Physical and biological measurements on the response of Bacillus megaterium spores to high vacuum treatments.

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Physical and Biological Measurements on the Response of *Bacillus megaterium* Spores to High Vacuum Treatments

T H E S I S

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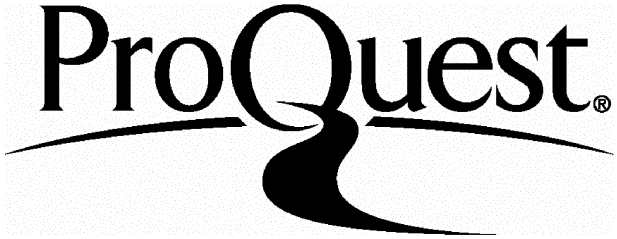
This research has been carried out in the School of Pharmacy of the Bath University of Technology under the supervision of D. J. G. Davies, M.Sc.,Ph.D., M.P.S.



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SUMMARY

The introduction includes a description of the physics of freeze-drying together with the methods used and the variables that have to be controlled. The properties of lyophilised bacteria are discussed, with particular reference to the factors that affect the heat and radiation resistance of freeze-dried spores and the way in which these findings have been used to develop hypotheses concerning the mechanisms of spore inactivation.

The experimental work is divisible into three parts. The first is concerned with physical measurements carried out on the drying system. The temperature and weight of the spore sample were monitored during drying. To explain the results obtained, a hypothesis of reversibly and irreversibly dehydrated sites within the spore has been introduced.

The second section is concerned with the effects of different drying treatments on the biological response of Bacillus megaterium spores. The criteria used were viability, the characteristics of outgrowth and germination, together with the subsequent growth rate of the vegetative cells produced, and the heat and radiation resistance of the spores. Log. survivor/heating time curves obtained in these experiments have been explained on the basis of the shoulder representing the time during which necessary structural changes occur in the spore before the lethal mechanism represented by the heat inactivation constant becomes operative. The effects of different drying treatments have been discussed in relation to this "model".

The final section is concerned with a detailed study of the E.P.R. spectra of dried spores. The development of the resonance signal during drying and during subsequent heating of the dried spores has been considered together with the effect of drying conditions,

oxygen, and rehydration on the resonance signal.

Findings from these experiments have been discussed in the light of current concepts of the mechanisms of spore inactivation and of the influence of water removal on these mechanisms.

C O N T E N T S

ORIGIN AND SCOPE OF THE WORK

Work by Tallentire and his co-workers (150,152,159,165), has been concerned with the investigation of the post-irradiation oxygen effect in spores. Throughout this work the experimental procedure involved gamma irradiation of spores dried in kaolin powders, re-equilibrated to known aqueous vapour pressures, in specific gaseous environments, followed by post irradiation treatment with oxygen, under controlled conditions. During this work it was found that unirradiated control samples of spores when heated at 353°K, in oxygen, exhibited a decrease in viability when in conditions of low moisture content. Furthermore, it was shown that storage over extended periods, of primary dried spore powders, in air at room temperature, induced changes in the radiation resistance of the contained spores (235).

It was therefore apparent that while bacterial spores could be subjected to treatments such as secondary drying, and re-equilibration, that are considered damaging to most biological materials, with generally no loss of viability, nevertheless, under certain conditions such treatments altered the resistance of spores to certain physical agents.

Work in this department (135) has confirmed that secondary drying causes damage to Bacillus megaterium spores, this damage being recognised as variable responses to a given heat treatment being shown by different samples. The fact that this variability only become apparent after heating in the presence of oxygen, and that the induced damage could be reduced by water and free radical scavengers such as Thiourea, is analogous to the findings obtained in studies of radiation damage in spores. It is known that oxygen dependent damage in spores is mediated through free radicals induced by the radiation, (157) and it seemed possible, therefore, that drying might also cause the formation of free radicals which in the absence of water are long-

lived and capable of reaction with oxygen.

It was clear from these findings that it was important for the elucidation of radiation and heat induced lethal mechanisms, to determine the type and extent of changes in spore resistance that could be produced by the secondary drying process, and to determine whether such factors as oxygen and water modify the effects of drying.

The present work was therefore centred on investigating the process of high vacuum drying, and the ways in which this process alters the biological response. It was hoped initially to devise a drying routine in which spores could be dried directly from distilled water suspension, without the use of a supposedly inert carrier such as kaolin. It was then intended to carry out physical measurements on the drying system, with particular reference to sample temperature and weight changes during drying, in an attempt to correlate observed biological effects with such physical parameters as freezing and drying rates. It was decided also, to study in detail the changes in spore integrity induced by drying, as shown by spore viability and germination characteristics, and the effects of the drying process on the resistance of the spores to inactivation by physical agents such as heat and radiation. It was felt that the results of these experiments may help in elucidating the mechanisms involved in the production of the shouldered log.survivor/heating time curves that have been demonstrated with dried Bacillus megaterium spores during previous work (135).

It was also decided to investigate more thoroughly the suggestion that drying might cause the induction of free radicals in the spore, by studying the E.P.R. spectra of dried spores, and the way in which these spectra are affected by the drying conditions, and by the

presence of water and oxygen.

Ultimately, it was hoped, through these studies, to be able to distinguish, when spores are subjected to drying processes, with subsequent re-equilibration or oxygen treatments, prior to heating or irradiation, the contribution of these preliminary processes to the total measured damage.

INTRODUCTION

Freeze-Drying

Freeze-drying or lyophilisation can be defined as the dehydration of a frozen aqueous material through the sublimation of ice. When a tissue or other hydrated biological material dries from the liquid state it suffers severe shrinkage, is often altered chemically, and becomes relatively insoluble, and when preservation for subsequent use is desired, one or more of these changes may render the final product unusable. Dehydration from the frozen state - freeze-drying - has three principle advantages over drying from the liquid state 1) the preservation of morphology, 2) the minimising of chemical changes, and 3) the maintenance of solubility.

The use of freeze-drying for the preservation of biological substances (1) and micro-organisms (2) dates from the first decade of this century, and detailed accounts of the history of its development and application have been given in reviews by Flosdorf (3,4,) and Fry (5).

This review will be mainly concerned with the factors affecting bacteria both during lyophilisation and during subsequent storage. The first sections will be concerned with a description of the physics of freeze-drying together with the methods used and the variables that have to be controlled. The influence of these variables on biological properties and the possible mechanisms by which damage is induced will then be discussed. Finally, the properties of lyophilised bacteria will be considered and in particular the heat and radiation resistance of freeze-dried bacterial spores.

Physics of the Freeze-Drying Process

The freeze-drying process can be subdivided into four distinct stages 1) the transfer of heat to the drying boundary, 2) the

sublimation of ice crystals, 3) the transfer of water vapour from the subliming ice crystal, through the already dried shell of the specimen to the specimen surface, and 4) the removal of the water vapour from the specimen surface. The stages in the drying process are analysed in detail below.

Sublimation

Each water molecule within the lattice of an ice crystal indulges in random motion, the magnitude of which determines the temperature of the crystal. There is a probability that this motion may be sufficient to overcome the restraining energy barrier and permit the molecule to escape from the surface of the crystal. Since random motion is a function of temperature, the probability of escape will increase with increasing temperature. When sublimation from ice takes place within a closed container, the concentration of water molecules in the space around the crystal will increase, and with it the probability of condensation of molecules at the crystal surface. The rate of condensation will be dependent on both temperature and vapour pressure, and at the equilibrium vapour pressure of the system will be equal to the rate of sublimation. The maximum rate of sublimation of ice at a given temperature in therefore

$$\frac{dm}{dt} = \frac{f \alpha P_S}{(2 \pi R T_S)^{\frac{1}{2}}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where $\frac{dm}{dt}$ is the rate of sublimation per unit area. T_S is the absolute temperature of the subliming interface, P_S is the saturated vapour pressure of ice at temperature T_S and R is the gas constant per gramme of water vapour. α is the coefficient of evaporation (6), and is a factor incorporated to allow for possible reduction in net recapture of molecules at the surface as a consequence of molecular rebounding during collisions.. f is often referred to as the drying

factor (7) and is the probability that an evaporated water molecule is removed from the system before it recondenses on the interface. In freeze-drying practice, where the subliming ice crystal is contained within the specimen being dehydrated, f is defined as

$$\frac{1}{f} = \frac{1}{f_s} + \frac{1}{f_e} - 1 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where f_s is the probability that an evaporated water molecule reaches the exterior surface of the specimen before it is recaptured and f_e is the probability that a water molecule which reaches the exterior surface is removed from the system before it re-enters the dried portion of the specimen.

Additional energy, the latent heat of sublimation, is acquired by each water molecule that makes the transition from the solid to the vapour state. Under normal circumstances this additional energy is derived from the ice crystal and its surrounding environment in the form of heat. This means that unless heat is supplied to the specimen, the temperature of the specimen will be reduced, and with it the rate of sublimation, until a temperature too low to support sublimation is reached.

Furthermore, if the escape of water vapour molecules following sublimation is impeded, and the vapour pressure in the vicinity of the subliming interface is increased, the concomitant increase in the amount of water vapour recondensing will decrease the rate of sublimation.

The specimen temperature, and thus the rate of sublimation is therefore dependent on the rate of heat input, and the rate of vapour transfer away from the drying boundary.

Heat transfer to the drying boundary

In order to maintain a constant rate of sublimation, energy must

be supplied to the interface between the dry shell and the frozen interior of the specimen, in the form of heat. If the specimen is uniformly heated from the bottom of the frozen portion, the rate H at which heat will reach the interface, through unit area of the frozen interior is given by

$$H = \frac{k_T \Delta T}{t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where k_T is the coefficient of thermal conductivity of the frozen layer, and ΔT is the absolute temperature drop across the layer of thickness t cms. If L is the latent heat of sublimation of ice, the rate of sublimation $\frac{dm}{dt}$ is given by

$$\frac{dm}{dt} = \frac{H}{L} = \frac{k_T \Delta T}{Lt} \quad \ldots\ldots\ldots\ldots\ldots (4)$$

The thermal conductivity k_T is usually quoted as 5.7 c 10^{-3} cal.cm^{-1} sec^{-1} cm^{-2} $^oC^{-1}$ for pure ice, with lower values being obtained in the presence of solids. Meryman (8) has found that for all practical purposes the thermal conductivities of frozen and unfrozen tissue can be considered to be the same, values of 2.6 x 10^{-3} and 2.8 x 10^{-3} cal.cm^{-1} sec^{-1} cm^{-2} $^oC^{-1}$ being obtained for frozen and unfrozen rabbit liver respectively. However, Kramer (9) has reported conductivities as high as 8.8 x 10^{-3} cal.cm^{-1} sec^{-1} cm^{-2} $^oC^{-1}$ in milk frozen at a rate of 8 x 10^{-1} ^oC min^{-1}, where the measurements were made in the same direction as the heat flow during freezing.

It can be seen from equation (4) that a temperature gradient must exist between the two faces of the frozen mass such that the interface temperature (T_S) will be lower than that of the lower surface of the frozen mass (T_C). This temperature gradient ΔT will decrease with decrease in t until $T_S = T_C$ when sublimation will be complete. However, it is essential that the temperatures T_S and T_C

should be such that a) the ice in contact with the heat source does not melt, and b) that the increased temperature has no deleterious effects on the specimen. Thus, the overall rate of heating will depend on the maximum safe temperature to which the specimen concerned can be heated.

Water vapour transfer through the dried shell

Under freeze-drying conditions, the water vapour produced by sublimation must pass by diffusion through a shell of dried material that increases in thickness with time. The factors controlling the flow of vapour through the dried shell are most readily understood if the shell is assumed to take the form of a number of parallel capillaries. The type of flow that will occur is dependent on the relationship between the mean free path (λ) of the water vapour molecules and the dimensions of the capillaries.

The mean free path is defined as the average distance which a molecule will travel before striking another molecule, and is expressed by the equation

$$\lambda = \frac{1}{\sqrt{2}\,\pi\, n \delta^2} \quad \ldots\ldots\ldots\ldots\ldots (5)$$

where δ is the diameter of the molecule and n is the number of molecules per square cm.

If the mean free path of the water vapour molecule is greater than the diameter of the capillary, the flow is molecular, and can be represented by the equation

$$G = \frac{4}{3} (2\pi RT)^{\frac{1}{2}} \frac{r^3}{\ell} (P_S - P_D) \ldots\ldots (6)$$

where G is the mass rate of flow through a capillary of length ℓ cm. and radius r. cm., P_D is the vapour pressure at the specimen surface, P_S is the vapour pressure at the drying boundary, R is the gas constant and T the absolute temperature. It should be noted that equation (6) is applicable to long tubes where r/ℓ is small and thus corrections

must be applied when the equation is applied to tubes where r/ℓ is large. Under the conditions of molecular flow it is apparent that the mass rate of water vapour flow depends on the pressure gradient across the dried shell.

When the mean free path of the molecules is very much less than the capillary diameter, flow will be viscous and can under isothermal conditions, be represented by Poiseuilles' Law where

$$G = \frac{\pi r^4}{16 \eta RT} (P_S^2 - P_D^2) \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

where η is the coefficient of viscosity. Under these conditions the mass rate of flow is dependent on the viscosity as well as the pressure gradient.

Between the extremes of free molecular flow and viscous flow there occurs a region of viscous slip flow. This is represented by a corrected form of Poiseuilles' Law as

$$G = \frac{\pi r^4}{8 \eta RT} \left[\frac{1}{2} (P_S^2 - P_D^2) + 4 \frac{\gamma}{r} (P_S - P_D) \right] \quad \ldots (8)$$

where γ is a constant and commonly called the coefficient of slip (9).

It has been shown that under certain circumstances the structure of freeze-dried meat (10) and milk (9) is comparable to a system of capillaries, and that the pore sizes would suggest that the flow regime for water vapour would be expected to lie between viscous and molecular in the region of viscous slip.

In general, however, capillaries will be tortuous, of uneven and non-circular cross section, and of different lengths. The dried shell may also have cracks or holes left by subliming ice crystals, that could provide intermittent low resistance pathways for water vapour. Several workers have considered that under these conditions it is possible to have, within the material, all three types of flow

occurring simultaneously (11,12,13). Sandall et al (11) and Mason et al (12) have derived mass transport equations for the freeze-drying process involving all three flow conditions and incorporating constants which characterise the geometry of the medium, and have shown good correlation between experimental results obtained with turkey meat slices, and the theoretical models. It must be borne in mind, however, that in cases where agreement has been demonstrated between theoretical models and experimental results, the materials investigated e.g. meat and vegetable samples, invariably have a high ratio of solids to water. It would be expected therefore that detailed modifications will be necessary when these models are applied to materials such as bacterial suspensions, where the ratio of solids to water is very small.

The overall importance of the resistance to water vapour flow imposed by the dried shell has been highlighted by Stephenson (7) who has demonstrated that a dried shell 1 mm thick reduces the drying rate by a factor in excess of 10^3 relative to the maximum rate of sublimation of ice at the same temperature.

Reduction of water vapour pressure at the specimen surface

It has been shown in the previous section, that the transfer of water vapour across the dried shell, for all types of flow, is dependent on the vapour pressure concentration gradient $P_S - P_D$. In order to achieve the maximum concentration gradient across the dried shell, it is clearly desirable to achieve as low a vapour pressure at the specimen surface as possible. The water vapour sublimed from the specimen must be ultimately removed from the freeze-drying system, either by condensation on a surface maintained at a temperature lower than that of the specimen, by absorption by a chemical or mechanical desiccant, or by being removed from the

closed system by means of vacuum pumps. These constitute the vapour removal surface, which, in a typical freeze-drying apparatus is separated from the specimen surface by a long tube of circular cross section. Thus, the factors affecting the flow of water vapour between these two surfaces are essentially the factors involved in the transfer of vapour through the theoretical capillary structure of the dried shell (see page 8). The ideal conditions, in theory, for the transfer of vapour are those of molecular flow, where

$$G_1 = \frac{4}{3} (2\pi RT)^{\frac{1}{2}} \frac{r_c^3}{\ell_c} (P_D - P_R) \quad \ldots\ldots\ldots\ldots\ldots\ldots (9)$$

where G_1 is the mass of water vapour flow between specimen and removal surfaces, separated by a tube of length ℓ_c cm. and radius r_c cm. P_D is the vapour pressure at the specimen surface, and P_R is the vapour pressure at the vapour removal surface. (Corresponding equations representing viscous and viscous slip flow can be derived also). It can be seen from these equations that in order to obtain the maximum value for G_1 under a given flow condition, it is necessary, in the design of the freeze-drying apparatus to maintain $\frac{r_c}{\ell_c}$ as large as possible. Furthermore, to achieve molecular flow the mean free path of the water vapour molecules must be as large as possible in comparison with the diameter of the tube. Since the mean free path is inversely proportional to the pressure, at constant temperature, it can be increased by reduction of the pressure within the drying apparatus by means of a vacuum pump. The motive force for the transfer of water vapour from the specimen surface to the vapour removal surface is the vapour pressure concentration gradient $(P_D - P_R)$. The design of the apparatus and the use of a vacuum system are important only in providing the minimum mechanical obstruction, and as long a mean free path as possible so that the transfer of water vapour away from the specimen surface is as effective as possible with the minimum number

of collisions of the vapour molecules en route.

Practical Aspects of Freeze-Drying

It has been shown in the previous section that the overall rate of sublimation is dependent on the rate of heat input and the rate of vapour transfer from the drying boundary, and thus the majority of attention has been focused on the practical implications of these aspects of the freeze-drying process.

Heat input

Three methods of introducing heat to a drying specimen have been employed in freeze-drying apparatus. The most common way is by conduction through the frozen mass from the heated surface on which the specimen is supported. The maximum amount of heat that can be delivered to the drying boundary, by this method, is limited by the maximum safe temperature (see page 7) for the specimen in question and the need to prevent drying taking place from the side of the specimen in contact with the heat source.

The second method utilises radiation from infra red sources, and has the advantage that the specimen may be suspended in the drying chamber, and irradiated and heated from all sides. However, because of the low thermal conductivity of the dried shell, there is a high probability that the maximum safe temperature for the specimen will be exceeded under the conditions of radiation heating.

The introduction of energy through high frequency electromagnetic radiation in the microwave region has also been suggested as a method of heat input (14,15) although the practical problems of its control and measurement have yet to be overcome.

Removal of water vapour

The methods used for the ultimate removal of water vapour from the freeze-drying apparatus, have employed the use of vacuum systems

in combination with a) desiccants, b) refrigerated condensers, and c) direct pumping.

The most commonly used desiccant is phosphorus pentoxide, which has an equilibrium vapour pressure of 10^{-23} torr at room temperature. However, it is difficult to maintain a sufficient exposure surface and to dissipate the heat and toxic vapours that are formed during the reaction with water (16). Hence, its use in practice is usually restricted to conditions where only small quantities of water vapour are removed over long periods, such as occur in secondary drying (see page 14). More recently it has been shown that synthetic zeolites, when used at liquid nitrogen temperatures can produce total pressures as low as 5×10^{-11} torr, and are capable of serving both as a water vapour trap and as a vacuum pump for other gases, thus obviating the need for mechanical pumps in the freeze-drying apparatus (17,18).

In modern freeze-drying equipment the refrigerated condenser is the most commonly used method for water vapour removal. The water vapour pressure at the condenser surface will decrease with reduction in condenser temperature and hence the lowest condenser temperatures will provide the most efficient water removal. However, although vapour pressure is related to temperature, this relationship is more nearly logarithmic than linear, and thus to obtain an equivalent vapour pressure differences, a larger temperature difference is required in the higher temperature ranges than in the lower ranges. In practice a temperature differential of 20 to 30^{o}K between specimen and condenser is usually maintained for primary drying. Mechanically refrigerated condensers at temperatures around 233^{o}K are frequently used in industrial freeze-driers, and the problems involved in their use have been discussed by Rowe(19). In small scale laboratory apparatus,

particularly where secondary drying to very low aqueous vapour pressures is required, solid carbon dioxide and liquid gas condensers are used. Since the condenser must have sufficient capacity to remove the heat released by condensation of ice, solid carbon dioxide is usually preferred since it has a higher latent heat of vapourisation than liquid nitrogen or liquid air.

Direct pumping of water vapour is generally inefficient since the capacity of most pumps is insufficient to cope with the large volumes of vapour produced at reduced pressures during freeze-drying. Furthermore, unless a mechanical pump is gas ballasted, the water vapour can cause corrosion and damage.

Secondary Drying

The removal of moisture from substances by sublimation from the ice phase, is often referred to as "primary" drying. However, a fraction of the total water in the sample will remain unfrozen during the initial freezing stage of the "primary" drying process, and will not therefore undergo sublimation. "Secondary" or "isothermal desorption" drying is the name given to the dehydration, under vacuum, of this non-freezable moisture which remains in the specimen after sublimation is complete. The passage of residual moisture from the interior of the specimen to the surface, under "secondary" drying conditions can be represented by Fick's first law of diffusion. This states that the mass of water $d\emptyset$ diffusing in the direction of the surface in a time dt, across an area A is proportional to the concentration gradient dc/dx at the plane in question, where x is the distance measured at right angles to the surface

$$d\emptyset = -DA \frac{dc}{dx} dt$$

The rate of change of concentration of water vapour $\frac{dc}{dt}$ at any given point is given by an exactly equivalent expression, Fick's second law of diffusion.

$$\frac{dc}{dt} = D\frac{d^2c}{dx^2}$$

where D is the coefficient of diffusion of moisture in the sample. The rate of drying is therefore dependent, in "secondary" drying as in "primary" drying, on the vapour pressure concentration gradient between the interior of the sample, the sample surface, and the vapour removal surface. At the end of "primary" drying the temperature will remain constant and will be that of the surrounding environment, and in theory the vapour pressure concentration gradient in the system can be increased by increasing this temperature still further. In practice however, it is usually necessary to maintain the temperature at a fixed level below ambient during the latter part of "primary" drying and throughout "secondary" drying to prevent damage to the specimen.

The vapour pressure concentration gradient can also be increased by obtaining as low an aqueous vapour pressure as possible at the removal surface. This may be achieved by the use of desiccants such as phosphorus pentoxide, or alternatively by refrigerated condensers containing either solid carbon dioxide, in acetone, or alcohol, at about $193^{o}K$, or liquid nitrogen at $77^{o}K$. Low total pressures are achieved by the use of vapour diffusion pumps which give pressures of 10^{-5} to 10^{-10} torr (20,21,22,23). The use of diffusion pumps requires the incorporation into the system of adequate trapping facilities to prevent backstreaming of the mercury or oil vapour onto the specimen.

Like "primary" drying, "desorptive" drying is dependent also on the thickness of the specimen, and the resistance of the dried

material to vapour flow, and thus increased rates of drying can be obtained if the specimen is presented as a thin film. It must be borne in mind also, that a certain amount of "desorptive" drying will take place during "primary" drying, so that only a proportion of the unfrozen water will remain when sublimation is complete.

Factors Affecting Survival of Organisms During Freeze-Drying

There are four principal stages of the freeze-drying process during which injury can occur: during initial freezing, during the drying, during subsequent storage, and on reconstitution. The overall damage caused by the process may result from injury occurring during any one of these stages, or it may be the cumulative effect of injuries occurring during several or all of the stages.

Initial freezing

Many types of cells have been shown to be capable of surviving super-cooling to as low as 263^{o}K with no apparent loss in viability (24,25,26,27,28,29). However, when freezing of the intra- or extracellular water occurs, damage is induced (30,31,32,33,34,35).

The formation of ice can have two main consequences, a) the concentrating of intracellular and extracellular solutes and b) the deposition of ice crystals. It was originally thought that the major effect of ice in causing injury was the mechanical damage induced by the ice crystals, which either compressed the cells, or speared them from outside (36,37). Haines (38), however, showed that at 271^{o}K the death rate for Pseudomonas aeruginosa was maximal, even though, at that temperature, unfrozen medium remained in which the cells were suspended out of contact with ice crystals. It would therefore appear that although extracellular ice may cause some cell injury, it is not responsible for the bulk of the damage caused by freezing.

Mazur (39) has accounted for the majority of low temperature injury in terms of two factors; the concentration of solutes or of a specific solute, either intracellularly or extracellularly, and the formation of large stable intracellular ice drystals. He has also suggested that these two factors may interact synergistically as well as exerting their effects independently, and additively. Evidence for the importance of concentration of extracellular solutes has been reported by Harrison (40). He showed that survival of Escherichia coli frozen to 251^{o}K, was much lower when the suspending medium was broth than when it was distilled water, and that dilution of the broth improved survival. Other workers using a variety of organisms have confirmed these findings and shown that survival was much higher in the total absence of solutes, when the bacteria were suspended in distilled water, than when sodium chloride, peptone, or broth was present in the suspending medium (41,42,43). Recent evidence suggests that death of Escherichia coli during freezing is likely to be due to the damaging effects of particular external solutes, especially sodium chloride, rather than the concentration of solutes in general (44,45). Solutions containing sodium chloride are usually the most lethal, but are much less lethal below their eutectic points. Furthermore, exposing cells to low temperatures in media containing sufficient sodium chloride to keep them unfrozen, is almost as harmful as cooling them to comparable temperatures in dilute solutions that freeze (46). These results would tend to suggest that the concentration of specific ions, such as Na^{+} and Cl^{-} may be involved in freezing damage, and that as a consequence cells should be suspended in water prior to freezing, if viability is to be maintained.

Evidence for the lethal effect of intracellular ice crystals

can be deduced from experiments in which the freezing rates have been compared (39,47,48). It has been demonstrated microscopically that a high rate of cooling induces the formation of intracellular ice crystals in bacteria, whereas low rates of cooling result in ice formation being restricted to the extracellular space, with subsequent dehydration of the cell (34). Many workers have shown that a higher proportion of cells in an aqueous suspension survive slow freezing than survive a rapid rate of freezing (30,31,32,33,34).

It has been further suggested that there is probably both an optimum rate of freezing (31,35) and a minimum safe temperature of freezing (32,33,49) for given cells in aqueous suspension and that these are modified in the presence of colloids or protective substances. The modifying effects of additives in the suspension during freezing has been highlighted by a number of workers who have shown that a variety of compounds including glucose, lactose, glycerol (31, 35,50) and dimethyl-sulphoxide (51) increased the percentage of cells surviving a rapid as well as a slow rate of freezing, although the mechanism of action of these compounds is not clear.

The overall effects of the initial freezing stage must be borne in mind in assessing the general efficiency of a freeze-drying process, as the damage induced by freezing can persist well into the drying stage, since the frozen, undried portions of the specimen are essentially in storage at the drying temperature until the drying boundary reaches them.

The drying process

Many workers have shown that damage to cells, as measured by loss of viability (53,54,55,56,57), or changes in enzyme systems (58), occurs during the drying process. It has been demonstrated, furthermore, that the majority of the damage is induced during

"secondary" drying when the unfrozen water is being removed by diffusive processes, rather than during "primary" drying, when water removal is by sublimation from the frozen state.(59,60,61). This has led a number of people to infer that the water not removed by sublimation is necessary for the biochemical integrity of the cell.

Wasserman and Hopkins (58) have postulated that an imbalance in enzyme systems brought about by lyophilisation is responsible for the damage induced in cells. They demonstrated differences in glucose oxidation and 2-ketogluconate oxidation enzymes during freeze-drying, and suggest that other, perhaps more vital, enzyme systems are likely to be similarly affected by removal of water from the cell.

An alternative hypothesis is that death of a microbial cell results from a physical change in the structure of an essential macromolecule, when the water bound to this molecule is removed during drying (62,63). Evidence for this theory is found in the demonstration that water molecules are responsible for the structural integrity of D.N.A.(64) and that this structural water is removed at about the same relative humidity as that at which the greatest changes in stability of freeze-dried cells occur (65).

An additional possible mechanism for injury during drying results from the recrystallisation of salts or hydrates formed from eutectic solutions inside the cell during water removal. Rey (66) and MacKenzie and Luyet (67) have demonstrated by means of time-lapse photomicrographs that salt or hydrate crystal growth occurs by recondensation and recrystallisation during drying, and that the size of crystal formed is directly related to the drying temperature. They suggest that considerable mechanical injury to structural elements might result from such crystal formation during the actual passage of the drying boundary through the specimen.

It is of interest that, as various micro-organisms show differences in resistance to deleterious agents such as heat, chemicals and radiation, so they exhibit differences in resistance to freeze-drying. Spores are usually extremely resistant, and are so readily preserved by drying that they are rarely considered in discussions on freeze-drying. It is however interesting that Haynes et al (68) were unsuccessful in preserving anaerobic sporeformers by lyophilisation. It is difficult and rather pointless to list the non-sporing organisms in order of increasing resistance to freeze-drying, although a number of authors have attempted to do so. A general picture of the relative resistance of various species to drying can, however, be obtained from the literature (69,70,71,72, 73).

The phase of growth of a culture is an important factor in determining resistance. Most organisms show the greatest resistance to freeze-drying at the end of the logarithmic phase of their growth cycle, and at the beginning of the stationary phase, and are most sensitive to drying during the logarithmic phase (55,74,75,76,77). There are, however, isolated reports of higher survival of organisms dried in the logarithmic phase (36,78) and of organisms whose survival is apparently unaffected by their stage of growth (57). How these results fit in with the various theories on mechanisms of damage is not clear.

A conflict of opinion exists in the literature as to the importance of cell concentration on the survival of organisms during freeze-drying (5). Some workers have indicated that high concentrations give a higher survival rate (79), while others have shown that increases in initial cell concentration either reduce (69) or have no apparent effect on the percentage survival (55). Furthermore,

an increase in the concentration of cells in the initial suspension will serve to decrease the concentration of solute per cell. This factor, together with the occurrence of growth or death in the initial culture can influence the response to drying and must be taken into account when assessing effects which appear to be due to differences in cell concentration.

The suspending medium was found, early on, to affect the survival of cells during freeze-drying, and consequently a great deal of attention has been focused on the nature and constituents of this medium. Much of this work has been qualitative, but it has shown that the drying of aqueous suspensions invariably results in poor survival of organisms, even though water has been shown to be suitable as a suspending medium during the initial freezing stage of the lyophilisation process (see page 17). A large number of additives has been suggested as giving protection to cells during freeze-drying among which glucose has been generally recommended for use with bacteria (55,80,81). The original use of this substance was as a supplement to broth, serum, or milk which in themselves were considered to be successful suspending media since they provided a protective colloid (82,82,84,85). As a consequence Fry and Greaves (55) developed a medium containing three parts of sterile serum, one part nutrient broth, and 7.5% glucose (Mist desiccans) which has been subsequently reported, by a number of workers, as giving good protection to cells (86,87,88). However, neither this medium, nor others that have been proposed have proved successful as a universal freeze-drying medium.

More recently Lion and Bergman (89,90) have investigated, on a quantitative basis, the relative efficiency of compounds in protecting Escherichia coli both during drying and during subsequent storage in

atmospheric oxygen. The compounds that exert a protective action can be conveniently divided into three groups, thiourea and its derivatives, sugars, and inorganic salts, in which 1.3×10^{-1}M thiourea, 1.1×10^{-1}M L-arabinose and 1.6×10^{-1}M sodium iodide respectively gave the greatest protection to the cells during drying. It was shown also that the substances giving greatest protection during drying did not necessarily give the best protection during exposure to and storage in atmospheric oxygen (see page 24) and hence the compounds needed to be assessed in terms of an overall effect after drying and storage of the organism. Furthermore, although a number of compounds have been shown to be protective, the mechanisms by which this protection is conferred are at present unknown.

Little work has been reported on the importance of the initial pH of the suspending medium, although Heckl(et al (91) have shown that survival of lyophilised Serratia marcescens was increased, with increase in pH of the medium over the range pH 4 to pH 10.5.

There is evidence that certain cells are themselves capable of liberating substances which can protect against freeze-drying damage, but little work has been carried out on the isolation and investigation of these substances. Record and Taylor (92) showed that, on being dried and reconstituted, Escherichia coli liberated a protective substance which not only protected Escherichia coli but also Serratia marcescens during freeze-drying. Similarly, it has been reported that cell free extracts of Brucella abortus were protective to cells of the same type (93,94). Although a lecithin type substance has been identified from these extracts, its mode of action has not yet been determined (94). Furthermore, it is possible that the protective effect of dead cells observed by Otten (79) may in part be the result of liberation of protective substances, although

as yet, such compounds have not been reported in extracts of other organisms.

Results of experiments in which the temperature of drying was varied require caution in interpretation since in the majority of cases, only the temperature of the outside of the drying vessel was recorded, and no measurements were made of the temperature of the sample in the vessel. Leach et al (95) were able to measure the temperature of the sample during the drying process, and showed that no significant differences in survival occurred when Pseudomonas fluorescens was freeze-dried at 293°K, 273°K and 253°K. Similarly, it has been demonstrated that survival of Brucella abortus is independent of drying temperature over the range 248°K to 238°K (96) and that drying temperatures between 233°K and 293°K did not greatly influence the survival of Escherichia coli (97). However, in contrast to these results, Muggleton (98) has shown that the lowest drying temperatures result in the highest survival of B.C.G. At 263°K to 258°K he reported 8% survival which increased to 51% at 242°K, the greatest increase in survival 12.5% to 45% occurring between 248°K and 245°K. However in the majority of cases it is difficult to determine whether these changes in survival are in fact a function of the drying temperature, or simply the result of differences in drying rate, or final moisture content.

The method of drying will also condition the temperature at which freeze-drying occurs. Although Fry (5) has implied that loss of viability would occur if the specimen was not pre-frozen, it has been shown, with a variety of organisms, that drying from liquid suspension can give a resultant viability comparable to, and in some cases superior to that obtained with freeze-drying (69,83,99, 100). Again, results indicate that such findings are by no means

universal, and hence drying from the frozen state is usually preferred.

Storage conditions

The influence of storage conditions on the survival of freeze-dried organisms was first demonstrated by Rogers (80) who showed that survival was highest in cultures stored under vacuum, and lowest in those stored in air or oxygen, whilst storage in atmospheres of hydrogen, nitrogen or carbon dioxide gave intermediate results. However, since details of the purification methods used were not given, it is possible that the results obtained with the latter gases, may have been due, in part, to the presence of trace amounts of oxygen. Other workers have since confirmed that dried bacteria undergo pronounced loss of viability when stored in air or oxygen, and have consequently recommended that cultures of dried bacteria should be stored in vacuo (36,74,76,101).

Of more useful application is the work of Lion and Bergman (89, 102) who have studied the effects of oxygen on Escherichia coli freeze-dried from distilled water. The authors showed that the maximum loss of viability did not occur during the drying process itself, but during the time of contact of the dried organisms with air between the primary and the secondary drying periods. They concluded that the lethality reported by other workers during lyophilisation of suspensions from distilled water was probably due almost entirely to the extreme sensitivity of the organisms to oxygen, and that careful protection of the cells from air resulted in high survival rates. Subsequent experiments showed that certain compounds not only protected the cell against damage during drying but also partly abolished the lethal effects of oxygen during storage. (See page 21). Thiourea and some of its derivatives, monosaccharides, and simple inorganic salts, in particular sodium iodide were effective in

increasing the survival of Escherichia coli exposed to oxygen (89). The reducing property of thiourea and other compounds was shown not to be involved in their mode of action since other similar reducing sulphydryl compounds were either ineffective (cysteamine) or even enhanced the deleterious effect of oxygen, (dithionite, cysteine, glutathione). Likewise, no correlation was found between the protective efficiency of sugars, and their chemical and steric structure, their fermentability by the bacteria, or their reducing power. More recent work by Lion (90) has shown that these substances interfere directly with the reaction between oxygen and some bacterial receptor, in the dry state, since addition of the agent to the resuspending medium does not confer protection against oxygen death.

Sodium glutamate has also been shown to give good storage protection to dried bacteria (103,104) and B.C.G. vaccine (105,106, 107,108), and it was further demonstrated that this stabilising effect was increased when the substance was combined with soluble starch, dextrin, or polyvinyl-pyrrolidone. Again, the mechanism of the protective action is not known, but studies by Morichi et al (109) on compounds allied to glutamic acid have shown that the presence of a hydrogen bond generating group (-OH, $-NH_2$, $>NH$) and two acid groups is characteristic of substances giving storage protection.

Some indication of the nature of the adverse effect of oxygen on dried bacteria has been provided by Lion et al (110). They showed that free radical production, as measured by an increase in the relative Electron Paramagnetic Resonance (E.P.R.) signal, occurred when dried Escherichia coli cells were stored in the presence of oxygen. Furthermore, a quantitative relationship appeared to exist between the ability of certain additives, such as thiourea and sodium iodide to suppress the E.P.R. signal of oxygen-exposed dried bacterial

cells, and their ability to protect these cells against the lethal effect of oxygen. A correlation between death and free radical production has also been shown to exist for Sarcina lutea, Streptococcus lactis and Serratia marcescens (111).

More recent work by Heckly and Dimmick (91) has been aimed at obtaining a direct correlation between free radical formation and survival of freeze-dried Serratia marcescens. These authors showed that the logarithm of the number of cells surviving storage was inversely related to the free radical concentration, and that both were similarly affected by temperature over the range 253°K to 310°K, and by oxygen concentrations between 10^{-2} and 1 atmosphere.

Observations of weak relatively sharp, electron spin resonance signals have been reported in a variety of lyophilised biological materials (112,113,114) and it was further shown that in the presence of oxygen these materials produced different, long-lived free radical species (115). Although the formation of free radicals in dried vegetative cells has been confirmed, there is still a diversion of opinion concerning radical formation in dried bacterial spores. Tanooka (116) has reported that dried Bacillus subtilis spores showed an E.P.R. signal which was not changed by storage either in nitrogen or in air. Conversely, Windle and Sachs (117) claimed to show a complete absence of free radicals in clean Bacillus megaterium spores even after long periods of storage in air, and attributed these findings to an extremely low level of metabolic activity in the dormant spore.

Dewald (118) has recently carried out kinetic studies on the destructive action of oxygen on lyophilised Serratia marcescens. He has shown that the degree of inactivation depends upon temperature, time, and the partial pressure of oxygen and has shown that such

inactivation follows the expression $\ln N/N_o = -k\left[O_2\right]^{\frac{1}{3}} t^{\frac{1}{2}}$ where N/N_o is the surviving fraction, $\left[O_2\right]$ is the oxygen concentration, t is time, and k is the rate constant. The role played by molecular oxygen in the inactivation process is at present unknown, although kinetic studies of this type would indicate that the mechanisms involved are not simple (110,118). Dewald has explained his results on the basis of a chain reaction involving radicals occurring during the inactivation process. Such a theory would also explain why only very small quantities of oxygen are required to cause death of freeze-dried organisms.

It is generally accepted that the higher the temperature of storage of freeze-dried organisms, the lower the survival rate is likely to be, although it is possible, by using a suitable suspending fluid and drying technique, to produce dried cultures of certain organisms which will remain stable at higher temperatures for a limited time (81,119).

Of more useful application is the use of elevated temperatures in accelerated storage tests, to predict the stability of freeze-dried organisms. Accelerated tests are based on the observation that a suitable parameter of the viability of the cells declines linearly with time, at selected temperatures, and that the logarithms of the rates of decline at the various temperatures are in a linear relationship with the reciprocals of absolute temperatures in accordance with the Arrhenius equation (see page 150). These requirements have been shown to exist with freeze-dried viruses (120,121), but there is some confusion as to whether such requirements are fulfilled by lyophilised bacteria (36,76,103,122). Having obtained a non-logarithmic order of thermal death for freeze-dried Neisseria gonorrhoeae exposed to temperatures of the order of 373°K,

Greaves (103) concluded that it was not possible to correlate resistance of dried bacteria at this elevated temperature with survival times at lower temperatures. Similar findings have been reported by Maister et al (76) who demonstrated that survival of freeze-dried Serratia marcescens at 353°K could not be correlated with survival at 323°K. In contrast, recent work by Damjanovic and Radulovic (122) has shown that the survival of Lactobacillus bifidus can be accurately predicted from accelerated storage tests. It is apparent therefore that one is not justified in making generalisations concerning the suitability of accelerated storage tests when applied to dried bacteria, and that one must judge each case individually.

It has been demonstrated that there is an optimum water content for maximum survival of freeze-dried cells, and that drying below this level causes lethality (55,123,124). In an attempt to overcome the problem of excessive drying some workers have added hygroscopic substances such as glucose to the suspending medium to assure some residual moisture (55,69,98,125). However, in the majority of cases no estimations of residual moisture were made to confirm this "moisture-binding" action of glucose, and often it was difficult to show that the beneficial effect was due to increased moisture, rather than to the presence of glucose per se (see page 21). The measurement of residual moisture in lyophilised bacteria has invariably proved difficult, since the errors inherent in the various methods employed are greatly increased when these methods are used to measure the very low concentrations of water found in dried materials, and hence it is often difficult to draw valid conclusions from these studies.

Scott (128) has overcome the problem of direct measurement of residual moisture by storing the dried organisms in the presence of

solutions of known water activity (a_w) and then estimating the survival as a function of the equilibrium a_w value. He found that the optimum a_w for survival varied with the composition of the suspending fluid and the presence or absence of air in the storage atmosphere. In particular he showed that with Salmonella newport dried from distilled water, survival was best at low moisture levels ($0.00a_w$) in vacuo, but at much higher a_w levels in air. However, using this technique of "re-equilibration" to a given moisture level, it is questionable whether the external water which then hydrates the cells behaves in the same manner as the original cellular water which was removed.

To obviate the need for re-equilibration, Nei et al (59) have described a modified freeze-drying technique by which they were able to obtain cells of a given moisture content (measured by weight determinations) by drying alone. Using this technique these workers have studied the effect of drying conditions and residual moisture content on the survival of freeze-dried Escherichia coli during the drying process and on subsequent storage. The results obtained on storage of the dried cells were in agreement with those of Scott (126). They showed that over the range of moisture contents from 0 to 20%, survival was highest after eight days storage in air at 303°K in cells with the highest residual moisture content. Conversely, for cells stored under similar conditions in vacuo or in nitrogen, the lowest residual moisture contents gave rise to the highest survival rates (61). These findings indicate a complex inter-relationship between water content, atmosphere, storage conditions, and cell viability, the mechanisms of which have yet to be elucidated.

Reconstitution conditions

Few workers in considering viability of freeze-dried micro-organisms have concerned themselves with the reconstitution process, although it can be deduced from the literature that differences in viability may arise from variation in rehydration conditions such as volume, composition and temperature of the hydrating fluid (55,59, 72,96,127,128,129).

Rehydration can produce high concentration gradients, momentary osmotic imbalances and may result in a final product which is radically different from the initial "fresh" material (130). Also, although the exact amount of water removed may be replaced, it cannot be replaced uniformly, and the concentration of solutes within the specimen may vary with time and location if distilled water is used as the reconstitution medium. The temperature and pH of the rehydration medium have also been shown to be important for the maintenance of viability, although reasons for their importance are not known (128). For example, Leach and Scott (128) have demonstrated that there is an optimum temperature for reconstitution and that this temperature is a characteristic of the species of organism. Using distilled water for rehydration, the highest temperature (310^{o}K) yielded the highest number of viable Serratia marcescens and Lactobacillus bulgaricus which confirmed the observation of previous workers (58,129). However, for rehydration of Vibrio metschnikovii and Escherichia coli the optimum temperatures were 273^{o}K and 293^{o}K respectively, and Staphylococcus aureus was apparently unaffected by the temperature of rehydration over the range 273^{o}K to 310^{o}K. Furthermore, the temperature effects were often obscured or eliminated if salt solutions or fluids other than distilled water were used for reconstitution.

The rate of reconstitution has also been shown in the few

studies undertaken, to be an important factor in obtaining the highest survival rate. Although slow vapour rehydration has been shown to be beneficial in the reconstitution of yeasts (131,132) and pine pollen (133), Leach and Scott (128) reported that the addition of moisture as a vapour to freeze-dried Vibrio metschnikovii was highly lethal. These authors showed that under the experimental conditions used, the best survival resulted when the mean rates of addition of water were between 10^{-2} and 10^{-1} mg.sec^{-1} mg. dry matter^{-1}.

The importance of the constitution of rehydration media has not received a great deal of attention, but results of Leach and Scott (128) and Bretz and Hartsell (134) would indicate that a high osmotic pressure is generally conducive to obtaining maximum recovery of lyophilised preparations, although again generalisations are not possible.

Heat Resistance of Dried Bacterial Spores

The factors influencing the heat resistance of bacterial spores, and the theories that have been proposed on the mechanisms of resistance have been discussed in detail in a previous review (135). The purpose of this section is to discuss the use of dried spores as a means of studying the factors such as moisture content, and gaseous environment, that affect microbial resistance to dry heat. It is well established that organisms are considerably more resistant to heat when dried than when in suspension (136,137,138) and the recognition of this fact has been utilised in the design of sterilisation procedures. However, it is only in recent years that work has centered on investigating more fully the effects of water and moisture content on the heat resistance of spores.

It was found, for example, that when spray dried powders containing Bacillus subtilis were exposed to atmospheres of increasing humidity, the spores remained viable and heat resistant below 30% moisture content. Over the range from 30% to 50% the spores remained viable but lost their heat resistance while at still higher moisture contents (80%) both heat resistance and viability were lost (139). More recent work has involved the use of spores dried under vacuum and re-equilibrated to known water activities (a_w). It was found that equilibration of bacterial spores with atmospheres of known humidity at 302^{o}K, and subsequent heating of the sealed ampoules without the a_w controlling solutions resulted in the greatest heat resistance when the spores were equilibrated at an a_w of 8×10^{-1} to 9×10^{-1} before heating. The maximum heat resistance was about ten to one hundred times the value obtained with very dry spores (a_w = 0.00) (140). Furthermore, it was noted that this treatment abolished the large difference in heat resistance between spores of Bacillus stearothermophilus and Clostridium botulinum type E that was apparent when the spores were heated in phosphate buffer. Unfortunately, however, the technique used in these studies was unsuitable for controlling the a_w value at the temperature of heating. Rapid heating and cooling are inevitably required when spores in sealed containers are exposed to lethal high temperatures for controlled periods of time. The temperature gradients during heating and cooling will result in vapour pressure gradients and cause transfer of water vapour. As a result, the spore moisture content, and the relative humidity of the system will be very much lower at the heating temperature than at the initial equilibration temperature. To prevent this change in spore moisture content during heating Murrell and Scott (141) utilised a technique in which, within the

sample tube containing the spores was sealed an open vessel containing a salt solution, which at the exposure temperature, yielded the same vapour pressure as that to which the spores were initially adjusted. The results of these experiments showed that spores exhibit the greatest heat resistance when adjusted to an a_w value of 2×10^{-1} to 4×10^{-1} corresponding to a water content of the order of 5.5 to 12.4% of the dry weight of the spores, depending on the species (142).

Another method of preventing spore water loss during heating has been developed by Angelotti et al (143) and entails encapsulating the spores in non-permeable plastics such as methylmethacrylate and epoxy. The plot of D value (see page 149) against water activity obtained using this technique was similar to that obtained by Murrell and Scott (141). Furthermore, the results confirmed that the maximum heat resistance was observed in spores equilibrated to a_w values in the range 2×10^{-1} to 4×10^{-1}. The authors also considered the dry heat resistance of spores located on steel and paper strips and located between stainless steel washers mated together under 12 inch-lb. and 150 inch-lb. torque. These results showed that differences in resistance displayed by the spores located in or on the various materials could be related to the rate at which spore moisture content was reduced during heating.

It is apparent therefore, that the dry heat resistance of bacterial spores is influenced not only by the initial spore moisture content, but also by the rate of spore desiccation during heating, the moisture retaining capacity of the material on or in which the spores are located, and the equilibrium vapour pressure of the system at the exposure temperature. A further point which must be borne in mind when assessing the results reported above is that in the majority

of experiments the spores were dried and re-equilibrated to a given moisture level, and under these conditions the water which then rehydrates the spores may not necessarily behave in the same manner as the original cellular water, particularly at the higher temperatures employed in heat resistance determinations.

