15.70, which is significant at $p = 0.05$ for 4, 4 degrees of freedom. In the 't-test' on the above means, the unknown variances are, therefore, not assumed to be equal. The 't-test' gave a computed value for t of 27.3 which, at $p = 0.05$, is significant for $n_1 \pm n_2 - 2$ (=8) degrees of freedom.

(b) The post-thaw percentage haemolysis results obtained from Nuttallia musculi-infected mouse blood are shown below.

Treatment	Mean	S.D.	No. Observations
DPPRAP	14.2	± 2.78	5
DAMCON	53.4	± 4.34	5

The variance ratio, $\frac{4.34^2}{2.78^2}$ is equal to 2.56, which is not significant at $p = 0.05$ for 4, 4 degrees of freedom. In the 't-test' on the above means, therefore, the unknown variances were assumed to be equal. The computed value for t was found to be 16.8 which, at $p = 0.05$, is significant for $n_1 + n_2 - 2$ (=8) degrees of freedom.

The indications are, therefore, from the above percentage haemolysis results, that both Babesia microti- and Nuttallia musculi-infected mouse bloods suffer less damage during cryopreservation when rapidly cooled in pp60 polythene tubing than they do when

slowly cooled in glass ampoules, in both cases with DMSO at a final concentration of 10% as cryoprotectant. These indications agree with those found in previous experiments for Babesia rodhaini-infected rat blood.

Infectivity titrations

(a) Babesia microti. The results of the Babesia microti-infected mouse blood prefreeze and post-thaw infectivity titrations are shown in Appendix Table 77. In each titration, thirty mice were injected, ten in each of three dilution groups. In the DMSO-diluted prefreeze and DMSO-protected rapidly cooled frozen and thawed blood titrations, the majority of animals became infected but in the DMSO-protected slowly cooled frozen and thawed blood titration less than half of the animals became infected.

The analyses of the prefreeze and post-thaw infectivity titrations are shown in Appendix Tables 78-84. The labels used in this experiment, in the titrations of blood infected with both parasites, are:

Label	Regression
	(i) Prefreeze bloods
OPPPWI	Buffer only diluted/Prepatent period/Untrimmed data
OPPPWO	Buffer only diluted/Prepatent period/Trimmed data
DPPPWI	DMSO-diluted/Prepatent period/Untrimmed data
DPPPWO	DMSO-diluted/Prepatent period/Trimmed data
	(ii) Post-thaw bloods
RAPPPPWI	Rapidly cooled/Prepatent period/Untrimmed data
RAPPPPWO	Rapidly cooled/Prepatent period/Trimmed data
CONPPPWI	Slowly cooled/Prepatent period/Untrimmed data
CONPPPWO	Slowly cooled/Prepatent period/Trimmed data

(1) Table 78 shows the results of a comparison between the regressions:

78 OPPPWI and OPPPWO

The results of this comparison indicate that the trimmed and untrimmed data prepatent period regressions of the buffer only-diluted prefreeze Babesia microti-infected mouse blood do not differ significantly in either slope or constant.

The following trimmed and untrimmed data regressions:

DPPPWI and DPPPWO

RAPPPPWI and RAPPPPWO

CONPPPWI and CONPPPWO

also do not differ significantly in either slope or constant. In view of these results, the use of trimmed data regressions in subsequent comparisons is justified.

(2) Table 79 shows the results of a comparison between the regressions:

79 OPPPWO and DPPPWO

The results of this comparison indicate that the prepatent period regressions of buffer only-diluted prefreeze and DMSO-diluted prefreeze Babesia microti-infected mouse blood do not differ significantly in either slope or constant.

(3) Tables 80 and 81 show the results of comparisons between the regressions:

80 OPPPWO and RAPPPPWO

81 DPPPWO and RAPPPPWO

The results of these comparisons indicate that:

(a) The prepatent period regressions of buffer only-diluted prefreeze and DMSO-protected, rapidly cooled frozen and thawed Babesia microti-infected mouse blood differ significantly in both slope and constant.

(b) The prepatent period regressions of DMSO-diluted prefreeze and DMSO-protected, rapidly cooled frozen and thawed Babesia microti-infected mouse blood differ significantly in constant but not in slope.

(4) Tables 82 and 83 show the results of comparisons between the regressions:

82 OPPPW0 and CONPPPW0

83 DPPPW0 and CONPPPW0

The results of these comparisons indicate that:

(a) The prepatent period regressions of buffer only-diluted prefreeze and DMSO-protected, slowly cooled frozen and thawed Babesia microti-infected mouse blood differ significantly in both slope and constant.

(b) The prepatent period regressions of DMSO-diluted prefreeze and DMSO-protected, slowly cooled frozen and thawed Babesia microti-infected mouse blood differ significantly in both slope and constant.

(5) Table 84 shows the results of a comparison between the regressions:

84 RAPPPPW0 and CONPPPW0

The results of this comparison indicate that the prepatent period regressions of DMSO-protected, rapidly cooled and slowly cooled Babesia microti-infected mouse blood differ significantly in both slope and constant.

(b) Nuttallia musculi. The results of the Nuttallia musculi-infected mouse blood prefreeze and post-thaw infectivity titrations are shown in Appendix Table 85. In each titration thirty mice were injected, ten in each of three groups. A majority of the animals became infected in all four prefreeze and post-thaw blood titrations.

The analyses of the prefreeze and post-thaw blood infectivity titrations are shown in Appendix Tables 86-92.

(1) Table 86 shows the results of a comparison between the regressions:

86 OPPPWI and OPPPWO

The results of this comparison indicate that the trimmed and untrimmed data prepatent period regressions of buffer only-diluted prefreeze Nuttallia musculi-infected mouse blood do not differ significantly in either slope or constant.

The following trimmed and untrimmed data regressions:

DPPPWI and DPPPWO

RAPPPPWI and RAPPPPWO

CONPPPWI and CONPPPWO

also do not differ significantly in either slope or constant. In view of these results, the use of trimmed data regressions in subsequent comparisons is justified.

(2) Table 87 shows the results of a comparison between the regressions:

87 OPPPWO and DPPPWO

The results of this comparison indicate that the prepatent period regressions of buffer only-diluted prefreeze and DMSO-diluted prefreeze Nuttallia musculi-infected mouse blood do not differ sig-

nificantly in either slope or constant.

(3) Tables 88 and 89 show the results of comparisons between the regressions:

88 OPPPW0 and RAPPPPW0

89 DPPPW0 and RAPPPPW0

The results of these comparisons indicate that:

(a) The prepatent period regressions of buffer only-diluted prefreeze and DMSO-protected, rapidly cooled frozen and thawed Nuttallia musculi-infected mouse blood differ significantly in both slope and constant.

(b) The prepatent period regressions of DMSO-diluted prefreeze and DMSO-protected, rapidly cooled frozen and thawed Nuttallia musculi-mouse blood differ significantly in constant and in slope.

(4) Tables 90 and 91 show the results of comparisons between:

90 OPPPW0 and CONPPPW0

91 DPPPW0 and CONPPPW0

The results of these comparisons indicate that:

(a) The prepatent period regressions of buffer only-diluted prefreeze and DMSO-protected, slowly cooled frozen and thawed Nuttallia musculi-infected mouse blood differ significantly in both slope and constant.

(b) The prepatent period regressions of DMSO-diluted prefreeze and DMSO-protected, slowly cooled, frozen and thawed Nuttallia musculi-infected mouse blood differ significantly in both slope and constant.

(5) Table 92 shows the results of a comparison between:

92 RAPPPPW0 and CONPPPW0

The results of this comparison indicate that the prepatent period regressions of DMSO-protected rapidly cooled and slowly cooled frozen and thawed Nuttallia musculi-infected mouse blood differ significantly in constant but not in slope.

Discussion. Percentage haemolysis

The percentage haemolysis results obtained in this experiment for both Babesia microti- and Nuttallia musculi-infected mouse blood are in general agreement with those obtained in earlier experiments with Babesia rodhaini-infected rat blood, in that DMSO-protected blood rapidly cooled in pp60 was less damaged during cryopreservation than that which was slowly cooled in glass ampoules.

Infectivity titrations

(a) Babesia microti. The results of the various comparisons of linear regression made on the prefreeze and post-thaw Babesia microti-infected mouse bloods show that:

(1) The DMSO-diluted prefreeze blood regression could not be shown to differ significantly from the buffer only-diluted prefreeze blood regression.

(2) The rapidly cooled frozen and thawed blood regression had a significantly greater value for constant than both the prefreeze blood regressions, usually indicative of a loss of infective organism numbers at injection due to the effects of cryopreservation. Interpretation of the results of the buffer only-diluted prefreeze and the rapidly cooled frozen and thawed blood regressions is complicated by the fact that a significant increase in the value of the slope in the latter was also shown. The derived figures shown

below for the effective numbers of infective organisms injected should help make this interpretation simpler.

(3) The slowly cooled frozen and thawed blood regression had a significantly greater value for constant than both prefreeze blood regressions. Significant differences in slope invalidate deductions concerning the implications of these differences in constant. The derived figures shown below help quantify these differences.

(4) The slowly cooled frozen and thawed blood regression was shown to have a significantly higher value for constant than the rapidly cooled frozen and thawed blood regression. A significant difference was also shown in slope. The derived figures shown below help quantify these differences.

(b) Nuttallia musculi. The results of the various comparisons of linear regression made on the prefreeze and post-thaw Nuttallia musculi-infected mouse blood regressions show that:

(1) The DMSO- and buffer only-diluted prefreeze blood regressions were shown not to differ significantly.

(2) The rapidly cooled frozen and thawed blood regression had a significantly greater value for constant than both the prefreeze blood regressions. Deductions from these results are difficult, however, since significant differences were also shown in slope. The derived figures shown below for the effective numbers of infective organisms injected help quantify these differences.

(3) The slowly cooled frozen and thawed blood regression also had a significantly higher value for constant than both prefreeze blood regressions. The existence of significant differences in slope invalidate the making of any deductions from these results,

a task which should be simplified by examination of the derived figures shown below.

(4) The slowly cooled frozen and thawed blood regression had a significantly higher value for constant than the rapidly cooled frozen and thawed blood regression. Since no significant difference was shown in the slopes of these regressions, it can be deduced that more infective organisms survived the cryopreservation processes when rapidly cooled than did when slowly cooled. This deduction is quantified below in the derived figures for effective numbers of infective organisms injected.

The derived figures for numbers of infective organisms present at onset of parasitaemia and effective numbers of infective organisms injected are shown below.

(a) From prefreeze buffer only-diluted blood regression:

ESTIMATE OF NUMBER OF ORGANISMS PRESENT AT ONSET OF PARASITAEMIA:

Babesia microti	Nuttallia musculi
3.34×10^7	2.68×10^7

(b) From treated blood regressions:

COMPUTED NUMBERS OF INFECTIVE ORGANISMS INJECTED

(Expressed as a percentage of 10^7; computed figures shown in brackets)

Treated blood	Babesia microti	Nuttallia musculi
PRED	64% (6433030)	38% (3848590)
DAMCON	2% (200180)	1.29% (129607)
DPPRAP	4.05% (405204)	2.52% (251764)

It can be seen that these results are similar to those obtained in Experiment 3 with Babesia rodhaini-infected rat blood,

in that rapidly cooled bloods give better results than slowly cooled bloods. Relative to their DMSO-diluted prefreeze bloods, the Babesia microti- and Nuttallia musculi-infected rapidly cooled frozen and thawed mouse bloods gave 6.3% and 6.54% survival figures respectively. For the slowly cooled frozen and thawed bloods, the survival figures are increased to 3.11% and 3.37% for Babesia microti and Nuttallia musculi respectively. The figures for the slowly cooled Babesia microti-infected blood, however, should be viewed with caution since only fourteen mice out of thirty injected showed parasites.

This reduction in numbers of animals becoming infected (and with Babesia rodhaini, dying) with less successful treatments has been a general trend throughout this study and is particularly noticeable in Experiments 3, 9 and 10 in which larger numbers of animals were initially injected. There are in the literature, several examples of studies in which the success or failure of a particular cryopreservation technique has been judged by a simple comparison of the relative proportions of animals becoming infected before and after freezing. This has been the case particularly where domestic animals, e.g. cattle, are involved and where full titration methods are economically impossible (Waddell, 1963; Frerichs et al, 1968; and Dalgliesh, 1972a for Babesia spp. in cattle; Dar, Ligthart and Wilson, 1972 for Trypanosoma vivax and T. congolense in cattle; Cunningham, Brown, Burridge and Purnell, 1973 for Theileria parva in cattle).

It has been a feature of the results obtained in this study that less animals have, in general, been infected with bloods shown by percentage haemolysis measurement to have been more damaged as

a result of one of the less successful cryopreservation techniques than were infected with bloods shown to have been less damaged as a result of the most successful cryopreservation technique, DPPRAP. It is not being suggested here that such a simple method for estimation of success be substituted for the more sophisticated techniques used in this study. It is, however, being stressed that examination of the actual proportion of animals which become infected (and die) might not only be useful as a rough estimate of the degree of damage suffered, but might act also as a guide to the amount of credibility which should be applied to any results obtained from the subsequent statistical analysis. For instance, a limited amount of credibility may be applied to the results obtained from the analyses in Experiment 9 above (Table 58). As indicated above, although forty animals were injected with the prefreeze buffer only-diluted 'control' blood, only thirty-two became infected and only twenty-six died. This considerable fall in numbers of animals becoming infected and dying indicated, even before the blood was subjected to the various treatments (e.g. dilution and cryopreservation) that, from the point of view of infectivity, this blood might behave atypically. The treated bloods in Experiment 9 suffered a further loss in numbers of animals infected, down to only twenty-three in the DAMRAP frozen and thawed blood. Whereas with numbers of animals dying, the DMSO-diluted prefreeze blood showed an increase over the 'control' blood, twenty-eight against twenty-six, the two frozen and thawed bloods showed a decrease to twenty-five and twenty for DPPRAP and DAMRAP respectively, possibly indicative of a loss of infectivity of these bloods.

The evidence from this experiment, however, would appear to

confirm that obtained in the previous experiments in which Babesia rodhaini-infected rat blood was studied in that rapid cooling of DMSO-protected Babesia microti- and Nuttallia musculi-infected mouse blood has been shown to give greater success, however estimated, than slow cooling.

CHAPTER 5

Discussion

In this chapter, points of interest, most of which have already been discussed to a greater or lesser extent, will be dealt with. Additional information, unpublished or not available at the time that the relevant experiment was carried out, will be introduced, conclusions will be drawn and further avenues of fruitful study will be suggested.

Of the three main cryoprotectants tested, glycerol, DMSO and PVP, the best results were obtained using DMSO (at a final concentration of 10%). This was perhaps not surprising, since DMSO had been previously used successfully for the cryopreservation of several Babesia spp. What was surprising, however, was the extent to which 10% DMSO was better than 7.5% glycerol (which had also previously been used successfully for the cryopreservation of several Babesia spp.) and 0.004M PVP, and the extent to which simple dilution with glycerol and DMSO appeared to reduce the effective numbers of infective organisms present in the prefreeze bloods.

Dalgliesh (1972b) reviewed the literature on the injurious effects of the two penetrating cryoprotectants, glycerol and DMSO. He pointed out that Joyner (1954) and Corrias, Marchisio and Molinari (1955) had utilised the toxic effects of glycerol for Trichomonas foetus to eliminate this parasite from infected bovine semen, by incubating the infected semen with added glycerol for about 24 hours at +5°C. Overdulve and Antonisse (1970b) found that 5% and 10%

DMSO slightly depressed the infectivity of Babesia rodhaini-infected mouse blood kept at 0°C for about two hours prior to cryopreservation, and Overdulve, Antonisse, Leeflang and Zwart (1970) showed that 8% DMSO was markedly toxic to Trypanosoma congolense after exposure for only 20 minutes. Dalgliesh also stated that he and Mellors had found that, when DMSO and glycerol were added to Babesia rodhaini-infected blood, and this blood was then exposed to various incubation conditions, infectivity was lost to a greater or lesser extent. Incubation with DMSO at +38°C for periods of 1-4 hours resulted in a complete loss of infectivity, whereas incubation at +4°C resulted in a slight though significant loss of infectivity. Glycerol, on the other hand did not suppress infectivity completely at either temperature, and reduced infectivity more at +4°C than at +38°C.

Dalgliesh (1972b) summarised the suggested causes for the toxicity of DMSO and glycerol before and during cryopreservation as follows:

(a) It had been noted that certain cells, e.g. Novikoff hepatoma ascites cells (Bickis, Kazaks, Finn and Henderson, 1967) and bovine erythrocytes (Dalgliesh, 1969) are less permeable to glycerol at +4°C than at +38°C. It was suggested that the resultant prolonged stress factors, imposed by the osmotic effects of extracellular glycerol, were injurious. Lumsden, Robertson and McNeillage (1966) showed that Trichomonas vaginalis suffered gross distortion immediately after suspension in 10% glycerol. It was suggested that this distortion was caused by osmotic stresses. No noted adverse effects were seen using DMSO in the same system.

It is also well known that erythrocytes containing glycerol cannot be transferred directly to a glycerol-free isotonic medium

without swelling and lysis of the cells due to the osmotic effects of intracellular glycerol (Hurn, 1968). To avoid such osmotic damage, the author has in this study always diluted cryoprotectant-containing blood suspensions with diluent containing the appropriate cryoprotectant at the appropriate concentration. Dalgliesh (1972a) showed that intravenous injection of blood containing high glycerol concentrations was equivalent to exposure to an isotonic environment, and that this route of inoculation suppressed the infectivity of glycerol-protected Babesia bigemina. It is thought unlikely that the intraperitoneal route used exclusively in this study would result in the same degree of damage.

(b) Herbert, Lumsden and French (1968) observed that Trypanosoma brucei-infected blood was less damaged during rapid cooling when frozen without a protective additive than it was when frozen in the presence of either DMSO or glycerol. Rapatz and Luyet (1965) showed that "at high cooling velocities", glycerol and DMSO have a detrimental effect on the survival of erythrocytes. These "high cooling velocities" were not, however, defined. It is suggested by Dalgliesh (1972b) that the same mechanism of damage applies in both systems, i.e. there is an increase in the amount of intracellular ice crystal formation within cells frozen rapidly when cryoprotectants are present, caused by the freezing of the intracellular water associated with the cryoprotectants (Rapatz and Luyet, 1963; Doebbler and Rinfret, 1965).

(c) There may also be some evidence that the presence of cryoprotectants, particularly DMSO, might have a detrimental effect on the metabolism of the parasite. Callow and Farrant (1973) showed that glycerol, in suspensions of Leishmania tropica inoculated into

fresh culture medium, inhibited growth by making the fresh medium unsuitable rather than by a permanent toxic effect or by osmotic disruption during inoculation.

Thus it is obvious that, when penetrating cryoprotectants are being used, some care should be taken, wherever possible, concerning the mode of addition, temperature of incubation, mode of dilution and injection route, as all of these have been shown to have some bearing on the subsequent infectivity of the parasites thus protected. One might suggest that since most of these injurious effects are osmotic, the better results obtained by using DMSO might have been expected since DMSO is, relative to glycerol, much faster penetrating and any toxic osmotic effects would as a result be of a shorter duration.

The third cryoprotectant used in this study, PVP, gave results which were extremely difficult to explain. At this point, the differences between the results obtained from percentage haemolysis estimations and infectivity measurement are seen perhaps to the greatest extent. There was little doubt that some of the best results obtained, as judged by percentage haemolysis, were those given by PVP-protected, rapidly cooled blood. PVP-protected, rapidly cooled blood was, however, completely non-infective after cryopreservation. It has been suggested in the discussion of Experiment 3, that one of the reasons for this apparent disagreement between the percentage haemolysis and infectivity results was simply that the percentage haemolysis resulting was completely misleading and that the red cells were heavily damaged. A translation of a Russian paper (Dobry, Livora, Sebestik and Jelinek, 1969), obtained by the author since the completion of this particular part of this

study, argues very strongly in favour of the percentage haemolysis results being valid. It will be recalled that in the discussion of Experiment 3, evidence was quoted from the literature to the effect that Doebbler et al (1971), who found that rapid cooling of red cells in the presence of PVP gave good survival when measured by percentage haemolysis, also found a similar level of success when that success was judged by measurement of posttransfusion viability. The Russian paper was translated by NASA in December, 1972 and obtained by the author in 1973. It describes a method for the cryopreservation of human red cells, using 24000 M.Wt. PVP as cryoprotectant at a final concentration of 0.004M. Dobry et al rapidly cooled the blood protected in this way to -196°C by plunging it, contained in the plastic tubing used in blood collecting and blood transfusion sets (inside diameter 3.6 mm; outside diameter, 4.6 mm) directly into liquid nitrogen. It can be seen that this method was very similar to that used by the author and labelled PPPRAP, with which the author obtained some of the best overall results, as estimated by percentage haemolysis measurement. Dobry et al, however, estimated success by both percentage haemolysis measurement and by posttransfusion viability. Figures obtained from the former were extremely good (less than 5% haemolysis) and were supported by the figures obtained from the latter (24-hour posttransfusion viability of 76 ± 5% of transfused cells).

As a result of these figures, it is now suggested by the author that the percentage haemolysis results obtained in Experiments 1-4 for PVP-protected, rapidly cooled, Babesia rodhaini-infected rat blood were probably valid. The implications are, therefore, that the red cells were protected and the parasites within those red cells were not. Recent work (Farrant, 1974, personal communication) has

shown that mixtures of intra- and extracellular cryoprotectants can yield good survivals of some cell types. It is, therefore, suggested that when any further work is carried out on the cryopreservation of Babesia spp., the examination of the potential of such cryoprotectant mixtures should be included. The evidence from this study has indicated that DMSO can protect the parasite effectively and PVP can protect the red cell. It is suggested that a mixture of both cryoprotectants could probably protect both cell types, thus giving better results.

One of the stated aims of this study was to attempt to find a simpler and less expensive method for the estimation of the degree of success of any particular method tested for the cryopreservation of piroplasms in particular, and possibly of intracellular parasites in general. The suggested alternative method was the estimation of percentage haemolysis of the host red cells. The results of early experiments were encouraging in that they showed that Babesia rodhaini-infected rat blood suffered varying degrees of haemolysis when subjected to a range of cryopreservation techniques. Subsequent examination of such results and comparison with infectivity test results, however, showed that percentage haemolysis results could not be relied upon to indicate degree of parasite survival. When considering the results of two cryopreservation methods in particular, i.e. those methods in which PVP-protected blood rapidly cooled in pp60 (PPPRAP) and in which unprotected blood slowly cooled in glass ampoules (OAMCON) were used, major differences were seen between the percentage haemolysis and infectivity results. The former (PPPRAP) has already been discussed adequately; the latter (OAMCON) is discussed below.