Rahn (136) was one of the first workers to differentiate between death by moist heat and death by dry heat. He stated that death by dry heat is primarily an oxidation process, whilst death by moist heat is due to coagulation of a protein. The realisation that a high temperature coefficient (Q_{10}) was a characteristic both of killing of bacteria by heat, and of denaturation of proteins, tended to support this theory (144). Furthermore, Amaha (145) has shown that energies (ΔE) and entropies (ΔS) of activation for the killing of several Bacilli are of a similar order to those for the thermal denaturation of haemoglobin, trypsin and pancreatic lipase. However, the significant effects of sporulation, heating, and recovery on the apparent dry heat resistance of spores points to a more complex cause of death. Hansen and Rieman (146) have cited an hypothesis by Precht and co-workers that attempts to explain the differences due to water in more detail. This hypothesis states that when wet proteins are heated free-SH-groups are released with the formation of smaller peptide chains. These chains are mobile, and by establishing new bonds between themselves, can form new complexes different from the original protein molecule, and so incapable of correct functioning. In the absence of water, the lack of water dipoles tends to reduce the activity of the polar group in the peptide chains, which in turn reduces their mobility. It requires, therefore, more energy to open the peptide molecules, hence the increased apparent resistance in the dry state.

If Rahn's hypothesis (136) is valid, and death by dry heat is primarily an oxidation process, it would be expected that the dry heat resistance of spores should be lowest in oxygen. However, Pheil et al (147) have tested the heat resistance of dried spores in various gases at temperatures ranging from 394°K to 433°K and have shown that the differences in resistance are small. For Bacillus subtilis spores, the gases in order of increasing heat resistance were carbon dioxide, air, oxygen, helium, and nitrogen. Neither oxygen content nor molecular weight of the gas appeared to have a marked influence on dry heat resistance of either Bacillus subtilis or Clostridium sporogenes spores. Unfortunately, these workers did not control the moisture content of the spores during the heat resistance determinations, neither did they dry the test gases employed. It is possible therefore, particularly in the results obtained with air and carbon dioxide, that the effects of residual moisture were masking the true influence of the gaseous environment on the heat resistance, especially since it has been shown that even very small amounts of water (a_w 3.4 x 10^{-7} at 393°K) can cause significant changes in heat resistance (135).

Previous work in this school has shown that when spores of Bacillus megaterium are heated in conditions of controlled relative humidity, they exhibit a greater sensitivity to heat in the presence of oxygen, than in vacuo, but this work has not yet been extended to a study of other gaseous environments.

A further important point to be considered in studies on the heat resistance of dried spores is the recent realisation that while spores can be subjected to treatments such as lyophilisation, with generally no loss of viability, nevertheless, under certain environmental conditions, such treatments may alter the resistance of the

spores to certain physical agents. Work in this school has shown that the drying process causes damage to Bacillus megaterium spores which results in variable responses to a given heat treatment, particularly when this treatment is carried out in the presence of oxygen (135). Variations in survivor counts after subjecting dried spores to heat in atmospheres of different water vapour content has also been noted by Jacobs et al (137). These authors assumed this variation to be a function of experimental technique, and were able to mask it by increasing the number of samples taken at each survivor level and discarding counts for which the log. viable count was outside the 95% confidence limits for the particular treatment. It seems more probable, however, that this variability, and that among replicate plate counts reported by Fox and Pflug (148) is a result of the freeze-drying process rather than the sampling technique, and that the average recovery after heating would be a composite both of damage produced by drying, and of the heat sensitivity at the particular water content.

These results would therefore suggest that interpretation of work on dry heat resistance of spores must be approached with caution if the work involves a lyophilisation process.

Radiation Resistance of Dried Bacterial Spores

The inherent characteristics of the bacterial spore make it an ideal "biological model system" for investigations aimed at the elucidation of mechanisms involved in the cellular actions of ionising radiations. In particular, the capability of the spore to withstand drying and re-equilibration processes that have been shown to be damaging to most biological materials (149,150,151,152) has proved useful in studying the effects of water on the lethal damage

induced by radiation. Furthermore the ability of spores to tolerate exposure to specific gaseous environments and elevated temperatures has enabled these parameters also to be investigated in relation to radiation damage.

In all these investigations the slope of the log.survivor/dose curves - the inactivation constant, k (see page 188) has been used as the criterion of radiation sensitivity. By comparing different values of the inactivation constant obtained for various physical and chemical treatments during and after irradiation, lethal damage has been divided into three main categories, Class I, II and III (153,154,155) and the effect of water on these has been elucidated.

The damage observed when spores are irradiated anoxically followed by treatment with nitric oxide, heat or hydrogen sulphide is designated Class I. This damage is due to oxygen independent mechanisms, and has been postulated by Powers and his co-workers as being brought about by short lived species or by radicals (153, 156). The values of k_I obtained show little change with equilibration vapour pressure over the range from 5×10^{-4} torr to 10^{-1} torr. Above 4.6 torr there is an increase in oxygen dependent damage to a maximum value (representing an increase of about 25%) at 8 torr. Class I damage is therefore divisible on the basis of the behaviour of k_I with changing water content into two components, one water dependent and one water independent. This sensitising action of water may be the result of reactions involving products formed by radiolytic decomposition of water (157). Furthermore, it has recently been demonstrated that the magnitude of k_I is reduced if H_2S is present during irradiation, and thus a further subdivision of Class I damage is possible in terms of H_2S independent

components (158). Therefore, in the fully wetted spore, the oxygen independent radiation effects are experimentally divisible into four separate classes.

When the spores irradiated anoxically are exposed directly to oxygen immediately after irradiation there is an increase in the value of the inactivation constant. This increase is referred to as the post irradiation oxygen effect. (Class III damage). Because prior exposure of the spores to free radical scavengers such as nitric oxide prevented this damage, it has been postulated that Class III damage is due to the presence of radiation-induced long-lived free radicals. The kinetics of the post irradiation oxygen effect have been investigated by Davies (152,159). It was shown that the magnitude of the post irradiation oxygen effect increased with increase in equilibrium vapour pressure from 5×10^{-4} torr to 2×10^{-3} torr and 5×10^{-2} torr, but that the increase in water content of the spores did not affect the rate of development of the oxygen effect. It was suggested that water exercises a protective effect under these circumstances, by removal of the free radicals formed during irradiation before oxygen can react with them. Once the oxygen/radical complex has been formed, the rate of reaction of this to give a lethal event is independent of water content over the range studied, and dependent only on the temperature of storage. Tallentire and Powers (160) have confirmed that the magnitude of the post irradiation oxygen effect is a function of spore moisture content. They obtained the highest values at 5×10^{-4} torr, with the values decreasing gradually to zero at around 22 torr.

Good evidence for the involvement of long-lived free radicals in the post irradiation oxygen effect has been obtained by E.P.R. studies on irradiated spores (161,162,163,164). These experiments

have demonstrated that free radicals produced during anoxic irradiation are in fact long lived, the resultant E.P.R. signal remaining unchanged at room temperature for periods of up to forty eight hours. However, in the presence of even small amounts of water or nitric oxide, the signal decayed rapidly. Furthermore, the introduction of oxygen to the spores changed the signal within a very short time, and a typical oxyradical signal was obtained.

When spores were irradiated in oxygen and stored in oxygen there is a further increase in the magnitude of the post irradiation oxygen effect over that observed in spores irradiated anoxically. Davies (152) considers that this increase is the result of reactions involving short-lived free radicals formed during irradiation. For these reactions to occur it follows that oxygen must be present during irradiation, to react with the short lived species soon after their formation. Davies therefore concludes that the magnitude of the post irradiation oxygen effect observed in spores irradiated anoxically is a measure of the rate of reaction involving long-lived radicals only, whereas the magnitude of the effect observed after irradiation in oxygen is a measure of reactions involving both long-lived and short-lived radicals, the rate of conversion of the short-lived species to the lethal form being slower than that for the long-lived radicals.

Davies (152) also showed that when oxygen was present during irradiation, the inactivation constant increased to a value approximately six times that observed when irradiation was carried out in anoxia. A similar increase has been demonstrated by Tallentire and Powers (160) and has been designated the "Immediate" oxygen effect (Class II damage). This operational "immediate" effect has been shown to vary with the re-equilibration vapour pressure, having

a value of 0·042 $krad^{-1}$ at 5×10^{-4} torr which decreased to a final value of 0·015 $krad^{-1}$ at 22 torr. The k_{II} component was thus similar to k_{III} in having its largest value in the driest state (5×10^{-4} torr) but differed from it in that it was observed in wet spores where k_{III} could not be detected. Since in these experiments the irradiation period was often of up to two hours duration, some of the damage occurring during irradiation must in fact have been post irradiation oxygen dependent damage (Class III). However, with the experimental techniques used it was impossible to arrive at a measure of this component. Similarly, it is not possible from these experiments to obtain a measure of the "immediate" oxygen effect, and that it exists at all, is by inference. It is felt that as an oxygen effect is observed in aqueous suspension, there is an "immediate" oxygen effect, and that it is likely that in the dry condition this "immediate" effect will be at least as large as that observed in the presence of water.

EXPERIMENTAL

BACTERIOLOGICAL MATERIALS AND METHODS

The Test Organism

Previous work in this department has shown that spores of Bacillus megaterium (A.T.C.C.No.8245), produced on solid potato extract medium, can be exposed to reduced pressures of the order of 10^{-5} torr, for prolonged periods, with subsequent re-equilibration to partial pressures of water vapour, ranging from 5×10^{-4} torr to 40 torr, with no apparent loss of viability (135). It has also been shown that Bacillus megaterium spores, can be stored in oxygen for periods at elevated temperatures, and are suitable for the quantitative study of the effects of X-radiation (160, 162) ionising radiation, (149, 150, 165) and heat (135,151) on the viability of spores dried from kaolin suspensions.

For this reason it was decided that Bacillus megaterium (A.T.C.C. No.8245) should be used in the present investigation. The organism was kindly provided by Dr. A. Tallentire of the University of Manchester.

Preparation of the Suspension of Bacillus megaterium Spores

The method used was that recommended by workers at the Argonne National Laboratory and described previously by Davies (152) and Chiori (151). The spores were grown on the surface of a potato extract agar medium, prepared as follows: 400g. of peeled and cut red potatoes were placed in 1000 ml. of glass-distilled water and boiled for 20 minutes. The resulting suspension was filtered through gauze, made up to 1000 ml. with water, and the pH was adjusted to 5.8 to 7.0 with IN sodium hydroxide solution. 15g. of agar (Oxoid No.3) was added to the suspension, and the medium was autoclaved for 1 hour at 392 - 393°K. 20 ml. quantities of sterile medium were then distributed into sterile 10 cm. petri dishes, and allowed to solidify.

A surface streak of a suspension of Bacillus megaterium spores was made on nutrient agar plating medium (see page 45) and a single colony produced after 24 hr. incubation at 308°K was inoculated into three 10 ml. volumes of sterile Oxoid MR-VP medium (prepared according to the manufacturers directions). These volumes were then incubated for 18 hours at 308°K after which the liquid culture was flooded onto the surface of one hundred and twenty potato extract agar plates. A large number of 10 cm. plates were used since attempts to cultivate the spores on large plates (25 cm. x 25 cm.) or in Roux bottles were unsuccessful, and resulted in lower yields of viable spores. After incubation of the plates for six days at 308°K, a microscopic examination showed that the upper layers of the surface growth consisted almost entirely of refractile spores. This upper fraction of the surface growth was removed from each plate by rinsing with two 5 ml. quantities of sterile glass-distilled water. The resultant 10 ml. volumes of suspension were mixed and washed three times by successive centrifugations, and replacement of the suspending liquid with similar volumes of sterile glass-distilled water. Centrifugations were carried out at 3,000 r.p.m. in a refrigerated centrifuge maintained at 273°K. The washed spores were finally suspended in 100 ml. of sterile glass-distilled water to yield a standard spore suspension designated SS6S, which was stored at 277°K.

The Estimation of the Total Number of Spores in Suspension

A platelet counting chamber, with improved Neubauer ruling, having a nominal depth of 0.1 mm. ± 0.001 mm. was used to estimate the total number of spores, in accordance with the recommendations of Cook and Lund (116). The cover glass was placed on the chamber in the recommended manner and a drop of a suitable dilution of suspension

SS6S was introduced into the chamber with the aid of a sterile Pasteur pipette. The slide was left for thirty minutes to allow sedimentation of the spores, and then observed at a magnification of x 400 under phase contrast. The number of spores (identified by their "bright" appearance) in 256 small squares of the counting chamber were counted and recorded. Five separate determinations of the total number of spores in the suspension SS6S were made and the results obtained are summarised in Table 1.

TABLE 1

Summary of data obtained in the estimation of the total number of spores in suspension SS6S

Sample No.	Dilution of SS6S	Mean Count/ Small Square	Total No. of Spores/ml. SS6S	Mean No. of Spores/ml. SS6S
1		7.9125	7.9125×10^{10}	
2		7.0375	7.0375×10^{10}	
3	2.5×10^{3}	7.855	7.855×10^{10}	7.5194×10^{10}
4		7.237	7.237×10^{10}	
5		7.555	7.555×10^{10}	

Performance of Viable Counts on Samples of Suspension

The technique involved consists of three basic operations,

(i) resuspension of samples of the spore suspension in water,

(ii) serial dilution of the resultant spore suspension where required,

(iii) inoculation of a growth medium with a measured volume of a suitable dilution of this suspension, followed by the counting of colonies formed after a standard incubation of the inoculated medium.

Soper (135) and Chiori (151) have carried out investigations to ascertain the optimum conditions necessary for the production of colonies from spores of Bacillus megaterium, using the surface spread technique. The recommendations have been followed throughout this work with regard to volume of inoculum, spore concentration in the inoculum, composition and volume of plating medium, temperature and duration for overdrying of plating medium, temperature and duration of incubation, and conditions for storage of suspensions prior to counting.

Materials

The pipettes - "Visu-Red Pipettes" calibrated to deliver 1 ml. (i.e. graduated 0 to 1 ml.), made from "Pyrex" glass and manufactured by Jobling and Co.Ltd., were used. Previous tests had shown that these pipettes were suitable for delivery of 0.3 ml. and 1 ml. quantities under experimental conditions, and fulfilled the requirements suggested by Jennison and Wadsworth (167) for pipettes suitable for the performance of viable counts (135).

Semi-automatic burette - a semi-automatic burette similar to the type described by Rawlins (168), was employed for the measurement and delivery of 4 ml., and 19 ml. volumes of sterile glass-distilled water used in the performance of serial dilutions. Previous tests had shown that this apparatus was suitable for its intended purpose under experimental conditions (135).

Dilution tubes - these were thin-walled, rimless glass tubes of two sizes, 6" x $\frac{3}{4}$" which were used for resuspension of samples, and for five-fold and ten-fold dilutions, and 6" x 1" which were used for twenty-fold dilutions.

Preparation of glassware - all glass apparatus was cleaned by immersion in "chromic acid mixture", followed by seven rinses with

tap water and two rinses with glass-distilled water. The glassware was dried in a hot air oven, and sterilised by heat at 433°K for 1 hour, or in the case of the semi-automatic burette, by autoclaving for 15 minutes at 399°K in a high vacuum autoclave.

The diluent - sterile glass-distilled water was used to re-suspend spore samples, and in the dilution of spore suspensions throughout this work.

The plates - these were sterile disposable plastic petri dishes (90 mm. x 15 mm.) supplied by Dyos Ltd.

The plating medium - this consisted of

Peptone (Oxoid)	2.0 per cent
Agar (Oxoid No.3)	1.5 per cent
Sodium Chloride (B.D.H. Analar)	0.5 per cent
Glass-distilled water to	100 per cent

pH adjustment to between 7.2 and 7.4

The constituents were placed in a flask fitted with a 20 ml. Kipps burette, together with enough IN Sodium Hydroxide solution to give a pH after autoclaving of between 7.2 and 7.4. The medium was autoclaved for 35 minutes at 394°K and the pH verified. After the heat treatment the medium was mixed and 20 ml. quantities were poured into petri dishes. The medium was allowed to solidify, and the surfaces of the plates were dried in a hot air incubator room for 1 hour at 308°K.

Methods

Accurately measured volumes of spore suspension were resuspended in 100 times their volume of sterile water. Serial dilutions, in the ratio of 1 in 5, 1 in 10 and 1 in 20 were performed when necessary to give a final dilution containing between 1.3×10^2 and 7×10^2 viable spores/ml., giving between 40 and 200 colonies per plate.

0.3 ml. samples of the final dilutions were placed on the surface of each of five nutrient agar plates, utilising the same pipette. The samples were spread over the surface of the medium by means of a sterile glass spreader, and the plates were left to stand for 10 minutes to allow the liquid to diffuse into the medium. The plates were incubated in the inverted position for 24 hours at 308^{o}K. The colonies were counted by eye with background and direct illumination, with magnification, using a semi-automatic colony counter supplied by New Brunswick Scientific Co.

Distribution of Viable Spores in Spore Suspension SS6S

In order that spore suspension SS6S may be used for the study of the effects of high vacuum drying on viability and subsequent inactivation of the contained spores by heat and ionising radiation, it was essential that different samples taken from the suspension at the same time should contain similar numbers of viable spores per unit volume, within the limits of normal sampling error.

To test this, viable counts were performed on five samples from suspension SS6S. The five resuspended samples were each diluted to the same extent, and the colony counts from the resultant final dilutions were subjected to statistical analysis. The colony counts for SS6S and the corresponding Analysis of Variance of the log.counts is shown in Table 2 . The reasons for the log. transformation of the data in the statistical analysis are given on page 105.

TABLE 2

Counts obtained from quintuplicate plating of five samples of suspension SS6S, with corresponding Analysis of Variance of the log. counts.

Sample No.	Dilution Factor	Colony Counts	Total	Mean	Mean number of viable spores/ ml. SS6S
1		132,130,145,133,145			
2		129,134,128,129,132			
3	10^8	139,132,131,131,129	3409	136.36	4.545×10^{10}
4		137,147,134,148,138			
5		144,141,136,143,147			

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	Components of Variance	Variance Ratio (F)
Between Samples	0.0053	m-1 = 4	0.001325	$n\sigma_A^2 - \sigma_B^2$	5.116
Within Samples	0.00518	mn-n = 20	0.000259	σ_B^2	
TOTAL	0.01048	mn-1 = 24			

m = Number of samples

n = Number of individuals in each sample.

σ_A^2 = Variance due to differences between samples.

σ_B^2 = Variance within samples

The Variance Ratio F with degrees of freedom $n_1 = 4$, $n_2 = 20$ has values of 2.9 at P = 0.05, 4.4 at P = 0.01 and 7.1 at P = 0.001

Between Sample Variance = 0.000213 Between Sample Coefficient of Variation = 0.68%

Within Sample Variance = 0.000259 Within Sample Coefficient of Variation = 0.75%

The Analysis of Variance shows that the Between Samples Mean Square is significantly greater than the Within Samples Mean Square at the 5% probability level. However, since the Between Sample Coefficient of Variation, and Within Sample Coefficient of Variation were each less than 5%, it was concluded that the spore suspension SS6S was suitable for use in the proposed experiments.

The mean number of viable spores/ml. of 4.545×10^{10} taken in conjunction with mean number of spores/ml. of 7.5194×10^{10} (Table 1) gives a value for the viability of spore suspension SS6S of 60.44%.

Preparation of Primary Dried Kaolin Powder Containing Viable Bacillus megaterium Spores

200 ml. of a suspension containing 20% B.D.H. "Collo Kaolin" in glass-distilled water was sterilised by autoclaving for 1 hour at 388 - 389°K. A calculated volume of SS1S containing a known number of viable spores was added to the sterile kaolin suspension and mixed. The resultant suspension of spores and kaolin was transferred to a sterile 2 litre round bottomed flask, and frozen in a thin even film, around the inside of the flask by rotating the flask in a bath of solid carbon dioxide/acetone mixture. The flask was attached to a refrigerated condenser containing solid carbon dioxide/acetone mixture at 195°K, and the pressure in the system was reduced to less than 0.1 mm. mercury by means of a single stage rotary high vacuum pump. (Edwards Speedivac ES.35). Drying was continued at room temperature until ice which had initially formed on the exterior of the flask had melted, and the surface of the flask was at room temperature. This drying period was usually of the order of four to five hours, after which, gentle tapping of the flask resulted in all the powder falling freely from the sides of the flask, indicating that the majority of the water had been removed from the powder.

The flask was removed from the condenser and the powder transferred to a sterile screw capped jar, containing sterile glass beads. The sealed jar was rotated slowly for 24 hours. This served to break down the flakes of powder formed during the drying process, and also to thoroughly mix the powder. The resultant powder is referred to subsequently as a primary dried powder.

For preliminary experiments a single primary dried powder was prepared, designated PDM4S. This was stored in the dark, at 293^{o}K $\pm$ 0.5^{o}K and 70 - 75% R.H. in a Fisons Controlled Environment Cabinet. The powder was transferred to a screw capped jar with a modified Millipore Swinnex - 25 Filter Unit containing a 25 mm. Type HA 0.45 filter, fitted into the top of the jar. This container, enabled the spore powder to equilibrate to the conditions of the cabinet, without risk of contamination.

It was shown from viable counts performed on the suspension before drying and on the resultant powder immediately after drying and at intervals during storage, that no loss of viability had occurred during the drying process or during storage over the experimental period.

Distribution of Viable Spores in the Primary Dried Powder PDM4S

In order that the primary dried powder PDM4S could be used for the study of heat inactivation of the contained spores, it was essential that different samples taken from the bulk powder at the same time should contain similar numbers of viable spores per unit weight, within the limits of normal sampling error. To test this, viable counts were performed on five samples of PDM4S. The technique used to determine the viable count of a powder sample is identical in principle to that used with samples of spore suspension, and differs only in the method of resuspension of the sample. The

accurately weighed powder samples of approximately 40 mg. were resuspended in volumes of water 200 times the weight of the powder i.e. 8 ml. water to 40 mg. of powder. The five reconstituted suspensions from primary dried powder PDM4S were all diluted to the same extent and the colony counts from the resultant final dilutions were subjected to statistical analysis.

Table 3 shows the colony counts obtained and the corresponding Analysis of Variance of the log. colony counts.

The Analysis of Variance shows that the Between Samples Mean Square is not significantly greater than the Within Sample Mean Square at the 5% probability level, and therefore the differences in colony counts between samples could be attributed to the normal errors of random sampling. Furthermore, since the total Coefficient of Variation was less than 5% it was concluded that primary dried powder PDM4S was suitable for use in the proposed experiments.

TABLE 3

Colony counts obtained from quintuplicate plating of five samples of primary dried powder PDM4S, with corresponding Analysis of Variance of the log. counts

Sample No.	Wt.of powder (g)	Volume of water (ml.)	Dilution factor	Colony Count	Total	Mean	Mean No. of viable spores/ 40 mg. PDM4S
1	0.0400	8.00		104,114,114, 97,118,	547	109.4	
2	0.0394	7.88		94,111,103,111,110,	529	105.8	
3	0.0408	8.16	5×10^3	108,113,106,100,105,	532	106.4	1.779×10^6
4	0.0406	8.12		99,108, 94,103,108,	512	102.4	
5	0.0397	7.94		104,118,114,104,108,	548	109.6	

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Between Samples	0.00934	4	0.002335	
Within Samples	0.05766	20	0.002883	1.235
TOTAL	0.066	24		

Variance Ratio F with degrees of freedom $n_1 = 20$, $n_2 = 4$ has a value of 5.85 at P = 0.05.

Total Coefficient of Variation = 5.1%

THE DRYING PROCESS

This is essentially a freeze-drying process. The physics of such a process and the practical considerations involved in its design are outlined in the Introduction, and have been implicated in the construction and operation of the apparatus described below.

Materials

The sample vessel - this was constructed from medium wall Pyrex glass tubing, 10 mm. external diameter, 7.5 mm. internal diameter. One end of the tube was sealed by fusion of the glass to give a closed tube of approximately 12 cm. length. A sample of spore suspension was measured into it and the open end was fused on to a B 10 ground glass socket. The vessel was then constricted to an internal diameter of about 2 mm. halfway along its length.

The vacuum line - this is illustrated in Figure 1. This was similar to the apparatus described by Davies (152) and Soper (135) and was constructed of "Pyrex glass". The main section of the apparatus was evacuated by a Speedivac Glass Mercury Diffusion Pump Model G.M.2., backed by a Rotary High Vacuum Pump (Edwards Model ES35) fitted with a P_2O_5 moisture trap. Provision was made for a circular manifold M on to which could be attached up to twelve samples at any time by means of B.10 cones mounted radially. The manifold could be isolated from the vacuum line by tap H. A solid carbon dioxide/acetone cold trap G, and a liquid nitrogen cold trap T, were incorporated in the vacuum line at distances of approximately 100 cms. and 150 cms. respectively from the samples. On the occasions that the vacuum line was operated without the Micro-Force Balance, Vacuum Head (see page 79) in position, a vessel of similar dimensions to the Vacuum Head was attached at joint L to maintain the pumped volume of the system.

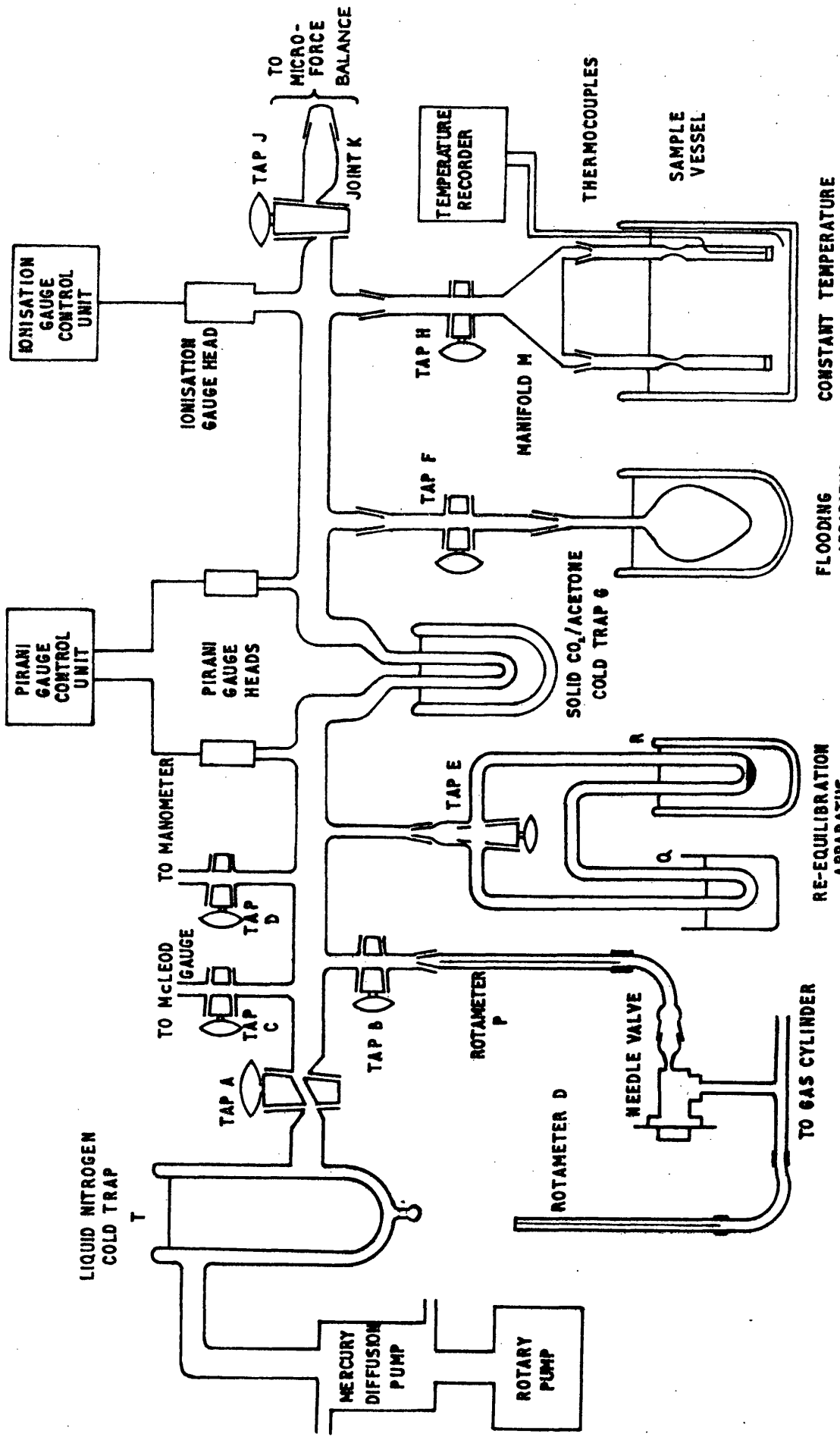


FIGURE. I. THE VACUUM APPARATUS

The pipettes - capillary pipettes B.S.1428, Pt.D4, Type 1 calibrated to deliver 0.1 ml. (i.e. graduated from 0 to 0.1 ml.) and manufactured by E - Mil, were used to measure samples of spore suspension into the sample vessels. Ten pipettes were taken at random and tested for suitability of use under experimental conditions. A 0.1 ml. volume of glass distilled water was delivered from each pipette into a vessel and weighed. No fixed immersion depth or drainage period was used, but the water used was maintained at 293°K. The procedure was carried out three times for each pipette. Triplicate weighings of 0.03 ml., and 0.06 ml. volumes of glass distilled water delivered from each pipette were also made.

The coefficient of variation for weights of water delivered from different pipettes was 3.56%, 0.58% and 0.73%, for 0.03 ml., 0.06 ml. and 0.1 ml. volumes respectively, and for weights of water delivered from the same pipette was 0.88%, 0.35%, and 0.35% for 0.03 ml., 0.06 ml. and 0.1 ml. volumes respectively. Furthermore, the percentage mean deviation from the theoretical weight of water at 293°K was calculated as 0.71% for 0.03 ml. volumes, 0.91% for 0.06 ml. volumes, and 0.23% for 0.1 ml. volumes. Since these weights lie within $\pm$ 1% of the theoretical weight at 293°K, it was decided to use the capillary pipettes for delivery of 0.03 ml., 0.06 ml., and 0.1 ml. quantities during this work.

Methods

Drying of spore samples - the moisture trap of the rotary pump was charged with phosphorus pentoxide. With the tap A to the main body of the vacuum line open, and taps to the McLeod Gauge (C), Mercury manometer (D), fine needle valve (B), equilibration apparatus (E), flooding apparatus (F), manifold (H) and Micro-Force Balance Vacuum Head (J) closed,

the rotary pump was switched on and the air admittance valve was slowly closed. When the pressure in the system had been reduced to between 5×10^{-2} torr and 7×10^{-2} torr as measured by the Pirani Gauge, the requisite number of sample vessels were attached to the manifold, of the vacuum line, and constant temperature baths were placed around the samples and the Vacuum Head (see page 67). After five minutes, thermocouple recordings showed that the samples had reached the temperature of the bath. The tap H to the manifold and J to the Vacuum Head were then slowly opened. The consequent reduction of the pressure in the sample vessels caused degassing and snap freezing of the samples to occur within one to two minutes of opening taps H and J. Five minutes after opening these taps the solid carbon dioxide/acetone mixture was placed around cold trap G. Drying was timed from the positioning of this cold trap. After a further period of fifteen minutes, the pressure in the system had again reached 5×10^{-2} torr to 7×10^{-2} torr as measured by the Pirani Gauge. Liquid nitrogen was placed in cold trap T, and the mercury diffusion pump was switched on. The Pirani Gauge was switched off and the pressure in the system was monitored at intervals by the McLeod Gauge and the Ionisation Gauge. The drying was continued at a pressure of less than 10^{-5} torr for the required period and the manifold and Vacuum Head were then isolated from the main body of the vacuum line by closing taps H and J, and the constant temperature baths were removed.

Re-equilibration of dried spore samples - after drying, samples were exposed to water vapour over water or ice at known temperatures, in the absence of permanent gases. The apparatus used is illustrated in Figure 1 and consists of a double vapour trap made of "Pyrex" glass and fitted with an L type three-way tap E. About 2 ml. sterile glass-distilled water was placed in arm Q. This was degassed by distilling it, under vacuum, over into arm R which was cooled by a solid carbon dioxide/ acetone mixture.

For re-equilibration, the cold trap G was removed from the vacuum line and a constant temperature bath was put around arm R of the re-equilibration apparatus in place of the cooling mixture. With the pumps isolated from the main body of the vacuum line by closure of tap A, tap E on the re-equilibration apparatus was opened to arm R. Taps H and J were opened allowing the water vapour to come into contact with the dried spores. The samples were left to re-equilibrate with the water vapour at the known aqueous vapour pressure for a period of 1 hour (see page 103). Aqueous vapour pressures of 10 torr and 5×10^{-2} torr were used during the course of this work. A constant temperature bath of water at $283^{o}K$ was used to obtain an aqueous vapour pressure of 10 torr, and a bath of melting chlorobenzene at a temperature of $228^{o}K$ was used to obtain an aqueous vapour pressure of 5×10^{-2} torr.

Exposure of samples to controlled gaseous environments - to obtain samples under anoxic conditions the sample vessels were sealed after completion of the drying period, or immediately after re-equilibration, by fusion of the glass at the constriction.

When spores were required in the presence of oxygen, taps H and J were closed at the termination of the drying process or when necessary, after the re-equilibration process. With tap A to the main body of the vacuum line open, a solid carbon dioxide/acetone bath was placed around the cold trap G, and tap B was opened to evacuate the rotameter P. Oxygen was allowed to flow freely from the cylinder, at a rate of 1 litre per minute as measured by rotameter O, to flush air from the connections. With tap A closed, the fine needle valve was opened and oxygen was bled into the vacuum line at a rate equivalent to 200 ml. per minute, as indicated by rotameter P, until atmospheric pressure was reached as indicated by the mercury manometer. The fine needle valve was then closed and the taps H and J were opened. The pressure in the system

was readjusted to atmospheric, by bleeding in additional oxygen via the fine needle valve. Taps H and J were then closed and the sample vessels sealed by fusion of the glass at the constriction.

Opening of sealed sample vessels - if an evacuated sample vessel is opened rapidly a large portion of the contents are carried out of the vessel as an aerosol. The technique used to open the sealed vessels was to mark the upper part with a file, and then to touch a piece of molten glass to the mark. It was observed that the small crack which was formed allowed air to enter slowly until atmospheric pressure was reached, when the tip could be lifted off safely without producing an aerosol.

Determination of a Suitable Sample Volume for Drying

In order that the drying process may be of use in the study of the effects of various drying conditions on bacterial spores it is essential that samples of suspension SS6S, when dried under different conditions, should at the end of the drying process contain similar numbers of spores to samples of undried suspension, within the limits of normal sampling error.

It is possible that spores could be lost from a sample at the commencement of drying or during the foaming which occurs with the evolution of dissolved gases from the suspension prior to snap-freezing. In addition, the high vacuum employed in the drying apparatus could remove dried spores from the surface of the sample during the drying process. With small volumes, a significant reduction in spore numbers may also occur as a result of incomplete removal of the dried sample from the vessel at the time of reconstitution.

In an experiment designed to establish a suitable drying volume, three volumes were tested. These were 0.03 ml., 0.06 ml. and 0.1 ml.

The upper limit of sample size was set by the capacity of the Micro-Force Balance (see page 78) i.e. 100 mg. A 0.1 ml. volume of spore suspension SS6S weighed approximately 100 mg. Four aliquots of each of the three volumes were dried for 6 hours at 298°K. At the termination of the drying process the sample vessels were sealed and removed from the manifold. The vessels were then opened and the contained spore samples resuspended in 100 times their volume of sterile water. Quantitative removal of the sample from the vessel was achieved with the aid of a sterile Pasteur pipette, using the five-fold transference method recommended by Davies (149). The total number of spores in each suspended sample was determined by the method described on page 42. Viable counts were also performed on each sample (see page 43) and the respective percentage viabilities calculated.

The data from this experiment are recorded in Table 4 together with the corresponding data for the original suspension SS6S.

Comparison of the mean total number of spores in the undried suspension with the mean number in each of the three sample volumes of dried spores reveals that in all cases a percentage of the original number of spores present in the sample is lost during the drying and/or recovery process. The 0.1 ml. samples, in which foaming prior to snap freezing was most pronounced, show the highest percentage loss of spores (14.7%) and it is therefore considered that the majority of the loss from these samples occurred during the initial stages of the drying process. The smallest samples (0.03 ml.) exhibited very little foaming during the pre-freezing stage of the drying, and thus most of the 9.7% loss of spores from these samples probably occurred either from removal of dried spores during the drying process, or from incomplete transference of the sample from the vessel at the time of reconstitution. A test for equality performed on the log. total counts of the 0.06 ml. samples of dried spores

TABLE 4

Total count, viable count and percentage viability of samples of spore suspension SS6S prior to drying and of 0.03 ml., 0.06 ml., and 0.1 ml., samples after drying for 6 hours at 298°K.

Sample Number	Total No. of Spores/ml.SS6S ($x10^{10}$)	No. of Viable Spores/ml.SS6S ($x10^{10}$)	Percentage Viability
A). Spore Suspension Prior to Drying			
1	7.9125	4.5667	57.72
2	7.0375	4.3467	61.76
3	7.8550	4.4133	5618
4	7.2370	4.6933	64.85
5	7.5550	4.7400	62.74
Mean	7.5194	4.5453	60.44
B). 0.03 ml. Sample After Drying			
1	6.4062	4.1333	64.52
2	6.7428	4.2700	63.33
3	6.8146	3.9767	58.36
4	7.2006	4.3667	60.64
Mean	6.7911	4.1867	61.65
Mean % Loss in Total Number of Spores After Drying 9.7%			
C). 0.06 ml. Sample After Drying			
1	6.7801	4.3300	63.86
2	7.0246	4.5500	64.77
3	7.3135	4.2433	58.02
4	7.5492	4.6633	61.77
Mean	7.1669	4.4467	62.04
Mean % Loss in Total Number of Spores After Drying 4.7%			
D). 0.1 ml. Sample After Drying			
1	6.7972	3.8567	56.74
2	6.5268	3.9400	60.37
3	6.2856	3.5667	56.74
4	6.0456	3.7833	62.58
Mean	6.4138	3.7867	59.04
Mean % Loss in Total Number of Spores After Drying 14.7%			

and of the samples of undried suspension gives a value of t of 0.692 which is less than the tabulated value at the 5% probability level of 2.45 and indicates that, in fact, the difference between the mean total number of spores in dried and undried 0.06 ml. samples (4.7%) is within the limits of error associated with normal sampling procedures. It was considered that 0.06 ml. was a suitable volume for use as the standard sample size in all experiments which involved a drying procedure.

A further test for equality performed on the log. percentage viability values obtained with samples of undried suspension and with 0.03 ml., 0.06 ml., and 0.1 ml. samples of dried spores gives a value of B of 1.073 in comparison with the tabulated value at the 5% probability level of 5.991 and indicates that, with respect to the percentage viability the dried and undried samples cannot be considered to have been derived from different populations. This shows that the loss observed with 0.03 ml. and 0.1 ml. sample is physical and not the result of changes in viability. The viability of the spores does not appear to be affected by the volume of spore suspension that is dried, or by the process of drying for a period of 6 hours at 298^{o}K with subsequent rehydration and resuspension.

The Effect of Freezing Spore Samples prior to Drying

A number of commercial and laboratory freeze-drying procedures utilise freezing of the preparation as the preliminary stage in the drying process. This ensures that drying takes place from the frozen state as opposed to the liquid state, and also serves to suppress the foaming which is frequently encountered during the degassing stage of the process. In theory, therefore, pre-freezing should also reduce the loss of spores from the sample during the initial stages of drying.

Ten 0.06 ml. samples of suspension SS6S were measured into sample

vessels. Five of the vessels were immersed in a bath of solid carbon dioxide/acetone mixture at 195°K for 5 minutes before being attached to the manifold, the remaining vessels being maintained at 298°K. All the samples were dried for 6 hours at 298°K. At the end of the drying period the sample vessels were sealed off under vacuum, and total and viable counts were performed on each resuspended spore sample. The counts obtained from the frozen and unfrozen samples after drying are recorded in Table 5 together with the corresponding percentage viabilities.

Statistical analysis of this data yields values of t of 0.72 and 0.48 for log. total counts and log. percentage viability respectively, in comparison with the tabulated value at the 5% probability level of 2.45 and shows that with respect to both total count and percentage viability, the frozen and unfrozen samples are unlikely to have been drawn from different populations.

It is apparent, therefore, that under the drying conditions used in these experiments, the inclusion of a pre-freezing treatment has no measurable beneficial effect on either the subsequent recovery of the dried spores or their viability. Furthermore, freezing per se is known to damage cells, the amount of damage induced being strongly influenced by the rate at which freezing occurs. Thus, unless the pre-freezing stage of the drying process can be carried out under constant and closely defined conditions, variations in the temperature or rate of freezing could give rise to variable amounts of damage being induced in different spore samples, the effects of which may well persist throughout the drying process. Any attempt to analyse changes in the characteristics of dried spores in terms of injury produced solely by the high vacuum drying process would then be extremely difficult.

It was therefore decided, that in all experiments described in

this thesis, in which a drying process is involved, 0.06 ml. samples of spore suspension would be snap-frozen prior to drying (after equilibration to the temperature at which the drying was to be carried out) and would not be subjected to a pre-freezing treatment.

TABLE 5

Total count, viable count and percentage viability of 0.06 ml. samples of spore suspension SS6S after drying for 6 hours at 298°K, a) pre-frozen prior to drying. b) maintained at 298°K prior to drying.

Sample Number	Total No. of Spores/ml.SS6S ($x10^{10}$)	No. of Viable Spores/ml.SS6S ($x10^{10}$)	Percentage Viability
A). Samples Pre-Frozen Prior to Drying			
1	6.8701	4.0033	58.27
2	7.0416	4.4567	63.29
3	7.1512	4.3633	61.01
4	7.3136	4.1967	57.38
5	7.6491	4.6233	60.44
Mean	7.2051	4.3287	60.08
B). Samples Maintained at 298°K Prior to Drying			
1	6.6911	4.3067	64.36
2	7.0041	4.2633	60.87
3	7.0662	4.0667	57.55
4	7.2216	4.5600	63.14
5	7.4171	4.4033	59.37
Mean	7.0800	4.3200	61.02

THE MEASUREMENT OF SAMPLE TEMPERATURE DURING DRYING

Introduction

Previous workers have presented strong evidence to indicate that the amount of damage observed in cells subjected to freezing and freeze-drying is influenced by the minimum temperature to which the cells are exposed and the rate at which this minimum is attained (see page 18). In the present series of experiments, although a standard drying treatment could be obtained with the high vacuum system employed, it was not possible to control precisely either the minimum sample temperature or the rate of cooling. To enable complete characterisation of the system, the changes in sample temperature that occur under the different drying conditions investigated, were determined.

The practical problems involved in the measurement of sample temperatures, and the choice of methods available have been appraised in a previous review (169) and the recommendations made have been considered in the experiments described in this section.

Materials

The temperature of the sample was monitored throughout the drying process by means of a thermocouple sealed into one of the sample vessels, (Figure 2). The thermocouple was of copper-constantan construction and was manufactured from 0·3 mm. plastic covered wire, sheathed in fibre glass. The soldered copper-constantan junction was located in the centre of the sample, and the leads from it were led out through the wall of the sample vessel, below the constriction to a cold junction maintained at 273 ^{o}K in ice water. The output from the thermocouple was fed directly into a Smiths' Servoscribe potentiometric recorder.

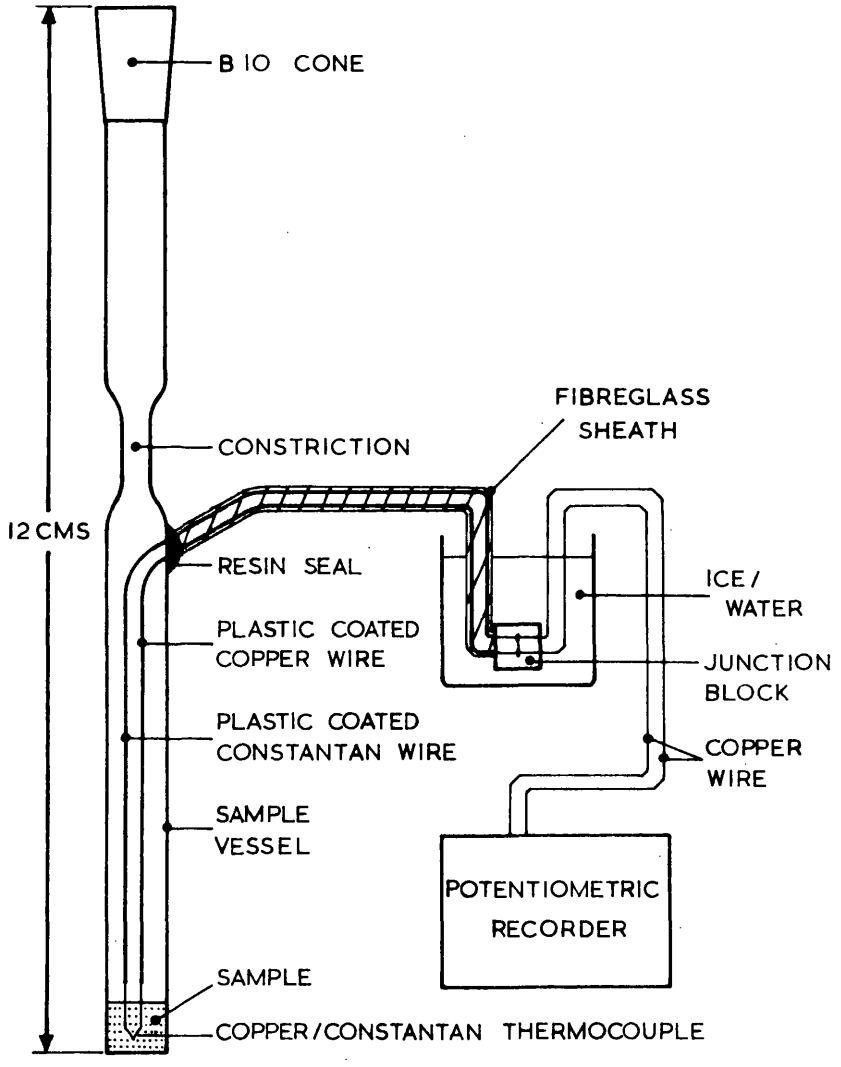


FIGURE 2.

APPARATUS FOR THE MEASUREMENT OF SAMPLE TEMPERATURE

Methods

Calibration of the Thermocouple

The sample vessel containing the thermocouple was attached to the vacuum drying apparatus and the pressure in the system was reduced to $< 10^{-5}$ torr. A constant temperature bath was placed around the sample vessel, and the recorder chart scale reading was recorded when the vessel and thermocouple were in equilibrium with the temperature of the bath. Oxygen was then admitted to the sample vessel, and the chart reading at temperature equilibrium was again recorded. The thermocouple was calibrated at six temperatures over the range 242°K to 333°K. The composition of the constant temperature baths employed is recorded in Table 6 together with the thermocouple calibration reading for each temperature expressed in numbers of recorder chart scale divisions. (0 - 100 divisions corresponds to a recorder imput of 5 mV from - 1.6 mV to + 3.4 mV.)

TABLE 6

Composition of constant temperature baths and corresponding thermocouple calibration readings.

Temperature (°K)	Composition of Constant Temperature Bath	Thermocouple Calibration (Chart Divisions)
242	Melting bromobenzone	8
257.4	Melting ethylene glycol	20
273	Melting ice	32
291.2	Melting glycerol	46.5
313.9	Melting phenol	51
333	Melting chloroacetic acid	89

The admission of oxygen to the sample vessel did not change the thermocouple calibration reading at any of the temperatures investigated. The calibration curve constructed from the data obtained in this experiment is illustrated in Figure 3.