It has been suggested that the good percentage haemolysis results obtained for blood frozen and thawed by the PPPRAP method were probably valid, and that the parasites within these surviving red cells were in some way rendered non-infective. In the early experiments in this study, it was suggested that the percentage haemolysis results obtained for blood frozen and thawed by the OAMCON method were falsely low, as a result of the removal of haemoglobin by crystallisation from the post-thaw supernatant fluids. Evidence contrary to that possible mechanism was found in Experiment 4, but in the discussion of that experiment, the author stressed that although only 60-70% haemolysis was found in the blood frozen and thawed by the OAMCON method, the macro- and microscopical appearance of the blood was indicative of very heavy destruction of red cells. No evidence of crystal formation was found in this blood and what had happened to the haemoglobin which should, as a result, have been found in the supernatant fluids was unexplained. Crystals were noted, however, in other thawed bloods in Experiment 4, and in some of the thawed bloods in Experiment 8. The existence of these crystals, often seen by the author in frozen and thawed rat bloods, suggests that the use of percentage haemolysis as an estimator of the success of particular cryopreservation techniques could be invalid when rat blood is involved. According to the experience of the author, and presumably of other workers, similar crystal structures do not occur as a result of cryopreservation in the bloods of other species. Investigation of the nature and mechanism of formation of these crystals in frozen and thawed rat blood could prove valuable.

An interesting feature of this study was the variation in the

degree of success measured for the most successful cryopreservation methods for Babesia rodhaini-infected rat blood, DPPRAP and to a lesser extent DAMRAP. In Experiments 3, 5, 7 and 8, the best frozen and thawed blood infectivity test results could be shown to be statistically separable, i.e. worse than, the prefreeze control blood infectivity test results. In Experiments 4 and 6, however, the best frozen and thawed blood infectivity test results could not be statistically separated from the prefreeze control blood infectivity test results. The results of Experiment 9 merit discussion on their own and will be discussed below.

Suggestions as to the causes of these discrepancies included:

(a) The effect of the stage of infection of the parasite in the donor rat.

(b) The effect of the level of parasitaemia in the donor rat.

(c) The possible effect of diluent instability.

(d) The effect of insufficient or unequal data trimming of the infectivity test results.

Experiments to test (a), (b) and (c) were carried out and led to the conclusions that stage of infection and level of parasitaemia in the donor rat and the use of the potentially unstable diluent, ABP8.0 had no effect on the degree of survival of Babesia rodhaini-infected rat blood during cryopreservation. Subsequent examination of the raw data which had been analysed to give the results in Experiments 4 and 6 showed that a possible explanation of these results was the existence of two or more 'outliers' in the raw data used in that prepatent period or survival time raw data. If only one outlier existed in the data, it would have been removed by the data trimming procedures employed. If two outliers existed, then the effect of

one of these outliers would still be noticeable, even after data trimming.

The author, therefore, suggests that, although DPPRAP was shown to be the best method tested for the cryopreservation of Babesia rodhaini-infected rat blood, it should in all cases have been shown to have been separable from the prefreeze controls. In two experiments, however, DPPRAP was shown not to be separable from the prefreeze controls. It is suggested that this was more a function of the prefreeze infectivity results being unusually poor due to insufficient data trimming, and not a result of the post-thaw infectivity results being unusually good.

It had been expected that the results of Experiment 9 of this study would confirm that, although it was the best method of those tested for the cryopreservation of Babesia rodhaini-infected rat blood, DPPRAP was not successful to the extent that the results given by it could not be statistically separated from prefreeze controls. The results of Experiment 9, however, analysed by comparison of linear regression, did not confirm this expectation. None of the comparisons of prepatent period and survival time linear regressions between the prefreeze and frozen and thawed bloods could be shown to be significantly different in either slope or constant. The derived figures for expected numbers of infective organisms at the onset of parasitaemia and at death and for the effective numbers of infected organisms injected, clearly indicated that, although the statistical techniques used had given credible results in Experiment 3, they did not give credible results in Experiment 9. The derived figures for expected numbers of infective organisms at the onset of parasitaemia and at death were found to be much too high at 5.786 x

10^{12} and 8.36×10^{20} respectively. When it is remembered that the average total number of red cells present in a 20g white mouse is only about 2×10^{10}, the invalidity of these derived figures becomes obvious.

The author, in the discussion of Experiment 9, discussed at length possible explanations for these erroneous derived figures, and these explanations need not be reiterated here at any length. The explanations referred to centred on the invalidity for non-adapted parasites of the statistical methods outlined by Overdulve and Antonisse (1970a and b), upon which the analysis of the results of Experiments 3 and 9 were based. It can be seen that the results obtained using these statistical methods in Experiment 3, in which the parasite used was highly adapted to the rat, were clearly credible. The parasite used in Experiment 9, however, was not obtained from passage rats, but from rats infected with stabilated material. With Babesia rodhaini, it had been noted that stabilation effectively 'de-adapted' the parasite with respect to its ability quickly to infect and kill the rat. The parasite, having been de-adapted, then required several passages in rats before it regained the degree of adaptation which it possessed prior to stabilation.

Stabilation had originally been intended to provide a consistent source of experimental material, it having been noted that passage Babesia rodhaini was becoming adapted to the rat to the extent that it was infecting and killing animals so quickly that full-scale infectivity titrations would not be able to be carried out successfully. It should be remembered at this point that, even though the parasite was so adapted to the rat (and to the mouse in infectivity titrations), an injected dose of 10^3 - 10^4 infective

organisms was still necessary to produce a patent infection and subsequent death. As a result, successful titrations could not be produced simply by reducing the range of doses from 10^7 - 10^4 to, say, 10^6 - 10^3 infective organisms, since the results obtained from the last two dilution groups could be complicated by the existence of an 'end-point'.

It was suggested by the author in the discussion of Experiment 9 that de-adaptation was manifested as two main effects:
(a) the existence of a 'lag phase', immediately after injection, in which little or no increase in parasite numbers takes place,
(b) the existence of a 'pathological changes' phase between the time of attainment of a critical number of infective organisms in the mouse and the death of that mouse.
For adapted parasites, these phases would be of negligible length and would have little or no effect on statistical analyses, as assumed by Overdulve and Antonisse (1970a and b) and by the author in Experiment 3.

The existence of either lag or pathological changes phases, or both, is in no way certain. Their existence is merely a mechanism by which the author has attempted to explain the results obtained in Experiment 9. In formulating this mechanism, the author has made several assumptions, which are listed. These assumptions, for which there is little but circumstantial evidence in support, include assumptions that the simplest situation which might apply, does apply, and as a result some or all might be invalid.

No mention has yet been made of the various computed numerical values for slope obtained in the three experiments in which infectivity titrations were carried out and in which linear regressions

were calculated from the resultant data. These values for slope, 'b', may be used to give estimates of the doubling time of the parasite concerned. The doubling time, 'Q', is given by the expression Q = blog 2. Shown below is a table of the various estimates for the doubling times of the three parasites studied, calculated from the results of Experiments 3, 9 and 10. The labels used are the same as those used in the appropriate experiment. The estimates are based on trimmed data regressions. Where a comparison of linear regression has previously shown that the regressions for prepatent period and survival time for a particular blood do not differ significantly in slope, the mean of the two observed values for slope is used in the calculation of doubling time.

Experiment 3. Estimated doubling times for Babesia rodhaini, in hours.

BPPPW0	GPPPW0	DPPPW0	PPPPW0	G2PPPW0	D2PPPW0
and	and	15.71	10.18	9.06	and
BSTW0	GSTW0	DSTW0	PSTW0	G2STW0	D2STW0
14.37	16.70	18.45	11.67	9.60	12.25

Experiment 9. Estimated doubling times for Babesia rodhaini, in hours.

BPPPW0	DPPPW0	ppPPPW0	APPPW0
6.31	and	8.90	8.66
BSTW0	DSTW0	ppSTW0	ASTW0
5.85	5.72	5.45	7.53

Experiment 10. Estimated doubling times for Babesia microti, in hours.

BPPPW0	DPPPW0	CONPPPW0	RAPPPPW0
14.03	16.10	32.50	16.15

Estimated doubling times for Nuttallia musculi, in hours.

BPPPWO	DPPPWO	CONPPPWO	RAPPPPWO
14.03	13.62	24.70	17.55

The estimations for the doubling time of rat passaged-Babesia rodhaini in mice derived from prefreeze blood regressions in Experiment 3 range from 10.18 to 18.45 hours. It is interesting to note that the prefreeze estimates in Experiment 10 are similar for Babesia microti in mice at 14.03 and 16.10 hours and for Nuttallia musculi in mice at 14.03 and 13.62 hours. In Experiment 3, cryopreservation using rapid cooling appeared, in general, to reduce the doubling time of Babesia rodhaini in mice. In Experiment 10, cryopreservation using rapid cooling had apparently little effect on the doubling time of Babesia microti in mice and lengthened the doubling time of Nuttallia musculi in mice. Slow cooling resulted in an increase in the doubling time by a factor of almost two for both parasites.

The values for the doubling time of Babesia rodhaini obtained in Experiment 9 reflect the suggested differences between the parasite used in that experiment and that used in other experiments (e.g. Experiment 3). The estimations of doubling time of Babesia rodhaini in mice obtained from prefreeze blood regressions, are markedly lower than those obtained from the corresponding regressions in Experiment 3, ranging as they do from 5.72 to 6.31 hours. It has been suggested above that the parasite used in Experiment 9 was non-adapted, or had been de-adapted to the rat and mouse. It was further suggested that this de-adaptation was manifested by the existence of 'lag' and 'pathological changes' phases. It appears,

from the figures above, that this de-adaptation is certainly not manifested, as might be expected, as an increase in doubling time, but rather is manifested as a decrease in doubling time. The doubling times derived from the slopes of three of the four post-thaw regressions are greater than the doubling times derived from the prefreeze regressions, in contrast with the trend towards a decrease in doubling time noted in Experiment 3.

In view of the many differences noted between the results obtained in Experiments 3 and 9, it is suggested that a study of the relationship between log. dose on one hand and prepatent period or survival time on the other hand for non-adapted, or de-adapted, parasites would be valuable inasmuch as it might allow the development of an infectivity test generally applicable to adapted, non-adapted or de-adapted parasites.

The results of this study have indicated that Babesia rodhaini-infected rat blood, B. microti-infected and Nuttallia musculi-infected mouse blood survive cryopreservation by a simple method involving the use of 10% final concentration DMSO as cryoprotectant and rapid cooling produced by plunging the material, in ampoules or in pp60 polythene tubing, directly into liquid nitrogen. This successful technique has been developed for volumes of infected blood of 1-2 ml. The use of pp60 in this study was intended as an experimental tool as it allowed rapid cooling and rapid thawing. Using the method described above of winding it on to metal templates, routine use of pp60 could prove cumbersome and expensive in storage space, and as a result it is suggested that, if large volumes of material are to be stored, they should be stored in heat-sealed glass ampoules, rapidly cooled.

It is possible to adapt the methods above for small volumes of heavily infected materials (up to 25 microlitres) either by cutting the pp60 into suitable lengths (Joyner, 1971) or by using heat-sealed capillary lymph tubes. It is extremely important, however, that when using glass capillary lymph tubes, they be not filled more than one third full, or they will almost certainly break upon plunging into liquid nitrogen. This method of stabilation, involving glass capillary lymph tubes, was used successfully for the stabilate of Nuttallia musculi, TREU 1189, used in Experiment 10.

Frerichs and Holbrook (1970) showed that Babesia rodhaini, B. microti and B. canis, protected with 11.6% glycerol were infective after cryopreservation by a method in which the material, in heat-sealed glass ampoules, was plunged directly into liquid nitrogen. No indication of the extent of this infectivity was given. Dalgliesh (1972b) indicated that he and Mellors had found that rapid rates of cooling were probably more successful in the cryopreservation of DMSO-protected Babesia rodhaini-infected mouse blood than slow rates of cooling. Dalgliesh and Mellors (1974), however, dealt with the cryopreservation of Babesia bigemina-infected bovine blood, and described the results obtained with several widely different cooling rates, from 3070°/min. to 0.73°/min. For DMSO-protected, Babesia bigemina-infected bovine blood, Dalgliesh and Mellors (1974) found an optimum cooling rate of 82°/min.

Thus there is an increasing amount of evidence to indicate that bloods infected with Babesia spp. should preferably be cooled rapidly during cryopreservation. Production of rates of cooling as exact as the 82°/min. recommended by Dalgliesh and Mellors is likely to

prove difficult in the absence of special equipment designed to produce such rates and, therefore, the author recommends that the infected blood, suitably protected (e.g. with 10% DMSO) and in suitable containers (e.g. heat-sealed glass ampoules or capillary tubes) be rapidly cooled simply by plunging into liquid nitrogen. The evidence from this study suggests that a survival of up to 4% of the total numbers of infective organisms present before cryopreservation would be achieved using this simple technique. It should be noted that rapid thawing (e.g. by agitation in a heated water bath) is, however, essential when using such a method.

Without doubt the most significant finding arising from this study is that which indicates that rapidly cooled DMSO-protected Babesia rodhaini-infected rat blood, Babesia microti-infected mouse blood and Nuttallia musculi-infected mouse blood are less damaged than the same bloods cooled slowly. This finding has, with regard to Babesia rodhaini-infected rat blood, been confirmed several times during the study. The universal applicability of such a cryopreservation method involving rapid cooling is now suggested for bloods infected with Babesia spp.

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APPENDIX

TABLE 1 EXPERIMENT 1

The results of a Duncan Multiple Range Test applied to the data obtained in Experiment 1. The labels used are:

Label	Treatment
PREFRE	Prefreeze control group
GAMCON	Glycerol/Ampoule/Conventional rate
GPPCON	Glycerol/pp60/Conventional
GAMRAP	Glycerol/Ampoule/Rapid rate
GPPRAP	Glycerol/pp60/Rapid
PAMCON	PVP/Ampoule/Conventional
PPPCON	PVP/pp60/Conventional
PAMRAP	PVP/Ampoule/Rapid
PPPRAP	PVP/pp60/Rapid
DAMCON	DMSO/Ampoule/Conventional
DPPCON	DMSO/pp60/Conventional
DAMRAP	DMSO/Ampoule/Rapid
DPPRAP	DMSO/pp60/Rapid
OAMCON	Zero cryoprotectant (ABP8.0 only)/ Ampoule/Conventional
OPPCON	Zero/pp60/Conventional
OAMRAP	Zero/Ampoule/Rapid
OPPRAP	Zero/pp60/Rapid

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	57205.4883	16	3575.3430	392.2405
WITHIN GROUPS	592.4866	65.	9.1152	
TOTAL	57797.9727	81.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRE	3.000	0.8200	4.	1
2	GAMCON	71.200	4.9200	5.	16
3	GPPCON	61.000	3.0000	5.	13
4	GAMRAP	42.400	3.1300	5.	10
5	GPPRAP	35.800	2.3900	5.	9
6	PAMCON	11.750	2.6300	4.	6
7	PPPCON	10.200	2.6800	5.	4
8	PAMRAP	15.800	3.5600	5.	7
9	PPPRAP	10.800	0.8400	5.	5
10	DAMCON	77.400	2.6100	5.	17
11	DPPCON	70.600	2.1900	5.	15
12	DAMRAP	7.200	1.7900	5.	3
13	DPPRAP	6.000	0.7100	5.	2
14	OAMCON	30.750	2.6300	4.	8
15	OPPCON	61.000	6.3600	5.	12
16	OAMRAP	68.000	1.8700	5.	14
17	OPPRAP	51.800	2.9500	5.	11

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRE	3.000	4.	1
2	DPPRAP	6.000	5.	13
3	DAMRAP	7.200	5.	12
4	PPPCON	10.200	5.	7
5	PPPRAP	10.800	5.	9
6	PAMCON	11.750	4.	6
7	PAMRAP	15.800	5.	8
8	OAMCON	30.750	4.	14
9	GPPRAP	35.800	5.	5
10	GAMRAP	42.400	5.	4
11	OPPRAP	51.800	5.	17
12	OPPCON	61.000	5.	15
13	GPPCON	61.000	5.	3
14	OAMRAP	68.000	5.	16
15	DPPCON	70.600	5.	11
16	GAMCON	71.200	5.	2.
17	DAMCON	77.400	5.	10

COMPUTED RANGES FOR ALPHA=0.05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO. OF GROUPS IN SUBSET	RANGE
2	2.8266
3	2.9722
4	3.0676
5	3.1363
6	3.1937
7	3.2403
8	3.2783
9	3.3095
10	3.3355
11	3.3573
12	3.3758
13	3.3917
14	3.4054
15	3.4174
16	3.4279
17	3.4371

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(OAMRAP , DPPCON , GAMCON)

(PREFRE , DPPRAP , DAMRAP)

(DAMRAP , PPPCON , PPPRAP)

(PPPCON , PPPRAP , PAMCON)

(OPPCON , GPPCON)

PAm AnP

TABLE 2 EXPERIMENT 2

The results of a Duncan Multiple Range Test on the data obtained from part 1 of Experiment 2. The labels used are:

Label	Treatment
PREFRO	Prefreeze undiluted control blood
PREFRP	Prefreeze, PVP-diluted control blood
OPPCON	Zero Cryoprotectant (ABP8.0 only)/ pp60/Conventional rate
OAMCON	Zero/Ampoule/Conventional
OPPRAP	Zero/pp60/Rapid rate
OAMRAP	Zero/Ampoule/Rapid
PPPCON	PVP/pp60/Conventional
PAMCON	PVP/Ampoule/Conventional
PPPRAP	PVP/pp60/Rapid
PAMRAP	PVP/Ampoule/Rapid

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	9310.9922	9	1034.5547	75.3130
WITHIN GROUPS	453.3123	33.	13.7367	
TOTAL	9764.3008	42.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	0.800	0.2100	5.	2
2	PREFRP	0.0	0.0	5.	1
3	OPPCON	13.900	0.4400	4.	3
4	OAMCON	18.100	4.4300	3.	4
5	OPPRAP	49.700	2.4500	4.	10
6	OAMRAP	21.700	1.5900	3.	5
7	PPPCON	28.680	6.2500	5.	8
8	PAMCON	26.250	6.0000	4.	7
9	PPPRAP	22.600	1.3800	5.	6
10	PAMRAP	36.040	5.4400	5.	9

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRP	0.0	5.	2
2	PREFRO	0.800	5.	1
3	OPPCON	13.900	4.	3
4	OAMCON	18.100	3.	4
5	OAMRAP	21.700	3.	6
6	PPPRAP	22.600	5.	9
7	PAMCON	26.250	4.	8
8	PPPCON	28.680	5.	7
9	PAMRAP	36.040	5.	10
10	OPPRAP	49.700	4.	5

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(OAMCON , OAMRAP , PPPRAP)

(PREFRP , PREFRO)

(OPPCON , OAMCON)

(OAMRAP , PPPRAP , PAMCON)

(PAMCON , PPPCON)

TABLE 3 EXPERIMENT 2

The results of a Duncan Multiple Range Test applied to the data obtained from part 2 of Experiment 2. The labels used are:

Label	Treatment
PREDIL	Prefreeze, undiluted control blood
PRE11N	Prefreeze control blood diluted with undialysed 11000 M.Wt. PVP
PRE11D	Prefreeze control blood diluted with dialysed 11000 M.Wt. PVP
PRE24N	Prefreeze control blood diluted with undialysed 24500 M.Wt. PVP
PRE24D	Prefreeze control blood diluted with dialysed 24500 M.Wt. PVP
PRE44N	Prefreeze control blood diluted with undialysed 44000 M.Wt. PVP
PRE44D	Prefreeze control blood diluted with dialysed 44000 M.Wt. PVP
11NPPC	11000 PVP, Undialysed/pp60/Conventional rate
11NAMC	11000 PVP, Undialysed/Ampoule/Conventional
11NPPR	11000 PVP, Undialysed/pp60/Rapid rate
11NAMR	11000 PVP, Undialysed/Ampoule/Rapid
11DPPC	11000 PVP, Dialysed/pp60/Conventional
11DAMC	11000 PVP, Dialysed/Ampoule/Conventional
11DPPR	11000 PVP, Dialysed/pp60/Rapid
11DAMR	11000 PVP, Dialysed/Ampoule/Rapid

Label	Treatment
24NPPC	24500 PVP, Undialysed/pp60/Conventional
24NAMC	24500 PVP, Undialysed/Ampoule/Conventional
24NPPR	24500 PVP, Undialysed/pp60/Rapid
24NAMR	24500 PVP, Undialysed/Ampoule/Rapid
24DPPC	24500 PVP, Dialysed/pp60/Conventional
24DAMC	24500 PVP, Dialysed/Ampoule/Conventional
24DPPR	24500 PVP, Dialysed/pp60/Rapid
24DAMR	24500 PVP, Dialysed/Ampoule/Rapid
44NPPC	44000 PVP, Undialysed/pp60/Conventional
44NAMC	44000 PVP, Undialysed/Ampoule/Conventional
44NPPR	44000 PVP, Undialysed/pp60/Rapid
44NAMR	44000 PVP, Undialysed/Ampoule/Rapid
44DPPC	44000 PVP, Dialysed/pp60/Conventional
44DAMC	44000 PVP, Dialysed/Ampoule/Conventional
44DPPR	44000 PVP, Dialysed/pp60/Rapid
44DAMR	44000 PVP, Dialysed/Ampoule/Rapid

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	10446.2656	30	348.2087	50.5419
WITHIN GROUPS	606.2761	88.	6.8895	
TOTAL	11052.5391	118.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREDIL	1.050	0.1000	4.	1
2	PRE11N	1.080	0.8000	4.	2
3	PRE11D	1.380	0.4800	4.	4
4	PRE24N	3.100	0.4100	4.	8
5	PRE24D	1.400	0.3500	4.	5
6	PRE44N	3.000	1.1600	4.	7
7	PRE44D	1.200	0.8000	4.	3
8	11NPPC	12.560	1.6400	4.	17
9	11NAMC	16.700	5.1100	4.	21
10	11NPPR	7.300	3.3100	3.	12
11	11NAMR	7.250	1.2800	4.	11
12	11DPPC	19.700	0.9000	4.	24
13	11DAMC	28.950	1.2000	2.	29
14	11DPPR	25.900	6.4400	4.	28
15	11DAMR	32.600	0.5500	3.	31
16	24NPPC	10.100	2.1600	4.	15
17	24NAMC	13.480	5.1900	4.	18
18	24NPPR	2.950	0.9500	4.	6
19	24NAMR	5.080	0.8000	4.	10
20	24DPPC	16.700	1.2400	4.	20
21	24DAMC	21.280	2.5900	4.	25
22	24DPPR	17.380	3.0300	4.	22
23	24DAMR	24.200	2.0500	4.	27
24	44NPPC	8.230	1.4300	4.	13
25	44NAMC	10.680	1.7500	4.	16
26	44NPPR	3.330	1.1600	4.	9
27	44NAMR	9.030	1.1000	3.	14
28	44DPPC	17.500	0.6600	4.	23
29	44DAMC	16.430	1.0900	4.	19
30	44DPPR	22.450	5.3000	4.	26
31	44DAMR	32.480	5.1900	4.	30

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREDIL	1.050	4.	1
2	PRE11N	1.080	4.	2
3	PRE44D	1.200	4.	7
4	PRE11D	1.380	4.	3
5	PRE24D	1.400	4.	5
6	24NPPR	2.950	4.	18
7	PRE44N	3.000	4.	6
8	PRE24N	3.100	4.	4
9	44NPPR	3.300	4.	26
10	24NAMR	5.080	4.	19
11	11NAMR	7.250	4.	11
12	11NPPR	7.300	3.	10
13	44NPPC	8.230	4.	24
14	44NAMR	9.030	3.	27
15	24NPPC	10.100	4.	16
16	44NAMC	10.680	4.	25
17	11NPPC	12.560	4.	8
18	24NAMC	13.480	4.	17
19	44DAMC	16.430	4.	29
20	24DPPC	16.700	4.	20
21	11NAMC	16.700	4.	9
22	24DPPR	17.380	4.	22
23	44DPPC	17.500	4.	28
24	11DPPC	19.700	4.	12
25	24DAMC	21.280	4.	21
26	44DPPR	22.450	4.	30
27	24DAMR	24.200	4.	23
28	11DPPR	25.900	4.	14
29	11DAMC	28.950	2.	13
30	44DAMR	32.480	4.	31
31	11DAMR	32.600	3.	15

THERE ARE 16 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PREDIL , PRE11N , PRE44D , PRE11D , PRE24D , 24NPPR , PRE44N , PRE24N , 44NPPR , 24NAMR)

(24NAMC , 44DAMC , 24DPPC , 11NAMC , 24DPPR , 44DPPC)

(11NAMR , 11NPPR , 44NPPC , 44NAMR , 24NPPC , 44NAMC)

(44DAMC , 24DPPC , 11NAMC , 24DPPR , 44DPPC , 11DPPC)

(24NPPR , PRE44N , PRE24N , 44NPPR , 24NAMR , 11NPPR)

(24NAMR , 11NAMR , 11NPPR , 44NPPC , 44NAMR)

(24DPPR , 44DPPC , 11DPPC , 24DAMC)

(44NPPR , 24NAMR , 11NAMR , 11NPPR)

(24NPPC , 44NAMC , 11NPPC , 24NAMC)

(44NAMR , 24NPPC , 44NAMC , 11NPPC)

(11NPPC , 24NAMC , 44DAMC)

(11DAMC , 44DAMR , 11DAMR)

(44DPPR , 24DAMR , 11DPPR)

(11DPPC , 24DAMC , 44DPPR)

(24DAMR , 11DPPR , 11DAMC)

(24DAMC , 44DPPR , 24DAMR)

TABLE 4 EXPERIMENT 2

The results of a Duncan Multiple Range Test applied to the data obtained from part 3 of Experiment 2. The labels used are:

Label	Treatment
PREDIL	Prefreeze undiluted control blood
PREF5N	Prefreeze control blood diluted with 5% undialysed 24500 M.Wt. PVP
PREF5D	Prefreeze control blood diluted with 5% dialysed 24500 M.Wt. PVP
PRE10N	Prefreeze control blood diluted with 10% undialysed 24500 M.Wt. PVP
PRE10D	Prefreeze control blood diluted with 10% dialysed 24500 M.Wt. PVP
PRE15N	Prefreeze control blood diluted with 15% undialysed 24500 M.Wt. PVP
PRE15D	Prefreeze control blood diluted with 15% dialysed 24500 M.Wt. PVP
THAW5N	Frozen and thawed blood protected with 5% undialysed 24500 M.Wt. PVP
THAW5D	Frozen and thawed blood protected with 5% dialysed 24500 M.Wt. PVP
THA10N	Frozen and thawed blood protected with 10% undialysed 24500 M.Wt. PVP
THA10D	Frozen and thawed blood protected with 10% dialysed 24500 M.Wt. PVP
THA15N	Frozen and thawed blood protected with 15% undialysed 24500 M.Wt. PVP
THA15D	Frozen and thawed blood protected with 15% dialysed 24500 M.Wt. PVP

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	3797.9402	12	316.4949	134.9201
WITHIN GROUPS	105.5608	45.	2.3458	
TOTAL	3903.5010	57.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREDIL	2.250	0.4700	4.	7
2	PREF5N	0.800	0.6500	4.	3
3	PREF5D	0.600	0.7700	4.	1
4	PRE10N	1.600	0.6500	4.	4
5	PRE10D	0.800	0.6500	4.	2
6	PRE15N	1.650	1.1900	4.	5
7	PRE15D	1.750	1.4500	4.	6
8	THAW5N	18.300	2.3400	5.	11
9	THAW5D	21.700	2.1500	5.	13
10	THA10N	21.100	2.4000	5.	12
11	THA10D	5.220	0.7200	5.	9
12	THA15N	3.000	0.9600	5.	8
13	THA15D	11.680	2.1100	5.	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREF5D	0.600	4.	3
2	PRE10D	0.800	4.	5
3	PREF5N	0.800	4.	2
4	PRE10N	1.600	4.	4
5	PRE15N	1.650	4.	6
6	PRE15D	1.750	4.	7
7	PREDIL	2.250	4.	1
8	THA15N	3.000	5.	12
9	THA10D	5.220	5.	11
10	THA15D	11.680	5.	13
11	THAW5N	18.300	5.	8
12	THA10N	21.100	5.	10
13	THAW5D	21.700	5.	9

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PREF5D , PRE10D , PREF5N , PRE10N , PRE15N , PRE15D , PREDIL)

(PRE10D , PREF5N , PRE10N , PRE15N , PRE15D , PREDIL , THA15N)

(THA10N , THAW5D)

TABLE 5 EXPERIMENT 3

The results of the prefreeze and post-thaw infectivity titrations, with and without data trimming. The labels used in Tables 5-31 are:

Label	Regression
	(1) Prefreeze bloods
BPPPWI	Buffer only diluted/prepatent period/ untrimmed data
BPPPWO	Buffer only diluted/prepatent period/ trimmed data
BSTWI	Buffer only diluted/survival time/untrimmed
BSTWO	Buffer only diluted/survival time/trimmed
DPPPWI	DMSO-diluted/prepatent period/untrimmed
DPPPWO	DMSO-diluted/prepatent period/trimmed
DSTWI	DMSO-diluted/survival time/untrimmed
DSTWO	DMSO-diluted/survival time/trimmed
GPPPWI	Glycerol-diluted/prepatent period/untrimmed
GPPPWO	Glycerol-diluted/prepatent period/trimmed
GSTWI	Glycerol-diluted/survival time/untrimmed
GSTWO	Glycerol-diluted/survival time/untrimmed
PPPPWI	PVP-diluted/prepatent period/untrimmed
PPPPWO	PVP-diluted/prepatent period/trimmed
PSTWI	PVP-diluted/survival time/untrimmed
PSTWO	PVP-diluted/survival time/trimmed

Label	Regression
	(2) Post-thaw bloods
G2PPPWI	Glycerol-protected/prepatent period/untrimmed
G2PPPWO	Glycerol-protected/prepatent period/trimmed
G2STWI	Glycerol-protected/survival time/untrimmed
G2STWO	Glycerol-protected/survival time/trimmed
D2PPPWI	DMSO-protected/prepatent period/untrimmed
D2PPPWO	DMSO-protected/prepatent period/trimmed
D2STWI	DMSO-protected/survival time/untrimmed
D2STWO	DMSO-protected/survival time/trimmed

r = the computed correlation coefficient

n = the number of observations

b = the slope of the regression line and m = its constant

All 'r' values are significant at p = 0.05, for n-2 degrees of freedom.

	BPPPWI	GPPPWI	DPPPWI	PPPPWI	BPPPWO	GPPPWO	DPPPWO	PPPPWO	G2PPPWI	D2PPPWI	G2PPPWO	D2PPPWO
r	-0.6541	-0.5553	-0.9025	-0.6077	-0.9695	-0.9686	-0.9403	-0.8492	-0.4029	-0.6429	-0.8067	-0.7217
n	39	35	34	40	35	31	30	36	22	28	19	25
b	-1.9113	-2.2461	-2.1928	-1.1400	-1.9004	-2.2404	-2.1736	-1.4111	-1.0548	-1.5555	-1.2562	-1.7264
m	14.6907	17.8923	17.7642	10.2700	14.5363	17.6707	17.3402	11.4000	13.5738	14.8888	14.3687	15.7075
7	1.3113	2.1692	2.4145	2.2900	1.2337	1.9878	2.1250	1.5222	6.1898	4.0000	5.5750	3.6226
5	5.1342	6.6618	6.8002	4.5700	5.0348	6.4687	6.4722	4.3445	8.2998	7.1113	8.0877	7.0755
4	7.0453	8.9076	8.9929	5.7100	6.9348	8.7090	8.6458	5.7555	9.3546	8.6668	9.3439	8.8019

	BSTWI	GSTWI	DSTWI	PSTWI	BSTWO	GSTWO	DSTWO	PSTWO	G2STWI	D2STWI	G2STWO	D2STWO
r	-0.8277	-0.8623	-0.9005	-0.7809	-0.9294	-0.9267	-0.9365	-0.8752	-0.8512	-0.5441	-0.8594	-0.5909
n	39	31	32	37	35	27	28	33	19	28	16	25
b	-2.0850	-2.2818	-2.5000	-1.7101	-2.0792	-2.2221	-2.5472	-1.6185	-1.3043	-1.5555	-1.3277	-1.6674
m	21.9304	23.7061	23.3750	19.2016	21.5223	23.1267	25.3805	18.3132	20.6956	20.8880	20.756	21.3514
7	7.3354	7.7330	5.8750	7.2302	6.9680	7.5722	7.5497	6.9834	11.5652	10.0000	11.462	9.6792
5	11.5054	12.2971	10.8750	10.6516	11.1268	12.0167	12.6485	10.2207	14.1741	13.1105	14.1175	13.0144
4	13.5902	14.5786	13.3750	12.3608	13.2056	14.2384	15.1915	11.8390	15.4784	14.6660	15.4452	14.6818

TABLE 6 EXPERIMENT 3

The results of a comparison of linear regression between BPPPWI (Gp 1) and BPPPWO (Gp 2), using group means.

GP 1 NO PTS:4

1	X :7.0	Y :1.30	
2	X :6.0	Y :3.20	
3	X :5.0	Y :5.22	
4	X :4.0	Y :7.00	

R SS	18.2786	MS	18.2786	F 3594.6300
E SS	0.0102	MS	0.0051	
T SS	18.2888			
SLOPE-	1.9120			

GP 2 NO PTS:4

1	X :7.0	Y :1.22	
2	X :6.0	Y :3.11	
3	X :5.0	Y :5.13	
4	X :4.0	Y :6.88	

R SS	18.0500	MS	18.0500	F 2791.5600
E SS	0.0129	MS	0.0065	
T SS	18.0629			
SLOPE-	1.9000			

C R DF	1.0000	SS	36.3282	MS	36.3282	F 6290.1100
CON DF	1.0000	SS	0.0181	MS	0.0181	F 3.1281
SL DF	1.0000	SS	0.0004	MS	0.0004	F 0.0727
ERR DF	4.0000	SS	0.0231	MS	0.0058	

TABLE 7 EXPERIMENT 3

The results of a comparison of linear regressions between BSTWI (Gp 1) and BSTWO (Gp 2), using group means.

GP 1 NO PTS:4

1	X :7.0	Y :7.80
2	X :6.0	Y :8.90
3	X :5.0	Y :11.10
4	X :4.0	Y :14.00

R SS	21.6320	MS	21.6320	F	52.8832
E SS	0.8181	MS	0.4091		
T SS	22.4501				
SLOPE-	2.0800				

GP 2 NO PTS:4

1	X :7.0	Y :7.22
2	X :6.0	Y :8.77
3	X :5.0	Y :10.88
4	X :4.0	Y :13.45

R SS	21.6320	MS	21.6320	F	165.9720
E SS	0.2607	MS	0.1303		
T SS	21.8926				
SLOPE-	2.0800				

C R DF	1.0000	SS	43.2642	MS	43.2642	F	160.4200
CON DF	1.0000	SS	0.2732	MS	0.2732	F	1.0131
SL DF	1.0000	SS	0.0003	MS	0.0003	F	0.0010
ERR DF	4.0000	SS	1.0788	MS	0.2697		

TABLE 8 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and BSTW0 (Gp 2). The data are shown below:

Gp 1

Dilution Group	Days
10^7	1, 1, 1, 1, 1, 1, 1, 2, 2.
10^6	2, 2, 3, 3, 3, 3, 4, 4, 4.
10^5	5, 5, 5, 5, 5, 5, 5, 6.
10^4	6, 6, 7, 7, 7, 7, 7, 7, 8.

Gp 2

10^7	6, 7, 7, 7, 7, 7, 8, 8, 8.
10^6	8, 8, 8, 8, 8, 9, 10, 10, 10.
10^5	10, 10, 10, 11, 11, 11, 12, 12.
10^4	12, 12, 13, 13, 13, 14, 14, 14, 16.

GP 1 NO PTS:35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				
SLOPE-	1.9004				

GP 2 NO PTS:35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

C R DF	1.0000	SS	354.2940	MS	354.2940	F	573.6460
CON DF	1.0000	SS	630.0000	MS	630.0000	F	1020.0500*
SL DF	1.0000	SS	0.7142	MS	0.7142	F	1.1564
ERR DF	66.0000	SS	40.7628	MS	0.6176		

TABLE 9 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and GPPPW0 (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	1, 2, 2, 2, 2, 2, 2, 3, 3.
10^6	3, 4, 4, 4, 4, 4, 4, 5, 5.
10^5	5, 6, 6, 6, 6, 7, 7, 7, 7.
10^4	9, 9, 9, 9.

GP 1 NO PTS: 35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				
SLOPE-	1.9004				

GP 2 NO PTS: 31

R SS	160.2970	MS	160.2970	F	440.9530
E SS	10.5422	MS	0.3635		
T SS	170.8390				
SLOPE-	2.2404				

C R DF	1.0000	SS	304.9890	MS	304.9890	F	907.2990
CON DF	1.0000	SS	9.2300	MS	9.2300	F	27.4581
SL DF	1.0000	SS	16.8941	MS	16.8941	F	50.2576
ERR DF	62.0000	SS	20.8413	MS	0.3362		

TABLE 10 EXPERIMENT 3

The results of a comparison of linear regressions between BSTWO (Gp 1) and GSTWO (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	7, 7, 7, 8, 8, 8, 8, 8.
10^6	9, 9, 9, 10, 10, 10, 10.
10^5	10, 11, 11, 12, 12, 13, 13, 13, 15.
10^4	14, 14, 14.

GP 1 NO PTS: 35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

GP 2 NO PTS: 27

R SS	134.2280	MS	134.2280	F	152.0570
E SS	22.0688	MS	0.8828		
T SS	156.2970				
SLOPE-	2.2221				

C R DF	1.0000	SS	319.2390	MS	319.2390	F	352.4660
CON DF	1.0000	SS	1.4952	MS	1.4952	F	1.6508
SL DF	1.0000	SS	8.4107	MS	8.4107	F	9.2861
ERR DF	58.0000	SS	52.5324	MS	0.9057		

TABLE 11 EXPERIMENT 3

The results of a comparison of linear regressions between GPPPWO (Gp 1) and GSTWO (Gp 2). The data are shown facing Tables 9 and 10 respectively.

GP 1 NO PTS: 31

R SS	160.2970	MS	160.2970	F	440.9530
E SS	10.5422	MS	0.3635		
T SS	170.8390				
SLOPE-	2.2404				

GP 2 NO PTS: 27

R SS	134.2280	MS	134.2280	F	152.0570
E SS	22.0688	MS	0.8828		
T SS	156.2970				
SLOPE-	2.2221				

C R DF	1.0000	SS	294.9440	MS	294.9440	F	488.3940
CON DF	1.0000	SS	445.9060	MS	445.9060	F	738.3700
SL DF	1.0000	SS	0.4191	MS	0.4191	F	0.6939
ERR DF	54.0000	SS	32.6109	MS	0.6039		

TABLE 12 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and DPPPW0 (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	1, 1, 1, 2, 2, 2, 2, 2, 3.
10^6	3, 4, 5, 5, 5, 5, 5, 5, 6.
10^5	6, 6, 6, 6, 6, 7, 7, 7, 8.
10^4	8, 8, 8.

GP 1 NO PTS:35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				
SLOPE-	1.9004				

GP 2 NO PTS:30

R SS	136.0670	MS	136.0670	F	214.0440
E SS	17.7995	MS	0.6357		
T SS	153.8670				
SLOPE-	2.1736				

C R DF	1.0000	SS	278.9460	MS	278.9460	F	605.5700
CON DF	1.0000	SS	7.3863	MS	7.3863	F	16.0350
SL DF	1.0000	SS	18.7074	MS	18.7074	F	40.6123
ERR DF	61.0000	SS	28.0987	MS	0.4606		

TABLE 13 EXPERIMENT 3

The results of a comparison of linear regressions between BSTWO (Gp 1) and DSTWO (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	7, 7, 7, 7, 7, 7, 8, 8, 8.
10^6	9, 9, 10, 10, 11, 12, 12.
10^5	11, 12, 12, 12, 13, 13, 13, 14, 15.
10^4	14, 15, 15.

GP 1 NO PTS:35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

GP 2 NO PTS:28

R SS	186.3120	MS	186.3120	F	185.4810
E SS	26.1165	MS	1.0045		
T SS	212.4290				
SLOPE-	2.5472				

C R DF	1.0000	SS	359.1940	MS	359.1940	F	374.5560
CON DF	1.0000	SS	5.3379	MS	5.3379	F	5.5662
SL DF	1.0000	SS	20.5406	MS	20.5406	F	21.4191
ERR DF	59.0000	SS	56.5802	MS	0.9590		

TABLE 14 EXPERIMENT 3

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and DSTW0 (Gp 2). The data are shown facing Tables 12 and 13 respectively.

GP 1 NO PTS: 30

R SS	136.0670	MS	136.0670	F	214.0440
E SS	17.7995	MS	0.6357		
T SS	153.8670				

SLOPE- 2.1736

GP 2 NO PTS: 28

R SS	186.3120	MS	186.3120	F	185.4810
E SS	26.1165	MS	1.0045		
T SS	212.4290				

SLOPE- 2.5472

C R DF	1.0000	SS	326.1500	MS	326.1500	F	401.0400
CON DF	1.0000	SS	498.2320	MS	498.2320	F	612.6360
SL DF	1.0000	SS	3.7708	MS	3.7708	F	4.6367
ERR DF	54.0000	SS	43.9160	MS	0.8133		

TABLE 15 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and PPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 8.

Gp 2

10^7	1, 1, 1, 1, 2, 2, 2, 2, 2.
10^6	2, 3, 3, 3, 3, 3, 3, 4, 4.
10^5	3, 4, 4, 4, 4, 4, 4, 4, 4
10^4	5, 5, 5, 6, 6, 6, 7, 8, 6

GP 1 NO PTS:35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				

SLOPE- 1.9004

GP 2 NO PTS:36

R SS	89.6055	MS	89.6055	F	182.4290
E SS	16.7001	MS	0.4912		
T SS	106.3060				

SLOPE- 1.4111

C R DF	1.0000	SS	245.4600	MS	245.4600	F	609.1200
CON DF	1.0000	SS	3.1046	MS	3.1046	F	7.7041
SL DF	1.0000	SS	5.7321	MS	5.7321	F	14.2246
ERR DF	67.0000	SS	26.9993	MS	0.4030		

TABLE 16 EXPERIMENT 3

The results of a comparison of linear regressions between BSTWO (Gp 1) and PSTWO (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	7, 7, 7, 7, 7, 7, 7, 7.
10^6	7, 8, 8, 8, 8, 9, 10, 12.
10^5	9, 9, 9, 10, 10, 10, 11, 11.
10^4	11, 11, 11, 11, 12, 12, 13, 13, 14.

GP 1 NO PTS:35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

GP 2 NO PTS:33

R SS	110.5010	MS	110.5010	F	101.5240
E SS	33.7412	MS	1.0884		
T SS	144.2430				
SLOPE-	1.6185				

C R DF	1.0000	SS	296.9580	MS	296.9580	F	296.0100
CON DF	1.0000	SS	5.5630	MS	5.5630	F	5.5453
SL DF	1.0000	SS	6.9657	MS	6.9657	F	6.9435
ERR DF	64.0000	SS	64.2049	MS	1.0032		

TABLE 17 EXPERIMENT 3

The results of a comparison of linear regressions between PPPPWO (Gp 1) and PSTWO (Gp 2). The data are shown facing Tables 15 and 16 respectively.

GP 1 NO PTS:36

R SS	89.6055	MS	89.6055	F	182.4290
E SS	16.7001	MS	0.4912		
T SS	106.3060				
SLOPE-	1.4111				

GP 2 NO PTS:33

R SS	110.5010	MS	110.5010	F	101.5240
E SS	33.7412	MS	1.0884		
T SS	144.2430				
SLOPE-	1.6185				

C R DF	1.0000	SS	213.1520	MS	213.1520	F	274.6740
CON DF	1.0000	SS	562.3170	MS	562.3170	F	724.6170
SL DF	1.0000	SS	13.0454	MS	13.0454	F	16.8106
ERR DF	65.0000	SS	50.4413	MS	0.7760		

TABLE 18 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and D2PPPW0 (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	2, 2, 2, 3, 3, 3, 6, 7, 7.
10^6	4, 4, 5, 5, 5, 5, 5, 5.
10^5	6, 7, 7, 7, 8, 8, 8, 8.
10^4	- = No animals infected.