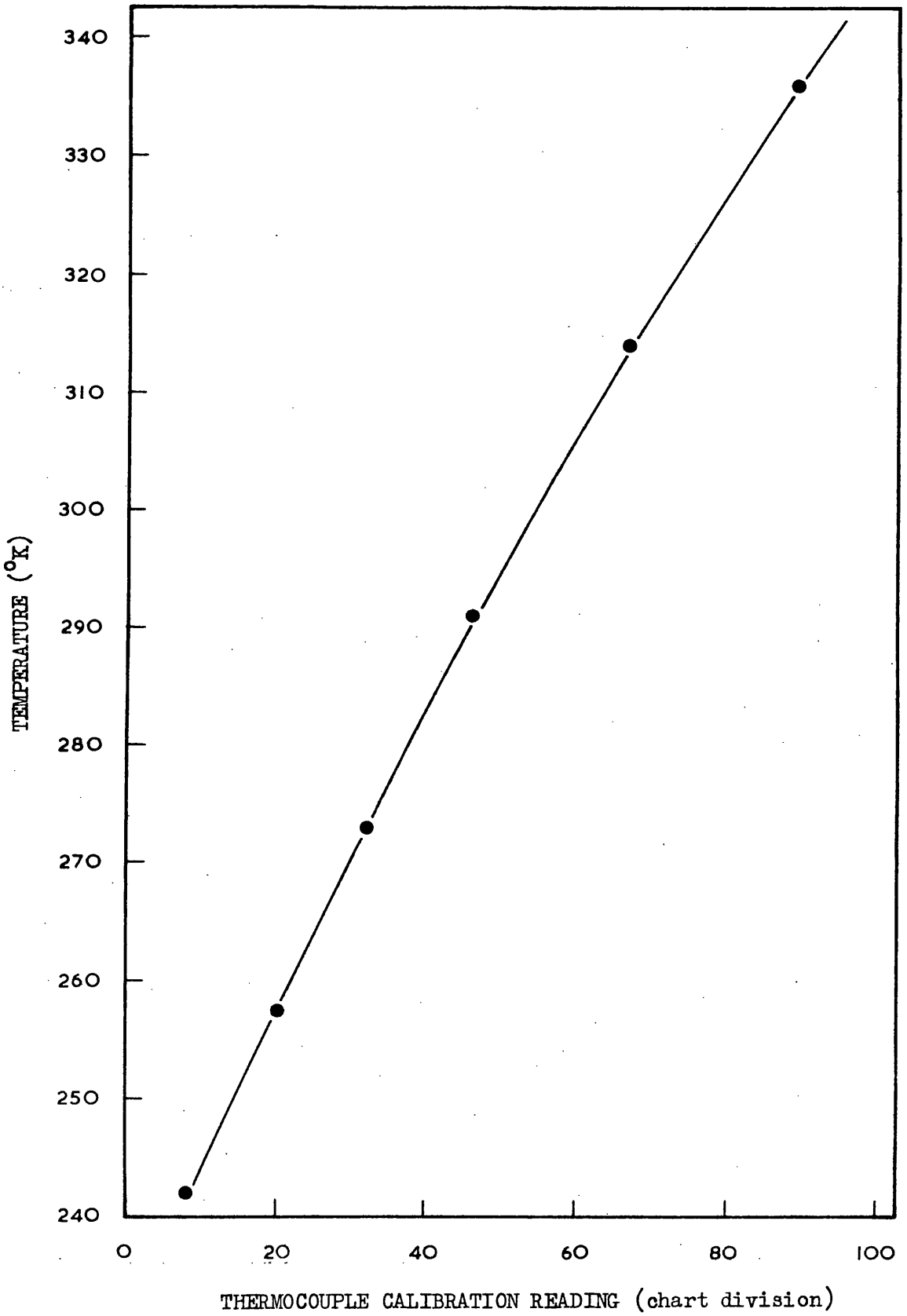


Figure 3. Calibration curve for the copper-constantan thermocouple. (Constructed from data in Table 6.)

Continuous Measurement of Sample Temperature During Drying at Different Temperatures

During a normal drying the sample vessel containing the thermocouple was attached to the manifold together with eleven other vessels each containing 0.06 ml. spore samples. The Micro-Force Balance was also operated to enable correlations to be made between weight changes and temperature changes in the samples during drying. The drying temperatures investigated in these experiments were 273°K, 288°K, 298°K., 308°K, 323°K, and 338°K. Samples were dried for 24 hours at each of the chosen temperatures, and at the termination of the drying process were re-equilibrated to 10 torr aqueous vapour pressure. A continuous record of the sample temperature was obtained throughout the drying period, and during subsequent re-equilibration and admission of oxygen to the samples.

Sample temperature/time profiles recorded during the first six minutes after the samples had been opened to the main body of the vacuum line are illustrated in Figures 4a and 4b for samples dried at 308°K and 338°K respectively. The curves obtained at all the drying temperatures investigated are divisible into three distinct sections, an initial cooling stage in which the sample temperature decreases rapidly with time, a period during which the sample remains at a relatively constant minimum temperature, and a warming up stage during which the sample temperature increases from the minimum up to that of the surrounding bath. The three main sections can be further subdivided, the degree of subdivision being dependent upon the drying temperature at which the profile is obtained. The sample temperature/time profile obtained at the highest drying temperature investigated, 338°K shows the greatest degree of differentiation and can be represented by the schematic diagram in Figure 5.

When the tap to the manifold is slowly opened to the main body

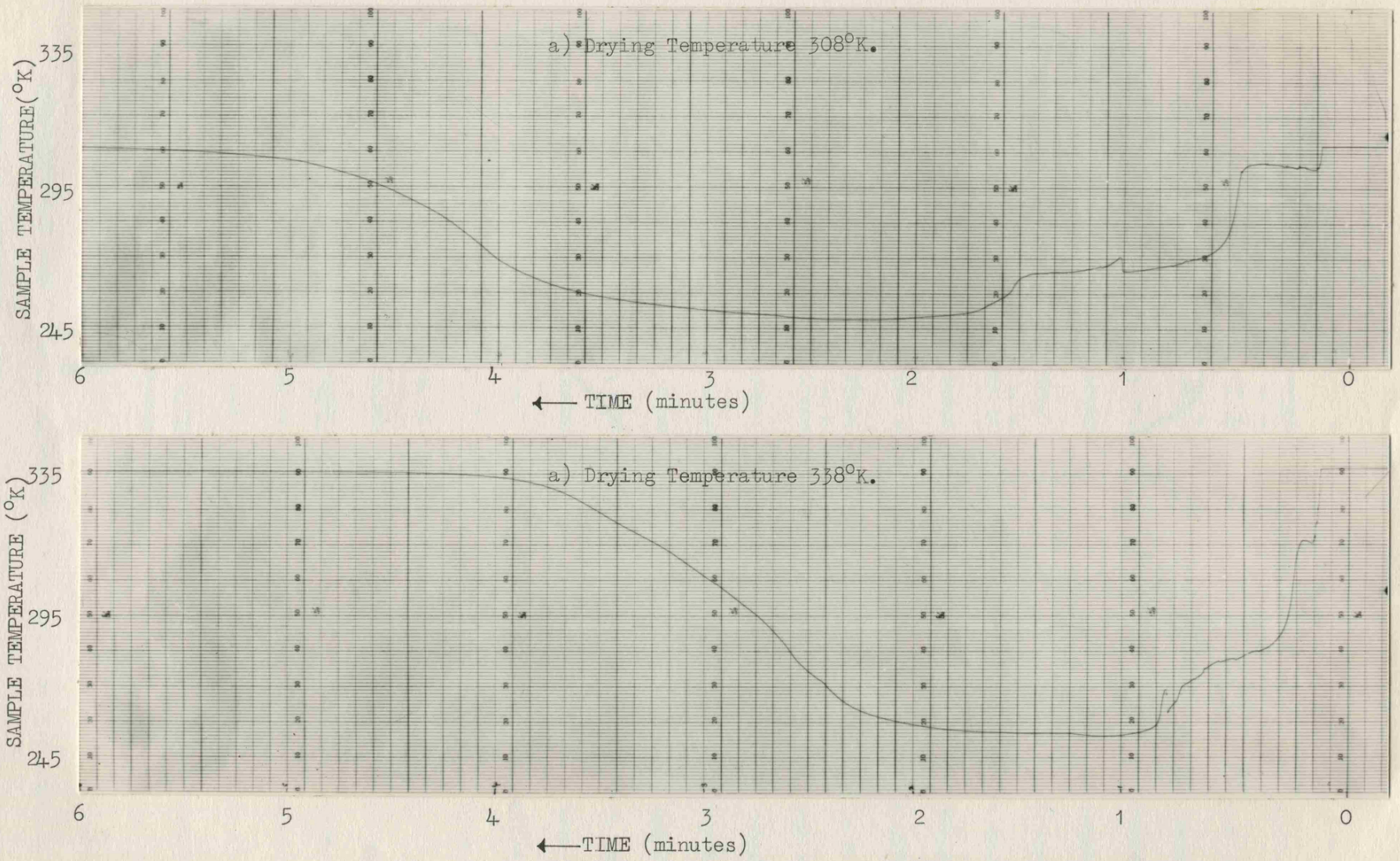


Figure 4. Sample temperature/time profiles recorded during the first six minutes after the samples of Bacillus megaterium spores had been opened to the main body of the vacuum line, a) drying temperature 308°K, b) drying temperature 338°K.

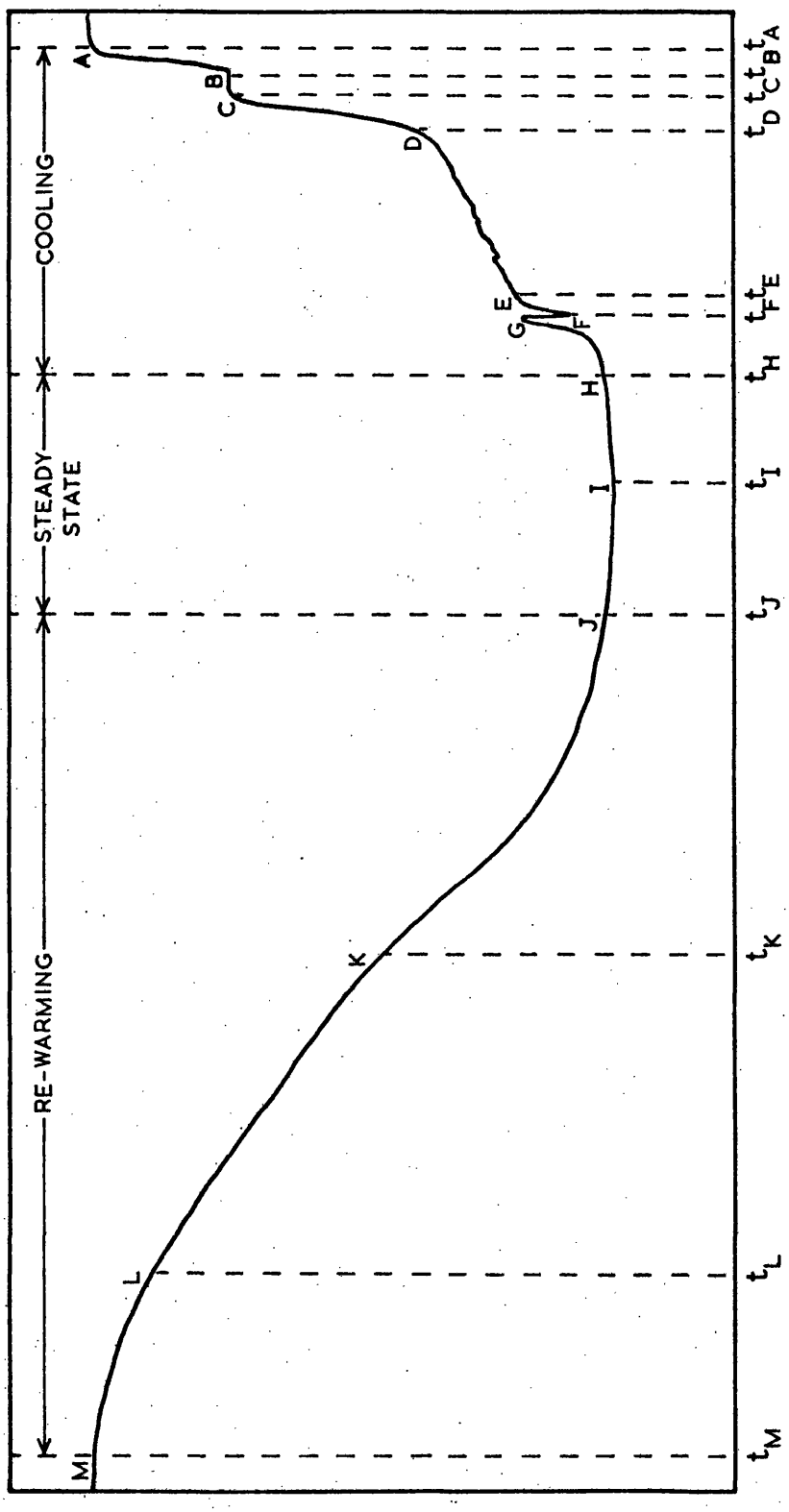


Figure 5. Schematic diagram of the sample temperature/time profile obtained during drying of Bacillus megaterium spores at 338°K.

of the vacuum line at point A, the corresponding decrease in pressure within the sample vessel results in an immediate rapid decrease in temperature (A - B). When the tap is fully open there occurs a short period of pressure re-equilibration (B - C) during which the sample temperature remains relatively stable. As the rate of pressure decrease becomes steady, the temperature again falls rapidly until a sample temperature of approximately 278 to 283^{o}K is reached (D). At this point the rate of decrease of sample temperature with time is markedly reduced and the curve becomes irregular in shape (D - E) probably as a result of the degassing and concomitant foaming which occurs within the sample. At the cessation of degassing the sample is still in the liquid state, and there follows a short period of rapid cooling to a temperature of approximately 260^{o}K (E - F). "Snap-freezing" of the supercooled sample then occurs, with a simultaneous instantaneous rise in sample temperature to approximately 270^{o}K (F - G). The temperature of the frozen sample then decreases rapidly at first, and then more slowly (G - I) to a minimum (I). After remaining at the minimum temperature $\pm$ 1^{o}K for a defined period (H - J), the sample warms up slowly to the temperature of the surrounding bath. Three stages are discernable in the warming up process: an initial stage with a relatively fast rate of warming (J - K), followed by a slower, constant rate period until the sample is within 5 - 10^{o} of the bath temperature (K - L), and a final, slow warming rate stage until the temperature of the sample is at equilibrium with that of the surrounding bath (L - M). The sample then remains at the chosen drying temperature throughout the drying process. Subsequent re-equilibration of the samples to 10 torr aqueous vapour pressure at the termination of the drying procedure and admission of oxygen to the vessels prior to sealing caused no observable changes in sample temperature.

Marked differences in the degree of subdivision were observed in sample temperature/time profiles obtained at drying temperatures below 338°K. These differences occurred only in the initial cooling stage of the profile, the steady state region and the rewarming region following a similar pattern under all the drying conditions investigated. At drying temperatures of 323°K, 308°K, and 298°K the rapid super-cooling stage E - F was not observed, and cooling of the sample to the minimum temperature prior to freezing (F) took place at a steady rate after point D was reached. This was accompanied by a corresponding increase in the duration of the pressure re-equilibration period (B - C). At the drying temperature of 273°K the initial cooling stages A - E observed at the higher temperature were non-existent. Super-cooling of the sample to approximately 260°K prior to "snap freezing" took place in one stage commencing immediately the manifold was opened to the vacuum line. The irregularity of this stage of the curve indicated that degassing of the sample probably occurred during the initial supercooling. Further differences were also observed, at different drying temperatures, in the behaviour of the sample immediately after "snap freezing". At 338°K and 323°K the sample temperature decreased rapidly to the minimum (region G - H) whereas at drying temperatures of 308°K and below, an initial slow rate and subsequent rapid rate of cooling were observed during the period in which the frozen sample cooled to the minimum temperature.

Five sample temperature/time profiles were recorded at each of the investigated drying temperatures, and the mean value for measurements carried out on these profiles are recorded in Table 7.

It can be seen from Table 7 that the total time taken from when the manifold is opened to the vacuum line until the sample attains the temperature of the surrounding bath is dependent upon

TABLE 7

Summary of characteristics of sample temperature/time profiles obtained with samples of spore suspension SS6S dried at different temperatures.

DRYING TEMPERATURE	273°K	283°K	298°K	308°K	323°K	338°K
Time for sample to freeze ($t_A - t_F$) (mins)	0.93	1	1.03	1	0.92	0.78
Minimum temperature of sample prior to freezing (F)	259°K	260°K	261.2°K	260°K	261.2°K	260.5°K
Minimum temperature of frozen sample (I)	239.5°K	243°K	246°K	247.5°K	250°K	251.5°K
Time for sample to reach minimum frozen temperature, ($t_A - t_I$) (mins)	10	5.5	3.5	2.25	1.5	1.08
Time during which the sample is maintained at the minimum temperature + 0.1°K ($t_H - t_J$) (mins)	6.27	2.67	1.42	0.83	0.88	0.58
Time for sample to warm up from minimum temperature to temperature of surrounding bath ($t_I - t_M$) (mins)	25	13	8.5	7.25	5	4.17
Time from start of process until sample has re-equilibrated to the temperature of surrounding bath ($t_A - t_M$) (mins)	35	18.5	12	9.5	6.5	5.25

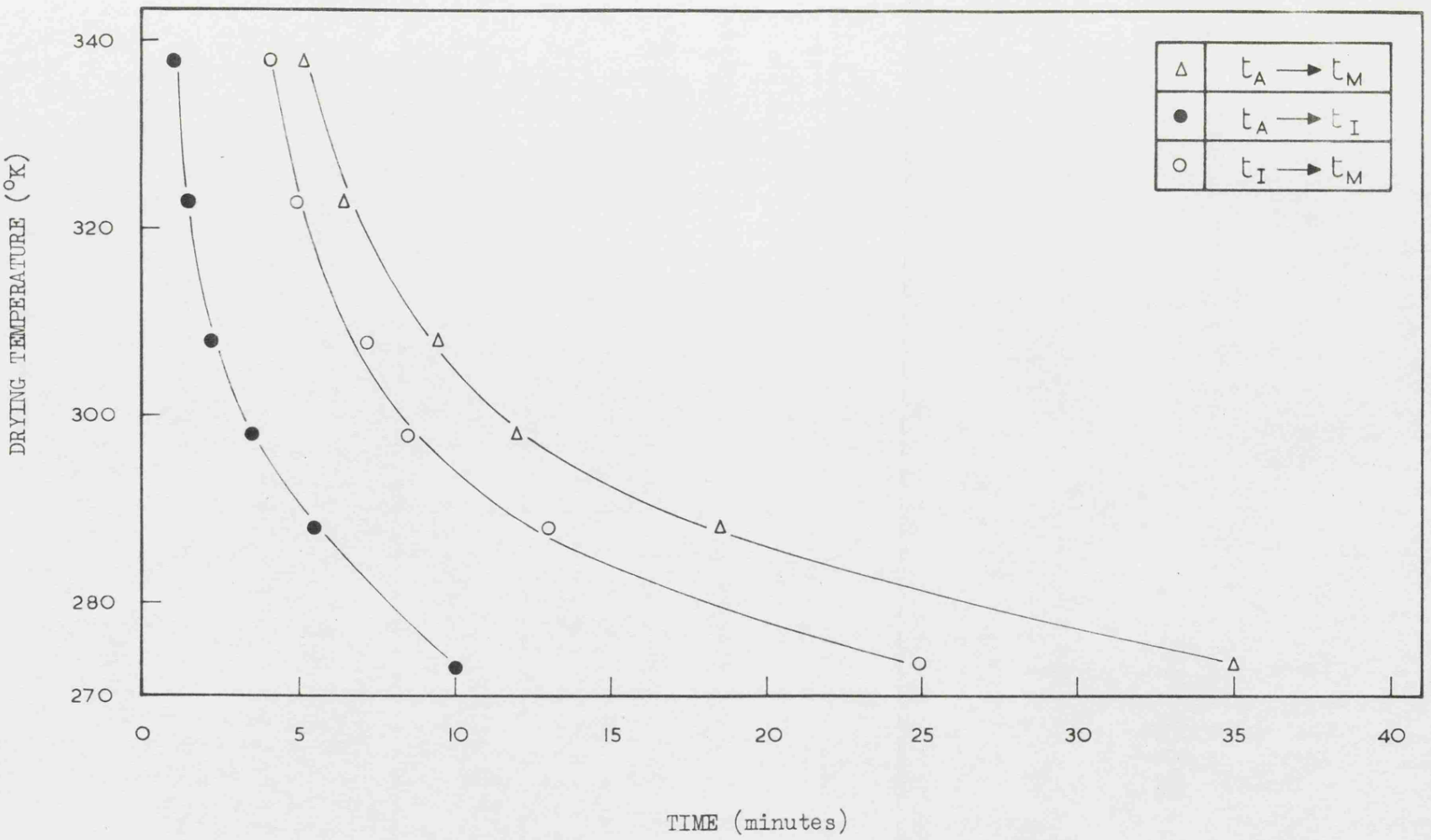


Figure 6. The relationship between "drying temperature" and the time taken for a sample of Bacillus megaterium spores to a). reach the minimum frozen temperature (●), b). warm up from the minimum frozen temperature to the drying temperature (○) and c). re-equilibrate to the drying temperature from the start of the process. (Δ).

the bath temperature, a longer time being required at low temperatures than at high temperatures. This relationship between the time interval ($t_A - t_M$) and the drying temperature is curvilinear as shown in Figure 6 .

The minimum temperature reached by the frozen sample during the drying process is also dependent upon the drying temperature employed, the relationship between these variables again being curvilinear. (Figure 7). The sample temperature differential that exists during the initial stage of the drying process is however directly related to the temperature at which the process is conducted, as shown in the plot of temperature differential (maximum sample temperature M - minimum sample temperature I) against drying temperature illustrated in Figure 7.

The temperature of drying also influences the time taken for the sample to reach the minimum temperature ($t_A - t_I$) as shown in Figure 6 Again the relationship is curvilinear, a greater increase in the $t_A - t_I$ interval occurring for the 15^oK decrease in drying temperature from 288^oK to 273^oK than occurs for an equivalent decrease in drying temperature from 338^oK to 323^oK.

In contrast, the minimum temperature to which the sample cools prior to freezing is independent of the drying temperature and has a value of $260^oK \pm 1.2^oK$. Thus, during the initial stage of the drying process, the spore sample undergoes approximately 12 to 14 degrees of supercooling, regardless of the temperature of drying over the range 273^oK to 338^oK. The time taken for the sample to reach 260^oK and to subsequently "snap freeze" ($t_A - t_E$) is also constant at 0.98 ± 0.05 minutes for drying temperatures from 273^oK to 323^oK, a slightly shorter time interval of 0.78 minutes being observed at 338^oK.

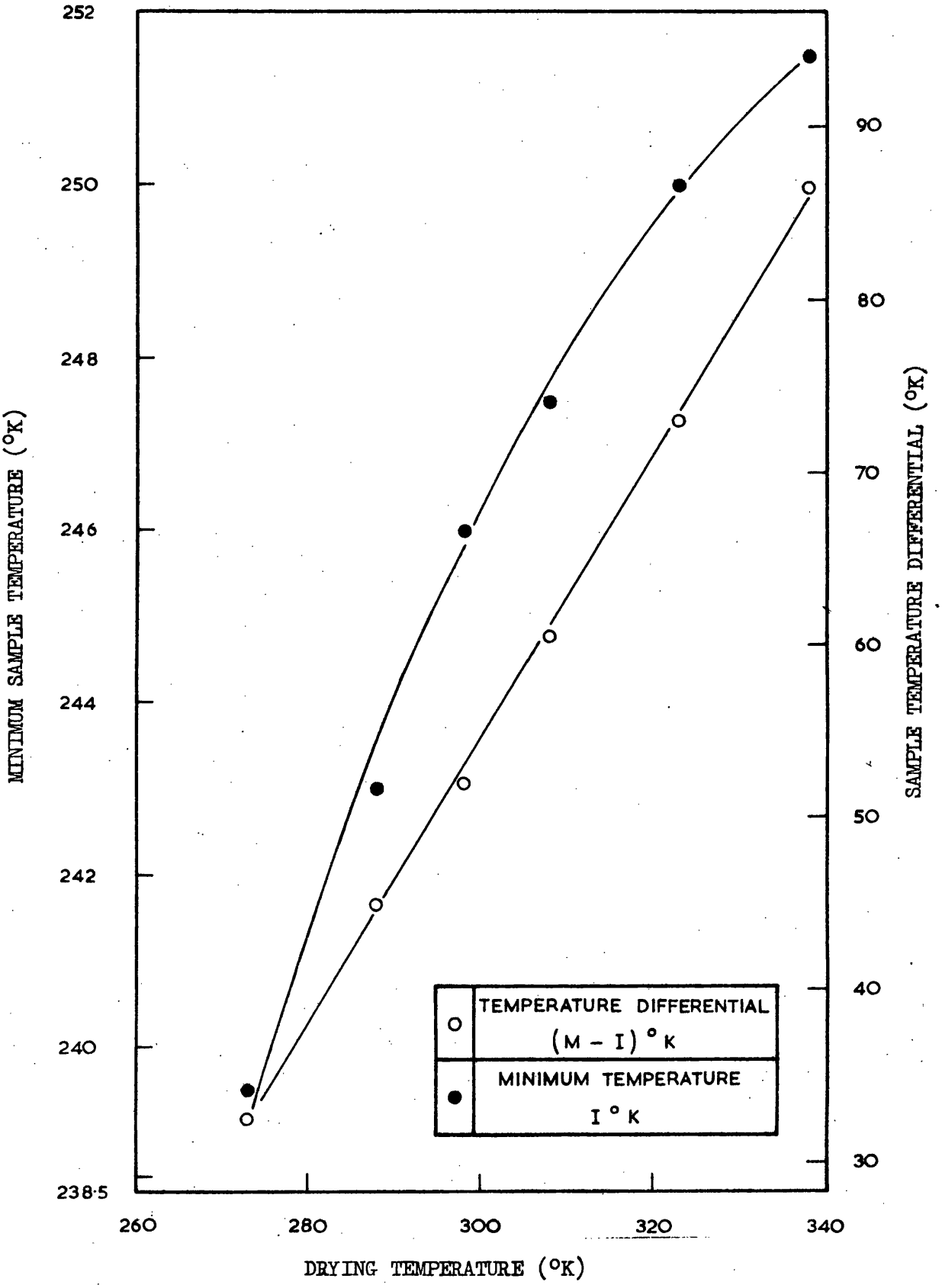


Figure 7. Plot of "minimum sample temperature (I)", and "sample temperature differential" (maximum sample temperature M - minimum sample temperature I) against "drying temperature" for samples of Bacillus megaterium spores.

It would thus appear that the differences observed in the time taken for the sample to re-equilibrate to the drying temperature result almost entirely from differences in the rate, after freezing, of cooling and of subsequent warming of the samples. (The time taken for the sample to re-equilibrate from the minimum temperature to that of the surrounding bath ($t_I - t_M$) also shows the expected curvilinear relationship with drying temperature as shown in Figure 6) The significance of these findings is discussed on page 244.

MEASUREMENT OF WEIGHT CHANGES IN SPORE SAMPLES DURING DRYING

Introduction

Previous workers have presented strong evidence to indicate that the stability of dried biological products, and in particular the survival and characteristics of freeze-dried bacteria, is generally influenced by their residual moisture content (see page 28). A number of techniques have been developed to determine the residual moisture content of dried specimens. However, since each method measures a different characteristic of the residual moisture, different values for water content can be obtained for a given sample depending on the particular method used. Moreover, since the mechanisms by which residual moisture causes deleterious effects are not known, it is not as yet possible to select a method of measurement which bears the most meaningful relationship to product damage.

Chemical methods based on the Karl Fischer iodometric titration principle (170, 171) have been applied with some success to the determination of the moisture content of dried bacteria (172). However, such methods measure only that part of the water which is "chemically active" in the context of the reaction, and since these measurements involve a destructive analysis they cannot be used for the continuous monitoring of moisture content.

Of more useful application is the use of gravimetric methods to monitor the moisture content of the specimen during the freeze-drying process, thus providing a measure of both the drying rate, and the residual moisture content. The first attempts to measure weight loss in samples during freeze drying utilised quartz fibre balances of the type devised by McBain (173) for studies on adsorption. Recently the development of electronic micro-force balances has enabled weights

between 1 ug. and 200 mg. to be recorded automatically and continuously, under conditions of high vacuum. McKenzie and Luyet (174) have successfully utilised such a balance to measure freeze-drying rates in tissue samples and have demonstrated the sensitivity and reproductibility of the technique.

In the experiments reported in this section, a similar technique and instrument to that used by McKenzie and Luyet has been employed to measure the changes in weight of samples of spore suspension during drying under different defined conditions.

Materials

The Micro-Force Balance - this was a Mark 2 Micro-Force Balance, Model B, manufactured by C.I. Electronics Ltd., having the following specifications.

Capacity: 1 gram sample, 1 gram counterweight.

Readout: Direct meter reading with provision for external recording.

Ranges: 5 in all. Direct switching.

Range 1	0	- 25	micrograms
Range 2	0	- 250	micrograms
Range 3	0	- 2.5	milligrams
Range 4	0	- 10	milligrams
Range 5	0	- 100	milligrams

Sensitivity: Weight changes of 0.5% on indicating meter, or less on recorder.

Reproducibility: $\pm$ 0.5 microgram.

Electrical tare: Coarse control 9 steps of 1.1 milligram.
Medium control 9 steps of 0.11 milligram.

The operating principles of the instrument are as follows:- The balance head unit electromagnetically balances the torque produced by the sample weight. A current flows through it in exact proportion to the applied weight, and this current operates the indicating meter and also provides an electrical output. Figure 8a illustrates the construction of the balance head unit. The balance arm carries a shutter interposed between a lamp and a pair of silicon photocells. A small displacement of this arm from its centre position causes excess current to flow through one photocell, and this is amplified and passed through the movement coil, restoring it to its original position.

In addition to generating a weight-proportional current, this servo action ensures a rapid response and makes the balance relatively insensitive to external vibration.

The simple bridge circuit of Figure 8b illustrates the principle. With the balance arm in its central position, the photocells have equal resistance and no bridge current flows through the movement coil and indicating meter. A slight arm movement changes the relative illumination on the photocells, unbalancing their resistances and causing a bridge current. This current, passing through the movement coil, rapidly produces a new equilibrium in which the residual bridge current just counteracts the external torque applied to the balance arm.

The complete circuit includes amplification of the photocell currents, and the output current, a stabilised power supply, zero adjustment, range switching and temperature compensation.

The Micro-Force Balance Vacuum Head - the Micro-Force Balance Head was remotely mounted in a "Pyrex" glass vacuum bottle. This enabled the weighing head to be operated in high vacuum (10^{-5} torr to 10^{-6} torr), so that changes in sample weight could be measured under

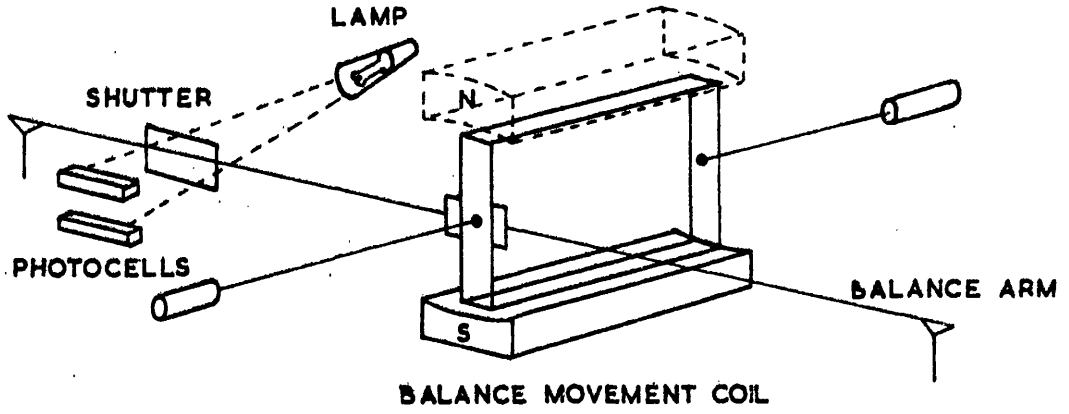


FIGURE 8a. CONSTRUCTION OF THE MICRO FORCE BALANCE HEAD UNIT

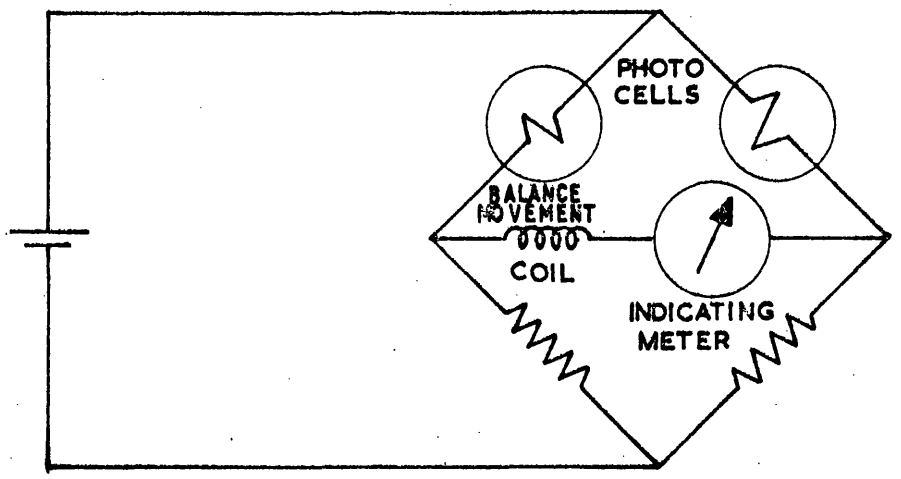


FIGURE 8b. BRIDGE CIRCUIT ILLUSTRATING PRINCIPLE OF OPERATION OF MICRO-FORCE BALANCE

conditions of controlled atmosphere. With this apparatus, illustrated in Figure 9, it was possible to follow weight changes in the spore sample during the drying process, and during subsequent re-equilibration.

Sample pan suspension rods - each sample pan was suspended at a distance of 30 cm. below the balance beam by means of two suspending rods. These were constructed from copper wire 0.48 mm. outside diameter, extended and heat annealed to maintain rigidity. The rods were 15 cm. long and were fitted with hooks at each end. A fine wire sample pan support was hooked onto the lower end of each pair of linked suspension rods.

Sample pans - these were constructed with identical internal diameters to the sample vessels used on the vacuum system (see page 52) The pans were flat bottomed cylinders, 7.5 mm. internal diameter, and 7 mm. height, manufactured from aluminium foil, and fitted with flanges at the open ends for attachment to the sample pan support. Sample pans were thoroughly cleaned, and sterilised prior to use by heating in a hot air oven at 433^{o}K for one hour.

Methods

Assembly - the Micro Force Balance Vacuum Head was connected to the vacuum apparatus by means of a greased cone and socket joint K, via a glass T-piece R and joint L. The Head was mounted rigidly in a level horizontal position by means of clamps around the ports L and N; and a spirit level was used to check the position of the balance. The location of the balance in the vertical plane was fixed with reference to a plumb-line, and was maintained by means of clamps fixed around the main body of the Vacuum Head. Horizontal and vertical location and subsequent rigidity were critical factors in obtaining accurate and reproducible results with the balance, and these locations

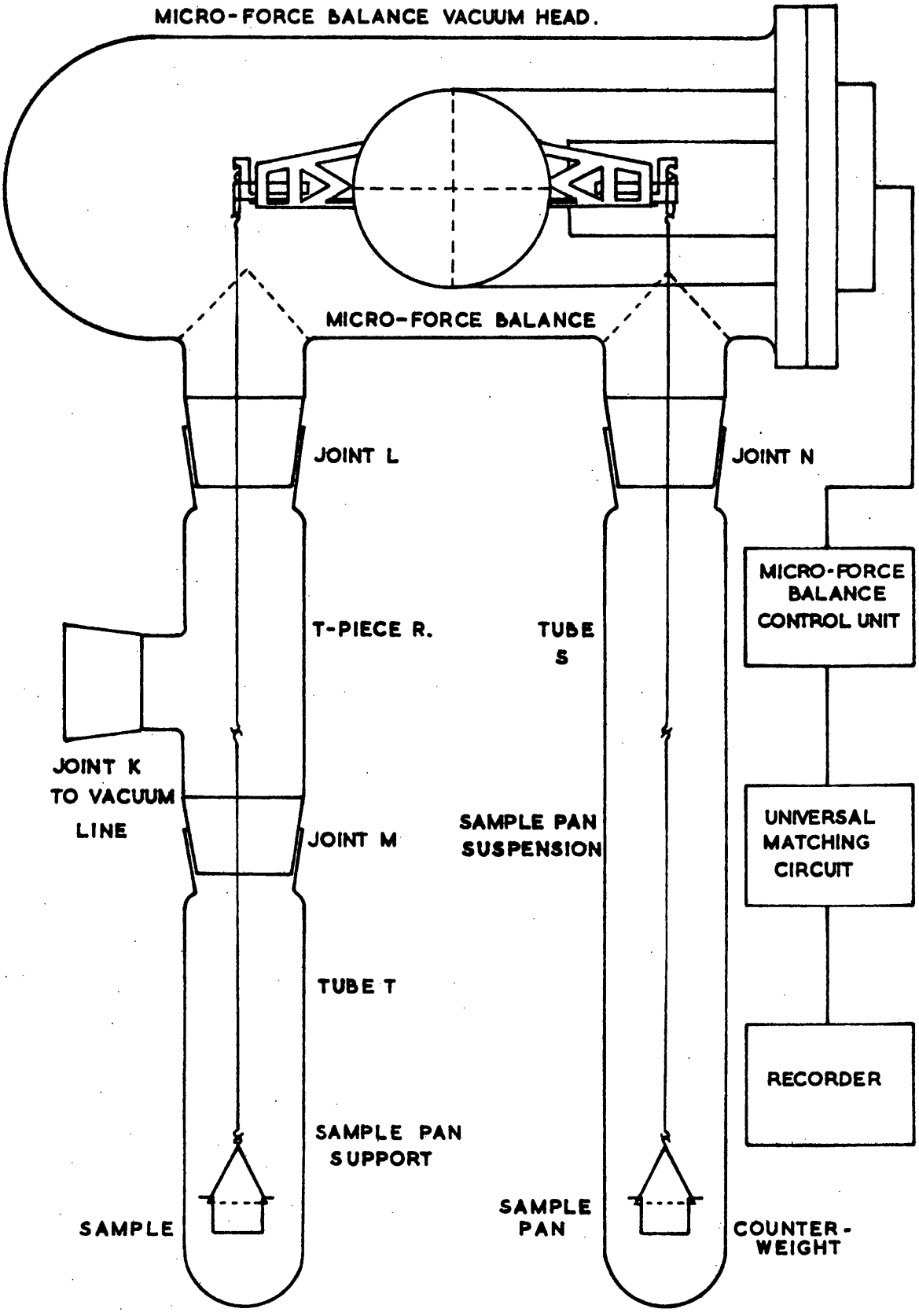


FIGURE 9. MICRO-FORCE BALANCE, FITTED IN A VACUUM HEAD

were checked at the beginning of each occasion that the balance was used and also at intervals during its use. The linked suspension rods were hung from each end of the balance arm, and to these were attached the sample pan supports and aluminium sample pans. Wide bore "Pyrex" glass tubes S and T fitted with ground glass sockets were fixed in position around the sample pans and attached to the Vacuum Head at joints N and M respectively. The Vacuum Head was isolated from the main body of the vacuum apparatus by means of tap J which had, fitted inside the bore of the tap, a glass construction of about 2 mm. internal diameter, such that the outlet diameter from the Vacuum Head was comparable in size to that from the sample vessels on Manifold M. With tap A open and the carbon dioxide/acetone trap G and the liquid nitrogen trap T in position, the rotary pump and the mercury diffusion pump were switched on and tap J was opened to enable the assembled Micro-Force Balance, and the Vacuum Head assembly to out-gas. When out-gassing was completed the balance was connected to the Control Cabinet, and the main supply to the cabinet switched on.

An external potentiometric recorder (Smiths Servoscribe) was used in conjunction with the Control Cabinet to give a permanent record of weighings and also to increase the accuracy of the measurements. The recorder was connected to the cabinet by way of a C.I. Universal Matching Circuit. This Circuit, in addition to enabling the balance to be used with a range of recorders, had provision for variable damping, and also for scale expansion by means of a five position Ratio Control. In operation, it was found that the 0-10 mV input range of the recorder gave the best reproducibility, and this range was used throughout the experiments reported in this section.

Zero adjustment - the balance unit was switched on and after a thirty minute "warming up" period the zero was adjusted in the manner recommended by the manufacturers, using counterweights in the place of the Coarse and Medium Controls on the Control Cabinet (see page 78) Zero adjustment was carried out with the balance open to the main body of the vacuum apparatus, with the pumps on and at a pressure of less than 10^{-5} torr as indicated by the McLeod Gauge. The apparatus and balance were operated for a period of 24 hours non-stop and the zero was checked at regular intervals. It was found that the zero did not change over the 24 hour drying period. Furthermore, it was shown that the zero remained stable when the sample tubes S and T were maintained at temperatures between 273°K and 338°K and drying was continued for periods of 6 hours, 12 hours and 24 hours.

Calibration - the five balance ranges were calibrated according to the manufacturers instructions. Calibration was carried out with the balance at a reduced pressure of 10^{-5} torr, and was found to be unaffected by periods of drying, at this pressure of 12 hours and 24 hours, with the sample tubes S and T maintained at temperatures between 273°K and 338°K and was also unaffected by admission of dry oxygen to the balance (see page 56) after drying. The calibration of the balance was checked at intervals throughout its use.

Operation - the Micro-Force Balance and Vacuum Head were maintained under vacuum, and only opened to the air for the minimum time necessary for introduction of the sample, or for calibration. The use of mechanical counterweights in place of the Coarse and Medium controls on the Control Cabinet during the zero adjustment process, enabled these controls to be used as an electrical tare, during experiments, to either maintain a positive needle reading, or for backing off purposes to enable extensive use to be made of the lower

balance ranges.

The required volume of spore suspension was pipetted into a sterile sample cup, and this was placed on the left hand support of the balance. A counterweight sample cup was placed on the left hand support of the balance. A counterweight sample cup was placed on the right hand support. Tubes S and T were fitted around the cups and attached to the Vacuum Head, and a constant temperature bath was positioned around them. The sample on the Micro-Force Balance was dried in conjunction with the requisite number of samples on the manifold of the vacuum apparatus. The procedure for drying the samples, and for subsequent re-equilibration and exposure to controlled gaseous environments is described in detail on page 52.

Preliminary Experiments

Determination of the Reproducibility of the Micro-Force Balance Technique for the Measurement of Weight Changes in Spore Samples During Drying

On three separate occasions the micro-force balance was operated during a normal drying at 308°K. Samples were dried for 24 hours and the change in weight of the spores was continuously recorded throughout the drying period. To obtain the most meaningful data, full use was made of the ranging facilities of the balance, thus enabling very slow drying rates of the order of 2×10^{-4} mg. min^{-1} to be detected. Because of the length of the recorded sample weight/drying time profiles it is not possible to illustrate a complete profile. Instead, a condensed version of a typical sample weight/drying time profile obtained during these experiments is shown in Figure 10.

It can be seen from Figure 10 that the sample loses weight at a slow steady rate in the five minutes during which it equilibrates to the temperature of the surrounding water bath. This is considered to be exaporative drying induced by the environment around the sample, and does not involve the high vacuum drying process. During the two to three minutes immediately following the opening of the balance to the main body of the vacuum apparatus, the loss of weight in the sample occurs more rapidly and at an uneven rate. The unevenness of drying at this stage is presumed to be the result of the pressure changes that take place during the initial stages of drying. After approximately three minutes exposure to the reduced pressure there occurs a very rapid drop in weight of the order of 6 mg. equivalent to a decrease of approximately 10% of the original sample weight. The sample then dries at a rate which shows little change until a

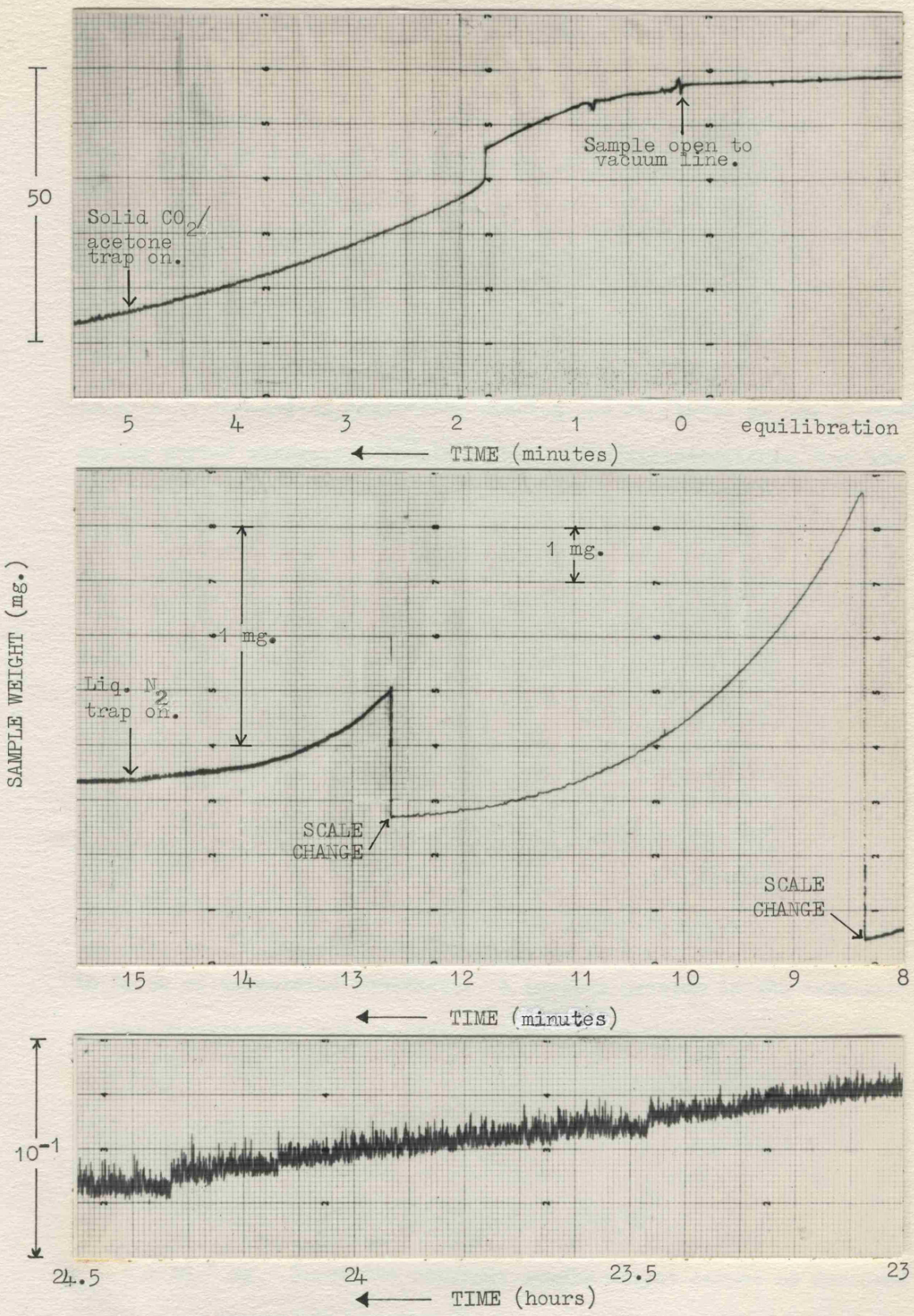


Figure 10. Condensed portions of a sample weight/drying time profile obtained with a sample of Bacillus megaterium spores during drying at 308°K.

weight of approximately 6 mg. is attained. This "steady rate" period is unaffected by the introduction of the solid carbon dioxide/acetone trap or the liquid nitrogen trap into the drying apparatus, or by the operation of the mercury diffusion pump. During the subsequent stages of the drying process the rate of weight loss decreases until the sample weight is approximately 3 mg. This point corresponds to an interval of about 40 minutes drying from the time of opening of the balance to the drying apparatus. During the remainder of the 24 hours drying period changes in sample weight are still detectable, but are extremely small.