GP 1 NO PTS:35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				
SLOPE-	1.9004				

GP 2 NO PTS:25

R SS	50.5481	MS	50.5481	F	25.0066
E SS	46.4920	MS	2.0214		
T SS	97.0400				
SLOPE-	1.7264				

C R DF	1.0000	SS	167.5090	MS	167.5090	F	165.1760
CON DF	1.0000	SS	21.8077	MS	21.8077	F	21.5039
SL DF	1.0000	SS	44.6254	MS	44.6254	F	44.0037
ERR DF	56.0000	SS	56.7911	MS	1.0141		

TABLE 19 EXPERIMENT 3

The results of a comparison of linear regressions between BSTWO (Gp 1) and D2STWO (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	8, 8, 8, 8, 9, 12, 13, 13, 14.
10^6	9, 9, 10, 10, 10, 10, 10, 11.
10^5	12, 13, 14, 14, 14, 14, 14, 15.
10^4	-

GP 1 NO PTS: 35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

GP 2 NO PTS: 25

R SS	47.1543	MS	47.1543	F	12.3404
E SS	87.8858	MS	3.8217		
T SS	135.0400				
SLOPE-	1.6674				

C R DF	1.0000	SS	190.6020	MS	190.6020	F	90.1880
CON DF	1.0000	SS	21.8087	MS	21.8087	F	10.3193
SL DF	1.0000	SS	49.9745	MS	49.9745	F	23.6467
ERR DF	56.0000	SS	118.3490	MS	2.1134		

TABLE 20 EXPERIMENT 3

The results of a comparison of linear regressions between D2PPPW0 (Gp 1) and D2STW0 (Gp 2). The data are shown facing Tables 18 and 19 respectively.

GP 1 NO PTS:25

R SS	50.5481	MS	50.5481	F	25.0066
E SS	46.4920	MS	2.0214		
T SS	97.0400				
SLOPE-	1.7264				

GP 2 NO PTS:25

R SS	47.1543	MS	47.1543	F	12.3404
E SS	87.8858	MS	3.8217		
T SS	135.0400				
SLOPE-	1.6674				

C R DF	1.0000	SS	97.6729	MS	97.6729	F	33.4352
CON DF	1.0000	SS	450.0000	MS	450.0000	F	154.0430
SL DF	1.0000	SS	0.0295	MS	0.0295	F	0.0101
ERR DF	46.0000	SS	134.3780	MS	2.9213		

TABLE 21 EXPERIMENT 3

The results of a comparison of linear regressions between BPPPW0 (Gp 1) and G2PPPW0 (Gp 2). The data are shown below:

Gp 1	As shown facing Table 8.
Gp 2	
10^7	5, 5, 5, 6, 6, 6, 6, 6, 6.
10^6	6, 6, 6, 6, 7, 7, 7, 8.
10^5	8, 9.
10^4	-

GP 1 NO PTS: 35

R SS	161.5870	MS	161.5870	F	517.7470
E SS	10.2992	MS	0.3121		
T SS	171.8860				
SLOPE-	1.9004				

GP 2 NO PTS: 19

R SS	13.2894	MS	13.2894	F	31.6783
E SS	7.1317	MS	0.4195		
T SS	20.4211				
SLOPE-	1.2562				

C R DF	1.0000	SS	81.7897	MS	81.7897	F	234.6120
CON DF	1.0000	SS	65.7859	MS	65.7859	F	188.7050
SL DF	1.0000	SS	93.0863	MS	93.0863	F	267.0160
ERR DF	50.0000	SS	17.4309	MS	0.3486		

TABLE 22 EXPERIMENT 3

The results of a comparison of linear regressions between BSTW0 (Gp 1) and G2STW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 8.

Gp 2

10^7	11, 11, 11, 11, 12, 12, 12.
10^6	12, 12, 13, 13, 13, 13, 14.
10^5	14, 14.
10^4	-

GP 1 NO PTS:35

R SS	193.4220	MS	193.4220	F	209.5260
E SS	30.4637	MS	0.9231		
T SS	223.8860				
SLOPE-	2.0792				

GP 2 NO PTS:16

R SS	13.1113	MS	13.1113	F	39.5715
E SS	4.6387	MS	0.3313		
T SS	17.7500				
SLOPE-	1.3277				

C R DF	1.0000	SS	115.2610	MS	115.2610	F	154.3270
CON DF	1.0000	SS	58.9932	MS	58.9932	F	78.9885
SL DF	1.0000	SS	91.2726	MS	91.2726	F	122.2090
ERR DF	47.0000	SS	35.1023	MS	0.7469		

TABLE 23 EXPERIMENT 3

The results of a comparison of linear regressions between G2PPPW0 (Gp 1) and G2STW0 (Gp 2). The data are shown facing Tables 21 and 22 respectively.

GP 1 NO PTS: 19

R SS	13.2894	MS	13.2894	F	31.6783
E SS	7.1317	MS	0.4195		
T SS	20.4211				
SLOPE-	1.2562				

GP 2 NO PTS: 16

R SS	13.1113	MS	13.1113	F	39.5715
E SS	4.6387	MS	0.3313		
T SS	17.7500				
SLOPE-	1.3277				

C R DF	1.0000	SS	34.3835	MS	34.3835	F	90.5570
CON DF	1.0000	SS	297.4060	MS	297.4060	F	783.2880
SL DF	1.0000	SS	7.9828	MS	7.9828	F	21.0244
ERR DF	31.0000	SS	11.7704	MS	0.3797		

TABLE 24 EXPERIMENT 3

The results of a comparison of linear regressions between GPPPW0 (Gp 1) and DPPPW0 (Gp 2). The data are shown facing Tables 9 and 12 respectively.

GP 1 NO PTS: 31

R SS	160.2970	MS	160.2970	F	440.9530
E SS	10.5422	MS	0.3635		
T SS	170.8390				
SLOPE-	2.2404				

GP 2 NO PTS: 30

R SS	136.0670	MS	136.0670	F	214.0440
E SS	17.7995	MS	0.6357		
T SS	153.8670				
SLOPE-	2.1736				

C R DF	1.0000	SS	296.3310	MS	296.3310	F	595.9720
CON DF	1.0000	SS	0.0816	MS	0.0816	F	0.1641
SL DF	1.0000	SS	0.0331	MS	0.0331	F	0.0665
ERR DF	57.0000	SS	28.3417	MS	0.4972		

TABLE 25 EXPERIMENT 3

The results of a comparison of linear regressions between GSTWO (Gp 1) and DSTWO (Gp 2). The data are shown facing Tables 10 and 13 respectively.

GP 1 NO PTS: 27

R SS	134.2280	MS	134.2280	F	152.0570
E SS	22.0688	MS	0.8828		
T SS	156.2970				
SLOPE-	2.2221				

GP 2 NO PTS: 28

R SS	186.3120	MS	186.3120	F	185.4810
E SS	26.1165	MS	1.0045		
T SS	212.4290				
SLOPE-	2.5472				

C R DF	1.0000	SS	318.0970	MS	318.0970	F	336.6790
CON DF	1.0000	SS	1.0206	MS	1.0206	F	1.0802
SL DF	1.0000	SS	2.4433	MS	2.4433	F	2.5860
ERR DF	51.0000	SS	48.1853	MS	0.9448		

TABLE 26 EXPERIMENT 3

The results of a comparison of linear regressions between GPPPW0 (Gp 1) and G2PPPW0 (Gp 2). The data are shown facing Tables 9 and 21 respectively.

GP 1 NO PTS: 31

R SS	160.2970	MS	160.2970	F	440.9530
E SS	10.5422	MS	0.3635		
T SS	170.8390				
SLOPE-	2.2404				

GP 2 NO PTS: 19

R SS	13.2894	MS	13.2894	F	31.6783
E SS	7.1317	MS	0.4195		
T SS	20.4211				
SLOPE-	1.2562				

C R DF	1.0000	SS	110.8100	MS	110.8100	F	288.4070
CON DF	1.0000	SS	28.7400	MS	28.7400	F	74.8019
SL DF	1.0000	SS	62.7757	MS	62.7757	F	163.3870
ERR DF	46.0000	SS	17.6739	MS	0.3842		

TABLE 27 EXPERIMENT 3

The results of a comparison of linear regressions between GSTWO (Gp 1) and G2STWO (Gp 2). The data are shown facing Tables 10 and 22 respectively.

GP 1 NO PTS:27

R SS	134.2280	MS	134.2280	F	152.0570
E SS	22.0688	MS	0.8828		
T SS	156.2970				
SLOPE-	2.2221				

GP 2 NO PTS:16

R SS	13.1113	MS	13.1113	F	39.5715
E SS	4.6387	MS	0.3313		
T SS	17.7500				
SLOPE-	1.3277				

C R DF	1.0000	SS	91.1059	MS	91.1059	F	133.0390
CON DF	1.0000	SS	40.3721	MS	40.3721	F	58.9541
SL DF	1.0000	SS	56.2336	MS	56.2336	F	82.1161
ERR DF	39.0000	SS	26.7074	MS	0.6848		

TABLE 28 EXPERIMENT 3

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and D2PPPW0 (Gp 2). The data are shown facing Tables 12 and 18 respectively.

GP 1 NO PTS:30

R SS	136.0670	MS	136.0670	F	214.0440
E SS	17.7995	MS	0.6357		
T SS	153.8670				
SLOPE-	2.1736				

GP 2 NO PTS:25

R SS	50.5481	MS	50.5481	F	25.0066
E SS	46.4920	MS	2.0214		
T SS	97.0400				
SLOPE-	1.7264				

C R DF	1.0000	SS	174.3730	MS	174.3730	F	138.3240
CON DF	1.0000	SS	4.0752	MS	4.0752	F	3.2327
SL DF	1.0000	SS	12.2421	MS	12.2421	F	9.7112
ERR DF	51.0000	SS	64.2915	MS	1.2606		

TABLE 29 EXPERIMENT 3

The results of a comparison of linear regressions between DSTWO (Gp 1) and D2STWO (Gp 2). The data are shown facing Tables 12 and 19 respectively.

GP 1 NO PTS:28

R SS	186.3120	MS	186.3120	F	185.4810
E SS	26.1165	MS	1.0045		
T SS	212.4290				
SLOPE-	2.5472				

GP 2 NO PTS:25

R SS	47.1543	MS	47.1543	F	12.3404
E SS	87.8858	MS	3.8217		
T SS	135.0400				
SLOPE-	1.6674				

C R DF	1.0000	SS	211.8490	MS	211.8490	F	91.0559
CON DF	1.0000	SS	5.3633	MS	5.3633	F	2.3053
SL DF	1.0000	SS	21.6179	MS	21.6179	F	9.2917
ERR DF	49.0000	SS	114.0020	MS	2.3266		

TABLE 30 EXPERIMENT 3

The results of a comparison of linear regressions between D2PPPW0 (Gp 1) and G2PPPW0 (Gp 2). The data are shown facing Tables 18 and 21 respectively.

GP 1 NO PTS:25

R SS	50.5481	MS	50.5481	F	25.0066
E SS	46.4920	MS	2.0214		
T SS	97.0400				
SLOPE-	1.7264				

GP 2 NO PTS:19

R SS	13.2894	MS	13.2894	F	31.6783
E SS	7.1317	MS	0.4195		
T SS	20.4211				
SLOPE-	1.2562				

C R DF	1.0000	SS	48.8211	MS	48.8211	F	36.4176
CON DF	1.0000	SS	12.7888	MS	12.7888	F	9.5397
SL DF	1.0000	SS	15.0164	MS	15.0164	F	11.2013
ERR DF	40.0000	SS	53.6237	MS	1.3406		

TABLE 31 EXPERIMENT 3

The results of a comparison of linear regressions between D2STWO (Gp 1) and G2STWO (Gp 2). The data are shown facing Tables 19 and 22 respectively.

GP 1 NO PTS:25

R SS	47.1543	MS	47.1543	F	12.3404
E SS	87.8858	MS	3.8217		
T SS	135.0400				
SLOPE-	1.6674				

GP 2 NO PTS:16

R SS	13.1113	MS	13.1113	F	39.5715
E SS	4.6387	MS	0.3313		
T SS	17.7500				
SLOPE-	1.3277				

C R DF	1.0000	SS	49.4432	MS	49.4432	F	19.7721
CON DF	1.0000	SS	11.6983	MS	11.6983	F	4.6781
SL DF	1.0000	SS	10.8224	MS	10.8224	F	4.3278
ERR DF	37.0000	SS	92.5244	MS	2.5007		

TABLE 32 EXPERIMENT 4

The results of a Duncan Multiple Range Test applied to the percentage haemolysis data obtained in Experiment 4. The labels used in Tables 32-36 are:

Label	Treatment
OPPCON	Zero cryoprotectant (ABP8.0 only)/ pp60/Conventional rate.
OAMCON	Zero/Ampoule/Conventional
OPPRAP	Zero/pp60/Rapid rate
OAMRAP	Zero/Ampoule/Rapid
PPPCON	PVP/pp60/Conventional
PAMCON	PVP/Ampoule/Conventional
PPPRAP	PVP/pp60/Rapid
PAMRAP	PVP/Ampoule/Rapid
DPPCON	DMSO/pp60/Conventional
DAMCON	DMSO/Ampoule/Conventional
DPPRAP	DMSO/pp60/Rapid
DAMRAP	DMSO/Ampoule/Rapid
GPPCON	Glycerol/pp60/Conventional
GAMCON	Glycerol/Ampoule/Conventional
GPPRAP	Glycerol/pp60/Rapid
GAMRAP	Glycerol/Ampoule/Rapid
SPPCON	Sucrose/pp60/Conventional
SAMCON	Sucrose/Ampoule/Conventional
SPPRAP	Sucrose/pp60/Rapid
SAMRAP	Sucrose/Ampoule/Rapid

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	98441.8125	19	5181.1445	570.9568
WITHIN GROUPS	825.7788	91.	9.0745	
TOTAL	99267.5625	110.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	SPPCON	90.200	2.7700	5.	19
2	SPPRAP	86.170	1.4700	6.	15
3	SAMCON	93.200	2.5800	5.	20
4	SAMRAP	82.670	1.2100	6.	14
5	GPPCON	77.400	2.0700	5.	13
6	GPPRAP	15.170	3.4300	6.	2
7	GAMCON	69.170	3.1900	6.	11
8	GAMRAP	32.800	3.0300	5.	6
9	DPPCON	55.200	4.9700	5.	8
10	DPPRAP	15.830	2.0400	6.	3
11	DAMCON	67.830	2.4800	6.	10
12	DAMRAP	23.000	1.7900	6.	5
13	OPPCON	86.800	3.0300	5.	16
14	OPPRAP	88.170	2.6400	6.	18
15	OAMCON	65.400	7.5700	5.	9
16	OAMRAP	87.670	1.2100	6.	17
17	PPPCON	77.200	2.1700	5.	12
18	PPPRAP	7.500	0.5500	6.	1
19	PAMCON	42.400	4.7200	5.	7
20	PAMRAP	17.830	0.7500	6.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PPPRAP	7.500	6.	18
2	GPPRAP	15.170	6.	6
3	DPPRAP	15.830	6.	10
4	PAMRAP	17.830	6.	20
5	DAMRAP	23.000	6.	12
6	GAMRAP	32.800	5.	8
7	PAMCON	42.400	5.	19
8	DPPCON	55.200	5.	9
9	OAMCON	65.400	5.	15
10	DAMCON	67.830	6.	11
11	GAMCON	69.170	6.	7
12	PPPCON	77.200	5.	17
13	GPPCON	77.400	5.	5
14	SAMRAP	82.670	6.	4
15	SPPRAP	86.170	6.	2
16	OPPCON	86.800	5.	13
17	OAMRAP	87.670	6.	16
18	OPPRAP	88.170	6.	14
19	SPPCON	90.200	5.	1
20	SAMCON	93.200	5.	3

THERE ARE 6 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GPPRAP , DPPRAP , PAMRAP)

(OAMCON , DAMCON , GAMCON)

(OPPCON , OAMRAP , OPPRAP , SPPCON)

(SPPRAP , OPPCON , OAMRAP , OPPRAP)

(SPPCON , SAMCON)

(PPPCON , GPPCON)

TABLE 33 EXPERIMENT 4

The results of a Duncan Multiple Range Test applied to the untrimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 4.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	854.0398	17	50.2376	10.8224
WITHIN GROUPS	524.5469	113.	4.6420	
TOTAL	1378.5867	130.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	2.300	1.7700	10.	1
2	PREFRP	2.400	1.3500	10.	2
3	PREFRG	3.670	1.8700	9.	6
4	PREFRD	3.600	2.6800	10.	5
5	PREFRS	7.710	1.1100	7.	13
6	ZERPPR	9.500	0.7100	2.	16
7	PAMCON	9.250	1.8900	4.	15
8	PPPCON	9.000	1.4100	2.	14
9	PAMRAP	7.380	0.5200	8.	11
10	GAMCON	7.110	4.9100	9.	10
11	GPPCON	10.200	4.9200	6.	17
12	GAMRAP	5.000	0.6300	6.	8
13	GPPRAP	4.300	0.6800	10.	7
14	DAMCON	7.440	1.4200	9.	12
15	DPPRAP	2.560	0.7300	9.	3
16	DAMRAP	3.000	1.0000	9.	4
17	DPPCON	6.330	1.2200	9.	9
18	SAMRAP	11.500	2.1200	2.	18

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	2.300	10.	1
2	PREFRP	2.400	10.	2
3	DPPRAP	2.560	9.	15
4	DAMRAP	3.000	9.	16
5	PREFRD	3.600	10.	4
6	PREFRG	3.670	9.	3
7	GPPRAP	4.300	10.	13
8	GAMRAP	5.000	6.	12
9	DPPCON	6.330	9.	17
10	GAMCON	7.110	9.	10
11	PAMRAP	7.380	8.	9
12	DAMCON	7.440	9.	14
13	PREFRS	7.710	7.	5
14	PPPCON	9.000	2.	8
15	PAMCON	9.250	4.	7
16	ZERPPR	9.500	2.	6
17	GPPCON	10.200	6.	11
18	SAMRAP	11.500	2.	18

THERE ARE 6 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GAMRAP , DPPCON , GAMCON , PAMRAP , DAMCON , PREFRS , PPPCON)

(DPPCON , GAMCON , PAMRAP , DAMCON , PREFRS , PPPCON , PAMCON , ZERPPR)

(PREFRO , PREFRP , DPPRAP , DAMRAP , PREFRD , PREFRG , GPPRAP)

(PREFRS , PPPCON , PAMCON , ZERPPR , GPPCON , SAMRAP)

(DPPRAP , DAMRAP , PREFRD , PREFRG , GPPRAP , GAMRAP)

(GPPRAP , GAMRAP , DPPCON)

TABLE 34 EXPERIMENT 4

The results of a Duncan Multiple Range Test applied to the trimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 4.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	516,2634	14	36,8759	43,1187
WITHIN GROUPS	81,2457	95,	0,8552	
TOTAL	597,5090	109,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	1,890	1,2700	9,	1
2	PREFRP	2,000	0,5000	9,	2
3	PREFRG	3,130	0,9900	8,	6
4	PREFRD	2,890	1,5300	9,	5
5	PREFRS	7,500	1,0500	6,	13
6	PAMRAP	7,290	0,4900	7,	12
7	PAMCON	8,330	0,5800	3,	15
8	GAMCON	5,500	0,9300	8,	9
9	GPPCON	8,200	1,1000	5,	14
10	GAMRAP	4,800	0,4500	5,	8
11	GPPRAP	4,110	0,3300	9,	7
12	DAMCON	7,130	1,1300	8,	11
13	DPPRAP	2,500	0,7600	8,	3
14	DAMRAP	2,750	0,7100	8,	4
15	DPPCON	6,000	0,7600	8,	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	1,890	9,	1
2	PREFRP	2,000	9,	2
3	DPPRAP	2,500	8,	13
4	DAMRAP	2,750	8,	14
5	PREFRD	2,890	9,	4
6	PREFRG	3,130	8,	3
7	GPPRAP	4,110	9,	11
8	GAMRAP	4,800	5,	10
9	GAMCON	5,500	8,	8
10	DPPCON	6,000	8,	15
11	DAMCON	7,130	8,	12
12	PAMRAP	7,290	7,	6
13	PREFRS	7,500	6,	5
14	GPPCON	8,200	5,	9
15	PAMCON	8,330	3,	7

THERE ARE 7 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(DAMCON , PAMRAP , PREFRS , GPPCON , PAMCON)

(PREFRO , PREFRP , DPPRAP , DAMRAP)

(DPPRAP , DAMRAP , PREFRD , PREFRG)

(PREFRP , DPPRAP , DAMRAP , PREFRD)

(GAMRAP , GAMCON)

(GPPRAP , GAMRAP)

(GAMCON , DPPCON)

TABLE 35 EXPERIMENT 4

The results of a Duncan Multiple Range Test applied to the untrimmed survival time data obtained from single-dilution infectivity tests in Experiment 4.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	556.7847	16	34.7990	10.1007
WITHIN GROUPS	297.9634	87.	3.4249	
TOTAL	854.7480	103.		

**

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	8.500	2.5900	10.	2
2	PREFRP	8.100	1.8500	10.	1
3	PREFRG	9.430	2.5100	7.	4
4	PREFRD	9.130	2.1600	8.	3
5	PREFRS	13.330	0.5900	3.	10
6	ZERPPR	16.500	0.7100	2.	17
7	PAMCON	15.250	1.5000	4.	16
8	PPPCON	14.500	0.7100	2.	15
9	PAMRAP	13.750	1.0400	8.	13
10	GAMCON	12.430	1.5900	7.	9
11	GPPCON	14.000	0.0	2.	14
12	GAMRAP	10.500	0.8400	6.	8
13	GPPRAP	10.130	0.5900	8.	7
14	DAMCON	13.400	1.1400	5.	12
15	DPPRAP	9.750	2.8100	8.	6
16	DAMRAP	9.440	1.4200	9.	5
17	DPPCON	13.400	2.0700	5.	11

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRP	8.100	10.	2
2	PREFRO	8.500	10.	1
3	PREFRD	9.130	8.	4
4	PREFRG	9.430	7.	3
5	DAMRAP	9.440	9.	16
6	DPPRAP	9.750	8.	15
7	GPPRAP	10.130	8.	13
8	GAMRAP	10.500	6.	12
9	GAMCON	12.430	7.	10
10	PREFRS	13.330	3.	5
11	DPPCON	13.400	5.	17
12	DAMCON	13.400	5.	14
13	PAMRAP	13.750	8.	9
14	GPPCON	14.000	2.	11
15	PPPCON	14.500	2.	8
16	PAMCON	15.250	4.	7
17	ZERPPR	16.500	2.	6

**

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GAMCON , PREFRS , DPPCON , DAMCON , PAMRAP , GPPCON , PPPCON)

(PREFRO , PREFRD , PREFRG , DAMRAP , DPPRAP , GPPRAP , GAMRAP)

(PREFRP , PREFRD , PREFRD , PREFRG , DAMRAP , DPPRAP)

(PREFRS , DPPCON , DAMCON , PAMRAP , GPPCON , PPPCON , PAMCON , ZERPPR)

(GAMRAP , GAMCON)

TABLE 36 EXPERIMENT 4

The results of a Duncan Multiple Range Test applied to the trimmed survival time data obtained from single-dilution infectivity tests in Experiment 4.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	384.2197	13	29.5554	25.8687
WITHIN GROUPS	79.9760	70.	1.1425	
TOTAL	464.1956	83.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	7.890	1.8300	9.	2
2	PREFRP	7.550	0.7300	9.	1
3	PREFRG	8.500	0.5500	6.	3
4	PREFRD	8.570	1.6200	7.	4
5	PREFRS	13.000	0.0	2.	12
6	PAMRAP	13.430	0.5300	7.	13
7	PAMCON	14.670	1.1500	3.	14
8	GAMCON	11.830	1.3300	6.	9
9	GAMRAP	10.200	0.4500	5.	8
10	GPPRAP	9.860	0.6900	7.	7
11	DAMCON	13.000	0.8200	4.	11
12	DPPRAP	8.860	0.6900	7.	5
13	DAMRAP	9.130	1.1300	8.	6
14	DPPCON	12.500	0.5800	4.	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRP	7.550	9.	2
2	PREFRO	7.890	9.	1
3	PREFRG	8.500	6.	3
4	PREFRD	8.570	7.	4
5	DPPRAP	8.860	7.	12
6	DAMRAP	9.130	8.	13
7	GPPRAP	9.860	7.	10
8	GAMRAP	10.200	5.	9
9	GAMCON	11.830	6.	8
10	DPPCON	12.500	4.	14
11	DAMCON	13.000	4.	11
12	PREFRS	13.000	2.	5
13	PAMRAP	13.430	7.	6
14	PAMCON	14.670	3.	7

THERE ARE 7 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GAMCON , DPPCON , DAMCON , PREFRS)

(DPPRAP , DAMRAP , GPPRAP , GAMRAP)

(PREFRO , PREFRG , PREFRD , DPPRAP)

(DPPCON , DAMCON , PREFRS , PAMRAP)

(PREFRP , PREFRO , PREFRG , PREFRD)

(DAMCON , PREFRS , PAMRAP , PAMCON)

(PREFRG , PREFRD , DPPRAP , DAMRAP)

TABLE 37 EXPERIMENT 5

The results of a Duncan Multiple Range Test applied to the percentage haemolysis data obtained in Experiment 5. The labels used in Tables 37-41 are:

Label	Treatment
PREFRD	Prefreeze DMSO-diluted control blood
PREFRG	Prefreeze glycerol-diluted control blood
DAMRAP	DMSO/Ampoule/Rapid rate
DAMCON	DMSO/Ampoule/Conventional rate
DPPRAP	DMSO/pp60/Rapid
DPPCON	DMSO/pp60/Conventional
GAMRAP	Glycerol/Ampoule/Rapid
GAMCON	Glycerol/Ampoule/Conventional
GPPRAP	Glycerol/pp60/Rapid
GPPCON	Glycerol/pp60/Conventional

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	12822.7773	7	1831.8252	248.2464
WITHIN GROUPS	118.0649	16.	7.3791	
TOTAL	12940.8398	23.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	GPPCON	63.330	2.5200	3.	5
2	GPPRAP	16.330	0.5800	3.	2
3	GAMCON	74.000	1.0000	3.	7
4	GAMRAP	47.000	4.5800	3.	3
5	DPPCON	69.670	0.5800	3.	6
6	DPPRAP	15.000	0.0	3.	1
7	DAMCON	78.000	1.7300	3.	8
8	DAMRAP	49.000	5.2000	3.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	DPPRAP	15.000	3.	6
2	GPPRAP	16.330	3.	2
3	GAMRAP	47.000	3.	4
4	DAMRAP	49.000	3.	8
5	GPPCON	63.330	3.	1
6	DPPCON	69.670	3.	5
7	GAMCON	74.000	3.	3
8	DAMCON	78.000	3.	7

THERE ARE 4 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(DPPRAP , GPPRAP)

(GAMRAP , DAMRAP)

(DPPCON , GAMCON)

(GAMCON , DAMCON)

TABLE 38 EXPERIMENT 5

The results of a Duncan Multiple Range Test applied to the untrimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 5.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	565,3667	9	62,8185	34,5744
WITHIN GROUPS	138,0851	76,	1,8169	
TOTAL	703,4517	85,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRD	1,600	1,3500	10,	1
2	PREFRG	2,100	2,1300	10,	2
3	DAMRAP	7,290	2,5600	7,	5
4	DAMCON	8,250	0,7100	8,	8
5	DPPRAP	5,880	0,9900	8,	3
6	DPPCON	7,440	0,5300	9,	7
7	GAMRAP	7,440	1,1300	9,	6
8	GAMCON	8,440	0,8800	9,	9
9	GPPRAP	6,900	0,8800	10,	4
10	GPPCON	9,500	1,0500	6,	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRD	1,600	10,	1
2	PREFRG	2,100	10,	2
3	DPPRAP	5,880	8,	5
4	GPPRAP	6,900	10,	9
5	DAMRAP	7,290	7,	3
6	GAMRAP	7,440	9,	7
7	DPPCON	7,440	9,	6
8	DAMCON	8,250	8,	4
9	GAMCON	8,440	9,	8
10	GPPCON	9,500	6,	10

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GPPRAP , DAMRAP , GAMRAP , DPPCON , DAMCON)

(DAMRAP , GAMRAP , DPPCON , DAMCON , GAMCON)

(DAMCON , GAMCON , GPPCON)

(DPPRAP , GPPRAP , DAMRAP)

(PREFRD , PREFRG)

TABLE 39 EXPERIMENT 5

The results of a Duncan Multiple Range Test applied to the trimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 5.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	572,3103	9	63,5900	35,0875
WITHIN GROUPS	96,0534	53,	1,8123	
TOTAL	668,3635	62,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRD	7,670	2,0000	9,	1
2	PREFRG	7,890	0,9300	9,	2
3	DAMRAP	13,200	1,1000	5,	4
4	DAMCON	14,830	0,7500	6,	8
5	DPPRAP	12,670	1,5100	6,	3
6	DPPCON	14,140	0,3800	7,	6
7	GAMRAP	14,200	1,4800	5,	7
8	GAMCON	15,570	1,4000	7,	10
9	GPPRAP	14,000	1,7900	6,	5
10	GPPCON	15,330	0,5800	3,	9

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRD	7,670	9,	1
2	PREFRG	7,890	9,	2
3	DPPRAP	12,670	6,	5
4	DAMRAP	13,200	5,	3
5	GPPRAP	14,000	6,	9
6	DPPCON	14,140	7,	6
7	GAMRAP	14,200	5,	7
8	DAMCON	14,830	6,	4
9	GPPCON	15,330	3,	10
10	GAMCON	15,570	7,	8

THERE ARE 4 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GPPRAP , DPPCON , GAMRAP , DAMCON , GPPCON , GAMCON)

(DAMRAP , GPPRAP , DPPCON , GAMRAP , DAMCON , GPPCON)

(DPPRAP , DAMRAP , GPPRAP , DPPCON , GAMRAP)

(PREFRD , PREFRG)

TABLE 40 EXPERIMENT 5

The results of a Duncan Multiple Range Test applied to the untrimmed survival time data obtained from single-dilution infectivity tests in Experiment 5.

The computed shortest significant ranges have been omitted.

BMD07V - MULTIPLE RANGE TESTS - REVISEDFEBRUARY 19, 1970
HEALTH SCIENCES COMPUTING FACILITY, UCLA

PROBLEM CODET3STWI
NUMBER OF TREATMENTS GROUPS 10
NUMBER OF VARIABLE FORMAT CARDS 0
DATA INPUT TAPE 5
NUMBER OF COEF CARD(S) 0

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	610.8755	9	67.8750	17.9732
WITHIN GROUPS	237.9167	63.	3.7765	
TOTAL	848.7920	72.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRD	8.400	2.9900	10.	2
2	PREFRG	8.400	1.8400	10.	1
3	DAMRAP	13.330	1.0300	6.	4
4	DAMCON	15.000	0.8200	7.	7
5	DPPRAP	12.860	1.4600	7.	3
6	DPPCON	14.880	2.1000	8.	6
7	GAMRAP	15.170	2.7100	6.	8
8	GAMCON	15.880	1.5500	8.	10
9	GPPRAP	14.290	1.8000	7.	5
10	GPPCON	15.500	0.5800	4.	9

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TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRG	8.400	10.	2
2	PREFRD	8.400	10.	1
3	DPPRAP	12.860	7.	5
4	DAMRAP	13.330	6.	3
5	GPPRAP	14.290	7.	9
6	DPPCON	14.880	8.	6
7	DAMCON	15.000	7.	4
8	GAMRAP	15.170	6.	7
9	GPPCON	15.500	4.	10
10	GAMCON	15.880	8.	8

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(DPPRAP , DAMRAP , GPPRAP , DPPCON , DAMCON , GAMRAP , GPPCON)

(GPPRAP , DPPCON , DAMCON , GAMRAP , GPPCON , GAMCON)

(PREFRG , PREFRD)

TABLE 41 EXPERIMENT 5

The results of a Duncan Multiple Range Test applied to the trimmed survival time data obtained from single-dilution infectivity tests in Experiment 5.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	550,8403	9	61,2045	122,1918
WITHIN GROUPS	33,0586	66,	0,5009	
TOTAL	583,8989	75,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRD	1,220	0,6700	9,	1
2	PREFRG	1,440	0,5300	9,	2
3	DAMRAP	6,330	0,5200	6,	4
4	DAMCON	8,140	0,6900	7,	8
5	DPPRAP	5,570	0,5300	7,	3
6	DPPCON	7,380	0,5200	8,	7
7	GAMRAP	7,250	1,0400	8,	6
8	GAMCON	8,250	0,7100	8,	9
9	GPPRAP	6,780	0,8300	9,	5
10	GPPCON	9,200	0,8400	5,	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRD	1,220	9,	1
2	PREFRG	1,440	9,	2
3	DPPRAP	5,570	7,	5
4	DAMRAP	6,330	6,	3
5	GPPRAP	6,780	9,	9
6	GAMRAP	7,250	8,	7
7	DPPCON	7,380	8,	6
8	DAMCON	8,140	7,	4
9	GAMCON	8,250	8,	8
10	GPPCON	9,200	5,	10

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(GPPRAP , GAMRAP , DPPCON)

(DPPRAP , DAMRAP)

(DAMCON , GAMCON)

(PREFRD , PREFRG)

(DAMRAP , GPPRAP)

TABLE 42 EXPERIMENT 6

The results of a Duncan Multiple Range Test applied to the percentage haemolysis data obtained in Experiment 6. The labels used in Tables 42-46 are:

Label	Treatment
	(i) Prefreeze bloods
PREALD	Prefreeze, DMSO-diluted blood/Diluent MAS
PREALG	Prefreeze, glycerol-diluted blood/Diluent MAS
PREABD	Prefreeze, DMSO-diluted blood/Diluent ABP8.0
PREABG	Prefreeze, glycerol-diluted blood/Diluent ABP8.0
	(ii) Frozen and thawed bloods
ALDPPC	DMSO/MAS/pp60/Conventional rate
ALDPPR	DMSO/MAS/pp60/Rapid rate
ALDAMC	DMSO/MAS/Ampoule/Conventional
ALDAMR	DMSO/MAS/Ampoule/Rapid
ALGPPC	Glycerol/MAS/pp60/Conventional
ALGPPR	Glycerol/MAS/pp60/Rapid
ALGAMC	Glycerol/MAS/Ampoule/Conventional
ALGAMR	Glycerol/MAS/Ampoule/Rapid
ABDPPC	DMSO/ABP8.0/pp60/Conventional
ABDPPR	DMSO/ABP8.0/pp60/Rapid
ABDAMC	DMSO/ABP8.0/Ampoule/Conventional
ABDAMR	DMSO/ABP8.0/Ampoule/Rapid
ABGPPC	Glycerol/ABP8.0/pp60/Conventional
ABGPPR	Glycerol/ABP8.0/pp60/Rapid
ABGAMC	Glycerol/ABP8.0/Ampoule/Conventional
ABGAMR	Glycerol/ABP8.0/Ampoule/Rapid

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	25761.1406	15	1717.4092	260.7405
WITHIN GROUPS	296.3997	45.	6.5867	
TOTAL	26057.5391	60.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	ALDPPC	48.750	0.9600	4.	14
2	ALDPPR	5.000	0.8200	4.	2
3	ALDAMC	41.250	2.5000	4.	13
4	ALDAMR	8.000	0.8200	4.	5
5	ALGPPC	33.750	2.8700	4.	11
6	ALGPPR	12.000	1.4100	4.	8
7	ALGAMC	39.250	2.2200	4.	12
8	ALGAMR	12.000	2.8300	2.	7
9	ABDPPC	61.000	5.2300	4.	15
10	ABDPPR	5.250	2.6300	4.	3
11	ABDAMC	67.750	5.3200	4.	16
12	ABDAMR	8.000	1.0000	3.	4
13	ABGPPC	32.500	1.2900	4.	10
14	ABGPPR	5.000	0.0	4.	1
15	ABGAMC	31.750	2.3600	4.	9
16	ABGAMR	10.000	1.4100	4.	6

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	ABGPPR	5.000	4.	14
2	ALDPPR	5.000	4.	2
3	ABDPPR	5.250	4.	10
4	ABDAMR	8.000	3.	12
5	ALDAMR	8.000	4.	4
6	ABGAMR	10.000	4.	16
7	ALGAMR	12.000	2.	8
8	ALGPPR	12.000	4.	6
9	ABGAMC	31.750	4.	15
10	ABGPPC	32.500	4.	13
11	ALGPPC	33.750	4.	5
12	ALGAMC	39.250	4.	7
13	ALDAMC	41.250	4.	3
14	ALDPPC	48.750	4.	1
15	ABDPPC	61.000	4.	9
16	ABDAMC	67.750	4.	11

THERE ARE 4 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(ABDAMR , ALDAMR , ABGAMR , ALGAMR , ALGPPR)

(ABGPPR , ALDPPR , ABDPPR , ABDAMR , ALDAMR)

(ABGAMC , ABGPPC , ALGPPC)

(ALGAMC , ALDAMC)

TABLE 43 EXPERIMENT 6

The results of a Duncan Multiple Range Test applied to the untrimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 6.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	379.6863	19	19.9779	11.0294
WITHIN GROUPS	289.6123	160.	1.8113	
TOTAL	669.3926	179.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREALD	3.000	2.0580	10.	4
2	PREALG	2.500	2.3300	8.	1
3	PREABD	2.000	1.9300	10.	2
4	PREABG	3.250	2.7600	8.	6
5	ALDPPC	5.200	1.8100	10.	12
6	ALDPPR	2.900	0.3200	10.	3
7	ALDAMC	5.500	1.5100	10.	14
8	ALDAMR	3.700	0.4800	10.	8
9	ALGPPC	7.000	0.9400	10.	20
10	ALGPPR	5.290	1.8900	7.	13
11	ALGAMC	6.000	0.5800	7.	16
12	ALGAMR	5.200	0.9200	10.	11
13	ABDPPC	6.110	0.6000	9.	17
14	ABDPPR	3.400	0.5200	10.	7
15	ABDAMC	5.600	1.2600	10.	15
16	ABDAMR	3.200	0.6300	10.	5
17	ABGPPC	6.630	0.5200	8.	18
18	ABGPPR	4.710	0.4900	7.	18
19	ABGAMC	6.890	0.7800	9.	19
20	ABGAMR	4.430	0.7900	7.	9

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREALG	2.500	8.	2
2	PREABD	2.000	10.	3
3	ALDPPR	2.900	10.	6
4	PREALD	3.000	10.	1
5	ABDAMR	3.200	10.	16
6	PREABG	3.250	8.	4
7	ABDPPR	3.400	10.	14
8	ALDAMR	3.700	10.	8
9	ABGAMR	4.430	7.	20
10	ABGPPR	4.710	7.	18
11	ALGAMR	5.200	10.	12
12	ALDPPC	5.200	10.	5
13	ALGPPR	5.290	7.	13
14	ALDAMC	5.500	10.	7
15	ABDAMC	5.600	10.	15
16	ALGAMC	6.000	7.	11
17	ABDPPC	6.110	9.	13
18	ABGPPC	6.630	8.	17
19	ABGAMC	6.890	9.	19
20	ALGPPC	7.000	10.	9

THERE ARE 8 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(ABGPPR , ALGAMR , ALDPPC , ALGPPR , ALDAMC , ABDAMC , ALGAMC , ABDPPC)

(ABGAMR , ABGPPR , ALGAMR , ALDPPC , ALGPPR , ALDAMC , ABDAMC , ALGAMC)

(PREALG , PREABD , ALDPPR , PREALD , ABDAMR , PREABG , ABDPPR , ALDAMR)

(ALGAMR , ALDPPC , ALGPPR , ALDAMC , ABDAMC , ALGAMC , ABDPPC , ABGPPC)

(PREALD , ABDAMR , PREABG , ABDPPR , ALDAMR , ABGAMR)

(PREABG , ABDPPR , ALDAMR , ABGAMR , ABGPPR)

(ALGAMC , ABDPPC , ABGPPC , ABGAMC , ALGPPC)

(ABDAMC , ALGAMC , ABDPPC , ABGPPC , ABGAMC)

TABLE 44 EXPERIMENT 6

The results of a Duncan Multiple Range Test applied to the trimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 6.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	381,8555	19	20,0977	28,3576
WITHIN GROUPS	99,2209	140,	0,7087	
TOTAL	481,0762	159,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREALD	2,670	1,8700	9,	4
2	PREALG	1,710	0,7600	7,	1
3	PREABD	2,220	0,6700	9,	2
4	PREABG	2,290	0,4900	7,	3
5	ALDPPC	4,670	0,7100	9,	11
6	ALDPPR	2,890	0,3300	9,	5
7	ALDAMC	5,110	0,9300	9,	14
8	ALDAMR	3,670	0,5000	9,	8
9	ALGPPC	6,780	0,6700	9,	20
10	ALGPPR	4,830	1,6000	6,	12
11	ALGAMC	5,830	0,4100	6,	16
12	ALGAMR	5,110	0,9300	9,	13
13	ABDPPC	6,000	0,5300	8,	17
14	ABDPPR	3,330	0,5000	9,	7
15	ABDAMC	5,330	1,0000	9,	15
16	ABDAMR	3,110	0,6000	9,	6
17	ABGPPC	6,570	0,5300	7,	18
18	ABGPPR	4,670	0,5200	6,	10
19	ABGAMC	6,750	0,7100	8,	19
20	ABGAMR	4,330	0,8200	6,	9

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREALG	1,710	7,	2
2	PREABD	2,220	9,	3
3	PREABG	2,290	7,	4
4	PREALD	2,670	9,	1
5	ALDPPR	2,890	9,	6
6	ABDAMR	3,110	9,	16
7	ABDPPR	3,330	9,	14
8	ALDAMR	3,670	9,	8
9	ABGAMR	4,330	6,	20
10	ABGPPR	4,670	6,	18
11	ALDPPC	4,670	9,	5
12	ALGPPR	4,830	6,	10
13	ALGAMR	5,110	9,	12
14	ALDAMC	5,110	9,	7
15	ABDAMC	5,330	9,	15
16	ALGAMC	5,830	6,	11
17	ABDPPC	6,000	8,	13
18	ABGPPC	6,570	7,	17
19	ABGAMC	6,750	8,	19
20	ALGPPC	6,780	9,	9

THERE ARE 10 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(ABGAMR , ABGPPR , ALDPPC , ALGPPR , ALGAMR , ALDAMC , ABDAMC)

(ALGAMC , ABDPPC , ABGPPC , ABGAMC , ALGPPC)

(ALGAMR , ALDAMC , ABDAMC , ALGAMC , ABDPPC)

(ALGPPR , ALGAMR , ALDAMC , ABDAMC , ALGAMC)

(ALDPPR , ABDAMR , ABDPPR , ALDAMR)

(PREALD , ALDPPR , ABDAMR , ABDPPR)

(PREABG , PREALD , ALDPPR , ABDAMR)

(PREABD , PREABG , PREALD , ALDPPR)

(PREALG , PREABD , PREABG)

(ALDAMR , ABGAMR)