The three profiles obtained in these experiments, under the standardised drying conditions employed, are superimposable and indicate a high degree of reproducibility, sufficient to enable the micro-force balance technique to be used to study the weight changes that occur under other defined drying conditions.

Treatment of Results

The sample weight/drying time profiles do not lend themselves to simple mathematical description, and cannot therefore be characterised in terms of calculated constants. A further problem in the treatment of the experimental data arises as a result of the basic design of the micro-force balance used in these experiments. On the highest range (range 1) weights up to 100 mg. can be measured on the recorder output from the balance to within $\pm$ 5 x 10^{-1} mg., whereas on the lowest range used (range 4) weights up to 2.5 x 10^{-1} mg. can be detected to within $\pm$ 2.5 x 10^{-3} mg. Since the original sample weight cannot be measured to the same degree of accuracy as the final sample weight after drying it is not possible to determine accurately the residual moisture content of the dried spore samples, or to calculate the decrease in the moisture

content of the samples during the drying process. For the same reason the accepted method of expressing drying curves in terms of drying rate as a function of moisture content, cannot be applied to the data from these experiments.

For comparative purposes it was therefore decided to express the sample weight/drying time profiles in the form of drying rate/drying time curves. This treatment has been used by Suzuki et al (175) to analyse data obtained with freeze-dried BCG suspensions. Drying rates, expressed in $mg.min^{-1}$, were determined from tangents drawn to the sample weight/drying time profiles at defined time intervals. The rates derived from the profiles obtained in the preliminary experiments are given in Table 8 , and the corresponding drying rate/drying time curves are illustrated in Figure 11.

The equality of the curves obtained for samples dried under identical drying conditions (24 hours at $308^{o}K$), on the three separate occasions confirms the suitability of the micro-force balance technique for use in further experiments.

TABLE 8

Drying rate as a function of the period of drying for triplicate samples of spore suspension dried at 308°K.

Time of Exposure to Reduced Pressure (minutes)	Drying Rate ($mg.min^{-1}$)		
	Sample A	Sample B	Sample C
After Equilibration	4.0×10^{-1}	4.2×10^{-1}	4.0×10^{-1}
1	2.38	2.4	2.36
3	2.6	2.585	2.525
5	2.714	2.667	2.591
10	2.455	2.412	2.444
15	2.286	2.286	2.3
18	2.133	2.133	2.125
19	1.933	2.051	1.799
20	5.125×10^{-1}	1.087	5.0×10^{-1}
20.5	2.542×10^{-1}	2.875×10^{-1}	2.25×10^{-1}
21	1.542×10^{-1}	1.333×10^{-1}	8.611×10^{-2}
22	4.688×10^{-2}	4.792×10^{-2}	2.104×10^{-2}
25	4.417×10^{-3}	4.845×10^{-2}	4.011×10^{-3}
29	1.165×10^{-3}	1.205×10^{-3}	1.05×10^{-3}
35	5.812×10^{-4}	6.117×10^{-4}	6.314×10^{-4}
40	5.25×10^{-4}	5.05×10^{-4}	4.752×10^{-4}
45	4.2×10^{-4}	3.815×10^{-4}	4.115×10^{-4}
55	3.415×10^{-4}	3.187×10^{-4}	3.05×10^{-4}
65	3.111×10^{-4}	2.876×10^{-4}	2.971×10^{-4}
125	2.75×10^{-4}	2.651×10^{-4}	2.615×10^{-4}
245	1.901×10^{-4}	1.846×10^{-4}	1.825×10^{-4}
365	1.615×10^{-4}	1.56×10^{-4}	1.665×10^{-4}

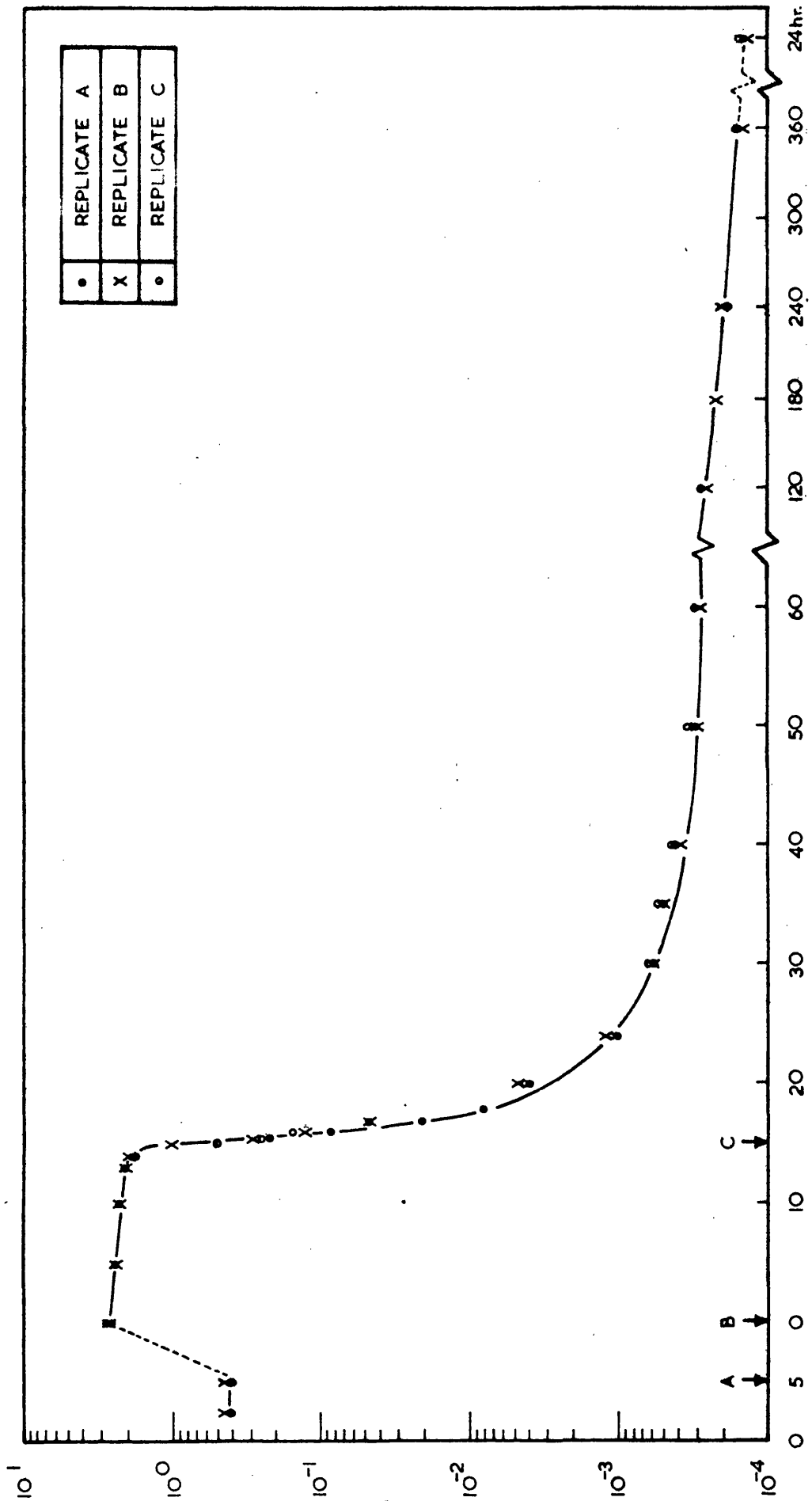


Figure 11. Plot of" drying rate" against "time" for replicate samples of Bacillus megaterium spores, dried at 308°K. A is the end of the equilibration stage, and the point at which samples are opened to the vacuum apparatus. B is the time of introduction of the solid CO_2 / acetone cold trap and is the point from which the drying process is timed. C is the time of introduction of the liquid nitrogen cold trap and the start of diffusion pumping.

Subsequent Experiments

Measurement of Weight Changes in Spore Samples During Drying at Different Temperatures

The drying temperatures investigated were 273°K, 288°K, 298°K, 308°K, 323°K and 338°K. Sample weight/drying time profiles were recorded over a period of 24 hours drying at each of the chosen temperatures. The profiles obtained at all drying temperatures are similar in shape to those obtained at 308°K in the preliminary experiments. The drying rate/drying time curves derived from these profiles are illustrated in Figure 12 . At all drying temperatures except 273°K, these curves follow a similar pattern. Each curve exhibits an initial "steady" drying rate stage during the five minute period in which the sample equilibrates to the temperature of the surrounding water bath. Since the sample temperature is changing during this part of the drying period, initial increases in drying rate would also be anticipated. However, the balance was not sufficiently sensitive in this range to detect such increases, if they did in fact exist. The drying rate at the end of the equilibration stage i.e. when the sample is at constant temperature is given in Table 9 , for each of the drying temperatures investigated. The dependency of this rate upon the temperature of the surrounding water bath is shown by the plot of log. drying rate against reciprocal of drying temperature which is linear with negative slope (Figure 13)

In theory the activation energy (see page 150)calculated from the slope of this plot should be equivalent to the heat of vaporisation of water if the decrease in weight is the result of removal of water from the sample by evaporation. The activation energy claculated from the data in Table 9 is 1.107×10^4 cal. $mole^{-1}$ $°K^{-1}$, compared with the

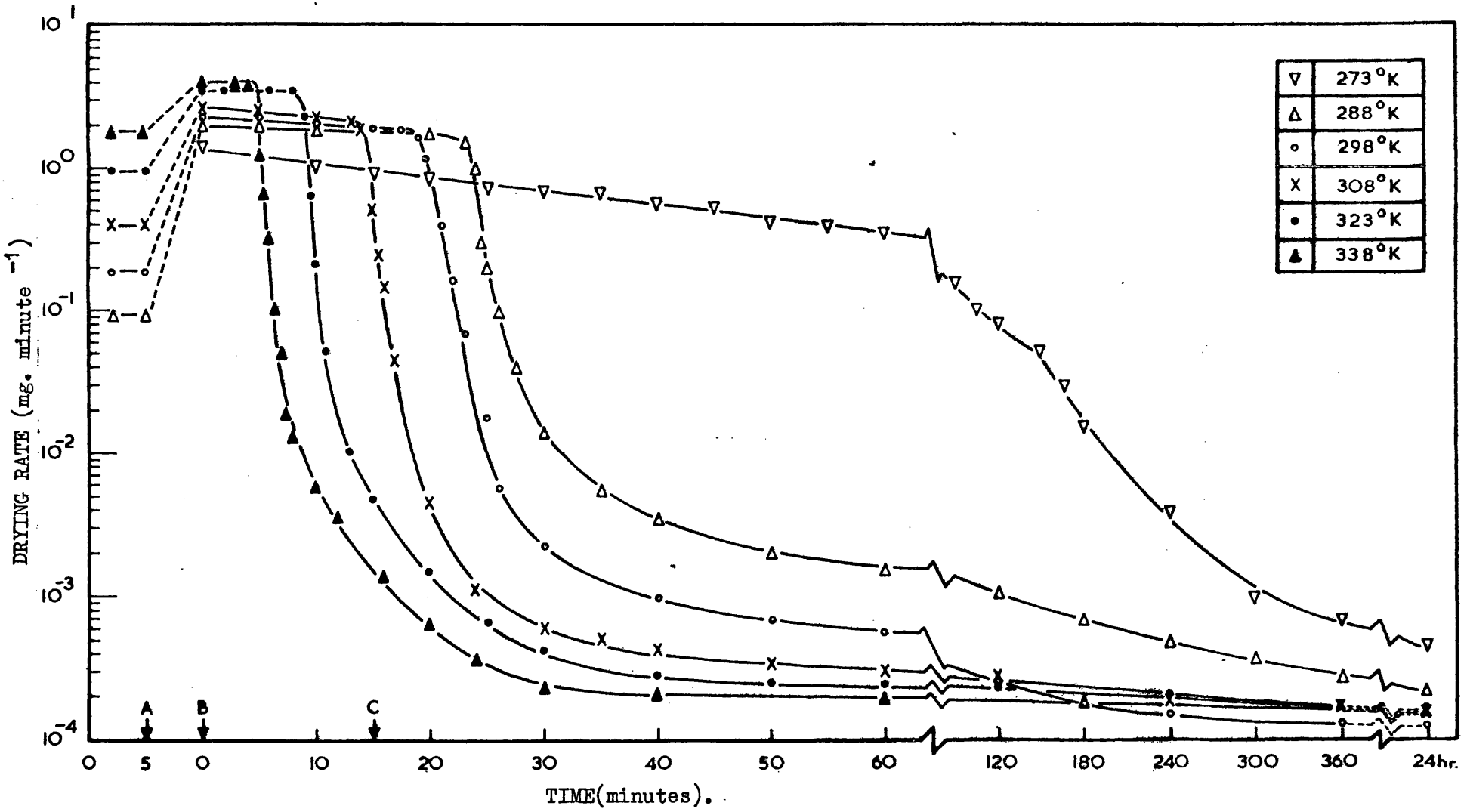


Figure 12. Plot of "drying rate" against "time" for samples of Bacillus megaterium spores, dried at different temperatures. A is the end of the equilibration stage, and the point at which samples are opened to the vacuum apparatus. B is the time of introduction of the solid CO_2 / acetone cold trap and is the point from which the drying process is timed. C is the time of introduction of the liquid nitrogen cold trap and the start of diffusion pumping.

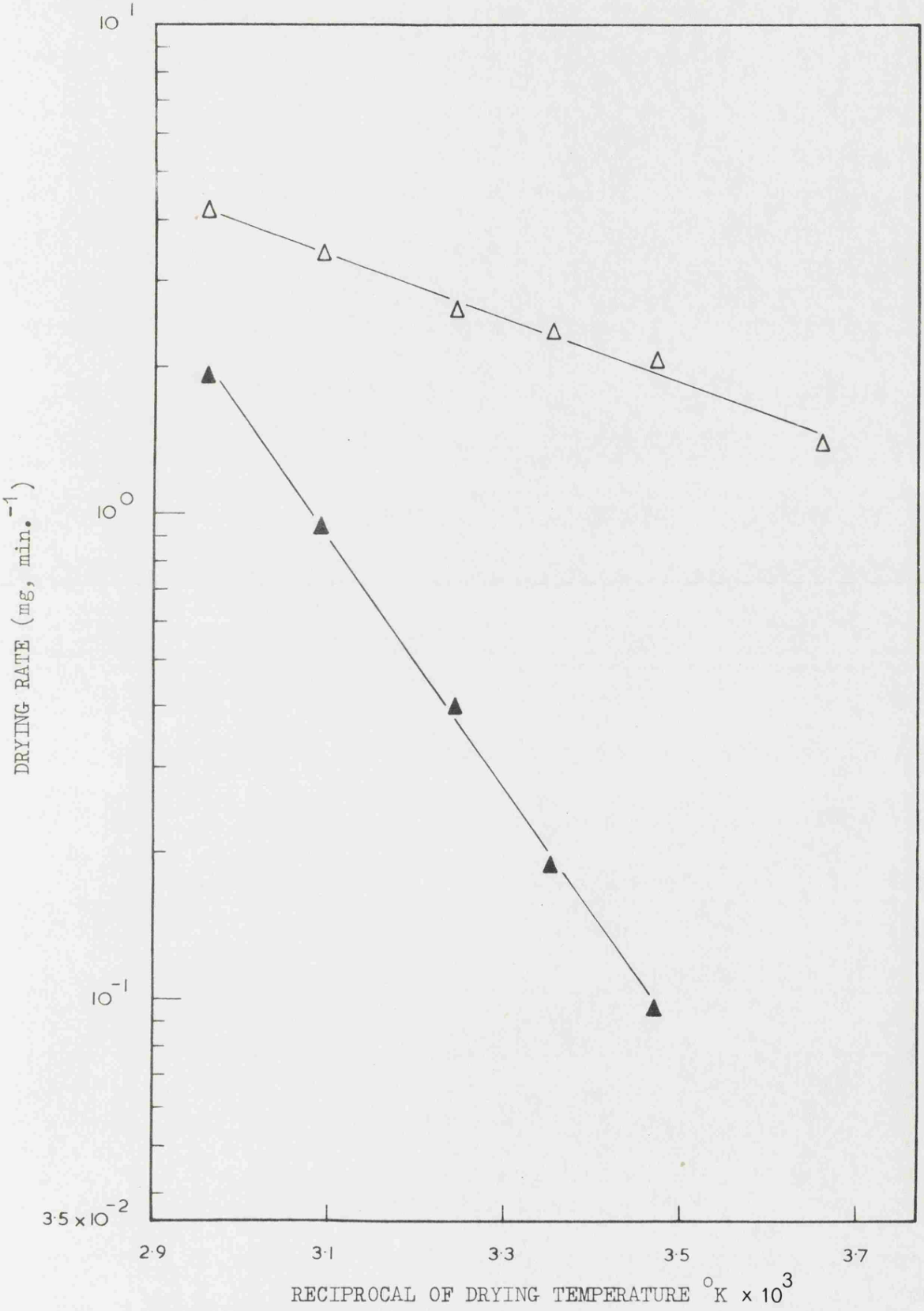


Figure 13. Plot of "drying rate" on a log. scale against the "reciprocal of the drying temperature" for Bacillus megaterium spores, at the end of the equilibration stage (▲), and at the point of introduction of the solid carbon dioxide/acetone cold trap into the system (Δ).

TABLE 9

Rates of drying of samples of spore suspension dried at different temperatures a) after five minutes equilibration to the temperature of the surrounding bath and b) five minutes after exposure to reduced pressure.

Drying Temperature (^{o}K)	Drying Rate ($mg.min^{-1}$)	
	After 5 mins. Equilibration to Temp.	After 5 mins. Exposure to Reduced Pressure
273	Not Measurable	1.41
288	9.588×10^{-2}	2.05
298	1.91×10^{-1}	2.35
308	4×10^{-1}	2.591
323	9.52×10^{-1}	3.432
338	1.88	4.16

value for the heat of vaporisation of pure water of 1.05×10^{4} cal. $mole^{-1}\ {}^{o}K^{-1}$ (176,177), indicating that the weight loss that occurs during this initial equilibration stage is almost certainly the result of simple evaporation of water from the sample.

When the samples are opened to the vacuum apparatus the drying rate is immediately increased. The drying period is timed from the point at which the solid carbon dioxide/acetone cold trap is introduced into the vacuum system i.e. 5 minutes after the taps to the micro-force balance and the manifold are opened to the main body of the vacuum apparatus (see page 55). At this point the drying rate is at its maximum, and the value of this rate at each drying temperature is given in Table 9 . These drying rates are again directly related to the

drying temperatures as shown by the linear plot of log. drying rate against reciprocal of drying temperature illustrated in Figure 13 The energy associated with drying (under the influence of reduced pressure) is reduced and at this point is 2.97×10^{3} cal.mole^{-1} $^{o}K^{-1}$, compared with the value during equilibration, when the samples are in air of 1.107×10^{4} cal.mole^{-1} $^{o}K^{-1}$.

The drying rate remains relatively unchanged for a defined period after which it falls rapidly until it again becomes constant. The final drying rate, athigh drying temperatures, is so slow that its precise measurement is not possible.

The time interval, from the introduction of the solid carbon dioxide/acetone cold trap into the drying system, during which the fast rate of drying is maintained is also a function of the reciprocal of the drying temperature, the relationship again being linear as illustrated in Figure 14.

The drying time required to attain the final (slowest) constant drying rate cannot be accurately determined, but it can be seen from Figure 12 that this time is a function of the drying temperature, being shortest at the highest temperature investigated ($338^{o}K$). It is also of interest that at drying temperatures above $298^{o}K$ there is no measurable change in the drying rate after 6 hours drying while at $288^{o}K$ changes in drying rate can still be detected after 24 hours drying.

In samples dried at $273^{o}K$ the rate of drying during initial equilibration is so slow that it cannot be detected with the technique employed. Furthermore, in contrast to the results obtained at higher drying temperatures, a period of constant high drying rate is not observed in samples dried at $273^{o}K$. At this temperature the drying rate is highest immediately after the samples are opened to the vacuum

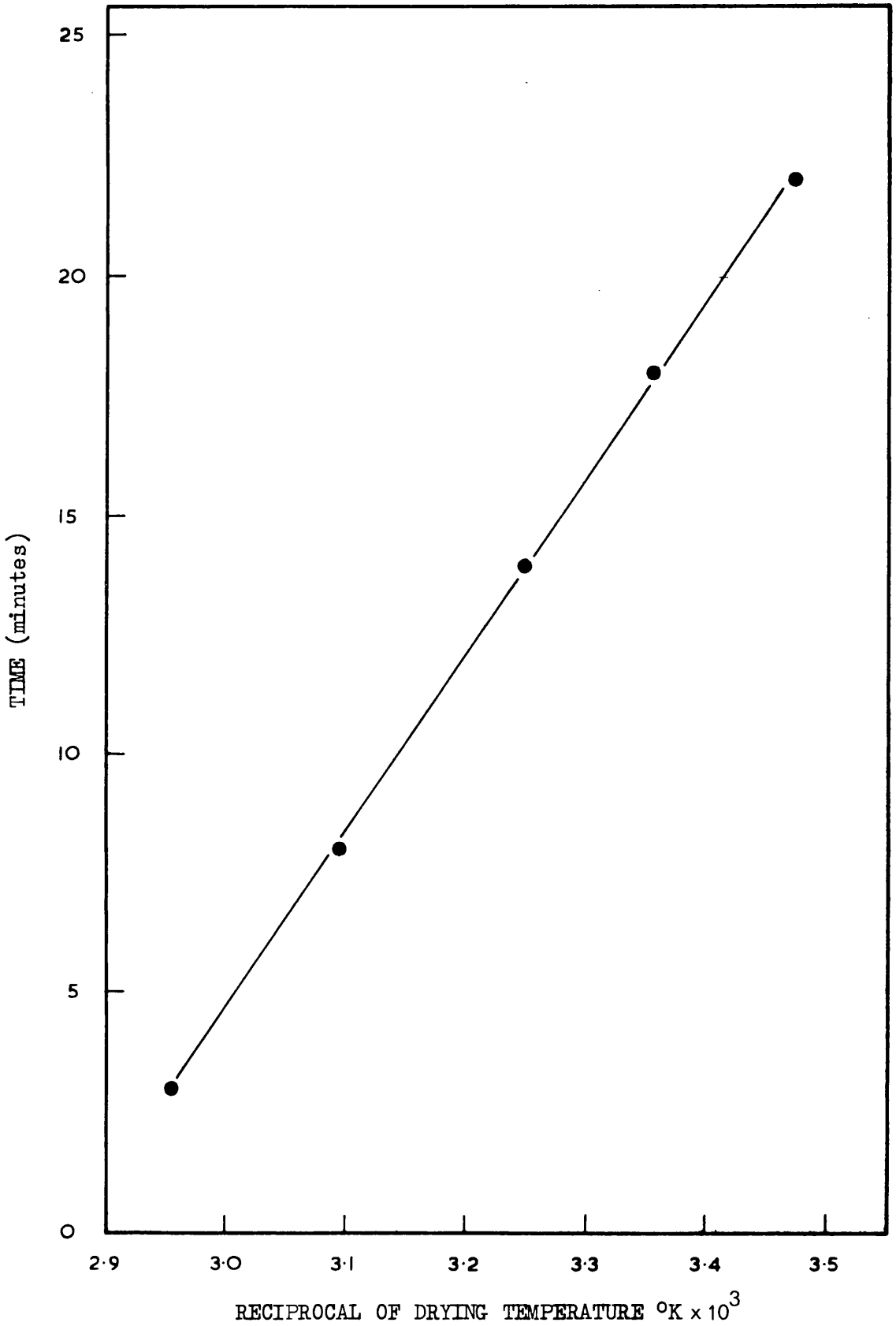


Figure 14. Plot of the time interval, from the introduction of the solid carbon dioxide/acetone cold trap, during which the fast drying rate is maintained, against the "reciprocal of the drying temperature" for Bacillus megaterium spores.

apparatus and decreases steadily over a subsequent drying period of approximately 2.5 hours. After this drying interval a decrease in rate is observed. This decrease is, however, much less than that observed at higher drying temperatures, and after 24 hours exposure a relatively high rate of drying of 4×10^{-4} mg.min^{-1} is still observed.

Although it is not possible to calculate the percentage weight loss of the sample if the lower ranges of the micro-force balance are employed, it is possible to estimate these losses if measurements are confined to the higher ranges of the instrument i.e. range 1, 0-100 mg. and range 2, 0-10 mg. Estimates can therefore be made of the percentage loss of weight of the sample during the initial stages of the drying process. The weight loss of samples and the percentage weight loss at a) the termination of the equilibration stage, b) the point of introduction of the solid carbon dioxide/acetone cold trap to the drying system, and c) the end of the constant high drying rate period is given in Table 10 for spores dried at different temperatures.

The weights recorded in Table 10 represent the means of values taken from a number of sample weight/drying time profiles recorded at each drying temperature. The mean weight of the 0.06 ml. sample of spore suspension was calculated from the experimental data as 59.98 mg. and this was used as the 100% value in calculating the percentage weight losses recorded in Table 10. The percentage weight loss data is illustrated graphically as a function of the reciprocal of the drying temperature in Figure 15.

It can be seen from Figure 15 that the log. percentage weight loss after the equilibration period, and five minutes after the samples are opened to the vacuum vessel (i.e. the point from which the drying is timed) exhibit a direct relationship with the reciprocal of the

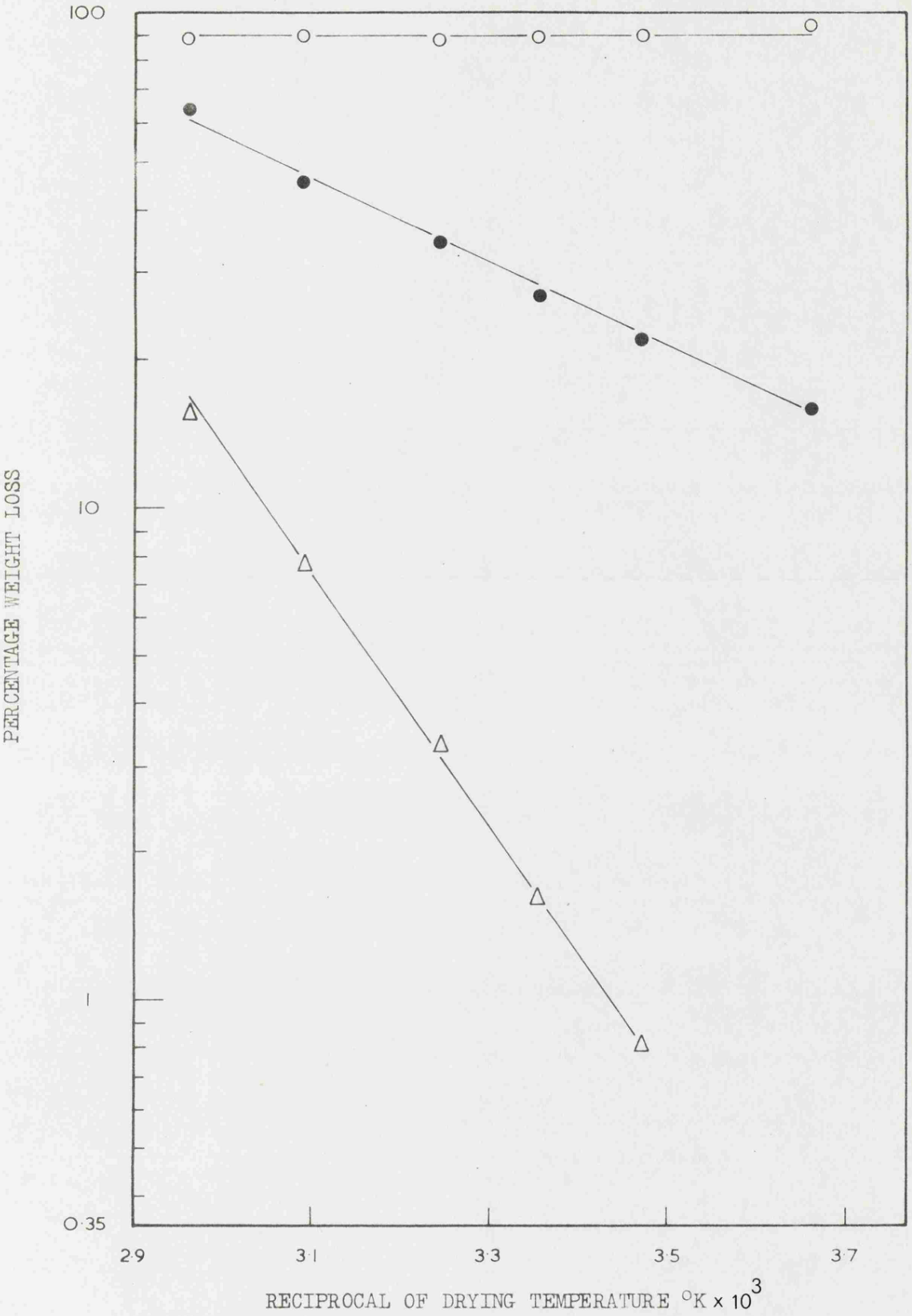


Figure 15. Plot of "percentage weight loss" on a log. scale against the "reciprocal of the drying temperature" for Bacillus megaterium spores at the termination of the equilibration stage (Δ), at the point of introduction of the solid carbon dioxide/acetone cold trap (●), and at the end of the constant high drying rate period (o).

of the drying temperature. In contrast, the percentage weight loss at the end of the constant high drying rate period is independent of the drying temperature over the range 288°K to 338°K and is relatively constant at 89.7% ± 0.9%, although the time taken to achieve this is directly proportional to the temperature (Fig. 14).

TABLE 10

Weight loss and percentage weight loss of spore samples at defined time intervals during the initial stages of drying at different temperatures.

Drying Time (°K)	Termination of Equilibration		Introduction of Solid Carbon Dioxide/Acetone Cold Trap		End of Constant High Drying Rate Period	
	Weight Loss (mg.)	% Weight Loss	Weight Loss (mg.)	% Weight Loss	Weight Loss (mg.)	% Weight Loss
273	-	-	9.62	16.04	56.6	94.36
288	0.48	0.8	13.32	22.21	54.17	90.57
298	0.95	1.58	16.3	27.18	54.32	90.32
308	2.0	3.33	20.82	34.71	53.63	89.21
323	4.66	7.77	27.42	45.72	53.84	89.76
338	9.4	15.67	38.68	64.49	53.24	88.76

Measurement of Weight Changes in Dried Spore Samples after Re-equilibration to Defined Aqueous Vapour Pressures

The aqueous vapour pressures investigated were 5×10^{-4} torr, 2×10^{-3} torr, 5×10^{-2} torr, 1 torr, and 10 torr. The composition of the constant temperature baths employed to attain 5×10^{-2} torr and 10 torr are described on page 56. Constant temperature baths containing solid carbon dioxide/acetone mixture at 195°K, melting 2-chloroethanol at 204°K and ammonium nitrate/ice mixture at 257°K were employed to obtain the re-equilibrium aqueous vapour pressures of 5×10^{-4} torr, 2×10^{-3} torr and 1 torr respectively.

On different occasions samples were dried for 6 hours and 24 hours at 273°K, 288°K, 298°K, 308°K, 323°K, and 338°K, and for 12 hours at 298°K, 308°K, and 338°K. At the termination of the drying period the samples were left to re-equilibrate with the water vapour at the lowest aqueous vapour pressure for a period of one hour, and the change in weight of the sample during this period was recorded. At the end of the re-equilibration period the taps to the manifold and the micro-force balance were closed and the constant temperature bath was changed. The taps were re-opened and the samples were exposed to the higher aqueous vapour pressure for a further period of one hour. The procedure was repeated for each stepwise increase in re-equilibration aqueous vapour pressure, and the weight changes in the sample during each re-equilibration were recorded. The sample weight/re-equilibration time profiles obtained with spores dried for 6 hours at 298°K are illustrated in Figure 16.

In all experiments, no change in sample weight was detected after re-equilibration of the dried spores to 5×10^{-4} torr. With the other re-equilibration aqueous vapour pressures studied the sample weight/re-equilibration time profiles follow a similar pattern. A rapid

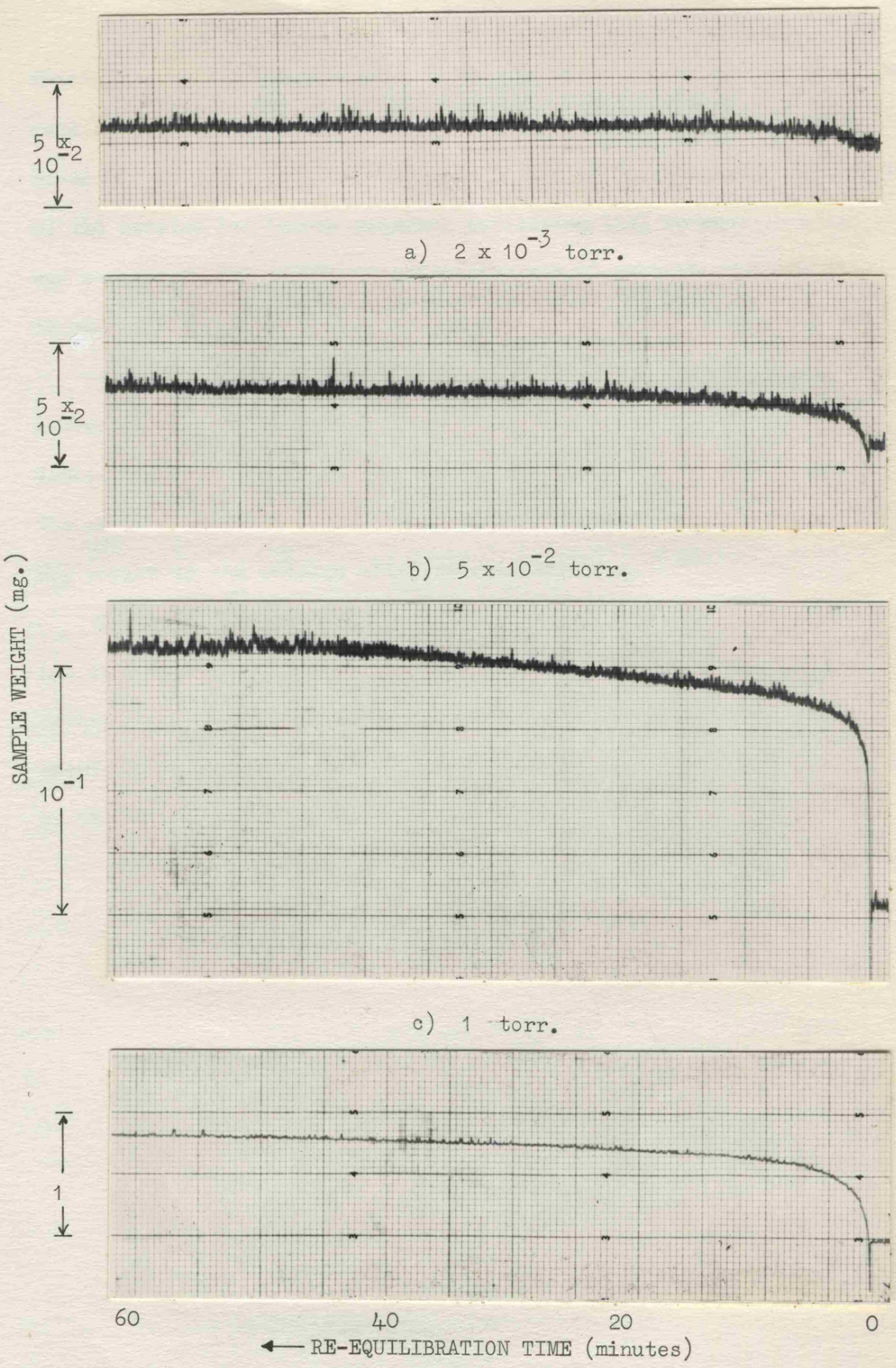


Figure 16. Sample weight/ re-equilibration time profiles obtained with samples of Bacillus megaterium spores dried for 6 hours at 298°K, and re-equilibrated to different aqueous vapour pressures.

initial increase in sample weight is followed by a levelling off in the rate of weight increase up to a constant value. After 60 minutes exposure to water vapour at the chosen aqueous vapour pressure the weight of the samples had become constant indicating that re-equilibration was complete. The sample weight at the end of each re-equilibration is shown in Table 11 for spores dried under different conditions.

It can be seen from Table 11 that the increase in weight of the spores on re-equilibration to a defined aqueous vapour pressure is independent of the drying treatment to which the spores are subjected. The weight increases were also shown to be unaffected by admission of dry oxygen to the samples after re-equilibration.

Re-equilibration of spores dried to different weight levels did not therefore increase the sample weight to a defined level, but rather resulted in the addition of a specific weight of water to the sample, irrespective of the dry weight of the spores. The significance of this finding is discussed fully on page 256.

TABLE 11

Weight increases in samples of spore suspension dried under different conditions, after re-equilibration to defined aqueous vapour pressures.

Drying Temperature (°K)	Drying Time (Hours)	Sample Weight Increase (mg)			
		2×10^{-3} torr	5×10^{-2} torr	1 torr	10 torr
273	6	4.96×10^{-3}	2.18×10^{-2}	1.26×10^{-1}	4.15×10^{-1}
	24	5.4×10^{-3}	2.15×10^{-2}	1.18×10^{-1}	4.2×10^{-1}
288	6	4.7×10^{-3}	2.23×10^{-2}	1.19×10^{-1}	4.53×10^{-1}
	24	4.76×10^{-3}	2.26×10^{-2}	1.24×10^{-1}	4.38×10^{-1}
298	6	5.24×10^{-3}	2.0×10^{-2}	1.31×10^{-1}	3.98×10^{-1}
	12	5.02×10^{-3}	2.13×10^{-2}	1.21×10^{-1}	3.8×10^{-1}
	24	4.76×10^{-3}	1.95×10^{-2}	1.15×10^{-1}	4.43×10^{-1}
308	6	5.35×10^{-3}	$2.\times10^{-2}$	1.26×10^{-1}	4.25×10^{-1}
	12	4.73×10^{-3}	2.23×10^{-2}	1.27×10^{-1}	3.88×10^{-1}
	24	4.95×10^{-3}	2.12×10^{-2}	1.16×10^{-1}	4.33×10^{-1}
323	6	5.25×10^{-3}	2.26×10^{-2}	1.12×10^{-1}	4.0×10^{-1}
	24	4.93×10^{-3}	$2.\times10^{-2}$	1.23×10^{-1}	3.78×10^{-1}
338	6	4.8×10^{-3}	$2.\times10^{-2}$	1.1×10^{-1}	3.94×10^{-1}
	12	4.72×10^{-3}	1.95×10^{-2}	1.15×10^{-1}	4.35×10^{-1}
	24	4.85×10^{-3}	2.25×10^{-2}	1.29×10^{-1}	3.82×10^{-1}
	MEAN	4.961×10^{-3}	2.121×10^{-2}	1.208×10^{-1}	4.121×10^{-1}
	Coefficient of Variation	5.22%	6.14%	5.71%	5.42%

THE EFFECT OF DIFFERENT DRYING TREATMENTS ON THE VIABILITY OF BACTERIAL SPORES

Introduction

Preliminary experiments have shown that 0.06 ml. samples of spore suspension SS6S can be dried for 6 hours at 298°K with no significant loss in viability, and that the difference between the total number of spores in dried and undried samples can be attributed to normal sampling error. This present series of experiments was designed to determine whether different, more extreme, drying conditions would affect the viability of spores.

Previous work in this Department has shown that the heat resistance of Bacillus megaterium spores varies with the aqueous vapour pressure to which the spores are re-equilibrated, a peak sensitivity being shown at 2×10^{-3} torr, and a peak resistance at between 10 and 22 torr. It was also shown that at all water levels heat sensitivity was increased in the presence of oxygen. It was considered that the damage induced by high vacuum drying would also become most apparent in the presence of oxygen, and that the effect of water on this damage would be greatest at a re-equilibrium aqueous vapour pressure of 10 torr. Thus, in the present series of experiments, the modifying effect of re-equilibration to 10 torr aqueous vapour pressure, and sealing in the presence of oxygen on the recovery and viability of spores dried under various conditions was investigated.

Treatment of Results

Throughout this thesis, statistical analysis of spore viability data, has been carried out on logarithmically transformed data, and it is pertinent at this stage to give some justification for the use of the transformation procedure.

In studies of the resistance of dried spores to heat and radiation

a logarithmic transformation of colony counts has been used to convert 'treatment' survival data to a linear regression (see pages 144 and 188). The regression analysis, in the form presented, uses an effective weighting factor equal to one, and requires that the natural logarithms of the colony counts should be normally distributed, with variance independent of the magnitude of the 'treatment', the dilution factor and the colony count. Dickinson (150) in analysing radiation dose:survival data for Bacillus megaterium spores has verified that, under clearly defined and standardised experimental conditions, the magnitude of the "between sample variance" detected at all dose levels was not governed by the magnitude of the radiation dose or the concomitant survival level when the natural logarithms of the colony counts were considered.

In the experiments described in this thesis, the manipulative techniques and mathematical treatments employed were essentially the same as those analysed by Dickinson, and it was therefore assumed that under these experimental conditions, the logarithms of the colony counts would be normally distributed at each defined treatment level. It was further considered, that for spores surviving defined drying procedures, the logarithms of both the colony counts and the total counts would also be normally distributed at each level of drying treatment. Thus, since logarithmic transformation of data was required for the routine treatment of results obtained in experiments on radiation and heat resistance, it was decided that, for the purpose of maintaining continuity, statistical analysis of viability data obtained with dried spore samples, where required, would be performed on the logarithmically transformed data.

The Effect of Drying at Different Temperatures with and without Subsequent Re-equilibration to 10 torr Aqueous Vapour Pressure on the Recovery and Viability of Bacillus megaterium Spores

The drying temperatures investigated were 273°K, 288°K, 298°K, 308°K, 323°K amd 338°K. Ten samples of spore suspension were dried for 6 hours at each of the six temperatures. At the termination of the drying process half of the samples were sealed in anoxia and removed from the manifold. The remaining samples were re-equilibrated to an aqueous vapour pressure of 10 torr, prior to sealing in anoxia. The sample vessels were then opened five minutes after sealing and the contained spore samples resuspended in 100 times their volume of sterile water. Total and viable counts were determined for each resuspended sample, and the percentage of viable spores in each was calculated. The experimental data obtained for unequilibrated spore samples and for samples re-equilibrated to 10 torr aqueous vapour pressure are recorded in Tables 12, 13, and 14.

A test for equality performed on the log. total counts from these experiments gave a value of B of 5.49 compared with the tabulated value at P = 0.05 of 19.67 indicating that the experimental values could not be considered to have been drawn from different populations. The quantitative recovery of the spores was not therefore influenced by the type of drying treatment to which they were subjected. However, when the log. percentage viability data was subjected to a similar analysis the value of B obtained was 21.56 indicating that the different drying treatments employed were having a significant effect on the viability of the dried spores. In order to assess which of the experimental factors present was having the greatest effect on spore viability, the data were subjected to a full Analysis of Variance. The groups of five replicates each give an estimate of the error variance, and since these estimates are not significantly different from one another it was considered that

TABLE 12

Viable and total counts for quintuplicate samples of spore suspension SS6S dried for 6 hours at 273°K and 288°K, with and without re-equilibration to 10 torr aqueous vapour pressure prior to sealing in anoxia.

Sample Number	Total Count/ml. SS6S x 10^{10}	Viable Count/ml. SS6S x 10^{10}	Percentage Viability
Dried at 273°K, unequilibrated			
1	6.776	4.38	64.64
2	6.904	4.2	60.83
3	7.655	4.327	56.53
4	7.045	4.273	60.65
5	7.255	4.353	60.0
Mean	7.127	4.307	60.43
Dried at 273°K, re-equilibrated to 10 torr			
1	6.706	4.52	67.4
2	7.612	4.38	57.54
3	7.046	4.447	63.11
4	7.005	4.32	61.67
5	7.408	4.32	58.32
Mean	7.155	4.387	61.31
Dried at 288°K, unequilibrated			
1	6.804	4.287	63.01
2	7.577	4.14	54.64
3	7.094	4.42	62.31
4	7.203	4.233	58.77
5	7.606	4.32	56.8
Mean	7.257	4.28	58.98
Dried at 288°K, re-equilibrated to 10 torr			
1	7.596	4.12	54.24
2	7.695	4.127	53.63
3	7.014	4.127	58.84
4	7.306	4.14	56.67
5	6.858	4.307	62.8
Mean	7.294	4.164	57.09

TABLE 13

Viable and total counts for quintuplicate samples of spore suspension SS6S dried for 6 hours at 298°K and 308°K, with and without re-equilibration to 10 torr aqueous vapour pressure prior to sealing in anoxia.

Sample Number	Total Count/ml. SS6S x 10^{10}	Viable Count/ml. SS6S x 10^{10}	Percentage Viability
Dried at 298°K, unequilibrated			
1	6.842	4.42	64.4
2	7.006	4.207	60.05
3	7.312	4.373	59.81
4	6.648	4.513	67.89
5	7.356	4.253	57.82
Mean	7.033	4.353	61.89
Dried at 298°K, re-equilibrated to 10 torr			
1	6.654	4.34	65.22
2	6.924	4.193	60.56
3	6.956	4.273	61.43
4	7.441	4.267	57.34
5	7.428	4.293	57.79
Mean	7.081	4.276	60.39
Dried at 308°K, unequilibrated			
1	6.645	4.307	64.82
2	6.704	4.313	64.33
3	7.004	4.38	62.54
4	7.254	4.433	61.11
5	7.465	4.28	57.33
Mean	7.014	4.343	61.92
Dried at 308°K, re-equilibrated to 10 torr			
1	7.015	4.32	61.58
2	6.851	4.34	63.35
3	7.365	4.2	57.03
4	7.295	4.467	61.23
5	7.811	4.453	57.01
Mean	7.267	4.356	59.94

TABLE 14

Viable and total counts for quintuplicate samples of spore suspension SS6S dried for 6 hours at 323°K and 338°K, with and without re-equilibration to 10 torr aqueous vapour pressure prior to sealing in anoxia.

Sample Number	Total Count/ml. SS6S x 10^{10}	Viable Count/ml. SS6S x 10^{10}	Percentage Viability
Dried at 323°K, unequilibrated			
1	7.415	4.573	61.67
2	6.901	4.707	68.21
3	7.831	4.42	56.44
4	7.065	4.67	66.1
5	7.345	4.66	63.44
Mean	7.311	4.605	62.99
Dried at 323°K, re-equilibrated to 10 torr			
1	6.464	4.453	68.89
2	6.835	4.4	64.37
3	6.765	4.293	63.46
4	7.024	4.293	61.12
5	7.368	4.433	60.17
Mean	6.891	4.375	63.49
Dried at 338°K, unequilibrated			
1	6.771	4.9	72.37
2	6.825	4.9	71.79
3	7.605	4.813	63.29
4	7.302	4.68	64.09
5	7.038	4.92	69.91
Mean	7.108	4.843	68.13
Dried at 338°K, re-equilibrated to 10 torr			
1	6.655	4.787	71.93
2	6.69	4.927	73.65
3	7.002	4.907	70.08
4	7.456	4.993	66.97
5	7.241	4.913	67.85
Mean	7.009	4.905	69.98

TABLE 15

Analysis of variance performed on 10^3 times the logarithm of percentage viability data recorded in Tables 12, 13 and 14.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Between temperatures	31,129.67	(5)	6,225.93	8.83(F=2.45)
linear	15,736.43	1	15,736.43	n.s.
residual	15,393.23	4	3,848.31	5.46(F=2.56)
Water vapour (absent or present)	322.02	1	322.02	n.s.
Temperature x Water vapour	1,529.68	5	305.94	n.s.
Residual	33,852.8	48	705.27	
Total	66,834.17	59		

n.s. non-significant.

the Analysis of Variance would be appropriate. The analysis (performed for ease of computation on 10^3 times the log. percentage viability values) is given in Table 15. Where appropriate, the tabulated value of the variance ratio at the 5% probability level is shown in brackets, in the table.

Inspection of Table 15 reveals that under the experimental conditions investigated, re-equilibration of the dried spores to 10 torr aqueous vapour pressure has no significant effect on viability. The influence of drying temperature on viability also is not affected by re-equilibration of the dried spores. The effect of drying temperature itself is, however, highly significant at both the 5% probability level (F=2.45) and the 1% probability level (F=3.38)as shown by the corresponding variance ratio of 8.83. The between temperatures sum of squares was

further analysed to determine whether a linear relationship existed between spore viability and drying temperature. The variance ratio corresponding to linear regression is not however significant at the 5% probability level. Furthermore, the between temperatures residual mean square is highly significant at both the 5% probability level (F=2.56) and the 1% probability level (F=3.76) showing that the relationship between the log. percentage viability and the drying temperature is not a linear one. From the plot of log. mean percentage viability against drying temperature illustrated in Figure 17 it would appear that two possible types of relationship could exist:

a) a curvilinear relationship between log. percentage viability and drying temperature with a minimum in the region of $288^{o}K$ to $293^{o}K$ or

b) a threshold effect at around $308^{o}K$ to $323^{o}K$ below which the drying temperature has no marked influence on spore viability, and above which a significant increase in viability occurs with increase in the temperature of drying.

It was considered to be unreasonable, at this stage, to attempt further investigation of these possibilities since it was felt that more experimental data over a wider range of drying temperatures would be required to enable valid conclusions to be drawn. It does appear, however, that a phenomenon, analogous to heat activation is manifest in spores subjected to a combination of high vacuum and high temperature treatments.

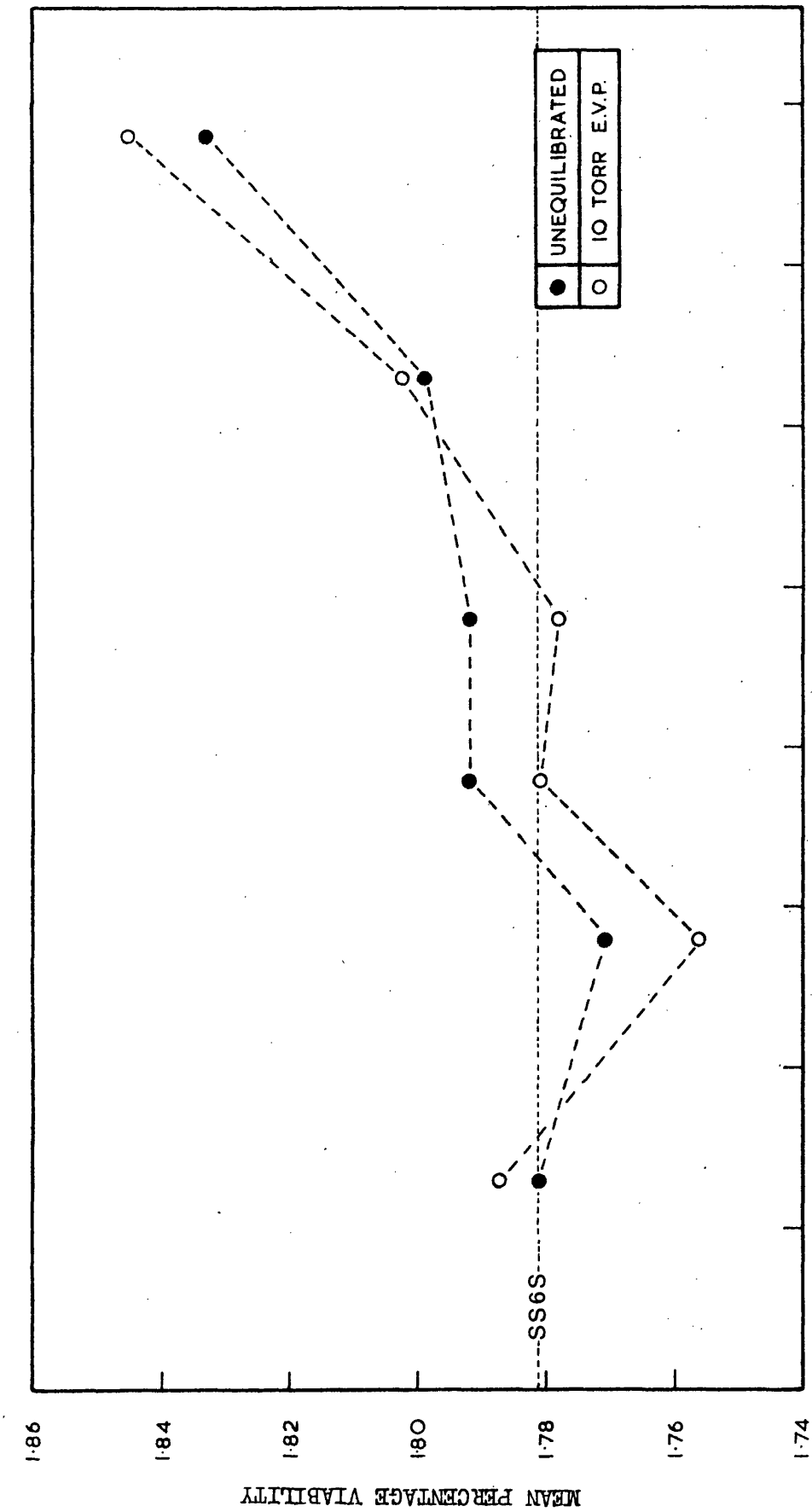


Figure 17. Plot of "mean percentage vilability" on a log. scale against "drying temperature" for Bacillus megaterium spores dried for 6 hours and sealed in anoxia.

The Effect of Oxygen on the Viability of Spores Dried at 298°K, With and Without Subsequent Re-equilibration to 10 torr Aqueous Vapour Pressure

Ten samples of spore suspension SS6S were dried for six hours at 298°K. At the completion of the drying period five of the samples were sealed in anoxia. Oxygen was admitted to the remaining vessels and these were then sealed off, in oxygen. Each dried sample was resuspended in sterile water and total and viable counts were performed on each resultant spore suspension.

In a repeat experiment the ten samples were re-equilibrated to 10 torr aqueous vapour pressure at the end of the six hour drying period. Half of the samples were sealed in anoxia, and the remainder in the presence of oxygen. Total and viable counts were performed on the resuspended samples.

The data obtained in these experiments is summarised in Table 16. In this series of experiments, two two-level factors are operating, i.e. presence or absence of oxygen and presence or absence of 10 torr aqueous vapour pressure and the corresponding Analysis of Variance carried out on 10^3 times the log. percentage viability data is illustrated in Table 17.

It can be deduced from Table 17 that the data obtained in this series of experiments confirm the previous observation that re-equilibration to 10 torr aqueous vapour pressure has no significant effect on the viability of dried spores. Furthermore, the analysis shows that there is no significant interaction between re-equilibration and the presence of oxygen, under the experimental conditions investigated. The variance ratio corresponding to the effect of oxygen per se is 4.33, compared with the tabulated value at the 5% level of significance of 4.49. Thus it must be concluded that in terms of biological changes in the spore leading to gross losses in viability, the action of oxygen is negligible, under the drying conditions investigated.

TABLE 16

Viable and total counts for quintuplicate samples of spore suspension SS6S dried for 6 hours at 298°K, with and without subsequent re-equilibration to 10 torr aqueous vapour pressure, prior to sealing in oxygen and in anoxia.

Sample Number	Total Count/ml. SS6S x 10^{10}	Viable Count/ml. SS6S x 10^{10}	Percentage Viability
a) Unequilibrated, sealed in anoxia			
1	6.842	4.42	64.6
2	7.006	4.207	60.05
3	7.312	4.373	59.81
4	6.648	4.513	67.89
5	7.356	4.253	57.82
Mean	7.033	4.353	61.89
b) Unequilibrated, sealed in oxygen			
1	7.953	4.473	56.24
2	7.287	4.253	58.36
3	7.606	4.273	56.18
4	7.91	4.48	56.64
5	7.088	4.433	62.54
Mean	7.569	4.383	57.91
c) Re-equilibrated to 10 torr, sealed in anoxia			
1	6.654	4.34	65.22
2	6.924	4.193	60.56
3	6.956	4.273	61.43
4	7.441	4.267	57.34
5	7.428	4.293	57.79
Mean	7.081	4.276	60.39
d) Re-equilibrated to 10 torr, sealed in oxygen			
1	7.015	4.34	61.87
2	7.195	4.607	64.03
3	7.750	4.253	54.88
4	7.746	4.353	56.2
5	7.399	4.273	57.75
Mean	7.421	4.365	58.82

TABLE 17

Analysis of variance performed on 10^3 times the logarithm of percentage viability data recorded in Table 16.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Oxygen	3,001.25	1	3,001.25	4.33(F=4.49)
Water Vapour	31.25	1	31.25	n.s.
Oxygen x Water Vapour	92.45	1	92.45	n.s.
Residual	11,095.6	16	693.48	
Total	14,220.55	19		

n.s. non significant.

The Effect of Different Drying Periods at 338°K, With and Without Subsequent Re-equilibration to 10 torr Aqueous Vapour Pressure on the Recovery and Viability of Bacillus megaterium Spores

Previous experiments have shown that the greatest increase in viability occurs in spores dried at 338°K (see page 110). It was therefore decided to utilise this drying temperature in a series of experiments designed to determine the effects of prolonged drying on spore viability.

Ten samples of spore suspension were dried, on different occasions, for 6 hours and 24 hours at 338°K. At the completion of the drying treatment half of the samples were sealed off in anoxia, the remainder being re-equilibrated to 10 torr aqueous vapour pressure prior to sealing in anoxia. The dried samples were resuspended in sterile water and total and viable counts performed on them. The data obtained from these determinations are recorded in Table 18.

In this series of experiments the two two-level factors operating are 6 hours or 24 hours drying time and presence or absence of 10 torr aqueous vapour pressure. The corresponding Analysis of Variance carried out on 10^3 times the log. percentage viability data is given in Table 19.

The analysis shows that in these experiments, as in previous ones, re-equilibration of the spores to 10 torr aqueous vapour pressure, after drying has no significant effect on their viability. Furthermore, the variance ratio corresponding to the interaction of re-equilibration with drying time is not significant at the 5% level, indicating that any changes in spore viability resulting from the increase in drying time, are also unaffected by re-equilibration of the dried spores to 10 torr aqueous vapour pressure. The variance ratio corresponding to the effect of drying time per se i.e. 8.9 is, however, highly significant at both the 5% level (F=4.49) and at the 1% level (F=8.53). It is thus

TABLE 18

Viable and total counts for quintuplicate samples of spore suspension SS6S dried for 6 hours and 24 hours at 338°K, with and without subsequent re-equilibration to 10 torr aqueous vapour pressure, prior to sealing in anoxia.

Sample Number	Total Count/ml. SS6S x 10^{10}	Viable Count/ml. SS6S x 10^{10}	Percentage Viability
a) 6 hours drying, unequilibrated			
1	6.771	4.9	72.37
2	6.825	4.9	71.79
3	7.605	4.813	63.29
4	7.302	4.68	64.09
5	7.038	4.92	69.91
Mean	7.108	4.843	68.13
b) 6 hours drying, re-equilibrated to 10 torr			
1	6.655	4.787	71.93
2	6.69	4.927	73.65
3	7.002	4.907	70.08
4	7.456	4.993	66.97
5	7.241	4.913	67.85
Mean	7.009	4.905	69.98
c) 24 hours drying, unequilibrated			
1	6.743	4.62	68.52
2	7.066	4.487	63.5
3	7.266	4.76	65.51
4	7.595	4.567	60.13
5	7.606	4.66	61.27
Mean	7.255	4.619	63.67
d) 24 hours drying, re-equilibrated to 10 torr			
1	6.527	4.453	68.22
2	7.396	4.46	60.3
3	6.919	4.46	64.46
4	6.602	4.473	67.75
5	7.045	4.64	65.86
Mean	6.898	4.497	65.19

TABLE 19

Analysis of variance performed on 10^3 times the logarithm of percentage viability data recorded in Table 18.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Drying Time	4,560.2	1	4,560.2	8.9 (F=4.49)
Water Vapour	627.2	1	627.2	n.s.
Drying Time x Water Vapour	3.2	1	3.2	n.s.
Residual	8,210.4	16	513.15	
Total	13,401.0	19		

n.s. non significant

apparent that an increase in the period of drying at 338°K from 6 hours to 24 hours results in a statistically measurable decrease (4-5%) in the percentage viability of the dried spores. The biological significance of this decrease is discussed on page 261, since at this stage it is clearly not possible to deduce how much of the loss in viability is the result of inactivation due to prolonged heating at the relatively high temperature of 338°K, and how much is the result of removal of biologically essential water from the spore during the prolonged drying.

THE EFFECT OF DIFFERENT DRYING TREATMENTS ON THE CHARACTERISTICS OF OUTGROWTH AND GERMINATION OF BACTERIAL SPORES AND ON THE SUBSEQUENT GROWTH RATE OF THE VEGETATIVE CELLS PRODUCED

Although the results obtained in the previous experiments indicate that spores can be subjected to intensive "secondary drying" with little apparent loss of viability, it was nevertheless considered possible that such treatments could induce changes in the integrity of the spores which would only be manifest as differences in germination characteristics and not as differences in overall viability. In the experiments reported in this section, a turbidimetric method was employed to determine the time required for outgrowth of spores subjected to defined drying treatments. The same technique was also used to measure the rate of growth of the vegetative cells formed after germination of the spores.

The turbidity of a bacterial suspension results from light being scattered by the component cells, during its passage through the suspension. Barer (178) considered that scattering in cellular suspensions occurred mainly as a result of refractive index gradients between the cell surface and the medium, although differences in refractive indices between the intracellular structures and the cytoplasm may also contribute.

Photoelectric measurements of turbidity can be made by direct determination of the light scattered i.e. nephelometry. Alternatively, the reduction in light transmitted, due to scattering, may be measured. The latter method, which assumes that there is no absorption of light, was used throughout this work.

The scattering of light by a suspension of bacteria obeys a relationship similar to that stated in Beer's Law.

$$E = \log \frac{Io}{I} = s\,a\,n\,l \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (12)$$

where E is the optical density (absorbance or extinction), Io is the intensity of the incident, parallel monochromatic beam of light,

I is the intensity of the light after passing through the suspension containing n organisms of area a perpendicular to the beam, s is the "turbidity coefficient" and l is the length of the light path through the suspension. The "turbidity coefficient" is a proportionality constant which depends upon the particular organism, the wavelength of light, the solvent used and the temperature at which the analysis is conducted.

This relationship applies only to low concentrations of organisms. Deviations occur with "optically dense" cultures which usually transmit more light than would be expected from the above relationship (179) Previous workers in this Department have shown that suspensions of several species of organism obey Beer's Law over the range of optical densities from 0.01 to 0.3 (180,181) and hence, in the experiments reported in this section, the highest optical density measured was 0.3.

Materials

The growth medium - this was formulated in a similar manner to the solid plating medium described on page 45 , the only difference being that the liquid medium did not contain agar. The concentrations of peptone and sodium chloride, the pH adjustment, and the sterilisation procedure were identical for both media.

The spectrophotometer - an S.P.600 spectrophotometer manufactured by Unicam Ltd., which measures log $\frac{Io}{I}$ directly was used for all optical density measurements.

The incubator - this was a "Shaking Reaction" Incubator manufactured by A. Gallenkamp & Co.Ltd., operating at $308^{o}K \pm 0.1^{o}K$.

Method

A suitable dilution of the spore suspension under investigation was prepared such that 0.01 ml. inoculated into 60 ml. of growth medium prewarmed to 308°K, gave a suspension having an optical density of approximately 0.02 at the wavelength employed. The suspension, contained in a 250 ml. aluminium capped conical flask was incubated at 308°K, and aerated by shaking at 120 oscillations per minute.

The growth of the culture was followed by taking 3 ml. samples at intervals and measuring their optical density at the chosen wavelength (see page 123) in a 1 cm. glass curette, against a reference curette containing sterile growth medium. During the later stages of the growth cycle when the optical density of the culture exceeded 0.3, 1 ml. samples of the suspension were diluted 10-fold or 100-fold with sterile growth medium so that all the optical density measurements were in the range 0.01 to 0.3, over which Beer's Law had been shown to be obeyed (see page 121). All measurements were carried out at room temperature (293°K - 295°K).

Preliminary Experiments

Unless otherwise stated, 0.01 ml. of a 1 in 100 dilution of spore suspension SS6S was used as the inoculum in the preliminary experiments.

Selection of the Wavelength of Incident Light

An inoculated culture was incubated at 308°K until the optical density of the suspension was between 0.15 and 0.2 when measured against growth medium at a wavelength of 370 mμ, this being the shortest wavelength for optical efficiency of the instrument. The optical density of the culture was then measured at a further 18 different wavelengths ranging from 370 mμ to 600 mμ.

In a repeat experiment 0.01 ml. of a 1 in 10 dilution of suspension SS6S was inoculated into 60 ml. of growth medium to give a spore suspension of optical density 0.15 to 0.2 at 370 mμ. The optical density of this suspension of non-germinated spores was also determined at different wavelengths over the range 370 mμ to 600 mμ.

The relationship between optical density and wavelength for suspensions of germinated and non-germinated spores is illustrated in Figure 18. It can be seen from these plots that neither spore suspensions nor cultures containing germinating spores and vegetative cells show any appreciable light absorption over the range of wavelengths investigated. Any wavelength between 370 mμ and 600 mμ could therefore be used for the optical density measurements of Bacillus megaterium cultures grown under the defined conditions employed in these experiments. Previous workers in this department have used 420 mμ for turbidimetric measurements of suspensions of a number of organisms in nutrient broth (180,181) and thus for the purposes of continuity it was decided to use a wavelength of 420 mμ in the experiments with Bacillus megaterium reported in this section.

Correlation of Optical Density at 420 mu with Viable Count

In order that the turbidimetric method could be applied to the study of spore germination and cell growth rates it was necessary to show that changes in the optical density of the suspension during incubation could be directly correlated with changes in the number of viable organisms.

Samples were taken from a culture at intervals during incubation and their optical densities were measured at 420 mμ. The viable count of the samples of known optical density was determined by the technique described on page 43. Where serial dilutions of samples

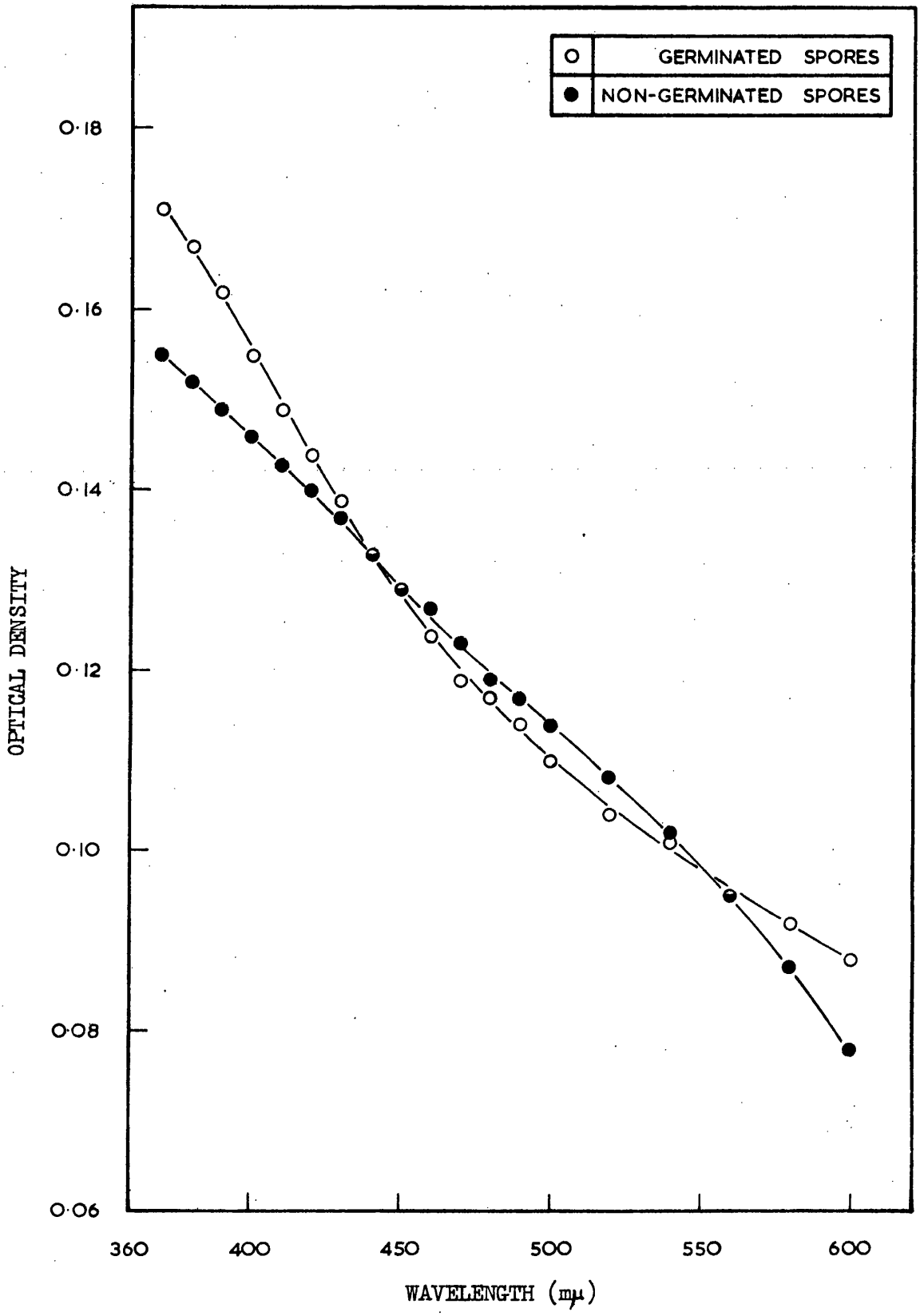


Figure 18. Plot of "optical density" against "wavelength" for Bacillus megaterium spores in growth medium.

were required, these were made in growth medium to minimise the death of vegetative cells as a result of osmotic shock.

Values of optical density and corresponding viable count for samples taken after various incubation periods are given in Table 20 and are presented graphically in Figure 19.

The log. optical density/ incubation time plot in Figure 19 is divisible into four sections:-

i) an initial lag period during which the optical density remains constant

ii) a logarithmic region where the log. optical density increases with time at a constant rate

iii) a region in which the rate of increase of log. optical density decreases with time and

iv) a region of slow decline in log. optical density with time.

The log. viable count/incubation time plot correlates with the log. optical density/incubation time plot over most of the curve, an increase in viable count giving rise to a concomitant increase in optical density. This correlation is further illustrated in Figure 20 in which a linear plot of slope 9.713×10^{-1} is obtained when log. optical density is plotted against log. viable count. Deviations from this linear relationship are shown to occur at the end of the initial lag period and in the region of slow decline. At the end of the lag period there occurs a period of incubation during which the optical density of the suspension increases but the viable count remains unchanged. This observation can be explained on the basis of this period representing the time for outgrowth of the spores. During outgrowth the surface area of the bacterial spore will increase. The value of a in the equation on page 120 will therefore increase and with it the optical density (E) of the suspension, although the number of organisms (n) will remain constant.

TABLE 20

Values of optical density at 420 mμ, and corresponding viable count, for a culture of Bacillus megaterium spores after different periods of incubation at 308°K

Incubation Time at 308°K (minutes)	Optical Density at 420 mμ	Viable Count per ml.
0	0.016	6.9×10^6
120	0.016	7×10^6
240	0.0165	6.93×10^6
255	0.017	6.83×10^6
270	0.02	7.65×10^6
285	0.033	1.017×10^7
300	0.053	1.57×10^7
330	0.128	3.847×10^7
360	0.26	9×10^7
390	0.5	1.663×10^8
420	0.83	2.64×10^8
480	1.95	6.693×10^8
540	3.15	1.16×10^9
600	4.75	1.6×10^9
660	6.62	2.347×10^9
720	8.05	2.517×10^9
900	9.75	2.607×10^9
1080	9.25	2.803×10^9
1440	8.68	1.78×10^9

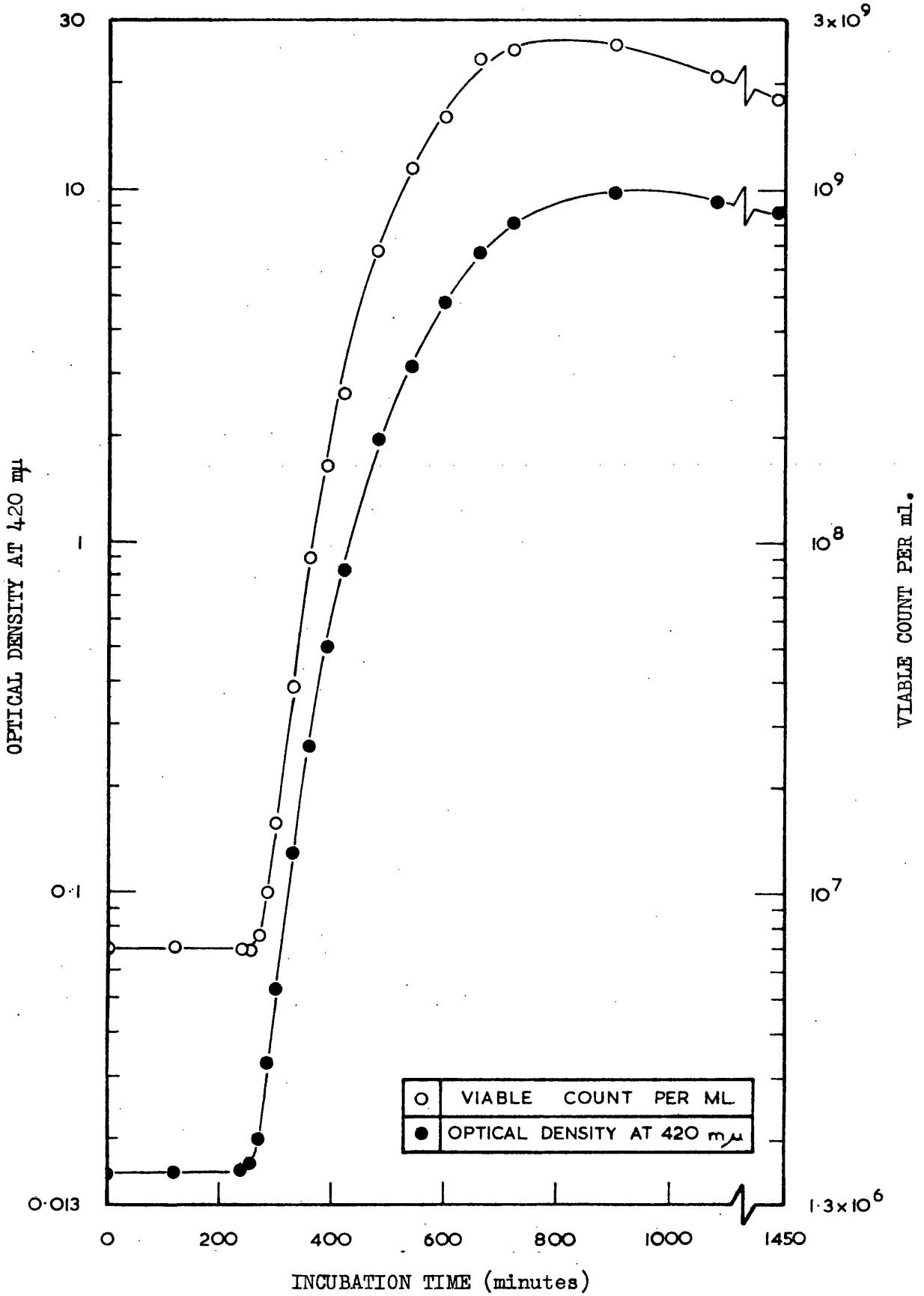


Figure 19. Plot of "optical density at 420 mμ" and "viable count per ml." on a log. scale against "incubation time" at 308°K in growth medium, for non-dried Bacillus megaterium spores.

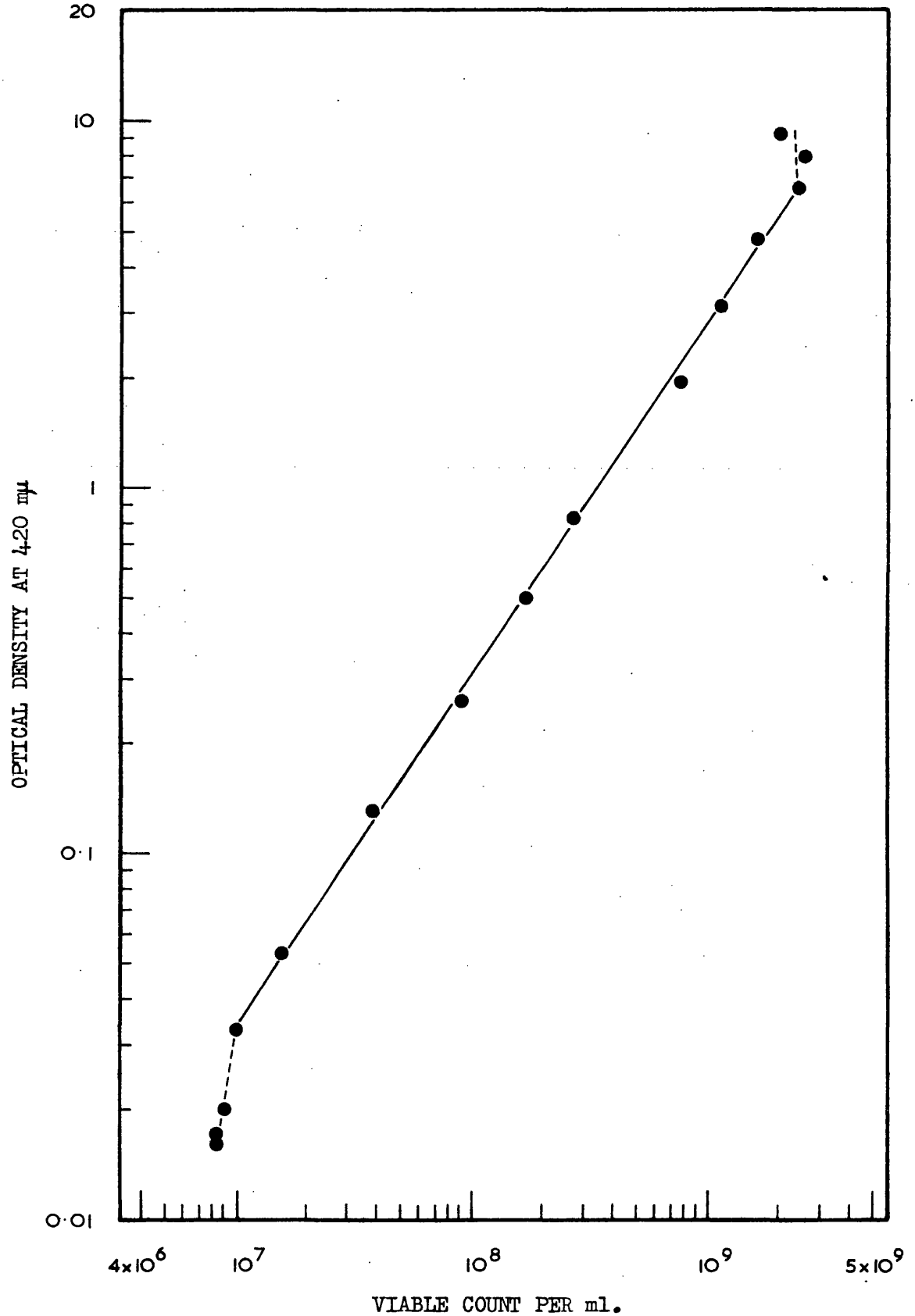


Figure 20. Plot of "optical density at 420 mμ" on a log. scale against "viable count per ml." on a log. scale for non-dried Bacillus megaterium spores incubated at 308°K in growth medium.

Only when outgrowth is complete will the vegetative cell formed be detected as a viable unit, and an increase in the viable count of the suspension occur. When all the viable spores have germinated the resultant vegetative cells will exhibit an initial logarithmic growth rate, and a direct relationship between optical density and viable count would be expected to exist as shown in Figure 20. The deviation from the linear relationship between log. optical density and log. viable count that exists at the beginning of the region of slow decline can be explained on the basis of death occurring without lysis of the cells. This death would not greatly affect the optical density of the culture, but would reduce the viable count. Only when subsequent lysis of the dead cells occurred would the optical density of the culture be reduced.

On the basis of this interpretation of results it was decided to utilise the time to reach the end of the lag period on the log. optical density/incubation time plot as a measure of the incubation time required for the commencement of outgrowth of the spores. The data from this part of the curve did not lend itself to precise mathematical analysis, and thus the time for commencement of outgrowth was estimated directly from the log. optical density/incubation time plot. The slope of this plot during the logarithmic phase was used as a measure of the growth rate constant for the vegetative cells formed during germination of the spores. The value of the rate constant and its standard error were calculated using a least squares analysis (see page 319). The values for the time of commencement of outgrowth and for the growth rate constant obtained in this experiment were 240 ± 10 minutes and $2.8545 \times 10^{-2} \pm 1.0739 \times 10^{-3}$ min.$^{-1}$ respectively.

Reproducibility of Log. Optical Density/Incubation Time Plots

Samples were taken from three replicate cultures, at intervals during incubation and their optical densities were measured at 420 mμ. Log. optical density/incubation time plots for the three cultures are illustrated in Figure 21 . For each culture the time for commencement of outgrowth was between 220 minutes and 240 minutes. The growth rate constants for the three cultures were $2.6952 \times 10^{-2} \pm 5.0824 \times 10^{-4}$ min.$^{-1}$, $2.6637 \times 10^{-2} \pm 4.6203 \times 10^{-4}$ min.$^{-1}$ and $2.6996 \times 10^{-2} \pm 6.2013 \times 10^{-4}$ min.$^{-1}$ respectively. A test for equality (see page 318) performed on these values gives a value of B of 0.269, which is less than the tabulated value at the 5% probability level of 5.991, and shows that the growth rate constants cannot be considered as being derived from different populations. The turbidimetric technique was therefore considered to be suitable, under the conditions employed, for use in subsequent studies on the effects of different drying treatments on the germination and growth characteristics of spores.

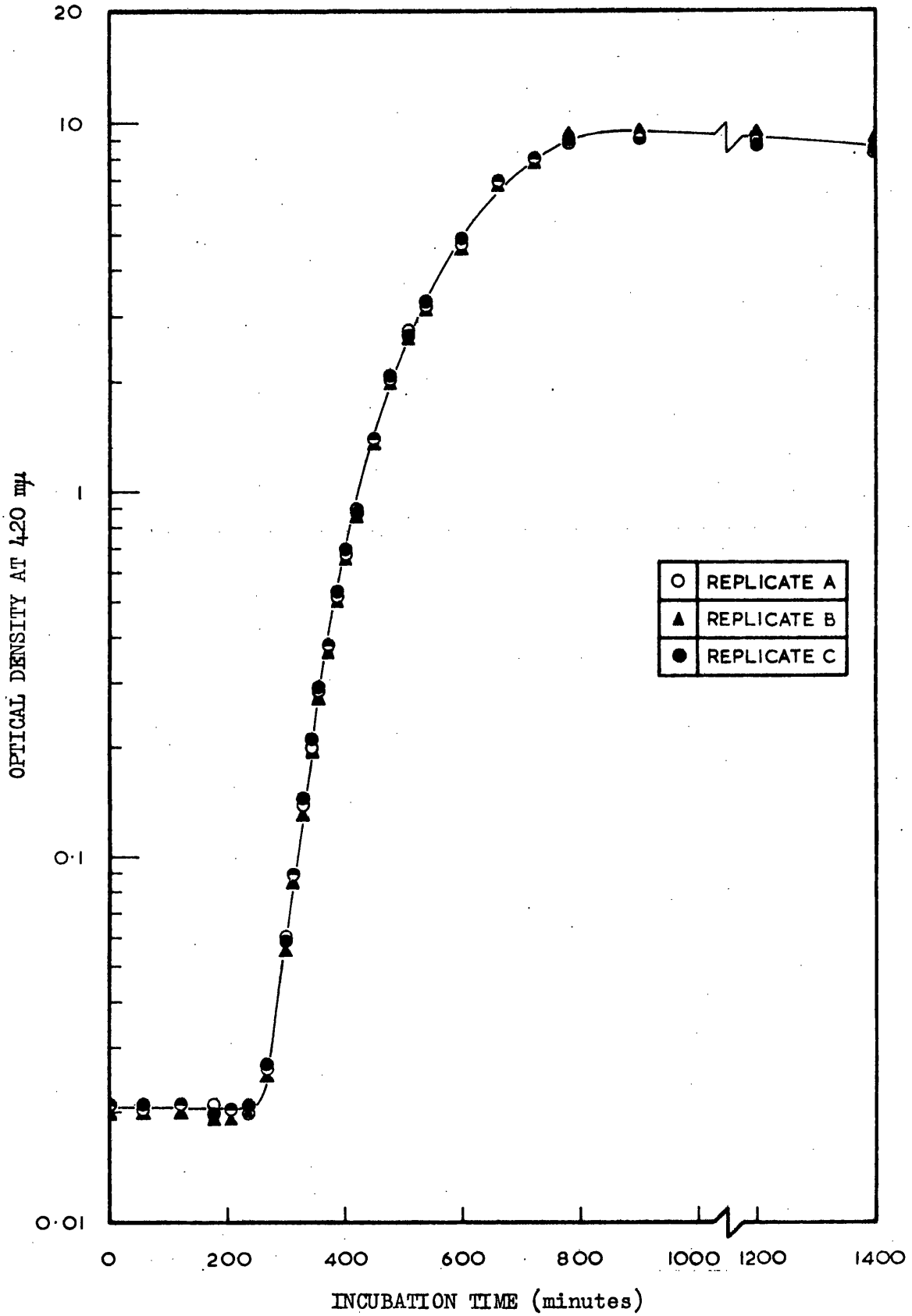


Figure 21. Plot of "optical density at 420 mμ" on a log. scale against "incubation time" at 308°K in growth medium, for replicate samples of non-dried Bacillus megaterium spores.

Subsequent Experiments

The Effect of Drying Temperature on the Germination and Growth of Dried Spores

Three experiments were performed to investigate the effect of drying temperature on the germination and growth of organisms. The drying temperatures studied were 273°K, 308°K and 338°K. In each experiment a sample of spore suspension SS6S was dried for a period of 6 hours at the chosen temperature. At the termination of the drying period the spore sample was sealed off in anoxia and reconstituted with 100 times its volume of sterile distilled water. A 0.01 ml. aliquot of the reconstituted spore suspension was inoculated into approximately 60 ml. of growth medium and the culture incubated at 308°K. Samples were taken at intervals for optical density determinations. The log optical density/incubation time plots obtained from these experiments are shown in Figure 22.

It can be seen from these plots that drying for 6 hours at the three temperatures investigated does not appear to influence the time required for commencement of outgrowth of the spores, this time being between 210 minutes and 220 minutes for all the samples. The values obtained for the growth rate constants were $2.5693 \times 10^{-2} \pm 3.4927 \times 10^{-4}$ $min.^{-1}$, $2.2676 \times 10^{-2} \pm 5.2448 \times 10^{-4}$ $min.^{-1}$, and $2.4874 \times 10^{-2} \pm 6.8617 \times 10^{-4}$ $min.^{-1}$, for samples of spores dried at 273°K, 308°K, and 338°K respectively. A test for equality performed on this data gives the value of t of 1.061 for spores dried at 273°K and 338°K. This is less than the tabulated values of 2.262 at the 5% probability level and 3.250 at the 1% probability level and indicates that the two constants cannot be considered to have been derived from different populations. The growth rate constant for spores dried at 308°K is,

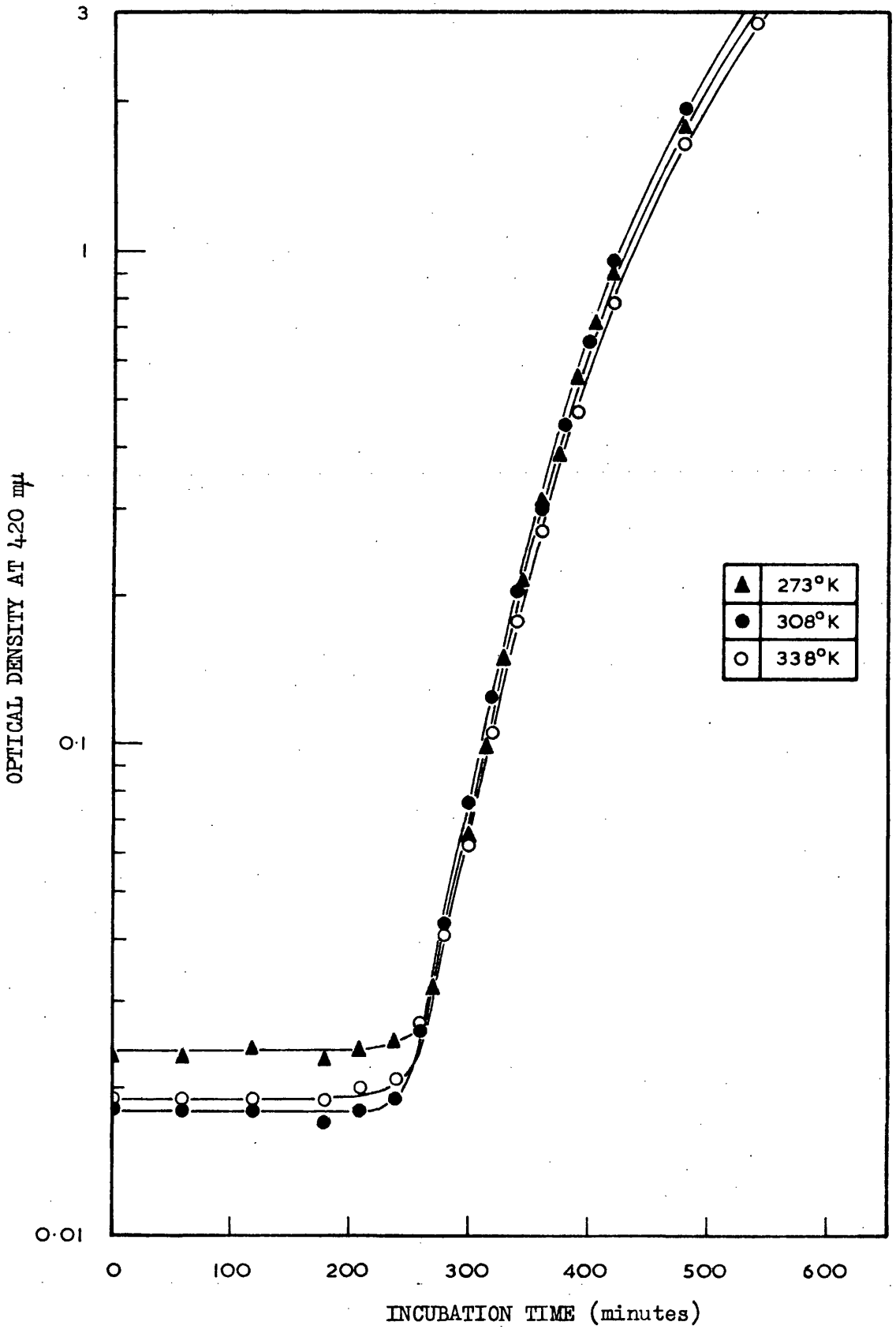


Figure 22. Plot of "optical density at 420mμ" on a log. scale against "incubation time" at 308°K in growth medium, for Bacillus megaterium spores dried for 6 hours at different temperatures and sealed in anoxia.

however, significantly different from that obtained for spores dried at 273°K and 338°K at both the 5% and the 1% probability levels, as shown by the t value of 4.788, and the significance of this is discussed on page 262.

The Effect of Different Periods of Drying at 338°K on the Germination and Growth Characteristics of Bacillus megaterium Spores

In order to investigate the effect of prolonged drying at the relatively high temperature of 338°K on the germination and growth of spores, two experiments were performed. Two samples of spores were dried at 338°K for 6 hours and 24 hours respectively, and sealed off in anoxia. After reconstitution, the dried spores were inoculated into growth medium and incubated in the manner described previously. Optical density determinations were made at intervals during the incubation period.

Least squares analysis of the logarithmic regions of the log. optical density/incubation time curves obtained in these experiments (Figure 23) gives values of $2.4874 \times 10^{-2} \pm 6.8617 \times 10^{-4}$ min^{-1} and $2.5174 \times 10^{-2} \pm 7.2338 \times 10^{-4}$ min^{-1} for the growth rate constants for spores dried for 6 hours and 24 hours respectively. When the values are subjected to the statistical analysis given in Appendix I page 318, the value of t obtained is 3.009×10^{-1} which is less than the tabulated value at the 5% probability level of 2.306, indicating that they were unlikely to have been derived from different populations. The times for commencement of outgrowth were 210 minutes for spores dried for 6 hours and 225 minutes for spores dried for 24 hours. However, it was considered that these times could not be considered significantly different within the limitations imposed by the technique employed for their estimation.