TABLE 45 EXPERIMENT 6

The results of a Duncan Multiple Range Test applied to the untrimmed survival time data obtained from single-dilution infectivity tests in Experiment 6.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	392,8105	19	20,6742	8,0712
WITHIN GROUPS	376,5391	147,	2,5615	
TOTAL	769,3496	166,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREALD	9,500	3,3700	10,	7
2	PREALG	8,570	2,0700	7,	1
3	PREABD	9,380	3,3400	8,	4
4	PREABG	9,500	2,6200	8,	6
5	ALDPPC	10,710	0,4900	7,	11
6	ALDPPR	8,600	0,5200	10,	2
7	ALDAMC	11,890	1,4500	9,	14
8	ALDAMR	9,670	0,7100	9,	8
9	ALGPPC	13,110	0,9300	9,	19
10	ALGPPR	10,830	1,1700	6,	12
11	ALGAMC	12,140	0,6900	7,	16
12	ALGAMR	11,800	1,3200	10,	13
13	ABDPPC	12,500	1,2000	8,	17
14	ABDPPR	9,500	0,9700	10,	5
15	ABDAMC	12,000	1,4900	10,	15
16	ABDAMR	8,900	0,7400	10,	3
17	ABGPPC	12,750	0,4600	8,	18
18	ABGPPR	10,330	0,5200	6,	9
19	ABGAMC	13,250	1,5800	8,	20
20	ABGAMR	10,570	0,5300	7,	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREALG	8,570	7,	2
2	ALDPPR	8,600	10,	6
3	ABDAMR	8,900	10,	16
4	PREABD	9,380	8,	3
5	ABDPPR	9,500	10,	14
6	PREABG	9,500	8,	4
7	PREALD	9,500	10,	1
8	ALDAMR	9,670	9,	8
9	ABGPPR	10,330	6,	18
10	ABGAMR	10,570	7,	20
11	ALDPPC	10,710	7,	5
12	ALGPPR	10,830	6,	10
13	ALGAMR	11,800	10,	12
14	ALDAMC	11,890	9,	7
15	ABDAMC	12,000	10,	15
16	ALGAMC	12,140	7,	11
17	ABDPPC	12,500	8,	13
18	ABGPPC	12,750	8,	17
19	ALGPPC	13,110	9,	9
20	ABGAMC	13,250	8,	19

THERE ARE 6 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(ABDAMR , PREABD , ABDPPR , PREABG , PREALD , ALDAMR , ABGPPR , ABGAMR , ALDPPC , ALGPPR)

(ALGAMR , ALDAMC , ABDAMC , ALGAMC , ABDPPC , ABGPPC , ALGPPC , ABGAMC)

(PREALG , ALDPPR , ABDAMR , PREABD , ABDPPR , PREABG , PREALD , ALDAMR , ABGPPR)

(ABGPPR , ABGAMR , ALDPPC , ALGPPR , ALGAMR , ALDAMC , ABDAMC , ALGAMC)

(ALGPPR , ALGAMR , ALDAMC , ABDAMC , ALGAMC , ABDPPC , ABGPPC)

(ALDPPC , ALGPPR , ALGAMR , ALDAMC , ABDAMC , ALGAMC , ABDPPC)

TABLE 46 EXPERIMENT 6

The results of a Duncan Multiple Range Test applied to the trimmed survival time data obtained from single-dilution infectivity tests in Experiment 6.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	422,4229	19	22,2328	21,8955
WITHIN GROUPS	133,8466	127,	1,0539	
TOTAL	556,2693	146,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREALD	8,560	1,6700	9,	5
2	PREALG	7,830	0,7500	6,	1
3	PREABD	8,430	2,1500	7,	3
4	PREABG	8,710	1,5000	7,	6
5	ALDPPC	10,670	0,5200	6,	12
6	ALDPPR	8,560	0,5300	9,	4
7	ALDAMC	11,630	1,3000	8,	14
8	ALDAMR	9,500	0,5300	8,	8
9	ALGPPC	12,880	0,6400	8,	20
10	ALGPPR	10,400	0,5500	5,	10
11	ALGAMC	12,000	0,6300	6,	16
12	ALGAMR	11,440	0,7300	9,	13
13	ABDPPC	12,290	1,1100	7,	17
14	ABDPPR	8,330	0,8700	9,	2
15	ABDAMC	11,670	1,1200	9,	15
16	ABDAMR	8,780	0,6700	9,	7
17	ABGPPC	12,710	0,4900	7,	18
18	ABGPPR	10,200	0,4500	5,	9
19	ABGAMC	12,860	1,2200	7,	19
20	ABGAMR	10,500	0,5500	6,	11

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREALG	7,830	6,	2
2	ABDPPR	8,330	9,	14
3	PREABD	8,430	7,	3
4	ALDPPR	8,560	9,	6
5	PREALD	8,560	9,	1
6	PREABG	8,710	7,	4
7	ABDAMR	8,780	9,	16
8	ALDAMR	9,500	8,	8
9	ABGPPR	10,200	5,	18
10	ALGPPR	10,400	5,	10
11	ABGAMR	10,500	6,	20
12	ALDPPC	10,670	6,	5
13	ALGAMR	11,440	9,	12
14	ALDAMC	11,630	8,	7
15	ABDAMC	11,670	9,	15
16	ALGAMC	12,000	6,	11
17	ABDPPC	12,290	7,	13
18	ABGPPC	12,710	7,	17
19	ABGAMC	12,860	7,	19
20	ALGPPC	12,880	8,	9

THERE ARE 8 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PREALG , ABDPPR , PREABD , ALDPPR , PREALD , PREABG , ABDAMR)

(ALGPPR , ABGAMR , ALDPPC , ALGAMR , ALDAMC , ABDAMC)

(PREABD , ALDPPR , PREALD , PREABG , ABDAMR , ALDAMR)

(ABGPPR , ALGPPR , ABGAMR , ALDPPC , ALGAMR)

(ALGAMR , ALDAMC , ABDAMC , ALGAMC , ABDPPC)

(ALDAMC , ABDAMC , ALGAMC , ABDPPC , ABGPPC)

(ALGAMC , ABDPPC , ABGPPC , ABGAMC , ALGPPC)

(ALDAMR , ABGPPR , ALGPPR , ABGAMR , ALDPPC)

TABLE 47 EXPERIMENT 7

The results of a Duncan Multiple Range Test applied to the percentage haemolysis data obtained in Experiment 7. The labels used in Tables 47-51 are:

Label	Treatment
	(i) Prefreeze bloods
NATU45	Prefreeze blood with a naturally occurring parasitaemia of 45%
NATU16	Prefreeze blood with a naturally occurring parasitaemia of 16%
NATU10	Prefreeze blood with a naturally occurring parasitaemia of 10%
NATUR5	Prefreeze blood with a naturally occurring parasitaemia of 5%
DILU45	Prefreeze blood with an artificially produced parasitaemia of 45%
DILU16	Prefreeze blood with an artificially produced parasitaemia of 16%
DILU10	Prefreeze blood with an artificially produced parasitaemia of 10%
DILUT5	Prefreeze blood with an artificially produced parasitaemia of 5%
	(ii) Frozen and thawed bloods
NA2T45	Frozen and thawed blood/Natural parasitaemia of 45%
NA2T16	Frozen and thawed blood/Natural parasitaemia of 16%
NA2T10	Frozen and thawed blood/Natural parasitaemia of 10%
NA2TU5	Frozen and thawed blood/Natural parasitaemia of 5%
DI2L45	Frozen and thawed blood/ Artificial parasitaemia of 45%
DI2L16	Frozen and thawed blood/ Artificial parasitaemia of 16%
DI2L10	Frozen and thawed blood/ Artificial parasitaemia of 10%
DI2LU5	Frozen and thawed blood/ Artificial parasitaemia of 5%

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	147,9038	7	21,1291	6,2622
WITHIN GROUPS	60,7336	18,	3,3741	
TOTAL	208,6374	25,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	NATU45	14,250	2,2200	4,	7
2	NATU16	12,750	0,9600	4,	6
3	NATU10	11,000	1,0000	3,	4
4	NATUR5	11,000	3,4600	4,	3
5	DILU45	17,750	0,9600	4,	8
6	DILU16	12,000	1,0000	3,	5
7	DILU10	11,000	0,0	2,	2
8	DILUT5	10,500	0,7100	2,	1

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	DILUT5	10,500	2,	8
2	DILU10	11,000	2,	7
3	NATUR5	11,000	4,	4
4	NATU10	11,000	3,	3
5	DILU16	12,000	3,	6
6	NATU16	12,750	4,	2
7	NATU45	14,250	4,	1
8	DILU45	17,750	4,	5

COMPUTED RANGES FOR ALPHA=0,05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO, OF GROUPS IN SUBSET	RANGE
2	2,9666
3	3,1144
4	3,2165
5	3,2748
6	3,3206
7	3,3559
8	3,3832

THERE ARE 2 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(DILUT5 , DILU10 , NATUR5 , NATU10 , DILU16 , NATU16)

(DILU10 , DILU16 , NATU16 , NATU45)

TABLE 48 EXPERIMENT 7

The results of a Duncan Multiple Range Test applied to the untrimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 7.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	456.1313	15	30.4088	11.5249
WITHIN GROUPS	348.2861	132.	2.6385	
TOTAL	804.4175	147.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	NATU45	2.400	2.2700	10.	6
2	NATU16	1.200	0.6400	10.	2
3	NATU10	1.800	2.2000	10.	3
4	NATUR5	1.000	0.0	10.	1
5	DILU45	1.900	1.2900	10.	4
6	DILU16	2.200	2.1000	10.	5
7	DILU10	2.500	2.9500	10.	7
8	DILUT5	2.600	2.2200	10.	8
9	NA2T45	5.670	1.1200	9.	15
10	NA2T16	5.500	0.5300	10.	14
11	NA2T10	5.400	0.5200	10.	12
12	NA2TU5	4.500	0.5300	8.	9
13	DI2L45	5.330	1.1200	9.	11
14	DI2L16	6.200	1.7900	5.	16
15	DI2L10	4.750	1.0900	9.	10
16	DI2LU5	5.500	2.0000	8.	13

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	NATUR5	1.000	10.	4
2	NATU16	1.200	10.	2
3	NATU10	1.800	10.	3
4	DILU45	1.900	10.	5
5	DILU16	2.200	10.	6
6	NATU45	2.400	10.	1
7	DILU10	2.500	10.	7
8	DILUT5	2.600	10.	8
9	NA2TU5	4.500	8.	12
10	DI2L10	4.750	9.	15
11	DI2L45	5.330	9.	13
12	NA2T10	5.400	10.	11
13	DI2LU5	5.500	8.	16
14	NA2T16	5.500	10.	10
15	NA2T45	5.670	9.	9
16	DI2L16	6.200	5.	14

THERE ARE 2 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(NA2TU5 , DI2L10 , DI2L45 , NA2T10 , DI2LU5 , NA2T16 , NA2T45 , DI2L16)

(NATUR5 , NATU16 , NATU10 , DILU45 , DILU16 , NATU45 , DILU10 , DILUT5)

TABLE 49 EXPERIMENT 7

The results of a Duncan Multiple Range Test applied to the trimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 7.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	438.1946	15	29.2130	25.0932
WITHIN GROUPS	135.0448	116.	1.1642	
TOTAL	573.2393	131.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	NATU45	1.890	1.6900	9.	7
2	NATU16	1.000	0.0	9.	2
3	NATU10	1.110	0.3300	9.	3
4	NATUR5	1.000	0.0	9.	1
5	DILU45	1.560	0.7300	9.	4
6	DILU16	1.670	1.3200	9.	5
7	DILU10	1.780	1.9900	9.	6
8	DILUT5	2.110	1.6900	9.	8
9	NA2T45	5.380	0.7400	8.	14
10	NA2T16	5.440	0.5300	9.	15
11	NA2T10	5.330	0.5000	9.	13
12	NA2TU5	4.430	0.5300	7.	9
13	DI2L45	5.000	0.5300	8.	12
14	DI2L16	5.750	1.7100	4.	16
15	DI2L10	4.500	0.7600	8.	10
16	DI2LU5	5.000	1.5300	7.	11

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	NATUR5	1.000	9.	4
2	NATU16	1.000	9.	2
3	NATU10	1.110	9.	3
4	DILU45	1.560	9.	5
5	DILU16	1.670	9.	6
6	DILU10	1.780	9.	7
7	NATU45	1.890	9.	1
8	DILUT5	2.110	9.	8
9	NA2TU5	4.430	7.	12
10	DI2L10	4.500	8.	15
11	DI2LU5	5.000	7.	16
12	DI2L45	5.000	8.	13
13	NA2T10	5.330	9.	11
14	NA2T45	5.380	8.	9
15	NA2T16	5.440	9.	10
16	DI2L16	5.750	4.	14

THERE ARE 2 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(NATUR5 , NATU16 , NATU10 , DILU45 , DILU16 , DILU10 , NATU45 , DILUT5)

(NA2TU5 , DI2L10 , DI2LU5 , DI2L45 , NA2T10 , NA2T45 , NA2T16 , DI2L16)

TABLE 50 EXPERIMENT 7

The results of a Duncan Multiple Range Test applied to the untrimmed survival time data obtained from single-dilution infectivity tests in Experiment 7.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	481,9670	15	32,1311	10,7741
WITHIN GROUPS	310,1545	104,	2,9823	
TOTAL	792,1216	119,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	NATU45	8,220	2,6300	9,	8
2	NATU16	6,330	0,5000	9,	2
3	NATU10	8,000	3,3900	9,	7
4	NATUR5	6,330	0,5000	9,	1
5	DILU45	7,110	1,1700	9,	3
6	DILU16	7,890	1,9000	9,	6
7	DILU10	7,550	2,4000	9,	5
8	DILUT5	7,440	2,1300	9,	4
9	NA2T45	11,500	0,5500	6,	15
10	NA2T16	11,670	1,0000	9,	16
11	NA2T10	11,290	0,4900	7,	13
12	NA2TU5	10,830	0,9800	6,	9
13	DI2L45	11,290	0,7600	7,	12
14	DI2L16	11,330	1,5300	3,	14
15	DI2L10	11,200	1,6400	5,	11
16	DI2LU5	11,000	0,7100	5,	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	NATUR5	6,330	9,	4
2	NATU16	6,330	9,	2
3	DILU45	7,110	9,	5
4	DILUT5	7,440	9,	8
5	DILU10	7,550	9,	7
6	DILU16	7,890	9,	6
7	NATU10	8,000	9,	3
8	NATU45	8,220	9,	1
9	NA2TU5	10,830	6,	12
10	DI2LU5	11,000	5,	16
11	DI2L10	11,200	5,	15
12	DI2L45	11,290	7,	13
13	NA2T10	11,290	7,	11
14	DI2L16	11,330	3,	14
15	NA2T45	11,500	6,	9
16	NA2T16	11,670	9,	10

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(NA2TU5 , DI2LU5 , DI2L10 , DI2L45 , NA2T10 , DI2L16 , NA2T45 , NA2T16)

(NATUR5 , NATU16 , DILU45 , DILUT5 , DILU10 , DILU16 , NATU10)

(DILU45 , DILUT5 , DILU10 , DILU16 , NATU10 , NATU45)

TABLE 51 EXPERIMENT 7

The results of a Duncan Multiple Range Test applied to the trimmed survival time data obtained from single-dilution infectivity tests in Experiment 7.

The computed shortest significant ranges have been omitted.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	526.7559	15	35.2504	6.7008
WITHIN GROUPS	631.2791	120.	5.2607	
TOTAL	1160.0349	135.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	NATU45	9.000	3.5000	10.	8
2	NATU16	6.700	1.2500	10.	2
3	NATU10	8.800	4.0800	10.	7
4	NATUR5	6.700	1.2500	10.	1
5	DILU45	7.600	1.9000	10.	3
6	DILU16	8.300	2.2100	10.	5
7	DILU10	8.400	3.5000	10.	6
8	DILUT5	7.900	2.4700	10.	4
9	NA2T45	12.000	1.4100	7.	15
10	NA2T16	12.100	1.6600	10.	16
11	NA2T10	11.500	0.7600	8.	11
12	NA2TU5	11.000	1.0000	7.	9
13	DI2L45	11.750	1.4900	8.	13
14	DI2L16	11.750	1.5000	4.	12
15	DI2L10	11.830	2.1400	6.	14
16	DI2LU5	11.500	1.3800	6.	10

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	NATUR5	6.700	10.	4
2	NATU16	6.700	10.	2
3	DILU45	7.600	10.	5
4	DILUT5	7.900	10.	8
5	DILU16	8.300	10.	6
6	DILU10	8.400	10.	7
7	NATU10	8.800	10.	3
8	NATU45	9.000	10.	1
9	NA2TU5	11.000	7.	12
10	DI2LU5	11.500	6.	16
11	NA2T10	11.500	8.	11
12	DI2L16	11.750	4.	14
13	DI2L45	11.750	8.	13
14	DI2L10	11.830	6.	15
15	NA2T45	12.000	7.	9
16	NA2T16	12.100	10.	10

THERE ARE 4 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(NATUR5 , NATU16 , DILU45 , DILUT5 , DILU16 , DILU10 , NATU10 , NATU45)

(NA2TU5 , DI2LU5 , NA2T10 , DI2L16 , DI2L45 , DI2L10 , NA2T45 , NA2T16)

(NATU45 , NA2TU5 , DI2LU5 , DI2L16)

(NATU10 , NATU45 , NA2TU5)

TABLE 52 EXPERIMENT 8

The results of a Duncan Multiple Range Test applied to the percentage haemolysis data obtained in Experiment 8. The labels used in Tables 52-56 are:

Label	Treatment
PREFRO	Undiluted prefreeze control blood
PREFRD	DMSO-diluted prefreeze control blood
PP6-79	Slowly cooled/pp60/Stored at -79°C
PP-196	Slowly cooled/pp60/Stored at -196°C
AMP-79	Slowly cooled/Ampoule/Stored at -79°C
AM-196	Slowly cooled/Ampoule/Stored at -196°C
DPPRAP	Rapidly cooled/pp60/Stored at -196°C
DAMRAP	Rapidly cooled/Ampoule/Stored at -196°C

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	6249,1406	5	1249,8281	265,8718
WITHIN GROUPS	79,9147	17,	4,7009	
TOTAL	6329,0547	22,		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	AMP=79	50,500	2,0800	4,	4
2	AM=196	51,000	3,7400	4,	5
3	PP6=79	46,250	2,2200	4,	3
4	PP=196	54,750	0,9600	4,	6
5	DPPRAP	13,750	1,5000	4,	1
6	DAMRAP	17,330	0,5800	3,	2

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	DPPRAP	13,750	4,	5
2	DAMRAP	17,330	3,	6
3	PP6=79	46,250	4,	3
4	AMP=79	50,500	4,	1
5	AM=196	51,000	4,	2
6	PP=196	54,750	4,	4

COMPUTED RANGES FOR ALPHA=0,05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO. OF GROUPS IN SUBSET	RANGE
2	2,9789
3	3,1266
4	3,2285
5	3,2960
6	3,3309

THERE ARE 1 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(AMP=79 , AM=196)

TABLE 53 EXPERIMENT 8

The results of a Duncan Multiple Range Test applied to the untrimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 8.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	323.4826	7	46.2804	66.3769
WITHIN GROUPS	38.2817	55.	0.6960	
TOTAL	361.6641	62.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	1.900	0.7400	10.	1
2	PREFRD	2.400	0.9700	10.	2
3	PP6=79	8.200	0.4500	5.	8
4	PP=196	7.600	0.8900	5.	6
5	AMP=79	7.800	0.8400	5.	7
6	AM=196	6.000	1.0000	9.	5
7	DPPRAP	3.300	0.8200	10.	3
8	DAMRAP	3.670	0.7100	9.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	1.900	10.	1
2	PREFRD	2.400	10.	2
3	DPPRAP	3.300	10.	7
4	DAMRAP	3.670	9.	8
5	AM=196	6.000	9.	6
6	PP=196	7.600	5.	4
7	AMP=79	7.800	5.	5
8	PP6=79	8.200	5.	3

COMPUTED RANGES FOR ALPHA=0.05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO. OF GROUPS IN SUBSET	RANGE
2	2.8357
3	2.9816
4	3.0781
5	3.1468
6	3.2026
7	3.2485
8	3.2857

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PREFRO , PREFRD)

(PP=196 , AMP=79 , PP6=79)

(DPPRAP , DAMRAP)

TABLE 54 EXPERIMENT 8

The results of a Duncan Multiple Range Test applied to the trimmed prepatent period data obtained from single-dilution infectivity tests in Experiment 8.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	256.5234	7	36.6462	79.2792
WITHIN GROUPS	21.7254	47.	0.4622	
TOTAL	278.2488	54.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	1.780	0.6700	9.	1
2	PREFRD	2.330	1.0000	9.	2
3	PP&=79	8.000	0.0	4.	8
4	PP=196	7.250	0.5000	4.	6
5	AMP=79	7.500	0.5000	4.	7
6	AM=196	5.750	0.7100	8.	5
7	DPPRAP	3.110	0.6000	9.	3
8	DAMRAP	3.500	0.5300	8.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	1.780	9.	1
2	PREFRD	2.330	9.	2
3	DPPRAP	3.110	9.	7
4	DAMRAP	3.500	8.	8
5	AM=196	5.750	8.	6
6	PP=196	7.250	4.	4
7	AMP=79	7.500	4.	5
8	PP&=79	8.000	4.	3

COMPUTED RANGES FOR ALPHA=0.05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO. OF GROUPS IN SUBSET	RANGE
2	2.8459
3	2.9921
4	3.0896
5	3.1568
6	3.2125
7	3.2574
8	3.2939

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PREFRO , PREFRD)

(PP=196 , AMP=79 , PP&=79)

(DPPRAP , DAMRAP)

TABLE 55 EXPERIMENT 8

The results of a Duncan Multiple Range Test applied to the untrimmed survival time data obtained from single-dilution infectivity tests in Experiment 8.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	268.5342	7	38.3620	19.3123
WITHIN GROUPS	99.3283	50.	1.9864	
TOTAL	367.8542	57.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	8.780	0.9700	9.	1
2	PREFRD	9.110	0.9300	9.	2
3	PP6=79	15.000	2.3400	5.	8
4	PP=196	14.250	0.9600	4.	6
5	AMP=79	14.600	1.6700	5.	7
6	AM=196	11.890	1.0500	9.	5
7	DPPRAP	10.446	1.6700	9.	3
8	DAMRAP	11.000	1.6000	8.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	8.780	9.	1
2	PREFRD	9.110	9.	2
3	DPPRAP	10.440	9.	7
4	DAMRAP	11.000	8.	8
5	AM=196	11.890	9.	6
6	PP=196	14.250	4.	4
7	AMP=79	14.600	5.	5
8	PP6=79	15.000	5.	3

COMPUTED RANGES FOR ALPHA=0.05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO OF GROUPS IN SUBSET	RANGE
2	2.8417
3	2.9677
4	3.0849
5	3.1624
6	3.2084
7	3.2537
8	3.2905

THERE ARE 5 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(PP=196 , AMP=79 , PP6=79)

(PREFRO , PREFRD)

(PREFRD , DPPRAP)

(DAMRAP , AM=196)

(DPPRAP , DAMRAP)

TABLE 56 EXPERIMENT 8

The results of a Duncan Multiple Range Test applied to the trimmed survival time data obtained from single-dilution infectivity tests in Experiment 8.