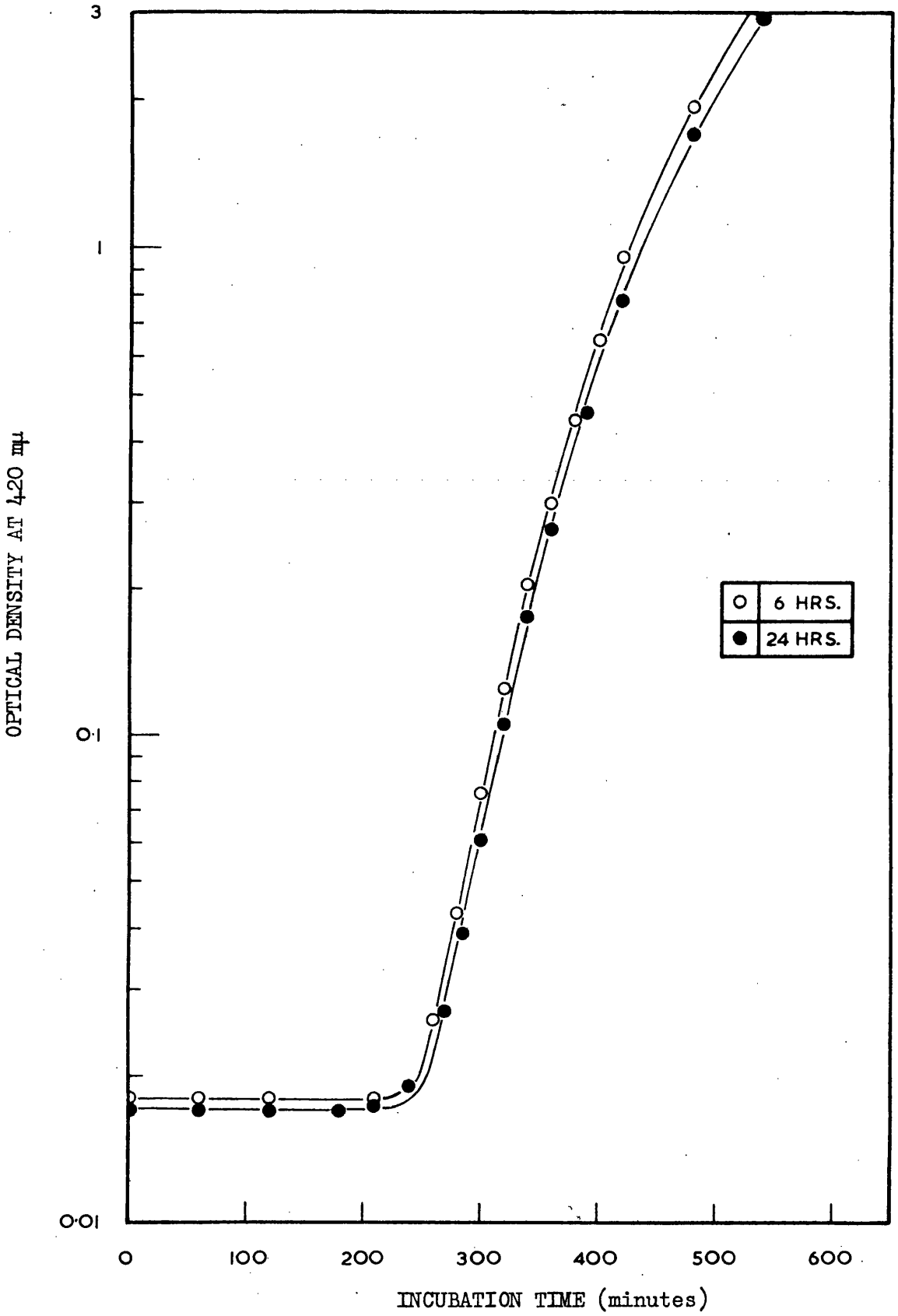


Figure 23. Plot of "optical density at 420mμ" on a log. scale against incubation time" at 308°K in growth medium, for Bacillus megaterium spores dried for 6 hours and 24 hours at 338°K and sealed in anoxia.

The Effect of Oxygen and Re-equilibration to 10 torr, on the Germination and Growth Characteristics of Spores Dried for 6 hours at 338°K

Two samples of spore suspension SS6S were dried for 6 hours at 338°K and one of these was sealed off in anoxia, the other being sealed off in oxygen. Incubation experiments were performed on these samples. The log. optical density/incubation time curves obtained are shown in Figure 24. Analysis of the growth rate constants $2.9866 \times 10^{-2} \pm 1.8254 \times 10^{-3}$ min^{-1} for spores in oxygen and $2.4874 \times 10^{-2} \pm 6.8617 \times 10^{-4}$ min^{-1} for spores in anoxia gives a value of t of 2.56 as compared to the tabulated values at the 5% and 1% probability levels of 2.365 and 3.499 respectively. However it was concluded that in view of the experimental conditions utilised in these investigations, the results of this analysis could not be considered as providing conclusive evidence of a significant difference between the two growth rate constants. Furthermore, as in the previous experiment, the method of estimation of the times for commencement of outgrowth would preclude any conclusions being drawn concerning the significance of the small apparent difference in these times of 225 minutes for spores sealed in oxygen and 215 minutes for spores sealed in anoxia.

A further experiment was performed to ascertain the effect of re-equilibration on the germination and growth characteristics of dried spores. In this case one of the two samples dried for 6 hours at 338°K was re-equilibrated to an aqueous vapour pressure of 10 torr, the other remaining un-equilibrated. Both samples were sealed off in anoxia and subjected to the incubation procedure. The data obtained are presented in Figure 25. For spores re-equilibrated to 10 torr after 6 hours drying at 338°K, the growth rate constant was $2.2572 \times 10^{-2} \pm 2.7947 \times 10^{-4}$ min^{-1} as compared with the $2.4874 \times 10^{-2} \pm 6.8617 \times 10^{-4}$ min^{-1} for the un-equilibrated spores. The value of t obtained from the analysis of these constants is 3.107 intermediate between the values at

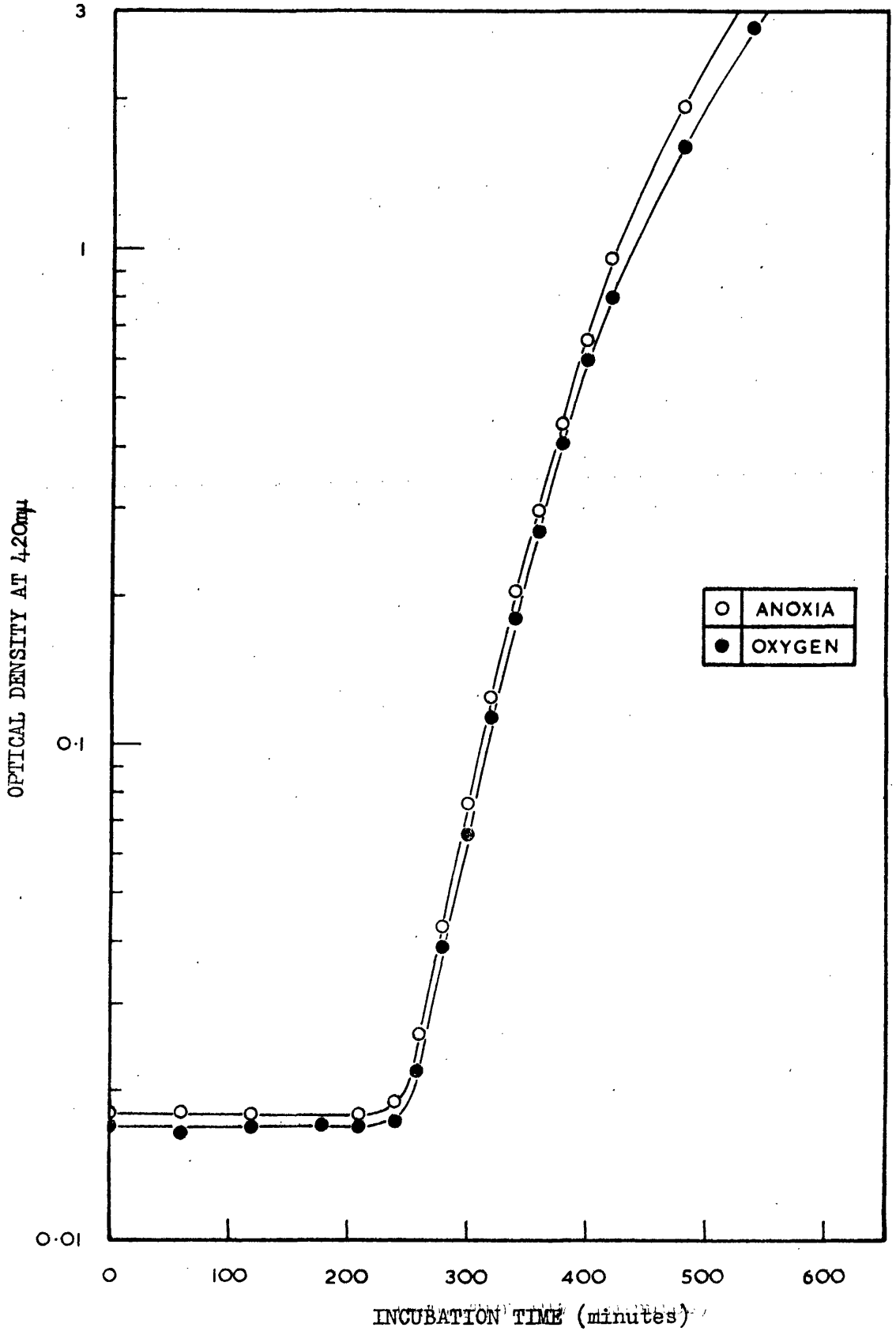


Figure 24. Plot of "optical density at 420mμ" on a log. scale against "incubation time" at 308°K in growth medium, for Bacillus megaterium spores dried for 6 hours at 338°K and sealed in anoxia and in oxygen.

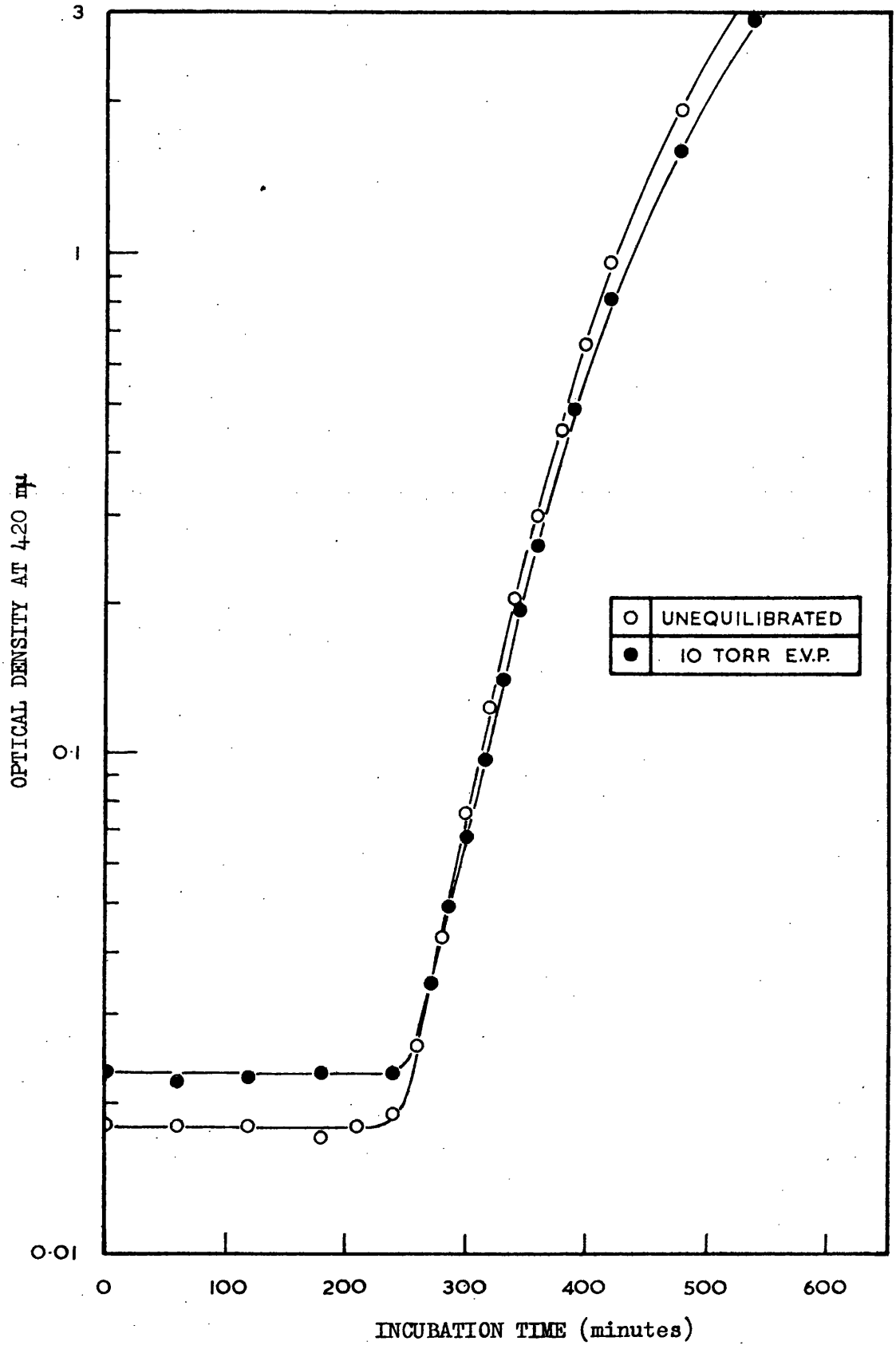


Figure 25. Plot of "optical density at 420mμ" on a log. scale against incubation time" at 308°K in growth medium, for Bacillus megaterium spores, dried for 6 hours at 338°K and sealed in anoxia.

the 5% and 1% probability levels of 2.262 and 3.25 respectively. The time for outgrowth of the re-equilibrated spores was approximately 220 minutes. Although the statistical analysis of these results showed that the growth rate constants were significantly different at the 5% probability level, the difference was considered negligible in terms of a change in an important biological characteristic of the spores, and certainly did not suggest that the addition of 10 torr of water vapour or the presence of oxygen were particularly damaging.

MEASUREMENT OF HEAT RESISTANCE

Materials

The heating bath - this was a thermostatically controlled bath supplied by Laboratory Thermal Equipment Ltd., and containing Shell Volute Oil 72. The bath was used for all temperatures employed during this work and over the range 363°K to 433°K was found to maintain the required temperature to within ± 0.1°K.

Methods

Determination of heating up times

A sample of spore suspension SS6S was dried for 6 hours at 298°K in the sample vessel containing the copper constantan thermocouple (see page 63) and sealed in anoxia. The vessel was immersed in the heating bath and the temperature of the sample was continuously recorded on a potentiometric recorder. The temperature/time profiles obtained over a range of heating bath temperatures between 363°K and 433°K are shown in Figure 26 . The time taken for the sample to attain the bath temperature was found to be 2.75 minutes in all cases.

In a repeat experiment the vessel containing the thermocouple and spore sample was sealed off in oxygen and heated at different temperatures between 363°K and 433°K. In this experiment the time taken to reach the bath temperature was found to be 2.5 minutes at all heating temperatures investigated.

In both experiments similar periods of time were required to cool the samples from the heating temperature to 273°K as were required to heat the samples to the temperature of the bath.

On the basis of these results a standard heating procedure was

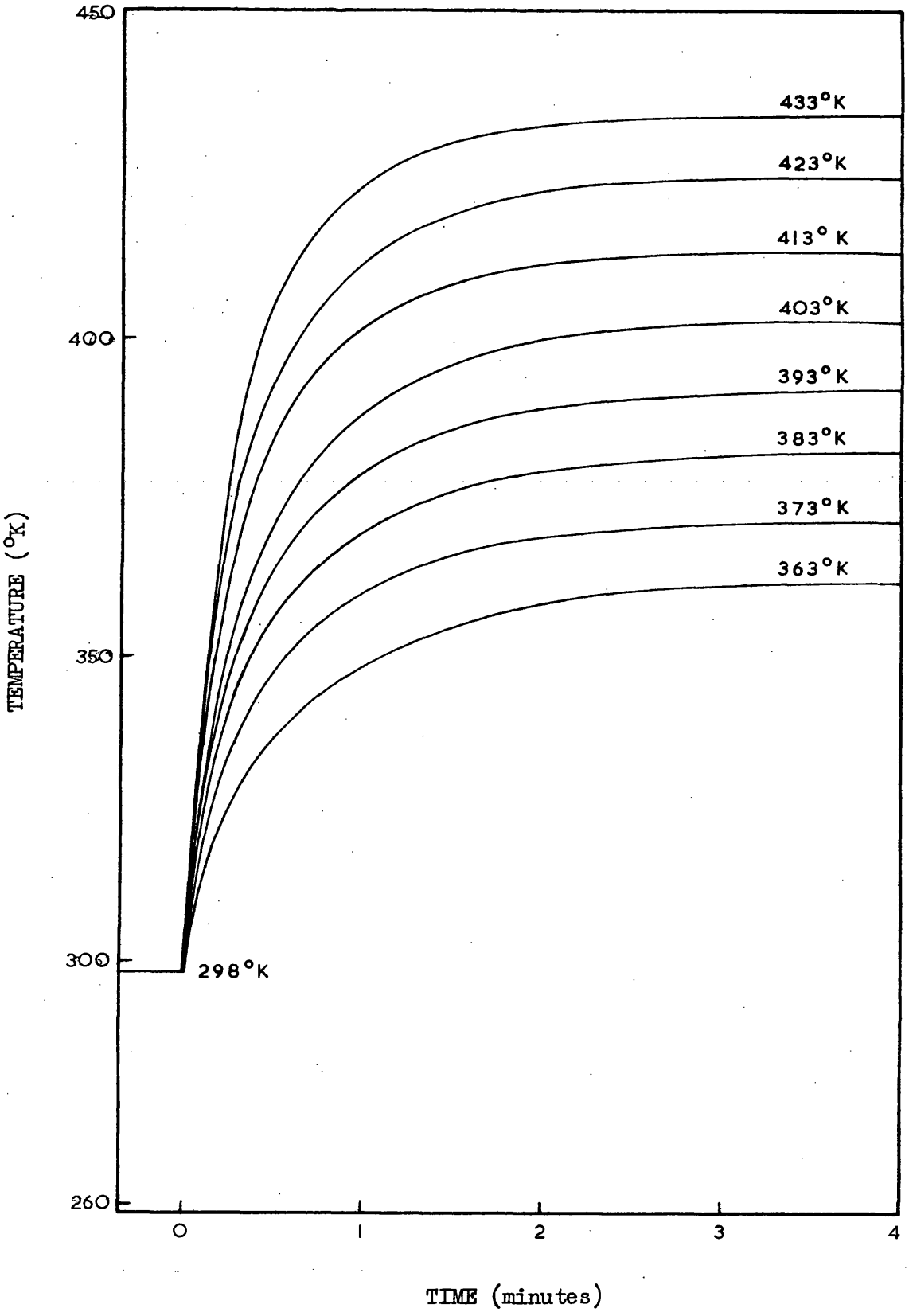


Figure 26. Sample temperature/time profiles for Bacillus megaterium spores dried for 6 hours at 298°K and heated in anoxia at different temperatures.

adopted. Samples of dried spore suspension sealed in sample vessels under defined conditions were placed in a wire basket which was lowered into the heating bath. The period of heating was timed after 2.75 minutes immersion for anoxically heated samples and after 2.5 minutes for samples heated in oxygen. After a defined heating peroid the samples were removed from the heating bath and immersed in a bath of ice water at 273^{o}K for 3 minutes. Immediately after this cooling period the vessels were opened and the contained samples reconstituted with the requisite volume of sterile water prior to the determination of the number of surviving spores (page 43).

Treatment of Results

When Bacillus megaterium spores are exposed to high temperature the log. survivor/heating time curves obtained exhibit a characteristic shape. The curves observed with primary dried spore powders heated in air at 403°K and 413°K are typical of the general shape and are illustrated in Figures 27 and 28. It can be seen that the log. survivor/heating time curves are sigmoidal having a prominent shoulder in the region of high surviving fraction i.e. they are type "b" curves. The curves appear to be linear below the 10^{-1} level of surviving fraction.

In radiobiological research the type "b" survivor curve has been described by a mathematical expression derived from the theoretical concept that inactivation results from a single event ('Hit') occurring in a number of sensitive regions ('Targets') of the cell. This expression is

$$\frac{N}{N_o} = 1 - (1 - e^{-kD})^n \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (13)$$

where $\frac{N}{N_o}$ is the fraction of spores surviving a dose of radiation D and k and n are constants (see page 188). This expression can be used in a non-interpretive way for type "b" curves, to obtain values for the two constants. When applied to log. survivor/heating time curves, the term D in equation 13 is replaced by t, the period of exposure to heat, and the equation for the curve becomes

$$\frac{N}{N_o} = 1 - (1 - e^{-kt})^n \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (14)$$

k is the slope of the log.survivor/heating time curve expressed in reciprocal minutes, and is named the heat inactivation constant. n is the intercept with the y axis of the extrapolated linear portion of the curve expressed in terms of surviving fraction.

Experimental data obtained with primary dried spore powders heated in air at 383°K, 393°K, 403°K, 413°K, 423°K, and 433°K were

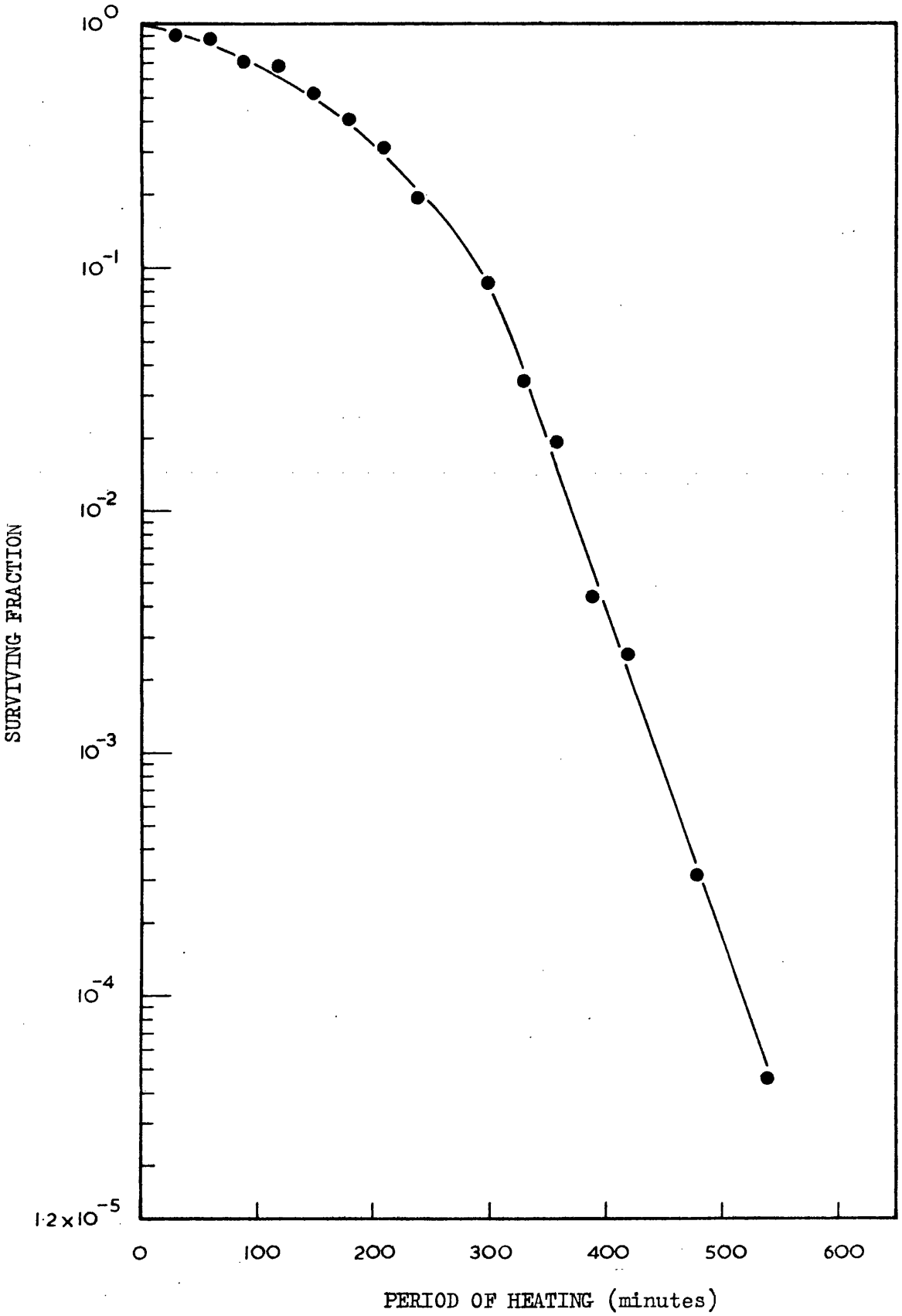


Figure 27. Plot of "surviving fraction" on a log. scale against "period of heating at 403°K "for primary dried Bacillus megaterium spores in PDM4S, heated in air.

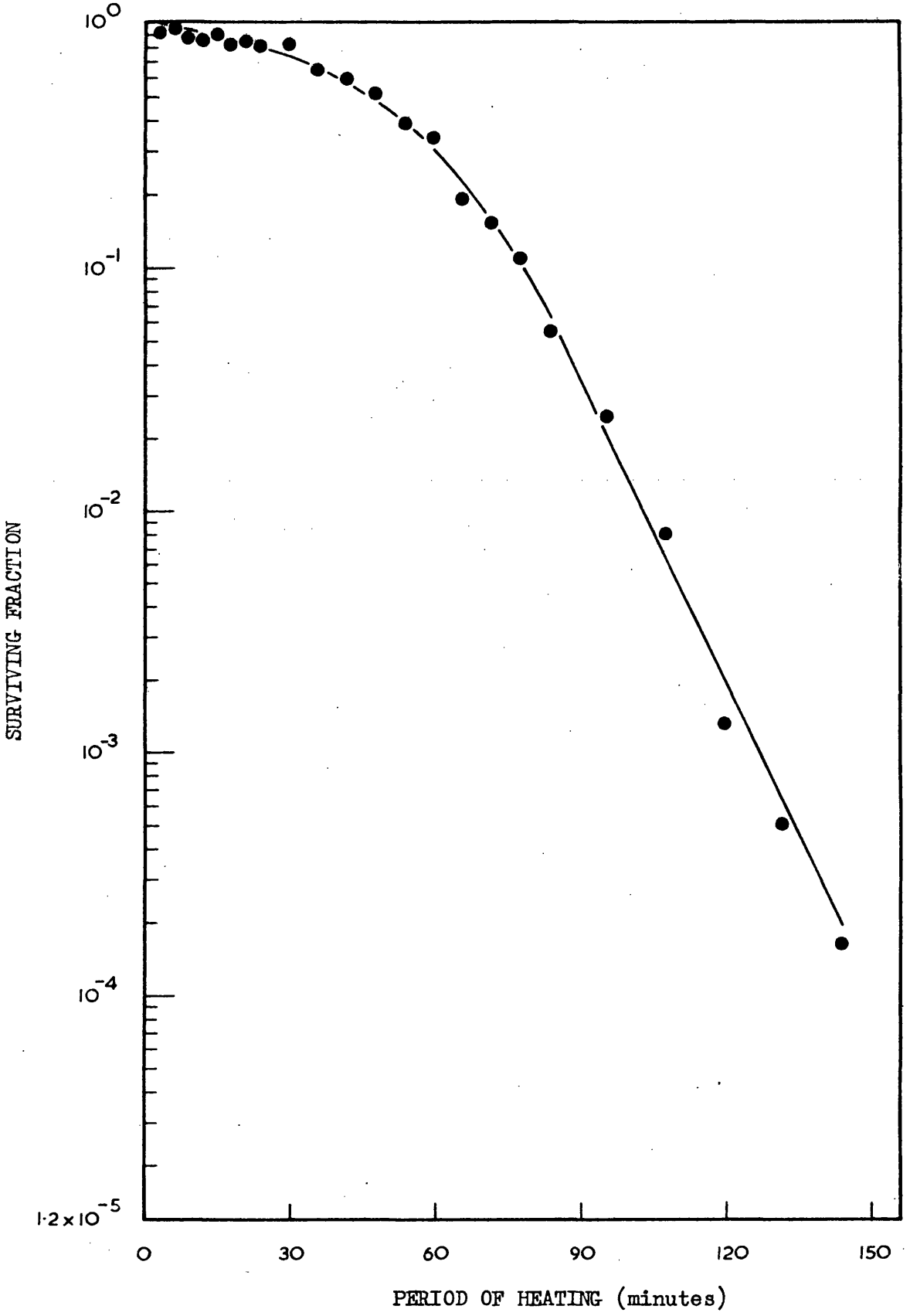


Figure 28. Plot of "surviving fraction" on a log. scale against "period of heating at 413°K" for primary dried Bacillus megaterium spores in PDM45, heated in air.

used to test the suitability of the above mathematical expression. The data were fitted to equation 14 by means of a Fortran computer program devised by Tyler and Dipert (182) This program produces least squares estimates and standard errors of the constants in this equation and also, for plotting purposes calculates the theoretical values for the surviving fraction at each heating time. The observed and computed values for the surviving fraction at each heating time, for spores heated at 403^{o}K, are plotted on a log.scale against time in Figure 29. The results obtained at 403^{o}K are typical of those obtained with primary dried spore powders at all the heating temperatures investigated.

It can be seen from Figure 29 that there is a significant difference in the shape of the observed and computed log.survivor/heating time curves. It is therefore concluded that equation 14 does not adequately represent the data obtained in experiments involving the heating of spores, and thus the constants k and n, when calculated with reference to this equation do not provide a realistic measure of the heat sensitivity of spores.

A simpler description of the linear portion of type "b" survivor curves is given by the expression

$$\frac{N}{N_o} = ae^{-kt} \qquad (15)$$

where k is the slope of the linear portion of the curve (at high values of t) and is again named the heat inactivation constant and expressed in reciprocal minutes. The intercept with the y axis of the extrapolated linear portion of the curve will give an estimate of the size of the shoulder, and is given by a, (this notation being chosen to avoid confusion with the constant n in expression 14) and is expressed in terms of surviving fraction. In practice it is more usual to use the logarithmic transformation of equation 15 , where the intercept

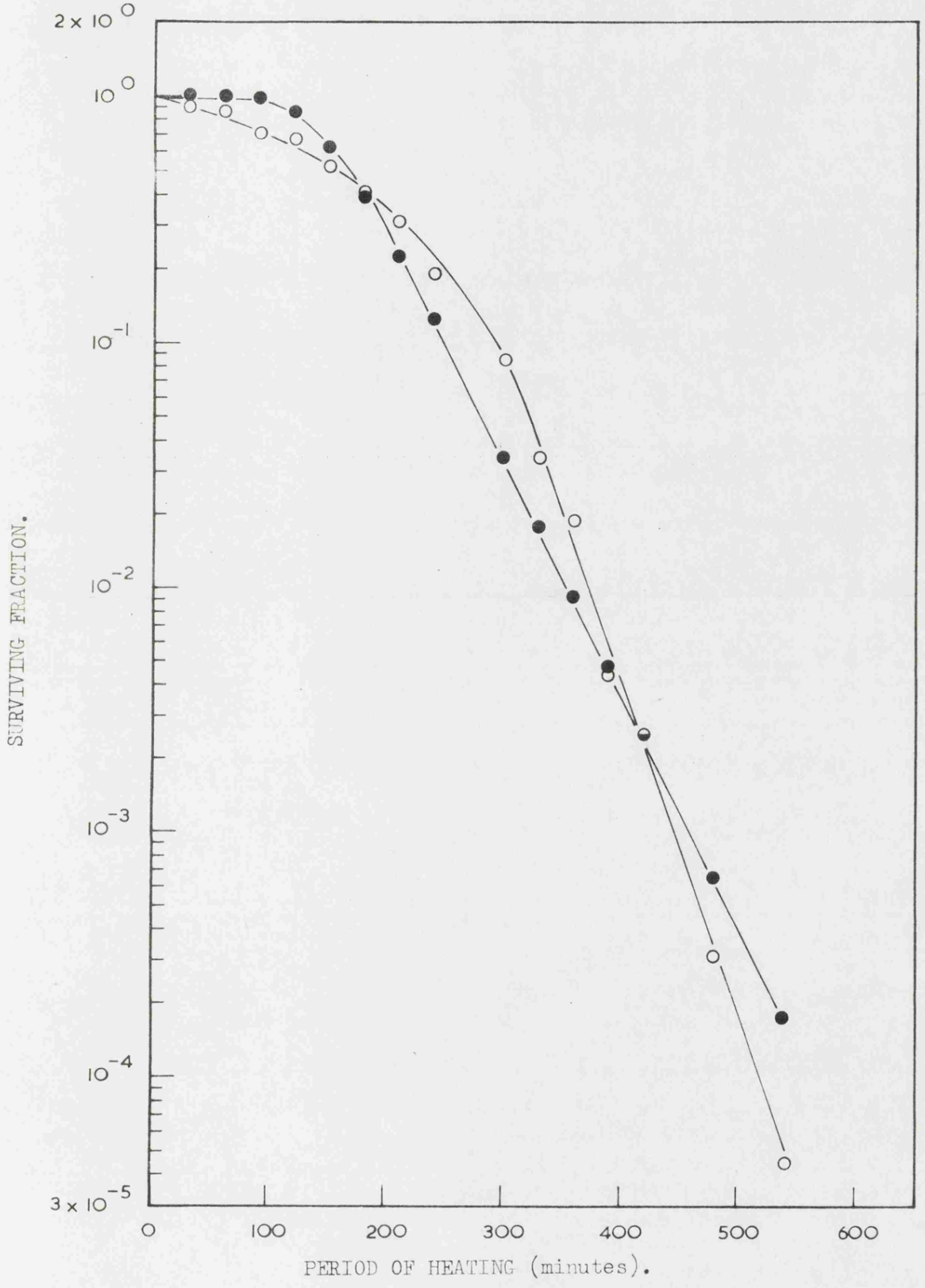


Figure 29. Plot of "surviving fraction" on a log. scale against "period of heating" at 403°K, in air, for primary dried Bacillus megaterium spores in PDM4S. (o) observed values of surviving fraction. (●) values of surviving fraction computed by fitting the experimental data to the equation $\frac{N}{N_0} = 1 - (1-e^{-kt})n$

is expressed as ln. surviving fraction.

Similar defined conditions were implicated in using equation 15 to obtain constants for log. survivor/heating time curves as were stipulated when an equivalent equation was used to characterise the survivor curves from radiation studies (see page 188) Thus, only data obtained from heating time levels resulting in a surviving fraction of below 10^{-1} were used in the computation of the values of k and a. The values of the constants and their standard errors were calculated using the least squares program in Appendix II.

The constant a in the logarithmic transform of equation 15 is a measure of the initial shoulder of the curve in terms of the y axis (ln. surviving fraction). A third constant was also introduced to express the shoulder in terms of the x axis (heating time). This was defined as the heating time beyond which the log. survivor/heating time curve was linear i.e. the heating time required to obtain a surviving fraction of 10^{-1}. This constant was called the shoulder constant s, and was expressed in minutes. Calculation of s involves the computed values of k and a as follows.

$$\ln \frac{N}{N_o} = \ln a - kt \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (16)$$

when $N_o = 10^{o}$ and $N = 10^{-1}$

$$\ln \frac{N}{N_o} = -2.3026 = \ln a - ks \quad \ldots\ldots\ldots\ldots\ldots \quad (17)$$

hence

$$s = \frac{-2.3026 - \ln a}{-k} \quad \ldots\ldots\ldots\ldots\ldots \quad (18)$$

N.B. The D value ,i.e. the period of heating required to reduce the surviving fraction by 1 log. cycle, is often used as a measure of heat resistance. When the log. surviving fraction is plotted against the period of heating the D value is numerically equal to 1/k.

Experimental

The Effect of Temperature on the Heat Resistance of Bacillus megaterium Spores

Introduction

The experiments reported in this section were instigated to investigate the characteristics of lethality resulting from exposure to high temperature, in spores subjected to different drying processes. The spore states examined were 1) undried, where the spores were in aqueous suspension, 2) primary dried, where water had been removed by sublimation from the ice phase, 3) secondary dried, where additional water had been removed by isothermal desorption, and 4) re-equilibrated, where water had been added to the spores after secondary drying.

It was decided to determine whether the mechanism of spore death had changed in its characteristics by using classical chemical methods of investigating the response to temperature. If it is assumed that heat inactivation represents a chemical event that obeys normal physico-chemical kinetic laws, then a quantitative relationship between the temperature and the rate of heat inactivation of spores can be expressed by the Arrhenius equation. This equation is

$$k = Ae^{-\frac{Ea}{RT}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (19)$$

where k is the heat inactivation constant, A is a constant associated with the entropy of inactivation and is known as the frequency factor, R is the gas constant (1.987 calories degree^{-1} mole^{-1}), Ea is the energy of activation and T is the temperature in ^{o}K.

In logarithmic form equation 19 is

$$\log k = \log A - \frac{Ea}{2.303R}\,\frac{1}{T} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (20)$$

and indicates that a plot of log k against $\frac{1}{T}$ should be linear and should give an apparent energy of activation (in calories mole^{-1})

While it is not possible to state what the mechanism of inactivation is, and therefore units of calories $mole^{-1}$ may be without significance, nevertheless, a change in the value of the energy of activation Ea with different pretreatments would probably indicate a change in mechanism. Likewise, a change in the frequency factor A would indicate a change in the susceptibility of the spore to lethal mechanisms, an increase in A indicating an increased probability of lethal reactions occurring at any given temperature.

Results

(a) Spores in aqueous suspension.

0.06 ml. samples of suspension SS6S were sealed in air, in sample vessels and heated for different times at 363°K, 373°K, 383°K, and 393°K, after which the numbers of surviving spores was determined. Log. survivor/heating time curves were constructed at each heating temperature. The data used in the construction of these curves were subjected to mathematical analysis as described on page 144 , and the calculated values of the constants k, ln.a, and s are recorded in Table 21.

(b) Primary dried spore powders.

Samples of primary dried spore powder PDM 4S contained in sample vessels were sealed in air and heated at 383°K, 393°K, 403°K, 413°K, 423°K, and 433°K. Log. survivor/heating time curves were constructed from the experimental data (Figures 27 and 28) and the values of k, ln.a, and s derived from these data are given in Table 21

(c) Secondary dried spores

Samples of spore suspension were dried for 6 hours at 298°K and sealed either in anoxia or in oxygen. Log. survivor/heating time curves were constructed at heating temperatures of 373°K, 383°K, 393°K and 403°K and the calculated values for the corresponding

TABLE 21

Values of k, ln.a and s, with associated standard errors for *Bacillus megaterium* spores heated in air at different temperatures a) in aqueous suspension, b) in primary dried powder.

Heating Temperature (°K)	k ($minutes^{-1}$)	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
a) Aqueous suspension SS6S					
363	4.2665×10^{-1}	2.7817×10^{-3}	2.3452	9.774×10^{-2}	1.089×10
373	1.3906	6.2349×10^{-3}	1.5177	6.5984×10^{-2}	2.75
383	4.3799	5.9831×10^{-2}	1.9092	1.8197×10^{-1}	9.6×10^{-1}
393	1.28×10	8.5974×10^{-2}	2.9877	1.1373×10^{-1}	4.1×10^{-1}
b) Primary dried powder PDM4S					
383	3.6919×10^{-3}	5.4429×10^{-5}	1.2678×10	3.1737×10^{-1}	4.0597×10^{3}
393	1.8×10^{-2}	6.8351×10^{-5}	1.0513×10	1.024×10^{-1}	1.0629×10^{3}
403	3.1651×10^{-2}	3.7263×10^{-4}	7.1396	1.7132×10^{-1}	2.983×10^{2}
413	1.0145×10^{-1}	1.8357×10^{-3}	5.8334	2.3173×10^{-1}	8.02×10
423	3.1994×10^{-1}	3.8593×10^{-3}	4.837	1.398×10^{-1}	2.23×10
433	7.9683×10^{-1}	1.1813×10^{-2}	3.5757	1.8703×10^{-1}	7.38

constants are listed in Table 22.

(d) Re-equilibrated spores.

Samples of spore suspension were re-equilibrated to 10 torr aqueous vapour pressure, after drying for 6 hours at 298°K, and were sealed in oxygen. The samples were heated at 383°K, 393°K, 403°K and 413°K, and the appropriate log.survivor/heating time curves were constructed. The computed values for the constants k, ln.a, and s obtained with the re-equilibrated spores are recorded in Table 22.

Figure 30 shows the plots of log.k against $\frac{1}{T}$ constructed from the data obtained in these experiments. In all cases the plots are linear with a negative slope showing that the Arrhenius relationship is obeyed, over the range of heating temperatures investigated, and thus the constants Ea and A can be used to characterise the relationship between heating temperature and spore inactivation. The values of the energy of activation and the frequency factor calculated from each of these plots are given on page 268 together with a detailed discussion of their biological significance. At this stage it is of interest to note that the different pre-treatments to which the spores are subjected prior to heating appear to have little effect on the slope of the log.k against $\frac{1}{T}$ plot i.e. the energy of activation, but result in significant changes in the value of the intercept i.e. the frequency factor.

The constant a also varies with the heating temperature, but the relationship between a and $\frac{1}{T}$ is non-linear. A similar non-linear relationship is shown when a is plotted against heating temperature. However, when the computed values of ln.a are plotted on a log. scale against $\frac{1}{T}$, a linear graph is obtained. This relationship between log. ln.a and $\frac{1}{T}$ is difficult to interpret in mechanistic terms, and does not appear to be a beneficial method of expressing the data. A

TABLE 22

Values of k, ln.a and s, with associated standard errors for Bacillus megaterium spores, dried for 6 hours at 298°K, with and without re-equilibration to 10 torr aqueous vapour pressure, and heated at different temperatures.

Heating Temperature (°K)	k ($minutes^{-1}$)	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
a) Non-equilibrated, heated in anoxia					
373	1.5441×10^{-2}	1.3389×10^{-4}	9.7755×10^{-1}	1.209×10^{-1}	2.124×10^{2}
383	4.1738×10^{-2}	4.9322×10^{-4}	1.5503	1.485×10^{-1}	9.23×10
393	1.0375×10^{-1}	1.6908×10^{-3}	2.0087	2.1818×10^{-1}	4.155×10
403	3.2289×10^{-1}	4.7252×10^{-3}	1.8192	1.6259×10^{-1}	1.277×10
b) Non-equilibrated, heated in oxygen					
373	1.9643×10^{-2}	2.1317×10^{-4}	2.0905	1.1003×10^{-1}	2.236×10^{2}
383	6.0592×10^{-2}	9.0645×10^{-4}	3.6335	1.9207×10^{-1}	9.797×10
393	1.974×10^{-1}	1.9092×10^{-3}	5.2198	1.6423×10^{-1}	3.811×10
403	5.9175×10^{-1}	3.2199×10^{-3}	3.3108	6.9247×10^{-2}	9.49
c) Re-equilibrated to 10 torr aqueous vapour pressure, heated in oxygen					
383	6.9921×10^{-3}	1.3178×10^{-4}	1.0938×10	3.7504×10^{-1}	1.8937×10^{3}
393	2.2966×10^{-2}	1.0478×10^{-4}	8.1886	8.173×10^{-2}	4.568×10^{2}
403	6.9806×10^{-2}	1.3721×10^{-3}	9.0738	3.5674×10^{-1}	1.6297×10^{2}
413	2.285×10^{-1}	8.2983×10^{-3}	9.3332	6.5472×10^{-1}	5.092×10

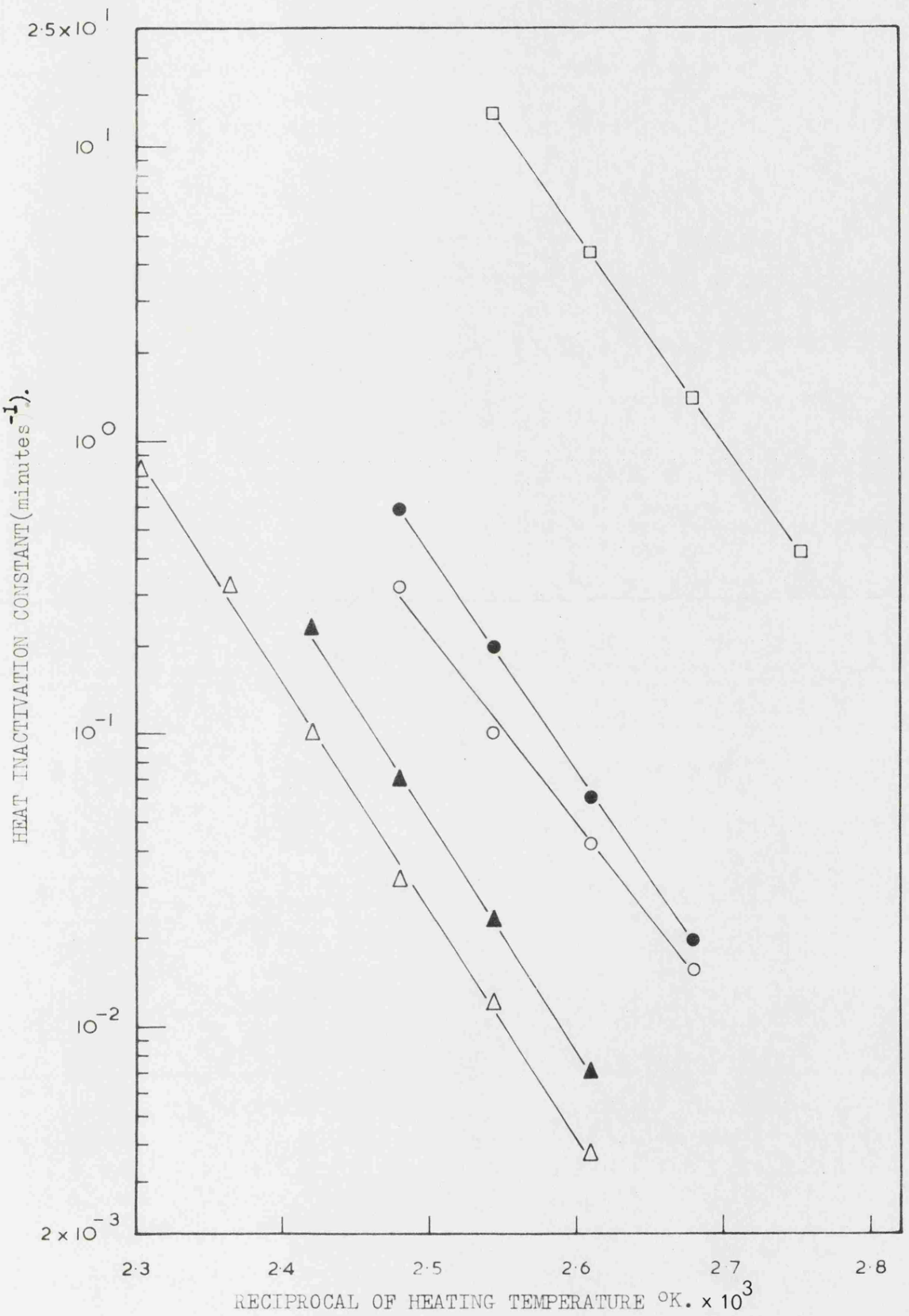


Figure 30. Plot of "heat inactivation constant, k," on a log. scale against the "reciprocal of the heating temperature" for Bacillus megaterium spores dried under different conditions. (□) undried suspension SS6S, (△) primary dried in PDM4S, heated in air, (○) 6 hours at 298°K, heated in anoxia.(●) 6 hours at 298°K, heated in oxygen, (▲) 6 hours at 298°K, re - equilibrated to 10 torr. heated in oxygen.

more sensible way would seem to be to attempt to express the shoulder in terms of a lag time, and hence the shoulder constant s, which is the heating time required to obtain a surviving fraction of 10^{-1}, has also been calculated. Figure 31 shows the plots of log.s against $\frac{1}{T}$ for spores subjected to different pretreatments before exposure to high temperature. In all cases a linear plot is obtained, the different pre-treatments having little effect on the slopes of these plots, but resulting in marked changes in the intercepts with the y axis. The biological significance of these findings is discussed on page 266.

On the basis of these results it was decided to use equation 15 to calculate the heat inactivation constant k and also to derive the shoulder constant s, in all heat sensitivity investigations reported in this thesis. The log.survivor/heating time curves obtained for different experimental conditions are described by k and s and hence these constants are used as a measure of the heat resistance of the spore population under defined environmental conditions.

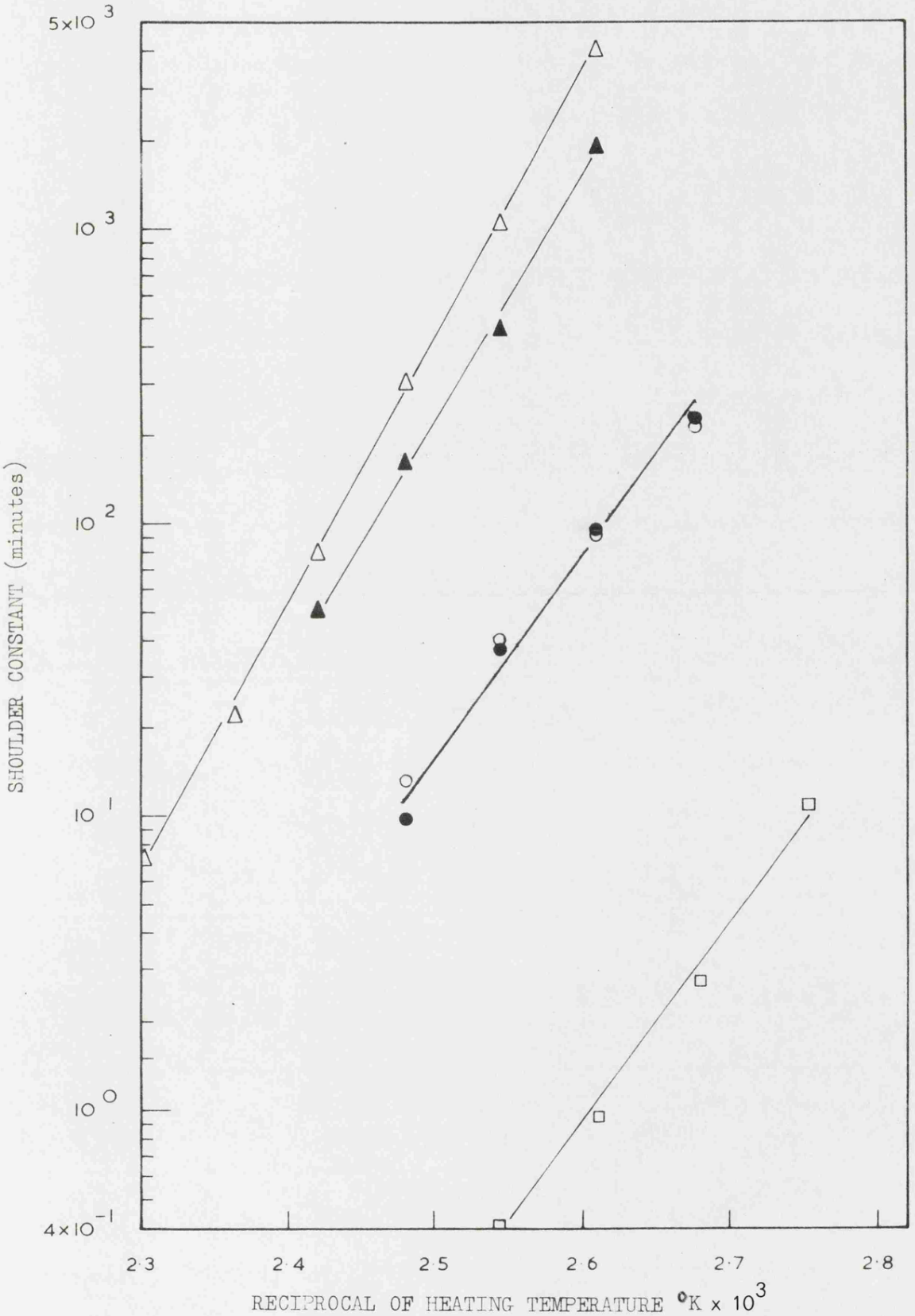


Figure 31. Plot of "shoulder constant,S," on a log. scale against the "reciprocal of heating temperature" for Bacillus megaterium spores dried under different conditions (□) undried suspension SS6S, (Δ) primary dried in PDM4S, heated in air, (o) 6 hours at 298°K, heated in anoxia, (●) 6 hours at 298°K, heated in oxygen, (▲) 6 hours at 298°K, re - equilibrated to 10 torr. heated in oxygen.

The Effect of Drying Temperature on the Heat Resistance of Bacillus megaterium Spores

The drying temperatures investigated were 273°K, 288°K, 298°K, 308°K, 323°K and 338°K. On different occasions 12 samples of spore suspension SS6S were dried for 6 hours at each of these temperatures. At the end of the drying period half of the samples were sealed in oxygen and the remainder in anoxia. Five samples from each set of six were then heated at 393°K, the sixth in each set being used as the unheated control. Log.survivor/heating time curves were constructed for spores dried under each experimental condition and are illustrated in Figures 32 and 33 for samples heated in anoxia and in oxygen respectively.

The data used in the construction of these curves were subjected to mathematical analysis as described on page 147 , and the calculated value for the constants k and s are recorded in Table 23. The relationship between these constants and the drying temperature is also represented graphically in Figures 34a and 34b.

It can be seen from these plots that the heat sensitivity of spores dried under all conditions investigated is greater in the presence of oxygen than in anoxia. Under both heating conditions the lowest heat sensitivity is observed in spores dried at 273°K. The heat inactivation constant k increases with increase in drying temperature up to 308°K and then remains relatively constant for temperatures between 308°K and 338°K. The shoulder constant s is also a function of the drying temperature, decreasing values of s being observed with increasing drying temperature over the range 273°K to 338°K.

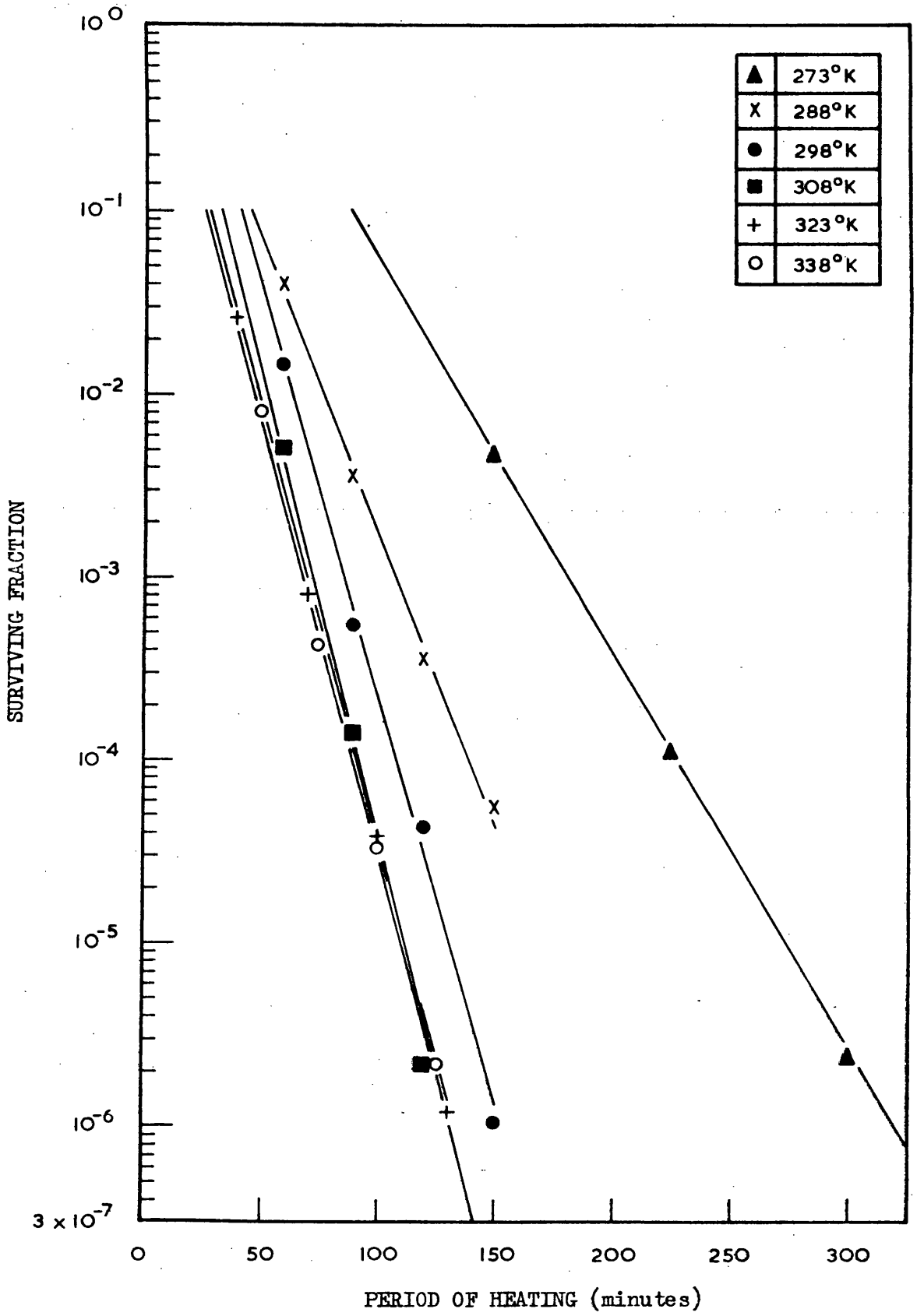


Figure 32. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for 6 hours at different temperatures, and heated in anoxia.

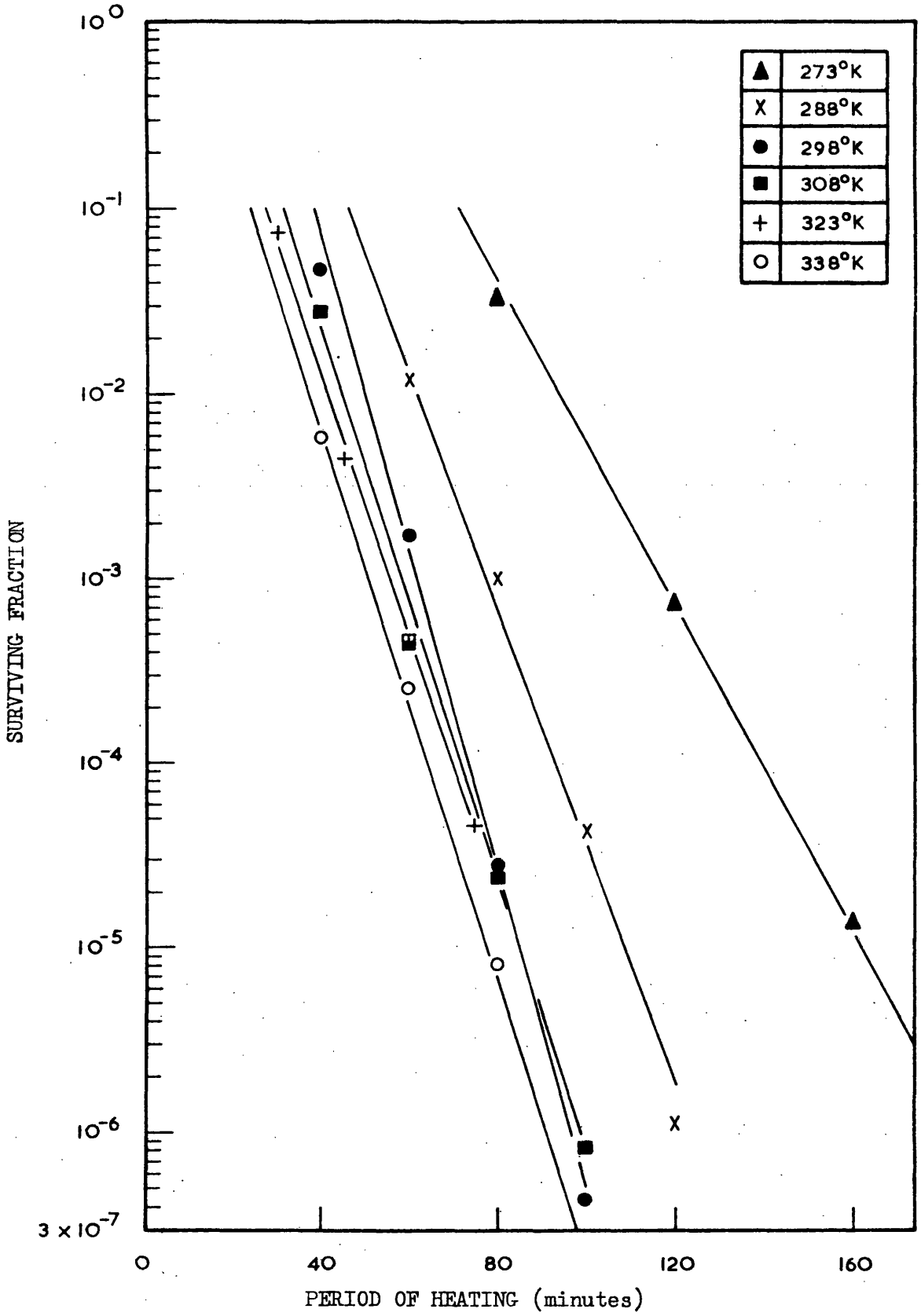
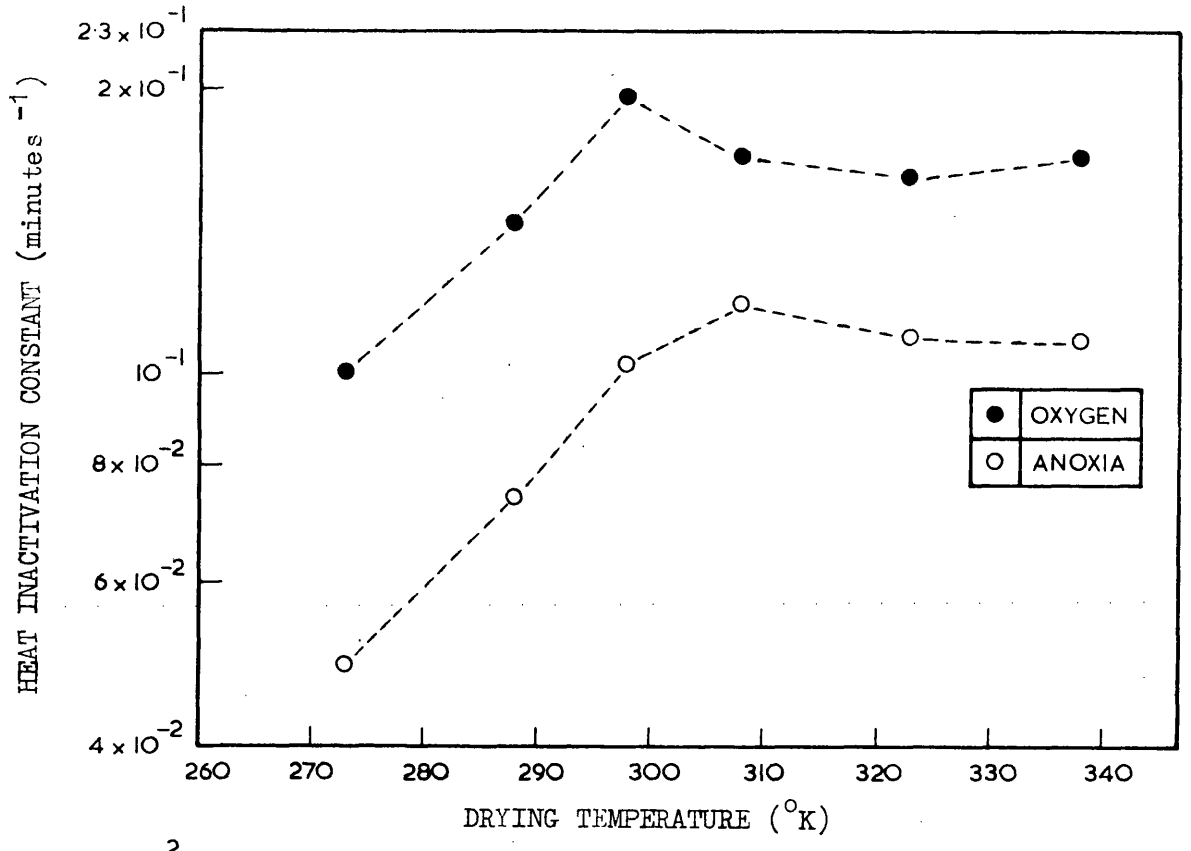


Figure 33. Plot of "surviving fraction" on a log. scale against "period of heating at 393° K" for Bacillus megaterium spores dried for 6 hours at different temperatures and heated in oxygen.

TABLE 23

Values of k, ln.a and s with associated standard errors for *Bacillus megaterium* spores dried for 6 hours at different drying temperatures and heated at 393°K, in anoxia, and in oxygen.

Drying Temperature (°K)	k (minutes^{-1})	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
Heated in anoxia					
273	4.9331×10^{-2}	3.5156×10^{-4}	2.0043	1.08×10^{-1}	8.731x10
288	7.3861×10^{-2}	1.1252×10^{-3}	1.1171	1.4518×10^{-1}	4.63 x10
298	1.0375×10^{-1}	1.6908×10^{-3}	2.0087	2.1818×10^{-1}	4.155x10
308	1.1794×10^{-1}	2.3373×10^{-3}	1.6674	3.0159×10^{-1}	3.366x10
323	1.0981×10^{-1}	7.2954×10^{-4}	6.9759×10^{-1}	8.2699×10^{-2}	2.753x10
338	1.0883×10^{-1}	8.5945×10^{-4}	5.5307×10^{-1}	9.2416×10^{-2}	2.624x10
Heated in oxygen					
273	1.017×10^{-1}	9.1289×10^{-4}	4.8908	1.5706×10^{-1}	7.073x10
288	1.4495×10^{-1}	1.9761×10^{-3}	4.4448	2.0345×10^{-1}	4.655x10
298	1.9742×10^{-1}	1.9057×10^{-3}	5.2216	1.6394×10^{-1}	3.811x10
308	1.709×10^{-1}	2.7062×10^{-3}	2.9943	2.3745×10^{-1}	3.099x10
323	1.6247×10^{-1}	2.1064×10^{-3}	2.126	1.359×10^{-1}	2.726x10
338	1.7023×10^{-1}	1.431×10^{-3}	1.7769	1.231×10^{-1}	2.396x10



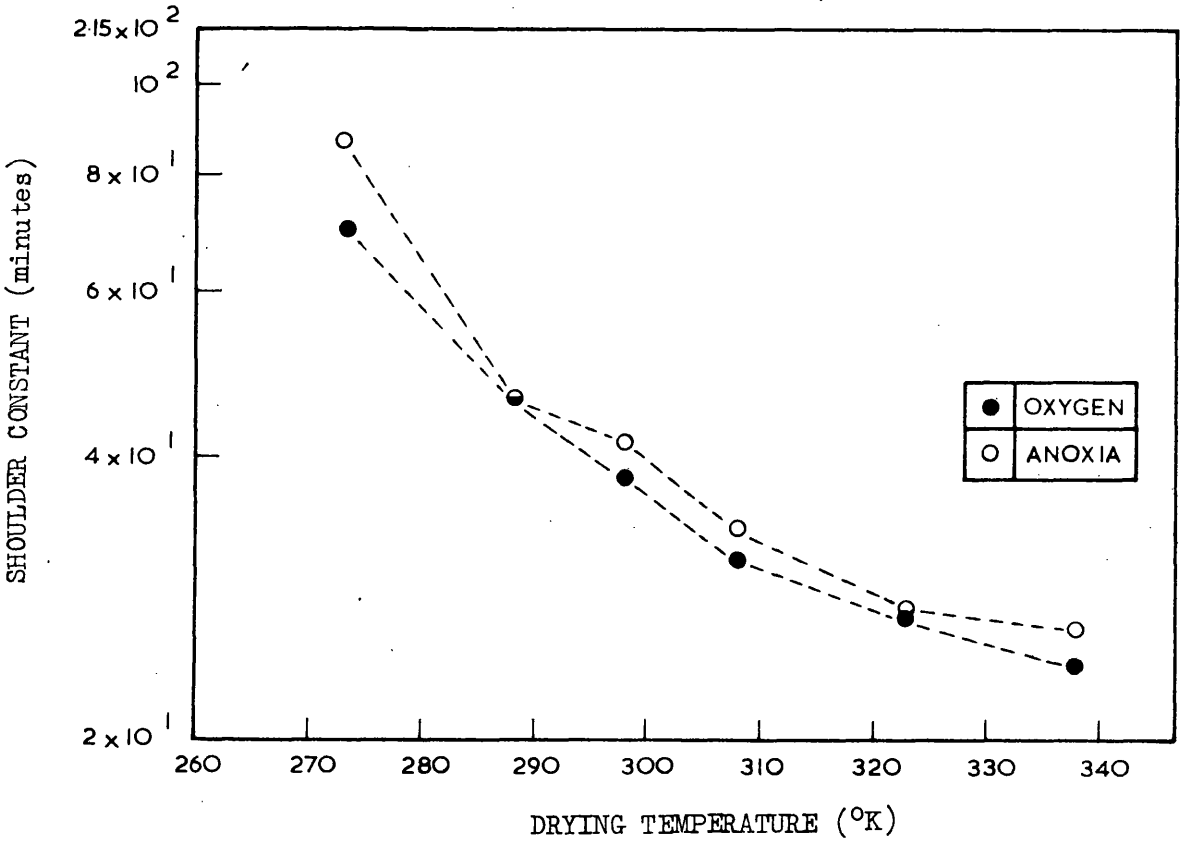


Figure 34. Plot of (a) "heat inactivation constant," and (b) "shoulder constant", on a log. scale, against "drying temperature", for Bacillus megaterium spores dried for 6 hours, and heated at 393°K (in oxygen and in anoxia.)

The Modifying Effect of Re-equilibration to 10 torr Aqueous Vapour Pressure on the Heat Resistance of Spores Dried at Different Temperatures

Samples were dried on different occasions for 6 hours at 273°K, 298°K, 308°K, 323°K and 338°K. At the termination of the drying process the samples were re-equilibrated to 10 torr aqueous vapour pressure prior to sealing in oxygen or in anoxia. Figures 35 and 36 show the log. survivor/heating time curves obtained in these experiments for spores heated at 393°K in anoxia and in oxygen respectively. The values of k and s computed from the experimental data are recorded in Table 24 and illustrated as a function of the drying temperature in Figures 37a and 37b.

Under all drying conditions investigated, re-equilibration of the dried spores to 10 torr aqueous pressure results in increased heat resistance. Heat sensitivity of re-equilibrated spores, as with non-equilibrated spores is greater in the presence of oxygen than anoxia. In contrast to non-equilibrated spores, the sensitivity of re-equilibrated spores is not influenced by the temperature of drying over the range 273°K to 323°K. Only in spores dried at 338°K prior to re-equilibration is any difference in heat resistance apparent, an increase in the value of k being observed in both oxygen and in anoxia, and a decrease in s in the presence of oxygen.

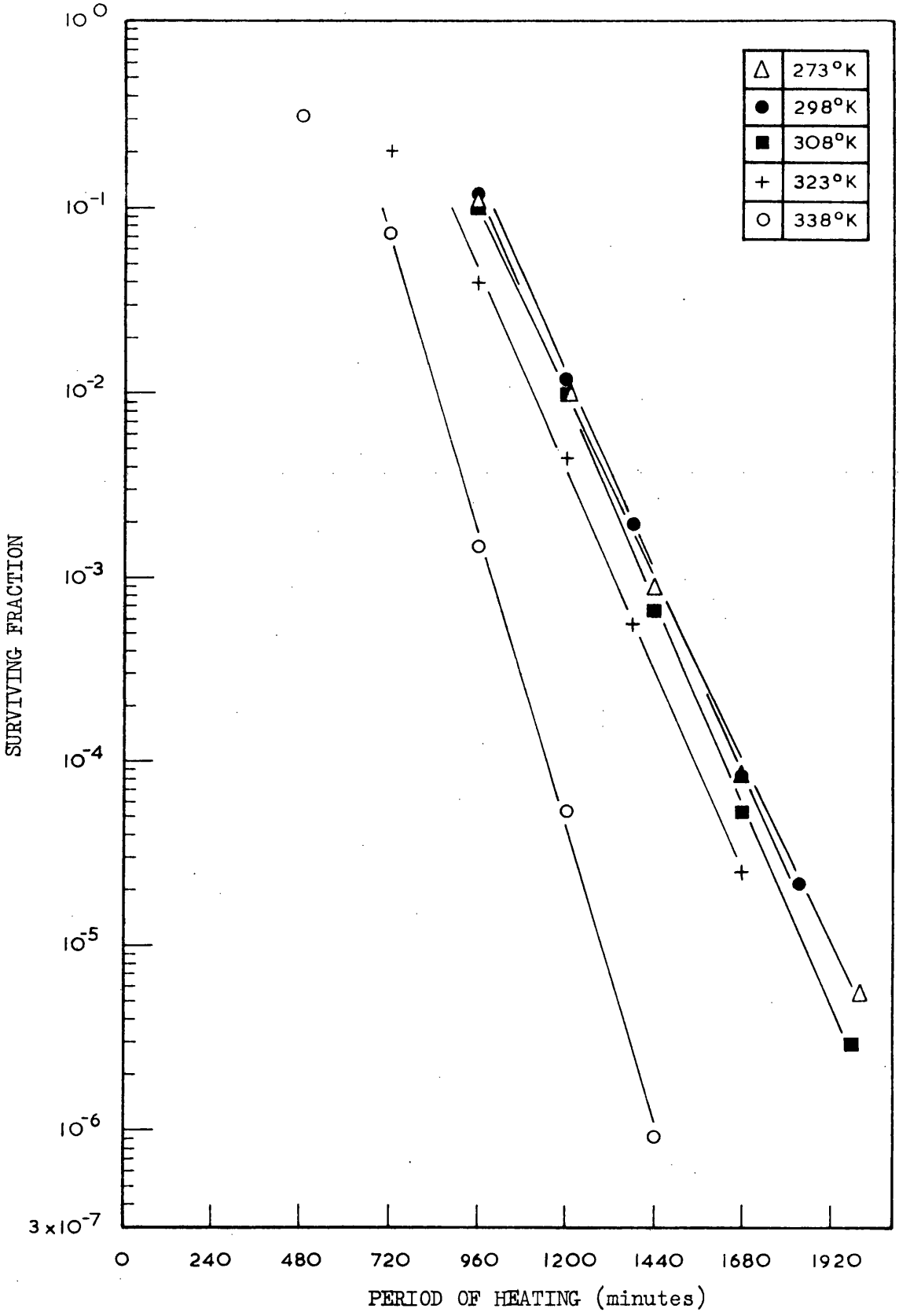


Figure 35. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for 6 hours at different temperatures, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in anoxia.

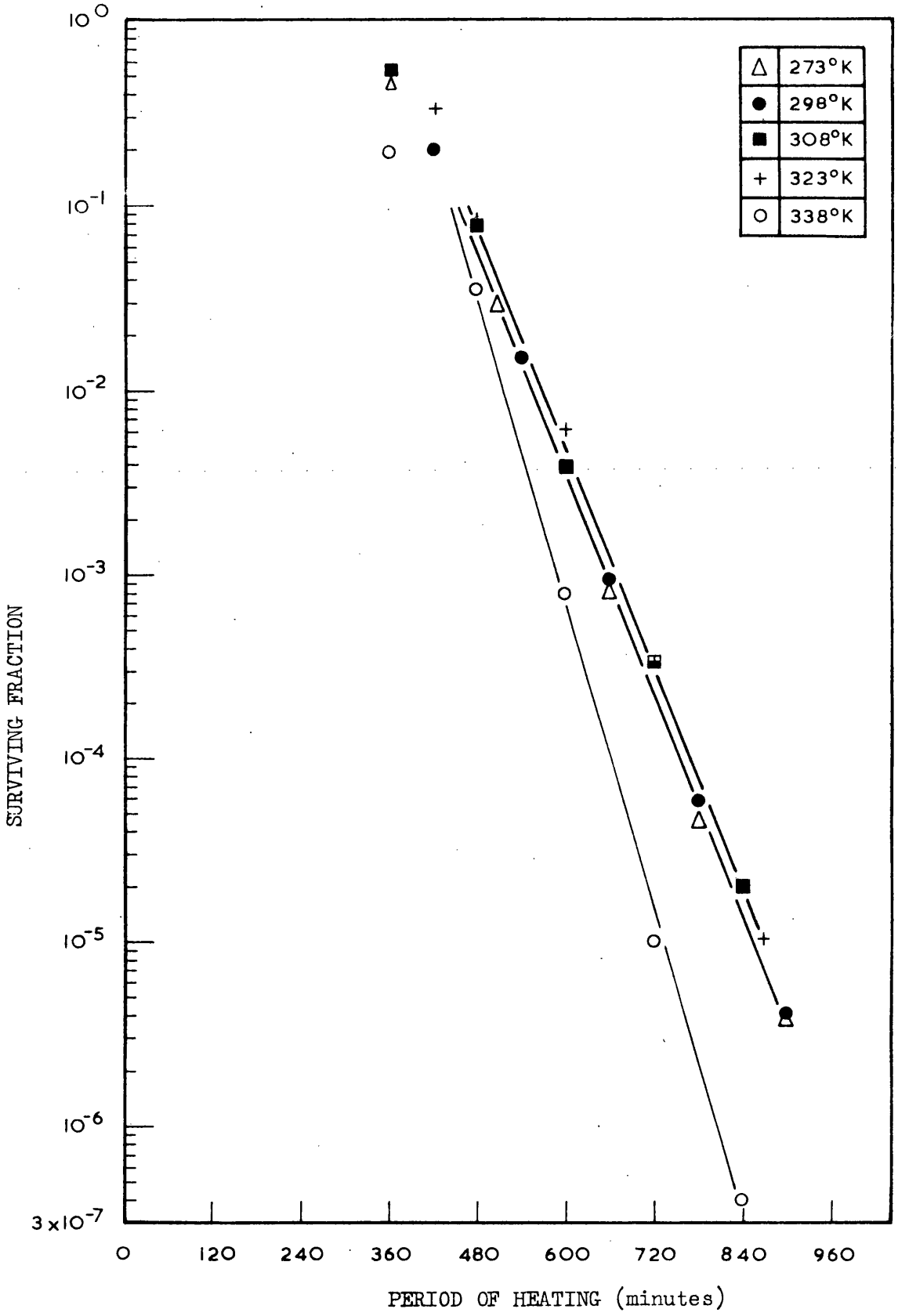
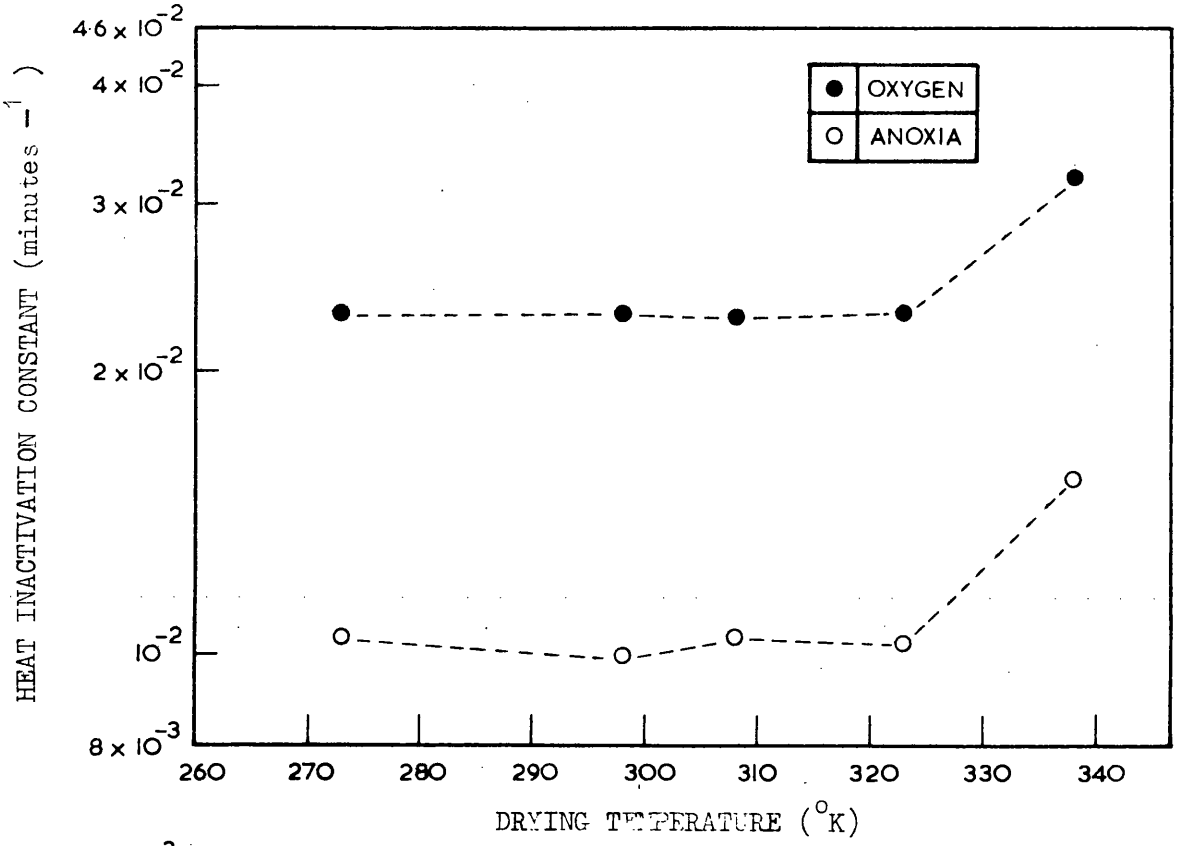


Figure 36. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for 6 hours at different temperatures, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in oxygen.

TABLE 24

Values of k, ln.a and s with associated standard errors for *Bacillus megaterium* spores dried for 6 hours at different drying temperatures, re-equilibrated to 10 torr aqueous vapour pressure, and heated at 393°K, in anoxia, and in oxygen.

Drying Temperature (°K)	k (minutes^{-1})	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
Heated in anoxia					
273	1.055×10^{-2}	7.3706×10^{-4}	9.0017	1.2602×10^{-1}	1.0715×10^{3}
298	1.0114×10^{-2}	7.8488×10^{-5}	7.7157	1.2711×10^{-1}	9.905×10^{2}
308	1.0473×10^{-2}	9.4917×10^{-5}	7.8731	1.6159×10^{-1}	9.716×10^{2}
323	1.0309×10^{-2}	1.154×10^{-4}	6.7992	1.6515×10^{-1}	8.829×10^{2}
338	1.5471×10^{-2}	1.4576×10^{-4}	8.5334	1.8009×10^{-1}	7.004×10^{2}
Heated in oxygen					
273	2.3019×10^{-2}	2.2274×10^{-4}	8.1144	1.7425×10^{-1}	4.525×10^{2}
298	2.2966×10^{-2}	1.0479×10^{-4}	8.1886	8.173×10^{-2}	4.568×10^{2}
308	2.2744×10^{-2}	2.4017×10^{-4}	8.2717	1.7412×10^{-1}	4.649×10^{2}
323	2.3208×10^{-2}	1.6085×10^{-4}	8.6984	1.1928×10^{-1}	4.74×10^{2}
338	3.2052×10^{-2}	4.4369×10^{-4}	1.1966×10	3.2167×10^{-1}	4.452×10^{2}



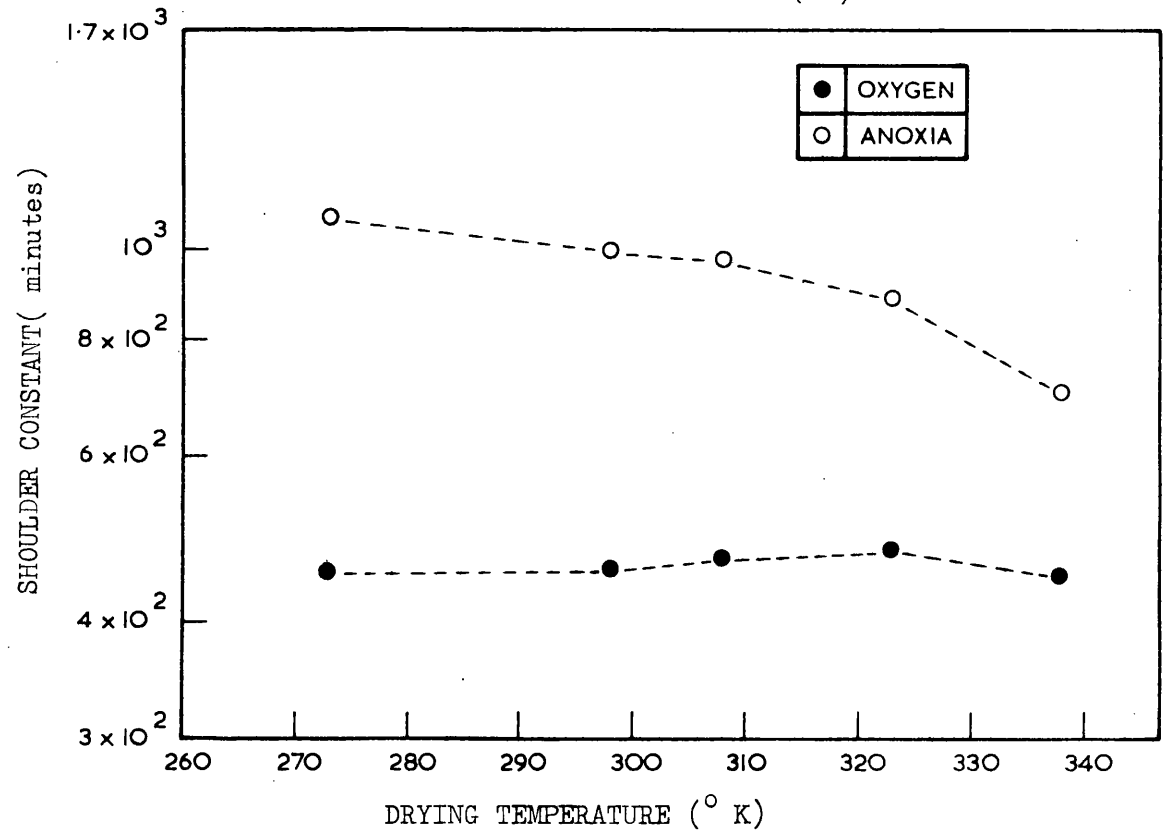


Figure 37. Plot of (a) "heat inactivation constant" and (b) "shoulder constant", on a log. scale, against "drying temperature", for Bacillus megaterium spores dried for 6 hours, re – equilibrated to 10 torr. aqueous vapour pressure and heated at 393^{o} K (in oxygen and in anoxia).

Effect of Different Drying Times on the Heat Resistance of Spores Dried at 298°K and 338°K

Samples of spore suspension were dried on different occasions for 6 hours, 7 hours, 9 hours, 12 hours and 24 hours at 298°K and sealed in anoxia and in oxygen. The dried samples were heated at 393°K and the log. survivor/heating time curves obtained are shown in Figures 38 and 39 for spores heated in anoxia and in oxygen respectively.

In a repeat series of experiments the effect of prolonged drying at the highest temperature studied (338°K) was investigated. Spores were dried for 6 hours, 12 hours and 24 hours at this temperature. Samples were heated at 393°K in anoxia and in oxygen and log.survivor/heating time curves were constructed.

The values of the heat inactivation constants and shoulder constants computed from the data from these experiments are recorded in Table 25.

Spores dried for different times at both of the temperatures investigated exhibit differences in the magnitudes of the corresponding constants k and s. Extension of the drying time from 6 hours to 12 hours results in a decrease in the value of s and an increase in the value of k. Further prolongation of the drying time from 12 hours to 24 hours has little effect on the observed anoxia and oxic heat resistance of spores dried at 298°K but results in a decrease in the value of s with spores dried at 338°K.

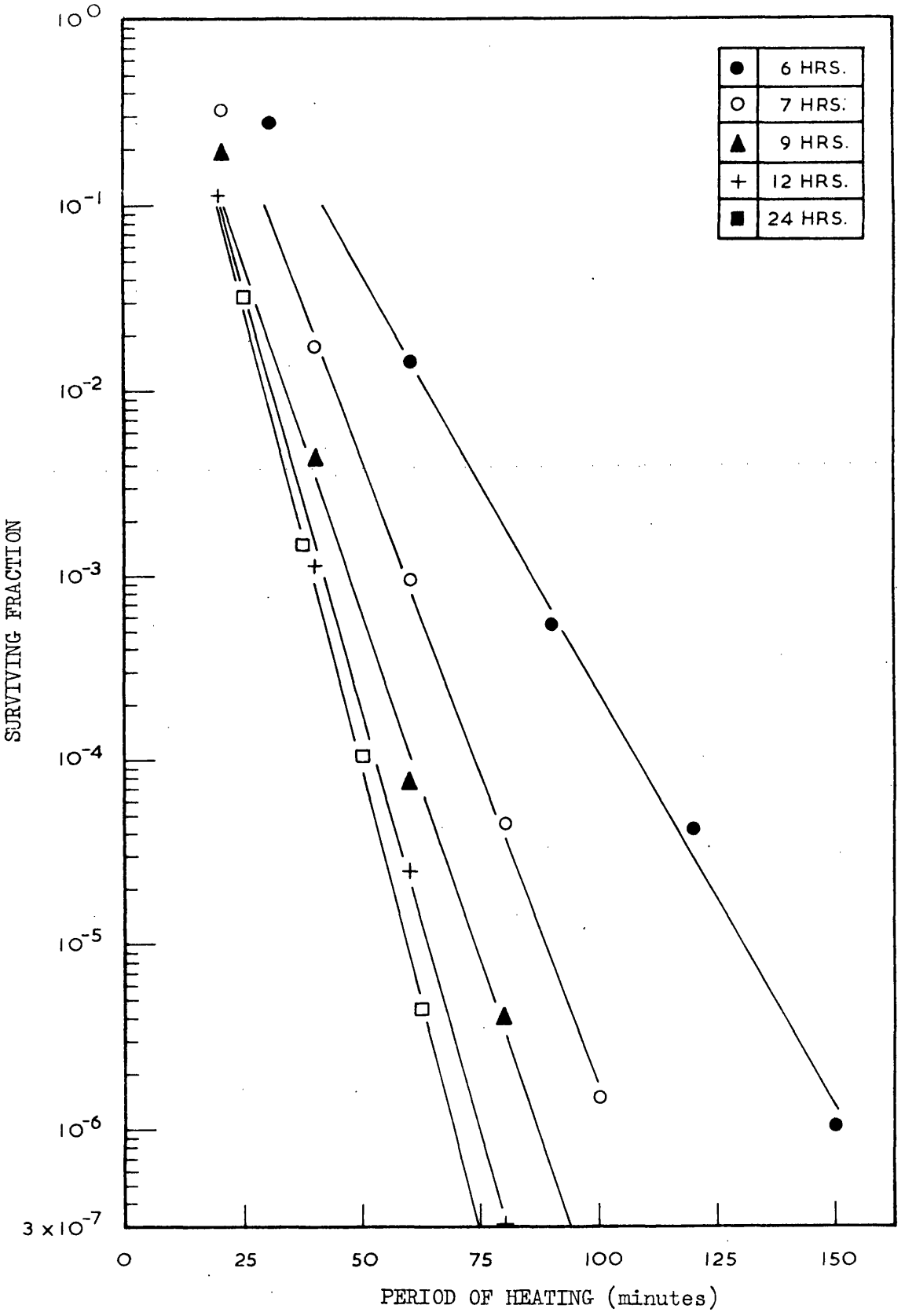


Figure 38. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores, dried for different times at 298°K, and heated in anoxia.

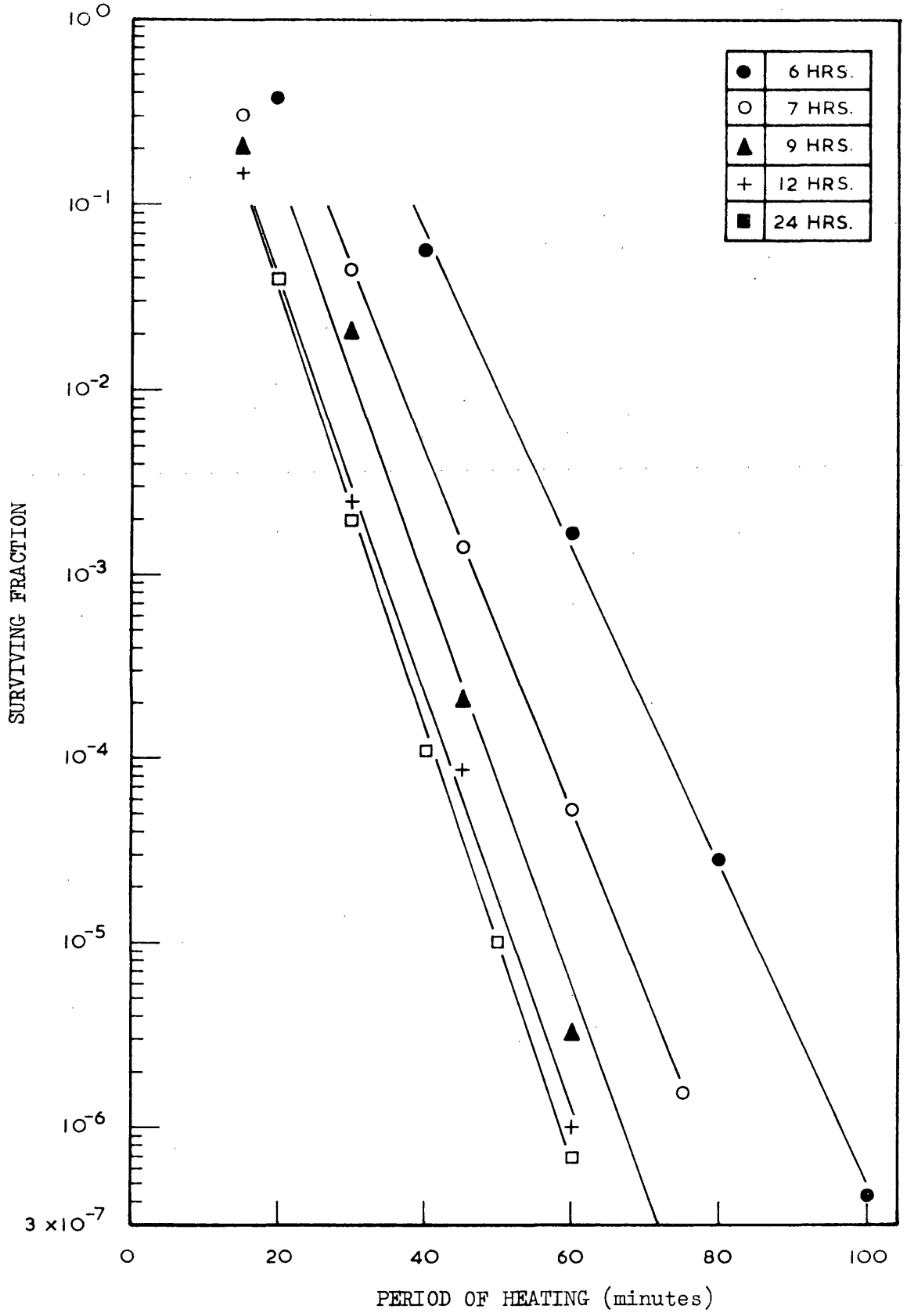


Figure 39. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for different times at 298°K, and heated in oxygen.

TABLE 25

Values of k, ln.a, and s with associated standard errors for Bacillus megaterium spores dried for different times at 298°K and 338°K, and heated at 393°K, in anoxia, and in oxygen.