ANALYSIS OF VARIANCE

	SUM OF SQUARES	DF	MEAN SQUARE	F RATIO
BETWEEN GROUPS	197.1402	7	28.1629	32.7975
WITHIN GROUPS	36.0650	42.	0.8587	
TOTAL	233.2053	49.		

TREATMENT MEANS IN ORIGINAL ORDER

TREATMENT NUMBER	LABEL	MEAN	STANDARD DEVIATION	NUMBER OF REPLICATIONS	RANK
1	PREFRO	8.500	0.5300	8.	1
2	PREFRD	8.880	0.6400	8.	2
3	PP6=79	14.000	0.8200	4.	8
4	PP=196	14.000	1.0000	3.	7
5	AMP=79	14.000	1.1500	4.	6
6	AM=196	11.750	1.0400	8.	5
7	DPPRAP	10.000	1.0700	8.	3
8	DAMRAP	10.870	1.1300	7.	4

TREATMENT MEANS IN RANKED ORDER

RANK	LABEL	MEAN	NUMBER OF REPLICATIONS	TREATMENT NUMBER
1	PREFRO	8.500	8.	1
2	PREFRD	8.880	8.	2
3	DPPRAP	10.000	8.	7
4	DAMRAP	10.870	7.	8
5	AM=196	11.750	8.	6
6	AMP=79	14.000	4.	5
7	PP=196	14.000	3.	4
8	PP6=79	14.000	4.	3

COMPUTED RANGES FOR ALPHA=0.05 (DUNCAN'S NEW MULTIPLE RANGE TEST)

NO. OF GROUPS IN SUBSET	RANGE
2	2.8543
3	3.0007
4	3.0991
5	3.1656
6	3.2205
7	3.2648
8	3.3035

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(DPPRAP , DAMRAP)

(PREFRO , PREFRD)

(AMP=79 , PP=196 , PP6=79)

*

TABLE 58 EXPERIMENT 9

The results of the prefreeze and post-thaw infectivity titrations, with and without data trimming. The labels used in Tables 58 to 76 are:

Label	Regression
	(1) Prefreeze bloods
OPPPWI	Buffer only-diluted/Prepatent period/Untrimmed data
OPPPWO	Buffer only-diluted/Prepatent period/Trimmed data
OSTWI	Buffer only-diluted/Survival time/Untrimmed
OSTWO	Buffer only-diluted/Survival time/Trimmed
DPPPWI	DMSO-diluted/Prepatent period/Untrimmed
DPPPWO	DMSO-diluted/Prepatent period/Trimmed
DSTWI	DMSO-diluted/Survival time/Untrimmed
DSTWO	DMSO-diluted/Survival time/Trimmed
	(2) Post-thaw bloods
ppPPPWI	Frozen in pp60/Prepatent period/Untrimmed
ppPPPWO	Frozen in pp60/Prepatent period/Trimmed
ppSTWI	Frozen in pp60/Survival time/Untrimmed
ppSTWO	Frozen in pp60/Survival time/Trimmed
APPPWI	Frozen in ampoules/Prepatent period/Untrimmed
APPPWO	Frozen in ampoules/Prepatent period/Trimmed
ASTWI	Frozen in ampoules/Survival time/Untrimmed
ASTWO	Frozen in ampoules/Survival time/Trimmed

* Table 57 has been included in the text. (p. 159)

	OPPPWI	DPPPWI	ppPPPWI	APPPWI	OPPPWO	DPPPWO	ppPPPWO	APPPWO
r	0.464	0.433	0.862	0.675	0.533	0.523	0.872	0.777
n	32	30	25	23	28	26	21	20
b	-0.8546	-0.6734	-1.3042	-1.0930	0.8750	-0.7188	-1.2366	-1.2007
m	11.2848	10.5515	14.1014	13.1627	11.1250	10.5328	13.5133	13.5945
7	5.3025	5.8374	4.9716	5.5116	5.000	5.5011	4.8566	5.1891
5	7.011	7.1843	7.5801	7.6976	6.750	6.9387	7.3300	7.5907
4	7.8664		8.8844	8.7967	7.625		8.5666	
	OSTWI	DSTWI	ppSTWI	ASTWI	OSTWO	DSTWO	ppSTWO	ASTWO
r	0.503	0.288	0.703	0.220	0.673	0.406	0.836	0.628
n	26	28	25	20	22	24	21	17
b	-0.7235	-0.7941	-0.7029	-0.8844	0.8091	-0.8750	-0.7557	-1.0419
m	16.8705	18.0294	16.9455	18.4741	16.927	17.9166	16.965	18.4311
7	11.8058	12.4705	12.0245	12.2828	11.263	11.7916	11.6750	11.1377
5	13.2530	14.0588	13.4305	14.0517	12.881	13.5416	13.1870	13.2215
4	13.9764		14.1335		13.690		13.042	

TABLE 59 EXPERIMENT 9

The results of a comparison of linear regressions between OPPPWI (Gp 1) and OPPPWO (Gp 2). The data are shown below:

Gp 1	
10^7	3, 4, 4, 4, 5, 5, 5, 6, 6, (7)
10^6	4, 5, 6, 6, 6, 6, 6, 9, 10, (10)
10^5	6, 6, 6, 6, 7, 7, 8, (8)
10^4	7, 7, 8, (10)

Gp 2 As for Gp 1 with the highest observation in each dilution (bracketed) removed by data trimming.

GP 1 NO PTS: 32

R SS	29.1142	MS	29.1142	F	12.4589
E SS	70.1046	MS	2.3368		
T SS	99.2188				
SLOPE-	0.9411				

GP 2 NO PTS: 28

R SS	20.9999	MS	20.9999	F	12.1332
E SS	45.0001	MS	1.7308		
T SS	66.0000				
SLOPE-	0.8750				

C R DF	1.0000	SS	50.4420	MS	50.4420	F	24.5407
CON DF	1.0000	SS	1.1088	MS	1.1088	F	0.5395
SL DF	1.0000	SS	0.3279	MS	0.3279	F	0.1595
ERR DF	56.0000	SS	115.1050	MS	2.0554		

TABLE 60 EXPERIMENT 9

The results of a comparison of linear regressions between OSTWI (Gp 1) and OSTWO (Gp 2). The data are shown below:

Gp 1

10^7	10, 11, 11, 12, 12, (15)
10^6	11, 12, 12, 12, 13, 13, (15)
10^5	12, 12, 12, 13, 13, 14, 14, (14)
10^4	12, 14, 15, (16)

Gp 2 As for Gp 1 with the highest observation in each dilution (bracketed) removed by data trimming.

GP 1 NO PTS:25

R SS	13.8846	MS	13.8846	F	7.9607
E SS	40.1154	MS	1.7441		
T SS	54.0000				
SLOPE-	0.7308				

GP 2 NO PTS:21

R SS	13.7156	MS	13.7156	F	17.1023
E SS	15.2375	MS	0.8020		
T SS	28.9531				
SLOPE-	0.8091				

C R DF	1.0000	SS	27.6659	MS	27.6659	F	20.9920
CON DF	1.0000	SS	1.8726	MS	1.8726	F	1.4208
SL DF	1.0000	SS	0.0657	MS	0.0657	F	0.0498
ERR DF	42.0000	SS	55.3529	MS	1.3179		

TABLE 61 EXPERIMENT 9

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and OSTW0 (Gp 2). The data are shown facing Tables 59 and 60 respectively.

GP 1 NO PTS:28

R SS	20.9999	MS	20.9999	F	12.1332
E SS	45.0001	MS	1.7308		
T SS	66.0000				
SLOPE-	0.8750				

GP 2 NO PTS:21

R SS	13.7156	MS	13.7156	F	17.1023
E SS	15.2375	MS	0.8020		
T SS	28.9531				
SLOPE-	0.8091				

C R DF	1.0000	SS	71.3940	MS	71.3940	F	53.3343
CON DF	1.0000	SS	415.2420	MS	415.2420	F	310.2030
SL DF	1.0000	SS	36.6785	MS	36.6785	F	27.4003
ERR DF	45.0000	SS	60.2376	MS	1.3386		

TABLE 62 EXPERIMENT 9

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and DPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 59.

Gp 2

10^7	4, 4, 4, 5, 5, 6, 6, 7, 8
10^6	6, 6, 6, 6, 6, 6, 6, 7, 8
10^5	6, 6, 7, 7, 7, 7, 7, 8
10^4	-

GP 1 NO PTS:28

R SS	20.9999	MS	20.9999	F	12.1332
E SS	45.0001	MS	1.7308		
T SS	66.0000				
SLOPE-	0.8750				

GP 2 NO PTS:26

R SS	8.7638	MS	8.7638	F	9.0368
E SS	23.2748	MS	0.9698		
T SS	32.0386				
SLOPE-	0.7188				

C R DF	1.0000	SS	28.4625	MS	28.4625	F	20.8440
CON DF	1.0000	SS	0.4986	MS	0.4986	F	0.3651
SL DF	1.0000	SS	1.3012	MS	1.3012	F	0.9529
ERR DF	50.0000	SS	68.2749	MS	1.3655		

TABLE 63 EXPERIMENT 9

The results of a comparison of linear regressions between OSTWO (Gp 1) and DSTWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 60.

Gp 2

10^7	9, 10, 12, 12, 12, 13, 13, 14
10^6	11, 12, 12, 12, 12, 13, 14
10^5	13, 13, 13, 13, 13, 14, 15, 15
10^4	-

GP 1 NO PTS:21

R SS	13.7156	MS	13.7156	F	17.1023
E SS	15.2375	MS	0.8020		
T SS	28.9531				
SLOPE-	0.8091				

GP 2 NO PTS:23

R SS	12.2500	MS	12.2500	F	8.2377
E SS	31.2285	MS	1.4871		
T SS	43.4785				
SLOPE-	0.8750				

C R DF	1.0000	SS	23.3488	MS	23.3488	F	20.0997
CON DF	1.0000	SS	0.5684	MS	0.5684	F	0.4893
SL DF	1.0000	SS	2.6168	MS	2.6168	F	2.2527
ERR DF	40.0000	SS	46.4660	MS	1.1617		

TABLE 64 EXPERIMENT 9

The results of a comparison of linear regressions between DPPPWO (Gp 1) and DSTWO (Gp 2). The data are shown facing Tables 62 and 63 respectively.

GP 1 NO PTS:26

R SS	8.7638	MS	8.7638	F	9.0368
E SS	23.2748	MS	0.9698		
T SS	32.0386				
SLOPE-	0.7188				

GP 2 NO PTS:23

R SS	12.2500	MS	12.2500	F	8.2377
E SS	31.2285	MS	1.4871		
T SS	43.4785				
SLOPE-	0.8750				

C R DF	1.0000	SS	25.8588	MS	25.8588	F	21.3500
CON DF	1.0000	SS	492.7530	MS	492.7530	F	406.8350
SL DF	1.0000	SS	4.8450	MS	4.8450	F	4.0002
ERR DF	45.0000	SS	54.5033	MS	1.2112		

TABLE 65 EXPERIMENT 9

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and ppPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 59.

Gp 2

10^7	3, 4, 4, 5, 5, 5, 6, 6
10^6	6, 6, 6, 6, 7, 7
10^5	7, 7, 7, 8
10^4	8, 8, 9, 9

GP 1 NO PTS:28

R SS	20.9999	MS	20.9999	F	12.1332
E SS	45.0001	MS	1.7308		
T SS	66.0000				
SLOPE-	0.8750				

GP 2 NO PTS:22

R SS	41.7085	MS	41.7085	F	75.3918
E SS	11.0645	MS	0.5532		
T SS	52.7730				
SLOPE-	1.2366				

C R DF	1.0000	SS	61.2220	MS	61.2220	F	50.2316
CON DF	1.0000	SS	1.2471	MS	1.2471	F	1.0232
SL DF	1.0000	SS	1.4864	MS	1.4864	F	1.2196
ERR DF	46.0000	SS	56.0646	MS	1.2188		

TABLE 66 EXPERIMENT 9

The results of a comparison of linear regressions between OSTWO (Gp 1) and ppSTWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 60.

Gp 2

10^7	11, 11, 11, 11, 12, 12, 12, 13
10^6	11, 12, 13, 13, 13, 13
10^5	13, 13, 13, 13
10^4	14, 14, 14, 14

GP 1 NO PTS:22

R SS	13.6029	MS	13.6029	F	34.4507
E SS	7.8971	MS	0.3949		
T SS	21.5000				
SLOPE-	0.7557				

GP 2 NO PTS:21

R SS	13.7156	MS	13.7156	F	17.1023
E SS	15.2375	MS	0.8020		
T SS	28.9531				
SLOPE-	0.8091				

C R DF	1.0000	SS	26.1819	MS	26.1819	F	44.1373
CON DF	1.0000	SS	0.1524	MS	0.1524	F	0.2568
SL DF	1.0000	SS	1.1366	MS	1.1366	F	1.9161
ERR DF	39.0000	SS	23.1345	MS	0.5932		

TABLE 67 EXPERIMENT 9

The results of a comparison of linear regressions between ppPPPW0 (Gp 1) and ppSTW0 (Gp 2). The data are shown facing Tables 65 and 66 respectively.

GP 1 NO PTS:22

R SS	41.7085	MS	41.7085	F	75.3918
E SS	11.0645	MS	0.5532		
T SS	52.7730				
SLOPE-	1.2366				

GP 2 NO PTS:22

R SS	13.6029	MS	13.6029	F	34.4507
E SS	7.8971	MS	0.3949		
T SS	21.5000				
SLOPE-	0.7557				

C R DF	1.0000	SS	40.5295	MS	40.5295	F	85.4984
CON DF	1.0000	SS	420.3640	MS	420.3640	F	886.7730
SL DF	1.0000	SS	14.7820	MS	14.7820	F	31.1832
ERR DF	40.0000	SS	18.9615	MS	0.4740		

TABLE 68 EXPERIMENT 9

The results of a comparison of linear regressions between OPPPWO (Gp 1) and APPPWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 59.

Gp 2

10^7	4, 4, 5, 5, 5, 6
10^6	6, 7, 7, 7, 7, 7, 8
10^5	6, 7, 7, 7, 8, 8, 8
10^4	-

GP 1 NO PTS:28

R SS	20.9999	MS	20.9999	F	12.1332
E SS	45.0001	MS	1.7308		
T SS	66.0000				
SLOPE-	0.8750				

GP 2 NO PTS:20

R SS	18.6717	MS	18.6717	F	27.3724
E SS	12.2785	MS	0.6821		
T SS	30.9502				
SLOPE-	1.2008				

C R DF	1.0000	SS	37.6951	MS	37.6951	F	28.9565
CON DF	1.0000	SS	2.3623	MS	2.3623	F	1.8147
SL DF	1.0000	SS	1.9765	MS	1.9765	F	1.5183
ERR DF	44.0000	SS	57.2786	MS	1.3018		

The results of a comparison of linear regressions between OSTWO (Gp 1) and ASTWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 60.

Gp 2

10^7	10, 11, 11, 11
10^6	12, 12, 13, 13, 14
10^5	12, 13, 13, 13, 13, 13, 14
10^4	-

GP 1 NO PTS:21

R SS	13.7156	MS	13.7156	F	17.1023
E SS	15.2375	MS	0.8020		
T SS	28.9531				
SLOPE-	0.8091				

GP 2 NO PTS:16

R SS	11.3308	MS	11.3308	F	18.8417
E SS	8.4192	MS	0.6014		
T SS	19.7500				
SLOPE-	1.0419				

C R DF	1.0000	SS	24.4225	MS	24.4225	F	34.0584
CON DF	1.0000	SS	0.0000	MS	0.0000	F	0.0000
SL DF	1.0000	SS	0.6239	MS	0.6239	F	0.8704
ERR DF	33.0000	SS	23.6567	MS	0.7169		

TABLE 70 EXPERIMENT 9

The results of a comparison of linear regressions between APPPW0 (Gp 1) and ASTW0 (Gp 2). The data are shown facing Tables 68 and 69 respectively.

GP 1 NO PTS:20

R SS	18.6717	MS	18.6717	F	27.3724
E SS	12.2785	MS	0.6821		
T SS	30.9502				
SLOPE-	1.2008				

GP 2 NO PTS:16

R SS	11.3308	MS	11.3308	F	18.8417
E SS	8.4192	MS	0.6014		
T SS	19.7500				
SLOPE-	1.0419				

C R DF	1.0000	SS	48.1172	MS	48.1172	F	74.3926
CON DF	1.0000	SS	275.8200	MS	275.8200	F	426.4380
SL DF	1.0000	SS	18.1147	MS	18.1147	F	28.0065
ERR DF	32.0000	SS	20.6976	MS	0.6468		

TABLE 71 EXPERIMENT 9

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and ppPPPW0 (Gp 2). The data are shown facing Tables 62 and 65 respectively.

GP 1 NO PTS:26

R SS	8.7638	MS	8.7638	F	9.0368
E SS	23.2748	MS	0.9698		
T SS	32.0386				
SLOPE-	0.7188				

GP 2 NO PTS:22

R SS	41.7085	MS	41.7085	F	75.3918
E SS	11.0645	MS	0.5532		
T SS	52.7730				
SLOPE-	1.2366				

C/R DF	1.0000	SS	47.7336	MS	47.7336	F	61.1626
CON DF	1.0000	SS	0.1885	MS	0.1885	F	0.2415
SL DF	1.0000	SS	2.7386	MS	2.7386	F	3.5091
ERR DF	44.0000	SS	34.3393	MS	0.7804		

TABLE 72 EXPERIMENT 9

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and APPPW0 (Gp 2). The data are shown facing Tables 62 and 68 respectively.

GP 1 NO PTS:26

R SS	8.7638	MS	8.7638	F	9.0368
E SS	23.2748	MS	0.9698		
T SS	32.0386				
SLOPE-	0.7188				

GP 2 NO PTS:20

R SS	18.6717	MS	18.6717	F	27.3724
E SS	12.2785	MS	0.6821		
T SS	30.9502				
SLOPE-	1.2008				

C R DF	1.0000	SS	26.1333	MS	26.1333	F	30.8720
CON DF	1.0000	SS	0.7505	MS	0.7505	F	0.8866
SL DF	1.0000	SS	1.3022	MS	1.3022	F	1.5383
ERR DF	42.0000	SS	35.5533	MS	0.8465		

TABLE 73 EXPERIMENT 9

The results of a comparison of linear regressions between ppPPPW0 (Gp 1) and APPPW0 (Gp 2). The data are shown facing Tables 65 and 68 respectively.

GP 1 NO PTS:20

R SS	18.6717	MS	18.6717	F	27.3724
E SS	12.2785	MS	0.6821		
T SS	30.9502				
SLOPE-	1.2008				

GP 2 NO PTS:22

R SS	41.7085	MS	41.7085	F	75.3918
E SS	11.0645	MS	0.5532		
T SS	52.7730				
SLOPE-	1.2366				

C R DF	1.0000	SS	59.6548	MS	59.6548	F	97.1122
CON DF	1.0000	SS	0.1817	MS	0.1817	F	0.2957
SL DF	1.0000	SS	0.7254	MS	0.7254	F	1.1809
ERR DF	38.0000	SS	23.3429	MS	0.6143		

TABLE 74 EXPERIMENT 9

The results of a comparison of linear regressions between DSTWO (Gp 1) and ppSTWO (Gp 2). The data are shown facing Tables 63 and 66 respectively.

GP 1 NO PTS:23

R SS	12.2500	MS	12.2500	F	8.2377
E SS	31.2285	MS	1.4871		
T SS	43.4785				
SLOPE-	0.8750				

GP 2 NO PTS:22

R SS	13.6029	MS	13.6029	F	34.4507
E SS	7.8971	MS	0.3949		
T SS	21.5000				
SLOPE-	0.7557				

C R DF	1.0000	SS	25.4788	MS	25.4788	F	26.6994
CON DF	1.0000	SS	0.1328	MS	0.1328	F	0.1392
SL DF	1.0000	SS	0.3742	MS	0.3742	F	0.3921
ERR DF	41.0000	SS	39.1256	MS	0.9543		

TABLE 75 EXPERIMENT 9

The results of a comparison of linear regressions between DSTWO (Gp 1) and ASTWO (Gp 2). The data are shown facing Tables 63 and 69 respectively.

GP 1 NO PTS:23

R SS	12.2500	MS	12.2500	F	8.2377
E SS	31.2285	MS	1.4871		
T SS	43.4785				
SLOPE-	0.8750				

GP 2 NO PTS:16

R SS	11.3308	MS	11.3308	F	18.8417
E SS	8.4192	MS	0.6014		
T SS	19.7500				
SLOPE-	1.0419				

C R DF	1.0000	SS	22.3525	MS	22.3525	F	19.7323
CON DF	1.0000	SS	0.5156	MS	0.5156	F	0.4552
SL DF	1.0000	SS	1.2283	MS	1.2283	F	1.0843
ERR DF	35.0000	SS	39.6477	MS	1.1328		

TABLE 76 EXPERIMENT 9

The results of a comparison of linear regressions between ppSTWO (Gp 1) and ASTWO (Gp 2). The data are shown facing Tables 66 and 69 respectively.

GP 1 NO PTS: 22

R SS	13.6029	MS	13.6029	F	34.4507
E SS	7.8971	MS	0.3949		
T SS	21.5000				
SLOPE-	0.7557				

GP 2 NO PTS: 16

R SS	11.3308	MS	11.3308	F	18.8417
E SS	8.4192	MS	0.6014		
T SS	19.7500				
SLOPE-	1.0419				

C R DF	1.0000	SS	24.0896	MS	24.0896	F	50.1983
CON DF	1.0000	SS	0.1455	MS	0.1455	F	0.3032
SL DF	1.0000	SS	0.8442	MS	0.8442	F	1.7591
ERR DF	34.0000	SS	16.3162	MS	0.4799		

TABLE 77 EXPERIMENT 10

The results of the prefreeze and post-thaw infectivity titrations, with and without data trimming, of Babesia microti-infected mouse blood. The labels used in Tables 77 to 92 are:

Label	Regression
	(i) Prefreeze bloods
OPPPWI	Buffer only diluted/Prepatent period/Untrimmed data
OPPPWO	Buffer only diluted/Prepatent period/Trimmed data
DPPPWI	DMSO-diluted/Prepatent period/Untrimmed data
DPPPWO	DMSO-diluted/Prepatent period/Trimmed data
	(ii) Post-thaw bloods
RAPPPPWI	Rapidly cooled/Prepatent period/Untrimmed data
RAPPPPWO	Rapidly cooled/Prepatent period/Trimmed data
CONPPPWI	Slowly cooled/Prepatent period/Untrimmed data
CONPPPWO	Slowly cooled/Prepatent period/Trimmed data

	OPPPWI	DPPPWI	CONPPPWI	RAPPPPWI	OPPPWO	DPPPWO	CONPPPWO	RAPPPPWO
r	-0.5947	-0.6100	-0.5953	-0.6328	-0.6875	-0.7129	-0.8204	-0.8448
n	30	29	14	27	27	26	12	24
b	-2.0500	-2.336	-3.8571	-2.0600	-1.9444	-2.224	-4.5000	-2.2393
m	15.7333	18.409	38.1428	19.3666	14.6296	17.163	41.5000	19.9658
7	1.3833	2.054	11.1428	4.9466	1.0185	1.591	10.000	4.2905
6	3.4333	4.393	15.000	7.0066	2.9632	3.819	14.5000	6.5300
5	5.4833	6.727		9.0666	4.9074	6.040		8.7692

TABLE 78 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between OPPPWI (Gp 1) and OPPPWO (Gp 2). The data are shown below:

Gp 1	
10^7	1, 1, 1, 1, 1, 1, 1, 1, 2, (4)
10^6	2, 2, 2, 2, 2, 2, 2, 2, 9, (9)
10^5	3, 4, 4, 4, 5, 5, 5, 6, 9, (10)

Gp 2 As for Gp 1 with the highest observation in each dilution (bracketed) removed by data trimming.

GP 1 NO PTS:30

R SS	84.0500	MS	84.0500	F	17.6527
E SS	133.3170	MS	4.7613		
T SS	217.3670				
SLOPE-	2.0500				

GP 2 NO PTS:27

R SS	68.0555	MS	68.0555	F	24.6909
E SS	68.9075	MS	2.7563		
T SS	136.9630				
SLOPE-	1.9444				

C R DF	1.0000	SS	152.0000	MS	152.0000	F	39.8370
CON DF	1.0000	SS	3.1442	MS	3.1442	F	0.8240
SL DF	1.0000	SS	0.1055	MS	0.1055	F	0.0277
ERR DF	53.0000	SS	202.2240	MS	3.8155		

TABLE 79 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between OPPPWO (Gp 1) and DPPPWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 78

Gp 2

10^7	1, 1, 1, 1, 1, 1, 1, 2, 2
10^6	1, 2, 3, 3, 4, 4, 7, 8, 9
10^5	5, 5, 5, 5, 5, 5, 7, 8

GP 1 NO PTS:27

R SS	68.0555	MS	68.0555	F	24.6909
E SS	68.9075	MS	2.7563		
T SS	136.9630				
SLOPE-	1.9444				

GP 2 NO PTS:26

R SS	83.9308	MS	83.9308	F	24.8118
E SS	81.1847	MS	3.3827		
T SS	165.1150				
SLOPE-	2.2245				

C R DF	1.0000	SS	149.5940	MS	149.5940	F	48.8374
CON DF	1.0000	SS	7.8084	MS	7.8084	F	2.5492
SL DF	1.0000	SS	2.3923	MS	2.3923	F	0.7810
ERR DF	49.0000	SS	150.0920	MS	3.0631		

TABLE 80 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and RAPPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 78.

Gp 2

10^7	3, 3, 3, 4, 4, 4, 4, 5, 6
10^6	6, 6, 6, 6, 7, 7, 8, 8, 10
10^5	8, 8, 8, 8, 9, 9

GP 1 NO PTS: 27

R SS	68.0555	MS	68.0555	F	24.6909
E SS	68.9075	MS	2.7563		
T SS	136.9630				
SLOPE-	1.9444				

GP 2 NO PTS: 24

R SS	73.3376	MS	73.3376	F	55.3256
E SS	29.1624	MS	1.3256		
T SS	102.5000				
SLOPE-	2.2393				

C R DF	1.0000	SS	119.1180	MS	119.1180	F	57.0875
CON DF	1.0000	SS	137.2820	MS	137.2820	F	65.7925
SL DF	1.0000	SS	22.2747	MS	22.2747	F	10.6751
ERR DF	47.0000	SS	98.0699	MS	2.0866		

TABLE 81 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and RAPPPPW0 (Gp 2). The data are shown facing Tables 79 and 80 respectively.

GP 1 NO PTS:26

R SS	83.9308	MS	83.9308	F	24.8118
E SS	81.1847	MS	3.3827		
T SS	165.1150				
SLOPE-	2.2245				

GP 2 NO PTS:24

R SS	73.3376	MS	73.3376	F	55.3256
E SS	29.1624	MS	1.3256		
T SS	102.5000				
SLOPE-	2.2393				

C R DF	1.0000	SS	144.9290	MS	144.9290	F	60.4162
CON DF	1.0000	SS	79.2047	MS	79.2047	F	33.0178
SL DF	1.0000	SS	12.3391	MS	12.3391	F	5.1438
ERR DF	46.0000	SS	110.3470	MS	2.3988		

TABLE 82 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between OPPPWO (Gp 1) and CONPPPWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 78.

Gp 2

10^7	8, 9, 10, 10, 11, 12
10^6	13, 13, 13, 15, 15, 18
10^5	-

GP 1 NO PTS:27

R SS	68.0555	MS	68.0555	F	24.6909
E SS	68.9075	MS	2.7563		
T SS	136.9630				
SLOPE-	1.9444				

GP 2 NO PTS:12

R SS	60.7500	MS	60.7500	F	20.5932
E SS	29.5000	MS	2.9500		
T SS	90.2500				
SLOPE-	4.5000				

C R DF	1.0000	SS	4.2667	MS	4.2667	F	1.5175
CON DF	1.0000	SS	716.5310	MS	716.5310	F	254.8440
SL DF	1.0000	SS	124.5390	MS	124.5390	F	44.2940
ERR DF	35.0000	SS	98.4075	MS	2.8116		

TABLE 83 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and CONPPPW0 (Gp 2). The data are shown facing Tables 79 and 82 respectively.

GP 1 NO PTS:26

R SS	83.9308	MS	83.9308	F	24.8118
E SS	81.1847	MS	3.3827		
T SS	165.1150				
SLOPE-	2.2245				

GP 2 NO PTS:12

R SS	60.7500	MS	60.7500	F	20.5932
E SS	29.5000	MS	2.9500		
T SS	90.2500				
SLOPE-	4.5000				

C R DF	1.0000	SS	16.5356	MS	16.5356	F	5.0794
CON DF	1.0000	SS	595.8980	MS	595.8980	F	183.0470
SL DF	1.0000	SS	128.1450	MS	128.1450	F	39.3635
ERR DF	34.0000	SS	110.6850	MS	3.2554		

TABLE 84 EXPERIMENT 10

Babesia microti

The results of a comparison of linear regressions between RAPPPW0 (Gp 2) and CONPPPW0 (Gp 1). The data are shown facing Tables 80 and 82 respectively.

GP 1 NO PTS:12

R SS	60.7500	MS	60.7500	F	20.5932
E SS	29.5000	MS	2.9500		
T SS	90.2500				
SLOPE-	4.5000				

GP 2 NO PTS:24

R SS	73.3376	MS	73.3376	F	55.3256
E SS	29.1624	MS	1.3256		
T SS	102.5000				
SLOPE-	2.2393				

C R DF	1.0000	SS	42.5633	MS	42.5633	F	23.2180
CON DF	1.0000	SS	288.0000	MS	288.0000	F	157.1020
SL DF	1.0000	SS	91.5242	MS	91.5242	F	49.9259
ERR DF	32.0000	SS	58.6624	MS	1.8332		

TABLE 85 EXPERIMENT 10

The results of the prefreeze and post-thaw infectivity titrations, with and without data trimming, of Nuttallia musculi-infected mouse blood.

	OPPPWI	DPPPWI	CONPPPWI	RAPPPPWI	OPPPWO	DPPPWO	CONPPPWO	RAPPPPWO
r	-0.6595	-0.4950	-0.7715	-0.4928	-0.8569	-0.6955	-0.8927	-0.5722
n	30	30	27	29	27	27	24	26
b	-1.7500	-1.7000	-3.1048	-2.375	-1.9444	-1.888	-3.4233	-2.4333
m	13.700	14.266	30.4104	22.375	14.4444	14.914	31.8913	21.9666
7	1.4500	2.366	8.6768	5.7500	0.8333	1.592	7.9275	4.9333
6	3.2000	4.066	11.7816	-	2.778	3.574	11.2975	-
5	4.9500	5.766	14.8864	10.500	4.7222	5.370	14.7743	9.8000

- = not computed

Nuttallia musculi

The results of a comparison of linear regressions between OPPPWI (Gp 1) and OPPPWO (Gp 2). The data are shown below:

Gp 1	
10^7	1, 1, 1, 1, 1, 1, 1, 1, 1, (7)
10^6	1, 1, 2, 2, 2, 2, 2, 4, 6, (7)
10^5	4, 4, 5, 5, 5, 5, 5, 5, 6, (7)

Gp 2 As for Gp 1 with the highest observation in each dilution (bracketed) removed by data trimming.

GP 1 NO PTS:30

R SS	61.2500	MS	61.2500	F	21.5588
E SS	79.5500	MS	2.8411		
T SS	140.8000				
SLOPE-	1.7500				

GP 2 NO PTS:27

R SS	68.0555	MS	68.0555	F	69.1308
E SS	24.6111	MS	0.9844		
T SS	92.6667				
SLOPE-	1.9444				

C R DF	1.0000	SS	128.9470	MS	128.9470	F	65.6118
CON DF	1.0000	SS	2.5333	MS	2.5333	F	1.2890
SL DF	1.0000	SS	0.3582	MS	0.3582	F	0.1823
ERR DF	53.0000	SS	104.1610	MS	1.9653		

TABLE 87 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and DPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 86.

Gp 2

10^7	1, 1, 1, 1, 1, 2, 2, 2, 6
10^6	1, 2, 2, 2, 2, 2, 2, 5, 8
10^5	5, 5, 5, 5, 6, 6, 6, 6, 7

GP 1 NO PTS:27

R SS	68.0555	MS	68.0555	F	69.1308
E SS	24.6111	MS	0.9844		
T SS	92.6667				
SLOPE-	1.9444				

GP 2 NO PTS:27

R SS	64.2222	MS	64.2222	F	23.4324
E SS	68.5186	MS	2.7407		
T SS	132.7410				
SLOPE-	1.8889				

C R DF	1.0000	SS	132.2500	MS	132.2500	F	71.0031
CON DF	1.0000	SS	6.6852	MS	6.6852	F	3.5892
SL DF	1.0000	SS	0.0278	MS	0.0278	F	0.0149
ERR DF	50.0000	SS	93.1298	MS	1.8626		

TABLE 88 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between OPPPW0 (Gp 1) and RAPPPPW0 (Gp 2). The data are shown below:

Gp 1 As shown facing Table 86.

Gp 2

10^7	3, 3, 3, 4, 4, 5, 5, 9, 9
10^6	6, 6, 7, 7, 7, 8, 9
10^5	9, 9, 9, 10, 10, 10, 11, 11

GP 1 NO PTS:27

R SS	68.0555	MS	68.0555	F	69.1308
E SS	24.6111	MS	0.9844		
T SS	92.6667				
SLOPE-	1.9444				

GP 2 NO PTS:24

R SS	100.3370	MS	100.3370	F	37.9521
E SS	58.1631	MS	2.6438		
T SS	158.5000				
SLOPE-	2.4324				

C R DF	1.0000	SS	156.0470	MS	156.0470	F	88.6049
CON DF	1.0000	SS	254.1280	MS	254.1280	F	144.2960
SL DF	1.0000	SS	12.3454	MS	12.3454	F	7.0098
ERR DF	47.0000	SS	82.7743	MS	1.7612		

TABLE 89 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and RAPPPPW0 (Gp 2). The data are shown facing Tables 87 and 88 respectively.

GP 1 NO PTS:24

R SS	100.3370	MS	100.3370	F	37.9521
E SS	58.1631	MS	2.6438		
T SS	158.5000				
SLOPE-	2.4324				

GP 2 NO PTS:27

R SS	64.2222	MS	64.2222	F	23.4324
E SS	68.5186	MS	2.7563		
T SS	132.7410				
SLOPE-	1.8889				

C R DF	1.0000	SS	153.4080	MS	153.4080	F	56.9156
CON DF	1.0000	SS	180.4460	MS	180.4460	F	66.9469
SL DF	1.0000	SS	11.1513	MS	11.1513	F	4.1372
ERR DF	47.0000	SS	126.6820	MS	2.6954		

TABLE 90 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between OPPPWO (Gp 1) and CONPPPWO (Gp 2). The data are shown below:

Gp 1 As shown facing Table 86.

Gp 2

10^7	6, 7, 8, 8, 8, 9, 9, 11
10^6	9, 10, 10, 11, 11, 11, 11, 12, 12
10^5	12, 14, 15, 16, 16, 16, 17

GP 1 NO PTS:27

R SS	68.0555	MS	68.0555	F	69.1308
E SS	24.6111	MS	0.9844		
T SS	92.6667				
SLOPE-	1.9444				

GP 2 NO PTS:24

R SS	175.3020	MS	175.3020	F	86.3623
E SS	44.6566	MS	2.0298		
T SS	219.9590				
SLOPE-	3.4233				

C R DF	1.0000	SS	202.6100	MS	202.6100	F	137.4760
CON DF	1.0000	SS	903.0610	MS	903.0610	F	612.7500
SL DF	1.0000	SS	40.7476	MS	40.7476	F	27.6433
ERR DF	47.0000	SS	69.2678	MS	1.4738		

TABLE 91 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between DPPPW0 (Gp 1) and CONPPPW0 (Gp 2). The data are shown facing Tables 87 and 90 respectively.

GP 1 NO PTS: 24

R SS	175.3020	MS	175.3020	F	86.3623
E SS	44.6566	MS	2.0298		
T SS	219.9590				
SLOPE-	3.4233				

GP 2 NO PTS: 27

R SS	64.2222	MS	64.2222	F	23.4324
E SS	68.5186	MS	2.7407		
T SS	132.7410				
SLOPE-	1.8889				

C R DF	1.0000	SS	199.5120	MS	199.5120	F	82.8543
CON DF	1.0000	SS	758.5950	MS	758.5950	F	315.0330
SL DF	1.0000	SS	40.0126	MS	40.0126	F	16.6166
ERR DF	47.0000	SS	113.1750	MS	2.4080		

TABLE 92 EXPERIMENT 10

Nuttallia musculi

The results of a comparison of linear regressions between RAPPPPWO (Gp 1) and CONPPPWO (Gp 2). The data are shown facing Tables 88 and 90 respectively.

GP 1 NO PTS:24

R SS	100.3370	MS	100.3370	F	37.9521
E SS	58.1631	MS	2.6438		
T SS	158.5000				
SLOPE-	2.4324				

GP 2 NO PTS:24

R SS	175.3020	MS	175.3020	F	86.3623
E SS	44.6566	MS	2.0298		
T SS	219.9590				
SLOPE-	3.4233				

C R DF	1.0000	SS	267.8350	MS	267.8350	F	114.6150
CON DF	1.0000	SS	188.0210	MS	188.0210	F	80.4602
SL DF	1.0000	SS	7.8045	MS	7.8045	F	3.3398
ERR DF	44.0000	SS	102.8200	MS	2.3368		

TABLE 93

The 5 per cent points of the F distribution.

Table 93 is taken from Table V of Fisher and Yates: Statistical Tables for Biological, Agricultural and Medical Research, published by Longman Group Ltd., London (previously published by Oliver and Boyd, Edinburgh), and by permission of the authors and publishers.

n_2 \ n_1	1	2	3	4	5	6	8	12	24	∞
1	161·4	199·5	215·7	224·6	230·2	234·0	238·9	243·9	249·0	254·3
2	18·51	19·00	19·16	19·25	19·30	19·33	19·37	19·41	19·45	19·50
3	10·13	9·55	9·28	9·12	9·01	8·94	8·84	8·74	8·64	8·53
4	7·71	6·94	6·59	6·39	6·26	6·16	6·04	5·91	5·77	5·63
5	6·61	5·79	5·41	5·19	5·05	4·95	4·82	4·68	4·53	4·36
6	5·99	5·14	4·76	4·53	4·39	4·28	4·15	4·00	3·84	3·67
7	5·59	4·74	4·35	4·12	3·97	3·87	3·73	3·57	3·41	3·23
8	5·32	4·46	4·07	3·84	3·69	3·58	3·44	3·28	3·12	2·93
9	5·12	4·26	3·86	3·63	3·48	3·37	3·23	3·07	2·90	2·71
10	4·96	4·10	3·71	3·48	3·33	3·22	3·07	2·91	2·74	2·54
11	4·84	3·98	3·59	3·36	3·20	3·09	2·95	2·79	2·61	2·40
12	4·75	3·88	3·49	3·26	3·11	3·00	2·85	2·69	2·50	2·30
13	4·67	3·80	3·41	3·18	3·02	2·92	2·77	2·60	2·42	2·21
14	4·60	3·74	3·34	3·11	2·96	2·85	2·70	2·53	2·35	2·13
15	4·54	3·68	3·29	3·06	2·90	2·79	2·64	2·48	2·29	2·07
16	4·49	3·63	3·24	3·01	2·85	2·74	2·59	2·42	2·24	2·01
17	4·45	3·59	3·20	2·96	2·81	2·70	2·55	2·38	2·19	1·96
18	4·41	3·55	3·16	2·93	2·77	2·66	2·51	2·34	2·15	1·92
19	4·38	3·52	3·13	2·90	2·74	2·63	2·48	2·31	2·11	1·88
20	4·35	3·49	3·10	2·87	2·71	2·60	2·45	2·28	2·08	1·84
21	4·32	3·47	3·07	2·84	2·68	2·57	2·42	2·25	2·05	1·81
22	4·30	3·44	3·05	2·82	2·66	2·55	2·40	2·23	2·03	1·78
23	4·28	3·42	3·03	2·80	2·64	2·53	2·38	2·20	2·00	1·76
24	4·26	3·40	3·01	2·78	2·62	2·51	2·36	2·18	1·98	1·73
25	4·24	3·38	2·99	2·76	2·60	2·49	2·34	2·16	1·96	1·71
26	4·22	3·37	2·98	2·74	2·59	2·47	2·32	2·15	1·95	1·69
27	4·21	3·35	2·96	2·73	2·57	2·46	2·30	2·13	1·93	1·67
28	4·20	3·34	2·95	2·71	2·56	2·44	2·29	2·12	1·91	1·65
29	4·18	3·33	2·93	2·70	2·54	2·43	2·28	2·10	1·90	1·64
30	4·17	3·32	2·92	2·69	2·53	2·42	2·27	2·09	1·89	1·62
40	4·08	3·23	2·84	2·61	2·45	2·34	2·18	2·00	1·79	1·51
60	4·00	3·15	2·76	2·52	2·37	2·25	2·10	1·92	1·70	1·39
120	3·92	3·07	2·68	2·45	2·29	2·17	2·02	1·83	1·61	1·25
∞	3·84	2·99	2·60	2·37	2·21	2·10	1·94	1·75	1·52	1·00

Lower 5 per cent. points are found by interchange of n_1 and n_2, *i.e.* n_1 must always correspond with the greater mean square.

TABLE 94.

The computer programme written by the author, for the comparison of linear regressions used in the statistical analysis of the data obtained in Experiments 3, 9 and 10. This programme is written in the Restricted FOCAL-12 language, for use with the 'DIGITAL' PDP-12 computer.

```
 W A
C FOCAL-12

01.10 E

02.05 S R=0
02.07 T !!!!!!!!!!!!!!!!!!,"  GP 1
02.11 A " NO PTS",N
02.20 S SX=0; S QX=0; S SY=0; S QY=0; S SP=0; S A=0
02.30 F A=1,N; DO 6

03.10 S WX=QX-SX*SX/N; S WY=QY-SY*SY/N; S WP=SP-SX*SY/N
03.23 S B=WP/WX; S RS=WP*B;  S ES=WY-RS;
03.27 S EM=ES/(N-2)
03.28 S QA=RS/EM
03.29 T !!!!,"  R SS",RS; T "   MS",RS; T "          F",QA
03.30 T !!,"  E SS",ES; T "   MS",EM
03.31 T !!,"  T SS",WY
03.32 T !!,"SLOPE",B

04.10 IF (R)4.2,4.2,4.42
04.20 S N1=N; S  R=R+1; S B1=B
04.25 S S1=RS; S S2=ES;   S M2=EM; S QB=QA
04.40 S S4=SX; S Q1=QX; S S5=SY; S Q2=QY; S P1=SP; S X1=WX; S W1=WP
04.41 T !!!!,"  GP 2"; G 2.11
04.42 S NT=N+N1; S XT=SX+S4; S YT=SY+S5; S PT=SP+P1
04.45 S TY=QY+Q2; S TX=QX+Q1
04.46 S SC=(PT-(XT*YT)/NT)↑2/(TX-XT↑2/NT); S SL=(RS+S1-SC)
04.47 IF (SL)4.48,4.48,4.49
04.48 S SL=SC-(RS+S1)
04.49 S ST=TY-YT↑2/NT;  S SS= ST-(SC+ES+SL+S2)

05.07 S ME=(ES+S2)/(NT-4); S QC=SC/ME; S QS=SS/ME
05.08 S QI=SL/ME
05.10 T !!!!,"C R DF",1; T "   SS",SC; T "   MS",SC; T "   F",QC
05.15 T !!,"CON DF",1; T "   SS",SS; T "   MS",SS; T "   F",QS
05.18 T !!,"  SL DF",1; T "   SS",SL; T "   MS",SL
05.19 T "   F",QI
05.20 T !!,"ERR DF",NT-4; T "   SS",ES+S2; T "   MS",ME
05.70 G 2.05

06.07 T !!,%2.0,A,"          ",%8.04
06.10 A "X  ",X,"       Y ",Y
06.20 IF (1000-X)6.6,6.7; IF (1000-Y)6.6,6.7
06.25 S SX=SX+X; S SY=SY+Y; S QX=QX+X*X; S QY=QY+Y*Y;
06.26 S SP=SP+X*Y; R
06.60 T "    CANCEL",; G 6.07
06.70 T !,"  RESTART"; DO 2.2
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