Drying Time (Hours)	k ($minutes^{-1}$)	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
Dried at 298°K, heated in anoxia					
6	1.0375×10^{-1}	1.6908×10^{-3}	2.0087	2.1818×10^{-1}	4.155x10
7	1.5596×10^{-1}	1.6975×10^{-3}	2.3094	1.4603×10^{-1}	2.957x10
9	1.8037×10^{-1}	2.5595×10^{-3}	1.6333	2.2018×10^{-1}	2.182x10
12	2.1123×10^{-1}	2.0008×10^{-3}	1.9356	1.4148×10^{-1}	2.006x10
24	2.3348×10^{-1}	1.362×10^{-3}	2.3836	9.0088×10^{-2}	2.007x10
Dried at 298°K, heated in anoxia					
6	1.9742×10^{-1}	1.9057×10^{-3}	5.2216	1.6394×10^{-1}	3.811x10
7	2.2776×10^{-1}	9.5818×10^{-4}	3.7456	6.182×10^{-2}	2.656x10
9	2.541×10^{-1}	5.2224×10^{-3}	3.1132	3.3693×10^{-1}	2.131x10
12	2.6014×10^{-1}	5.9431×10^{-3}	1.9952	3.045×10^{-1}	1.652x10
24	2.7257×10^{-1}	2.6388×10^{-3}	2.07	1.3963×10^{-1}	1.604x10
Dried at 338°K, heated in anoxia					
6	1.0883×10^{-1}	8.5945×10^{-4}	5.5307×10^{-1}	9.2416×10^{-2}	2.624x10
12	1.8621×10^{-1}	2.1548×10^{-3}	1.2661	1.7238×10^{-1}	1.916x10
24	2.0302×10^{-1}	3.2062×10^{-3}	9.1795×10^{-1}	1.9237×10^{-1}	1.594x10
Dried at 338°K, heated in oxygen					
6	1.7023×10^{-1}	1.431×10^{-3}	1.7769	1.231×10^{-1}	2.396x10
12	2.1165×10^{-1}	1.2694×10^{-3}	2.2511	8.0873×10^{-1}	2.152x10
24	2.251×10^{-1}	9.0653×10^{-4}	1.084	4.5327×10^{-2}	1.504x10

The Modifying Effect of Re-equilibration to 10 torr Aqueous Vapour Pressure on the Heat Resistance of Spores Dried for Different Times at 298°K and 338°K

On different occasions samples were dried for 6 hours, 12 hours and 24 hours at 298°K, and 338°K. At the end of the drying period the samples were re-equilibrated to 10 torr aqueous vapour pressure prior to sealing in anoxia or in oxygen. The dried spores were heated at 393°K and log. survivor/heating time curves were constructed. The anoxic and oxic curves obtained with spores dried at 298°K are shown in Figures 40 and 41.

Values of k and s computed from the data used in the construction of these curves is given in Table 26. Plots of these constants as a function of the drying time at each temperature are illustrated in Figures 44a and 44b.

The data from these experiments show that the increase in heat sensitivity that is observed when spores are dried for increased periods of time, at constant temperature, without re-equilibration, is eliminated if the spores are re-equilibrated after drying. The addition of 10 torr water vapour to the dried spores results in an increase in the value of s and a decrease in the value of k. Increased heat resistance is also observed when the spores are subjected to prolonged (24 hour) drying, prior to re-equilibration, but this increase when expressed in terms of changes in the values of k and s is complex, and is dependent upon the drying temperature. The significance of these results and those obtained in the previous heating experiments is considered in more detail in the Discussion (page 280).

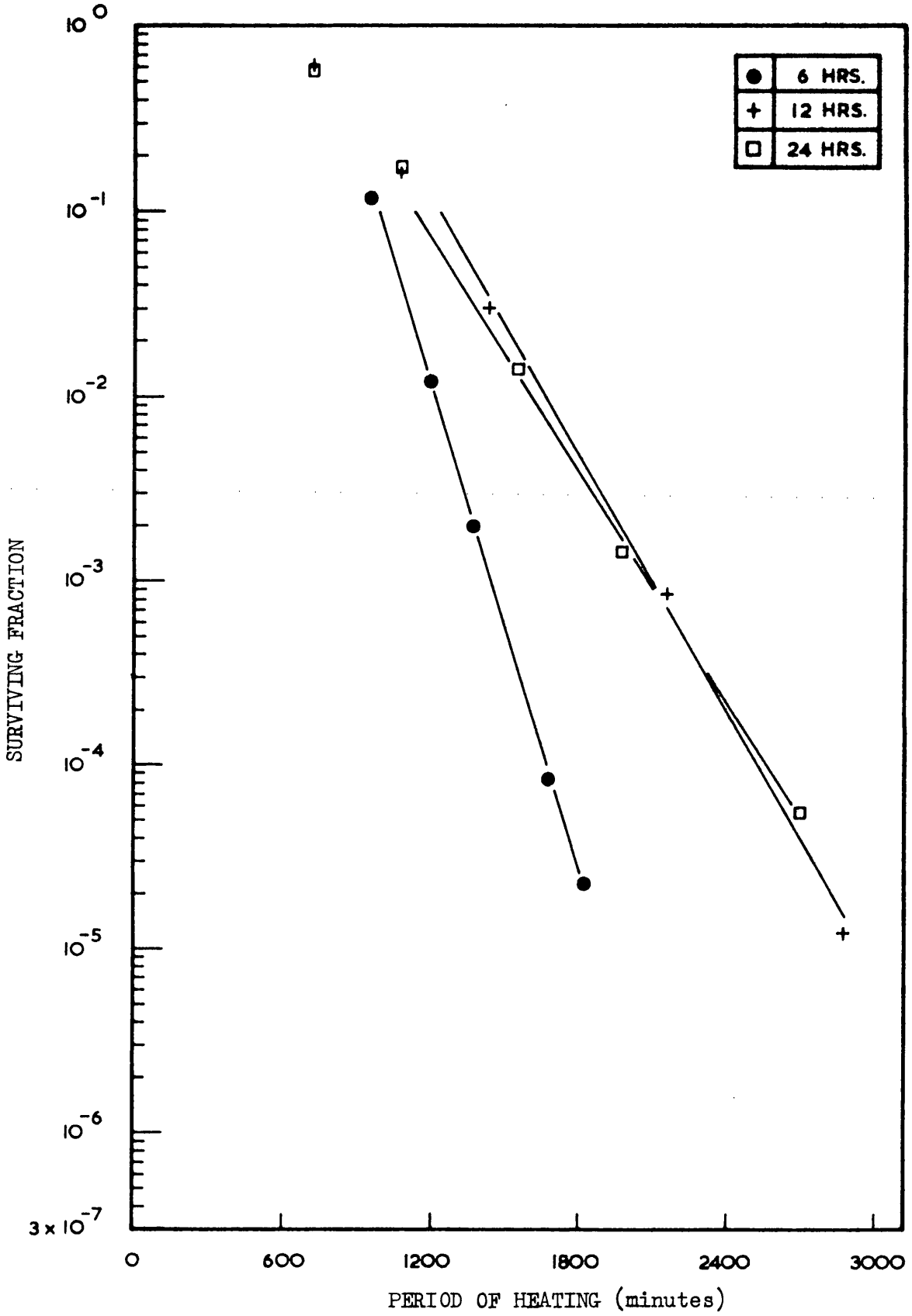


Figure 40. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for different times at 298°K, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in anoxia.

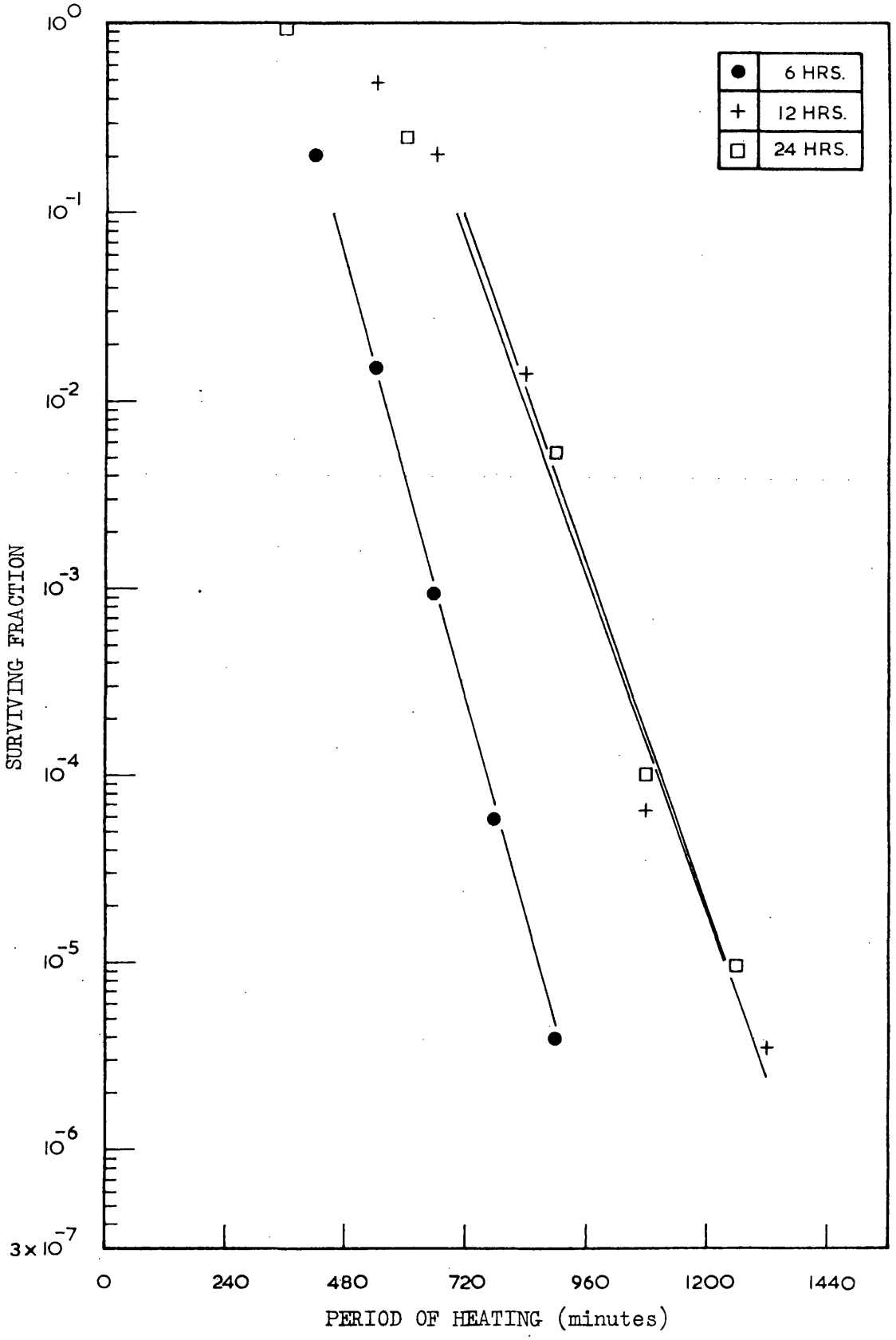


Figure 41. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K for Bacillus megaterium spores dried for different times at 298°K, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in oxygen.

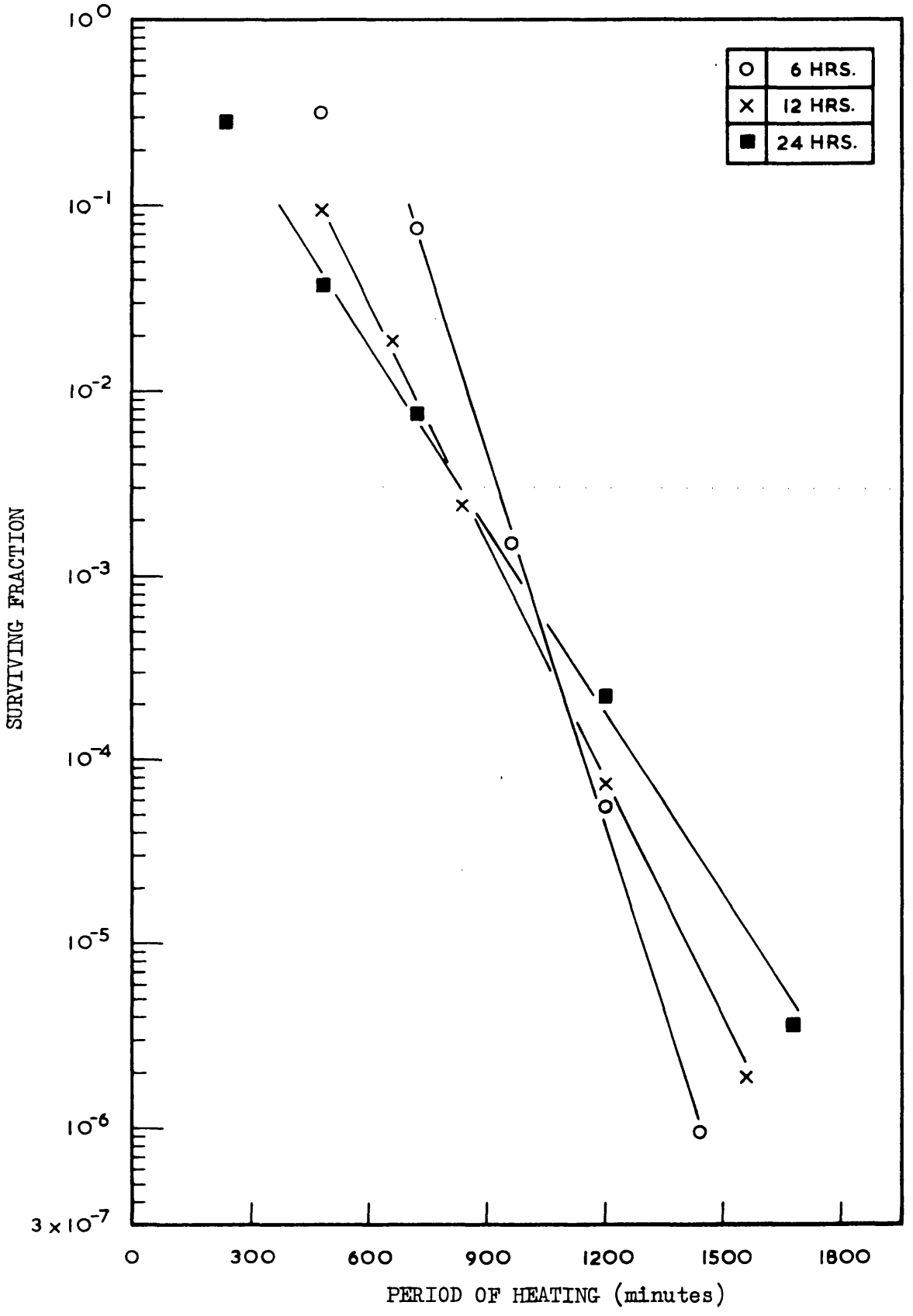


Figure 42. Plot of "surviving fraction" on a log. scale against "period of heating at 393°K" for Bacillus megaterium spores dried for different times at 338°K, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in anoxia.

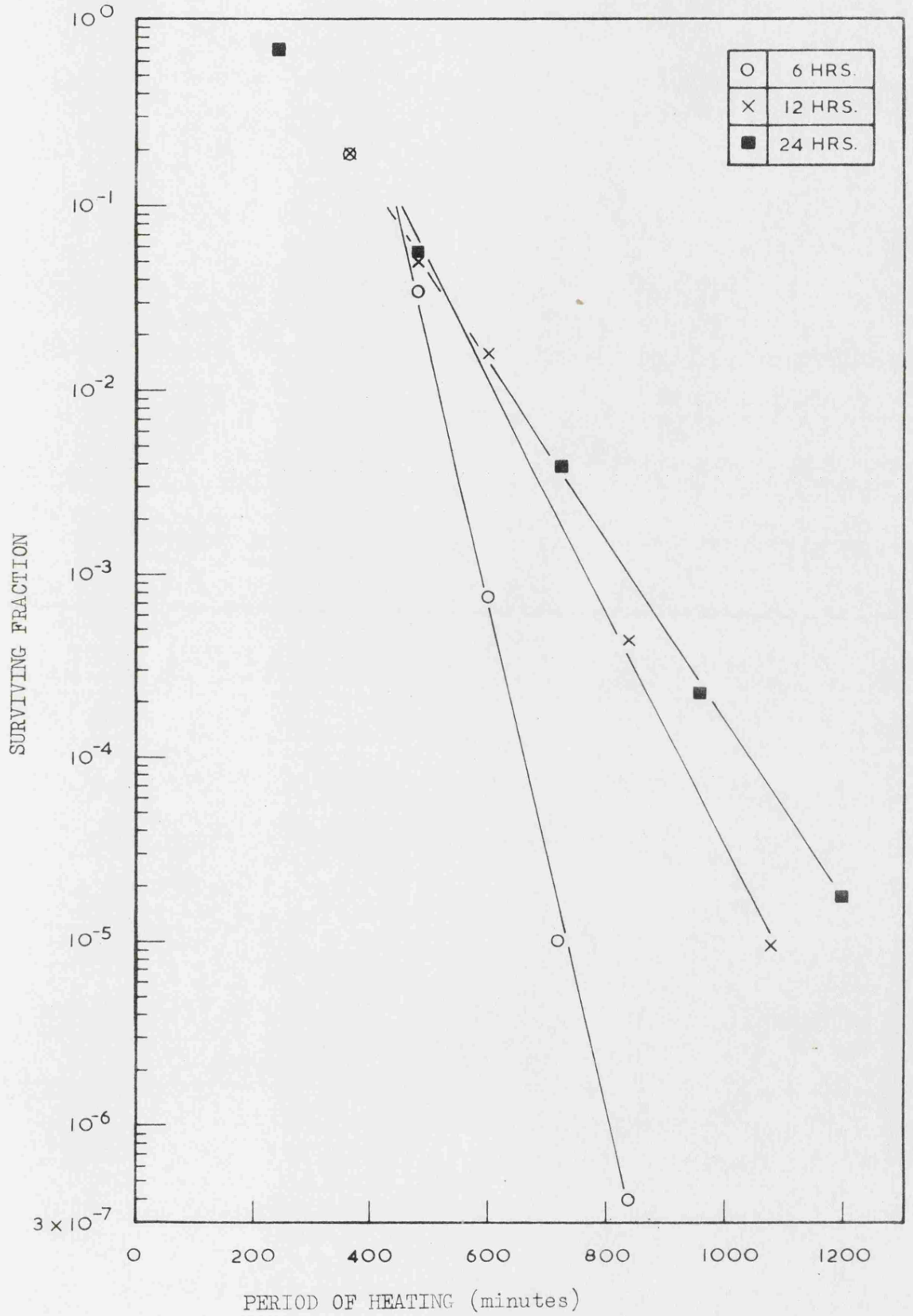
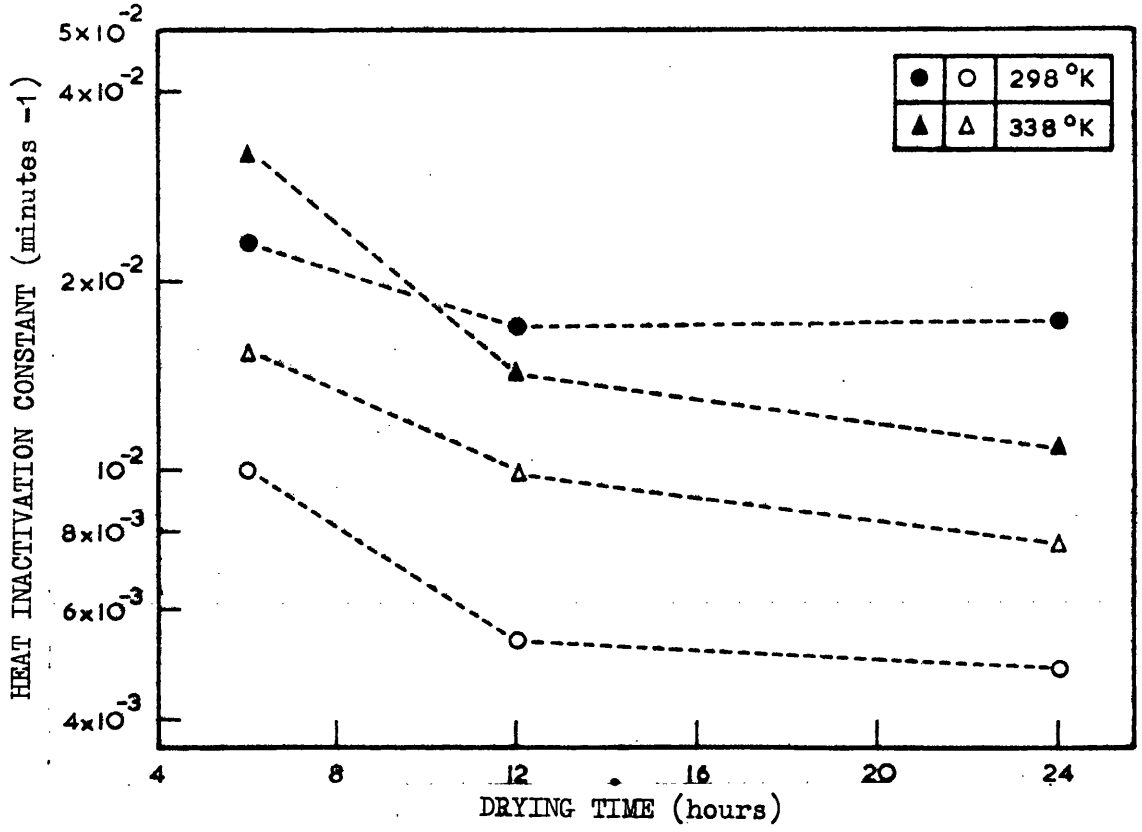


Figure 43. Plot of " surviving fraction" on a log. scale against " period of heating at 393° K" for Bacillus megaterium spores dried for different times at 338° K, re - equilibrated to 10 torr. aqueous vapour pressure, and heated in oxygen.

TABLE 26

Values of k, ln.a, and s, with associated standard errors for *Bacillus megaterium* spores dried for different times at 298°K and 338°K, re-equilibrated to 10 torr aqueous vapour pressure, and heated at 393°K in anoxia, and in oxygen.

Drying Time (Hours)	k ($minutes^{-1}$)	Standard Error of k	ln. a	Standard Error of ln. a	s (minutes)
Dried at 298°K, heated in anoxia					
6	1.0114×10^{-2}	7.8488×10^{-5}	7.7157	1.2711×10^{-1}	9.905×10^{2}
12	5.4207×10^{-3}	7.6307×10^{-5}	4.4247	1.8766×10^{-1}	1.241×10^{3}
24	4.8544×10^{-3}	6.6378×10^{-5}	3.2311	1.5155×10^{-1}	1.14×10^{3}
Dried at 298°K, heated in oxygen					
6	2.2966×10^{-2}	1.0479×10^{-4}	8.1886	8.173×10^{-2}	4.568×10^{2}
12	1.7243×10^{-2}	8.2557×10^{-4}	9.7928	9.4851×10^{-1}	7.015×10^{2}
24	1.7564×10^{-2}	7.2229×10^{-4}	1.0297×10	8.0844×10^{-1}	7.174×10^{2}
Dried at 338°K, heated in anoxia					
6	1.5471×10^{-2}	1.4576×10^{-4}	8.5334	1.8009×10^{-1}	7.004×10^{2}
12	9.9672×10^{-3}	7.1979×10^{-5}	2.4044	9.659×10^{-2}	4.722×10^{2}
24	7.696×10^{-3}	1.0091×10^{-5}	5.6967×10^{-1}	1.4632×10^{-1}	3.732×10^{2}
Dried at 338°K, heated in oxygen					
6	3.2052×10^{-2}	4.4369×10^{-4}	1.1966×10	3.2167×10^{-1}	4.452×10^{2}
12	1.4485×10^{-2}	2.5708×10^{-4}	4.2564	2.3393×10^{-1}	4.528×10^{2}
24	1.1192×10^{-2}	7.1755×10^{-5}	2.457	7.4071×10^{-2}	4.253×10^{2}



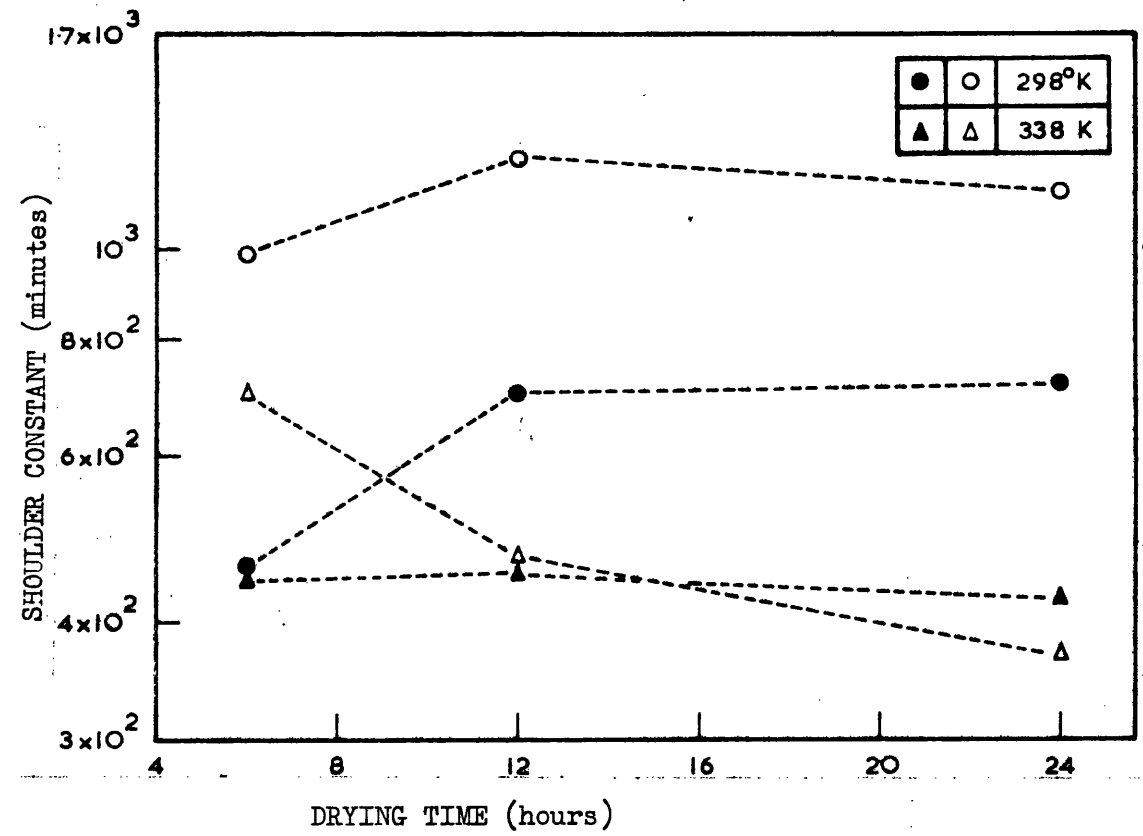


Figure 44. Plot of (a) "heat inactivation constant", and (b) "shoulder constant", on a log. scale, against "drying time", for Bacillus megaterium spores, dried at 298°K and 338°K, and heated at 393°K, in oxygen (closed symbols), and anoxia (open symbols).

Comparison of the Heat Resistance of Re-equilibrated Dried Spores with that of Spores Dried Directly to the Equivalent Water Level

The use of the technique of re-equilibration of dried spores to a given moisture level, as a means of obtaining cells of a given moisture content raises the question of whether the external water which then rehydrates the cells behaves in the same manner as the original cellular water which is removed. Experiments were therefore carried out to compare the heat resistance of spores re-equilibrated to a given water level after drying, with that of spores dried directly to the same water level. From measurements of weight changes in the sample during drying it is possible to determine the drying time at a given temperature at which the weight of the spores is equivalent to that attained after 6 hours drying and subsequent re-equilibration to a defined water level.

Previous heating experiments have studied the resistance of dried spores re-equilibrated to 10 torr aqueous vapour pressure. However, when attempts were made to dry spores directly to a weight equivalent to this re-equilibration level it was found that the rate of drying at the required drying time was so fast that large variations in the water content of successive samples were invariably introduced during the time interval necessary for removal of these samples from the manifold. A re-equilibration aqueous vapour pressure of 5×10^{-2} torr was therefore used in this series of experiments, since the drying rate at the required drying time was sufficiently slow to enable reproducible samples to be obtained.

Experiments reported in a previous section (page 77) have shown that the time taken for spores to dry to a weight level equivalent to that reached after 6 hours drying and subsequent re-equilibration to 5×10^{-2} torr aqueous vapour pressure was 2.5 hours, 3 hours and 4.5 hours at drying temperatures of 298°K, 308°K and 338°K respectively.

On different occasions, samples were dried at each of these three temperatures. After the required drying time half of the samples were sealed off in anoxia, and removed from the manifold. The remaining samples were dried for six hours and re-equilibrated to 5×10^{-2} torr aqueous vapour pressure prior to sealing in anoxia. The Micro-Force Balance was also operated during these experiments to confirm that the partially dried samples and the re-equilibrated samples were at the same weight level.

Samples were heated at $393^{o}K$ and the log.survivor/heating time curves obtained with both partially dried and re-equilibrated spores are shown in Figure 45. The calculated values for the heat inactivation constant k and the shoulder constant s derived from the experimental data are recorded in Table 27. Detailed consideration of these results in terms of the physical and biological integrity of the spore is reserved until the Discussion (page 287).

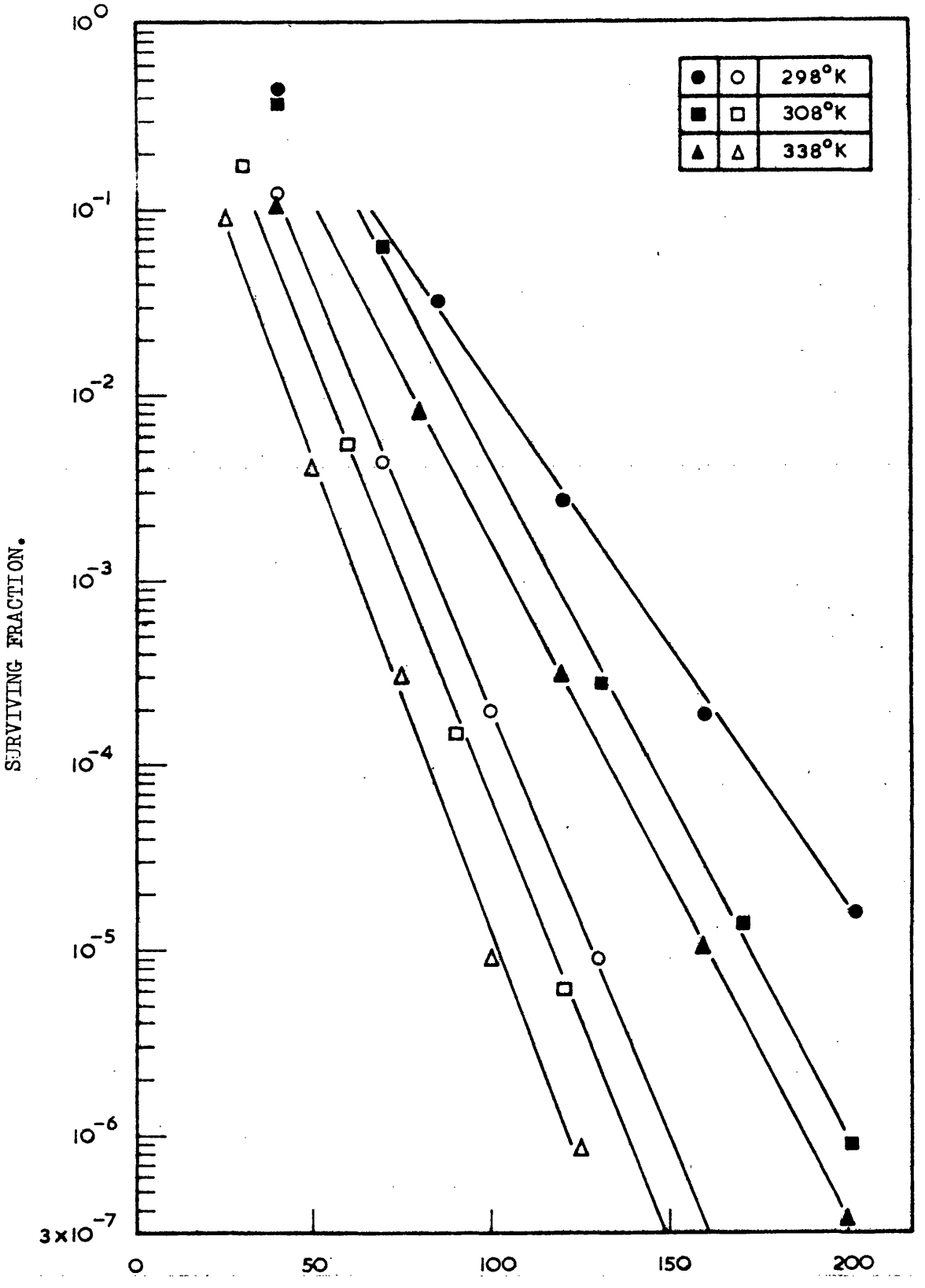


Figure 45. Plot of "surviving fraction" on a log. scale against "period of heating" at 393°K in anoxia, for Bacillus megaterium spores dried at different temperatures. a) dried for 6 hours and re - equilibrated to 5×10^{-2} torr. aqueous vapour pressure (closed symbols) b) dried directly to an equivalent water level (open symbols).

TABLE 27

Values of k, ln.a, and s, with associated standard errors, for Bacillus megaterium spores dried at 298°K, 308°K and 338°K and heated in anoxia at 393°K a) dried for 6 hours and re-equilibrated to 5×10^{-2} torr aqueous vapour pressure b) dried directly to the equivalent water (weight) level.

Drying Time (Hours)/Drying Temperature (°K)	k ($minutes^{-1}$)	Standard Error of k	ln.a	Standard Error of ln. a	s (minutes)
a) Re-equilibrated to 5×10^{-2} torr aqueous vapour pressure					
6 (298	6.5014×10^{-2}	6.0983×10^{-4}	1.9697	1.0516×10^{-1}	6.57 x10
6 (308	8.4929×10^{-2}	9.1161×10^{-4}	3 087	1.3727×10^{-1}	6 346x10
6 (338	8.4022×10^{-2}	3.7363×10^{-4}	1.9641	6.4281×10^{-2}	5.078x10
b) Dried directly to an equivalent weight					
2.5/298	1.072×10^{-1}	9.4058×10^{-4}	2.1554	1.2914×10^{-1}	4.159x10
3 /308	1.1084×10^{-1}	1.0602×10^{-3}	1.3455	1.368×10^{-1}	3.291x10
4.5/338	1.1714×10^{-1}	1.4117×10^{-3}	4.5307×10^{-1}	1.6173×10^{-1}	2.352x10

MEASUREMENT OF RADIATION RESISTANCE

Introduction

It has been shown by Tallentire and Powers (160) that spores are more sensitive to radiation in the dry state than in the presence of water. They further demonstrated that the greatest sensitivity was exhibited at an equilibrium vapour pressure of below 10^{-4} torr, and in the presence of oxygen. Davies (152) has shown that spores of Bacillus megaterium exhibit their maximum sensitivity to radiation when they are irradiated in the presence of oxygen, and subsequently stored under conditions which permit the development of the total oxygen-dependent damage. He has also demonstrated that the rate of development of the maximum post-irradiation oxygen effect is strongly temperature dependent, and has estimated the times necessary for the completion of this oxygen effect, for different storage temperatures.

It was considered that the most meaningful data would be obtained by studying the influence of drying, on spores irradiated in conditions under which the maximum sensitivity to radiation has been observed, i.e. where both oxygen dependent and oxygen independent mechanisms are operative. Thus it was decided to use a standard experimental technique, of irradiation in the presence of oxygen, followed by post irradiation storage, in oxygen, for 18 hours at $323^{o}K$.

Materials

Source of gamma rays - this was a Super Hotpoint "self contained" source, containing a 4724 curie Cobalt 60 source, and housed at the Agricultural Research Council, Meat Research Institute, at Langford, Bristol. The source consists of a ring of eight fixed rods of Cobalt 60 positioned inside a large lead and concrete drum. A sample cage 3" in diameter can be raised or lowered into the source by means of a pulley

connected to an electric motor. The cage can be lowered to any depth inside the source, and at its lowest position is exposed to gamma rays from the eight Cobalt 60 rods which are spaced equidistantly around the cage. The sample cage is constructed from $1\frac{1}{2}$" diameter steel tube, fitted with a steel platform at the lower end, and with ports cut out of the sides for the introduction of irradiation jigs.

The irradiation jig - this is illustrated in Figure 46 and consists of a block of nylon 40mm. thick, shaped to fit into the sample cage. Ten holes each 10mm. in diameter were bored out of the block to a depth of 34mm. to hold the sample vessels in a fixed position during irradiation.

Methods

Determination of the dose rate of the Cobalt 60 source

The dose rate in the sample vessel was estimated by using the Dewhurst (183) modification of the Ficke ferrous sulphate dosimeter (184).

Using "Analar" grade reagents a stock solution of 8N sulphuric acid was prepared by making up 213ml. of sulphuric acid to 1 litre with glass-distilled water, and a stock solution of 10^{-2} molar sodium chloride by dissolving 9.355g. of sodium chloride in 1 litre of glass-distilled water.

25ml. quantities of these two stock solutions were taken together with approximately 98.5mg. of ferrous ammonium sulphate, $Fe(SO_4)_2(NH_4)_2$, $6H_2O$ and made up to 250ml. with glass-distilled water. 1.6ml. samples of the dosimetric solution were measured into ten clean sample vessels, which were then closed with clean aluminium caps and the solutions were irradiated for 2 minutes.

Following irradiation, the solution was transferred from the sample vessel to one of a matched pair of clean 0.5cm. quartz cells. In the

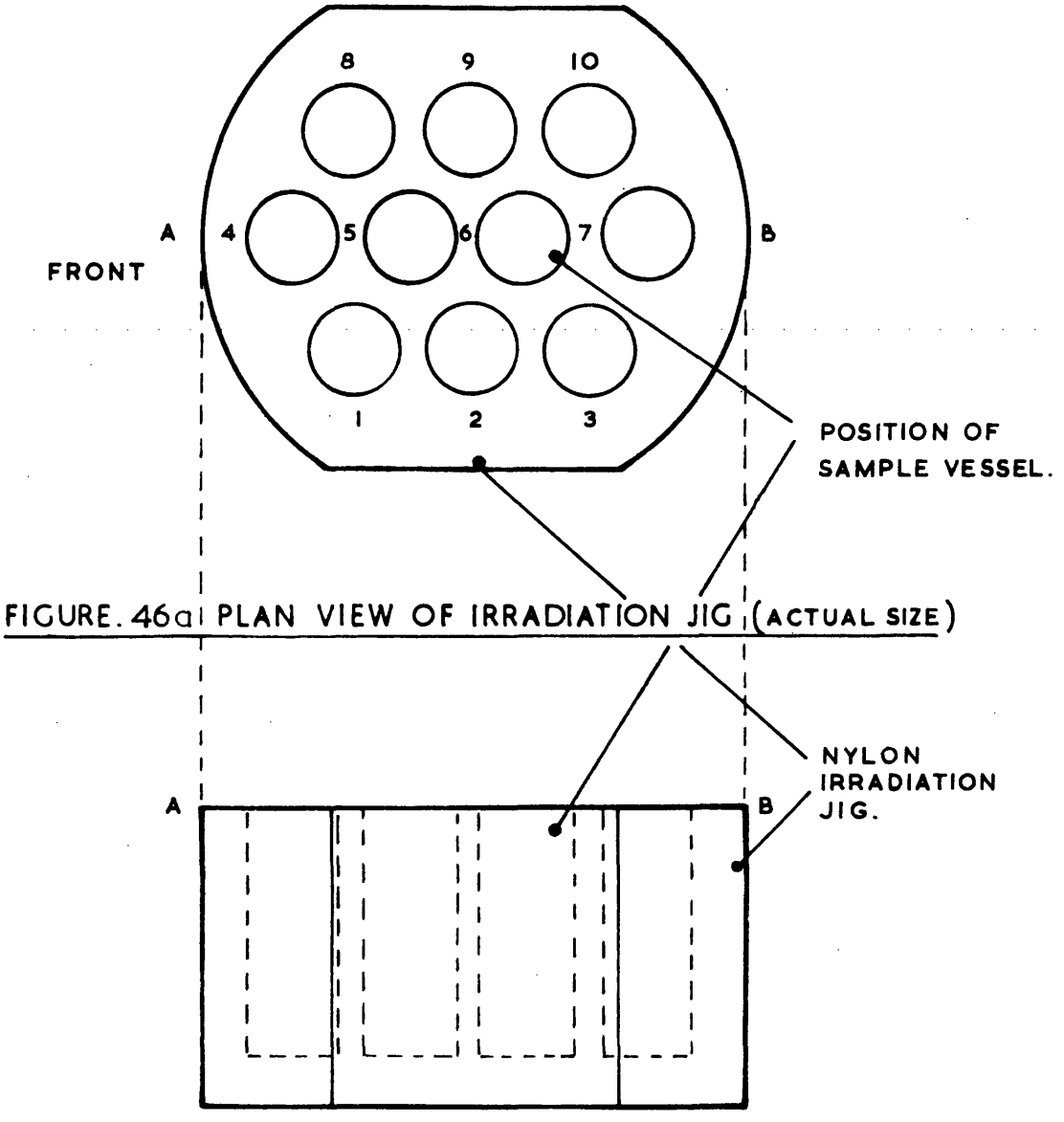


FIGURE. 46a PLAN VIEW OF IRRADIATION JIG (ACTUAL SIZE)

FIGURE. 46 b SIDE VIEW OF IRRADIATION JIG (ACTUAL SIZE)

other cell was placed 1.6ml. of a solution containing 0.8N sulphuric acid and 10^{-3}M sodium chloride in glass-distilled water for comparative readings. The yield of ferric ion in the solutions was determined by measuring the optical density at the λ_{max} of 303 mμ (185) and at a temperature of 298°K, using a Unicam S.P.500 Spectrophotometer fitted with a constant temperature cell. A control reading was taken of an unirradiated sample of dosimetric solution, and the experimental reading was corrected for the amount of ferric ion present in the unirradiated solution. The corrected optical densities for solutions irradiated in each of the positions (numbered 1 to 10) in the jig are recorded in Table 28. All dosimetry determinations and experiments were carried out with the sample cage at its lowest position in the source during irradiation.

The dose rates were calculated using a G value of 15.5 (186) Thus the dose rate in rads per minute was calculated as follows:-

$$\text{Number of rads per minute} = \frac{\text{O.D.} \times 10^{23} \times 6.02 \times 1.6}{0.5 \times 2.24 \times 10^{4} \times 15.5 \times 10^{14} \times t}$$

where O.D. is the optical density at 303 mμ, and t is the time of irradiation.

The dose rates used in the experiments reported in this section were corrected to the same date using the decay factor for the Cobalt 60 isotope obtained from Murray (187). Previous workers (185,188) have shown that the sample cage returns to the same position on each occasion that it is lowered into the source, and that the dose rate at each position in the jig is reproducible. It is apparent from Table 28 that owing to the construction and geometry of the source, sample vessels placed in different positions in the jig receive different dose rates. Therefore, during irradiation, all ten positions in the jig were filled with sample vessels or similar glass tubes, the jig was aligned to a particular position on the cage, to ensure that samples would receive

the dose of irradiation calculated from the experimental results given in Table 28 and the times necessary to give the required doses were determined independently for each position in the jig.

TABLE 28

Optical densities at 303 mμ of dosimetric solutions irradiated in numbered positions in the irradiation jig, and corresponding dose rates.

Position in jig	Corrected Optical Density Readings at 303 mμ	Mean Optical Density Reading	Number of Rads per minute
1	0.446, 0.443, 0.443	0.444	12.31×10^3
2	0.419, 0.415, 0.414	0.416	11.54×10^3
3	0.436, 0.430, 0.434	0.435	12.07×10^3
4	0.466, 0.464, 0.464	0.465	13.17×10^3
5	0.423, 0.421, 0.420	0.421	11.68×10^3
6	0.414, 0.414, 0.413	0.414	11.48×10^3
7	0.460, 0.457, 0.457	0.458	12.71×10^3
8	0.440, 0.440, 0.439	0.440	12.2×10^3
9	0.418, 0.416, 0.416	0.417	11.52×10^3
10	0.441, 0.440, 0.440	0.440	12.2×10^3

Treatment of Results

Examination of log.survivor/dose curves obtained with Bacillus megaterium spores indicates that they are sigmoidal having a small shoulder at high survival levels, i.e. they are type "b" curves. By inspection the curves are, however, linear below the 5×10^{-1} level of surviving fraction.

The type "b" curve can be described by the expression

$$\frac{N}{N_o} = 1 - (1 - e^{-kD})^n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (21)$$

where $\frac{N}{N_o}$ is the fraction of spores surviving a dose of radiation D, k is the slope of the log.survivor/dose curve, expressed in reciprocal kilorads, n is the intercept with the y axis of the extrapolated linear portion of the curve, expressed in terms of surviving fraction. In practice it is more usual to use the logarithmic transformation of equation(21) where n is expressed as ln. surviving fraction.

The log.survivor/dose curves obtained for different experimental conditions can be described by k and n and hence these constants are a measure of the radiation resistance of the spore population under defined experimental conditions.

Dickinson (150) has shown that the errors involved in using the equation $\frac{N}{N_o} = ne^{-kD}$ in place of expression(21) to determine the values of k and n for type "b" curves are insignificant under certain defined practical conditions. The conditions he has recommended are that

(a) five observations occur at each dose level,

(b) the minimum dose (other than zero dose) yields a surviving fraction of below 5×10^{-1}

(c) other dose levels are related to the minimum in the ratio of 1:2:3:4:5: or 1:2:4:6:8.

It was thus decided to implicate these recommendations and use the logarithmic transformation of the expression $\frac{N}{N_o} = ne^{-kD}$ to determine

the constants for all log.survivor/dose curves obtained during this work. An estimate of the number of survivors at a single dose level was calculated from individual plate counts obtained from quintuplicate platings. Using these values and those obtained from unirradiated controls estimates were made of the ln. surviving fraction at a single dose level. Thus each estimate from individual plate counts was used as a single observation. It also follows that only data obtained from dose levels resulting in a surviving fraction of below 5×10^{-1} were used in the computation of values of k and n. The number of observations used in the estimation of the constants was therefore generally twenty and never less than fifteen. It is assumed for the purposes of this thesis, that the ln. number of survivors are normally distributed at each survivor level, and consequently no weighting factor was used to adjust the significance of individual observations with different doses.

The values of the constants and their standard errors were calculated using a least squares analysis programmed on an I.B.M. Series 4-50 medium speed digital computer. The analysis program, written in Series 4-50 Algol, is included in Appendix II.

The Effect of Drying Time and Temperature on the Radiation Resistance of Bacillus megaterium Spores

The general technique employed in irradiation experiments was as follows. Six samples of spore suspension SS6S were dried for a chosen duration at a defined drying temperature. At the termination of the drying process the samples were sealed off in the presence of oxygen. Five of the samples were irradiated with different radiation doses in the ratio of 1:2:3:4:5, in the presence of oxygen, the sixth being used as the unirradiated control. After irradiation, all the samples were stored for 18 hours, in oxygen, at 323°K. Following this post irradiation treatment the spores were subjected to aqueous reconstitution and the standard counting technique described previously (see page 43).

In a series of experiments to determine the effect of drying time and temperature on the radiation sensitivity of spores, three times and three temperatures were used. The drying temperatures investigated were 273°K, 298°K, and 338°K. Samples of spore suspension were dried, on different occasions, for periods of 6, 12, or 24 hours at each of these three temperatures. At the end of the required drying treatment the spores were irradiated in the presence of oxygen and subjected to the standard post irradiation storage treatment. Log.survivor/dose curves were constructed for spores dried under each experimental condition, and are illustrated in Figures 47, 48, 49 and 50. The data used in the construction of these curves were subjected to mathematical analysis as described on page 188, and the calculated values of the constants k and n, together with their standard errors are recorded in Table 29.

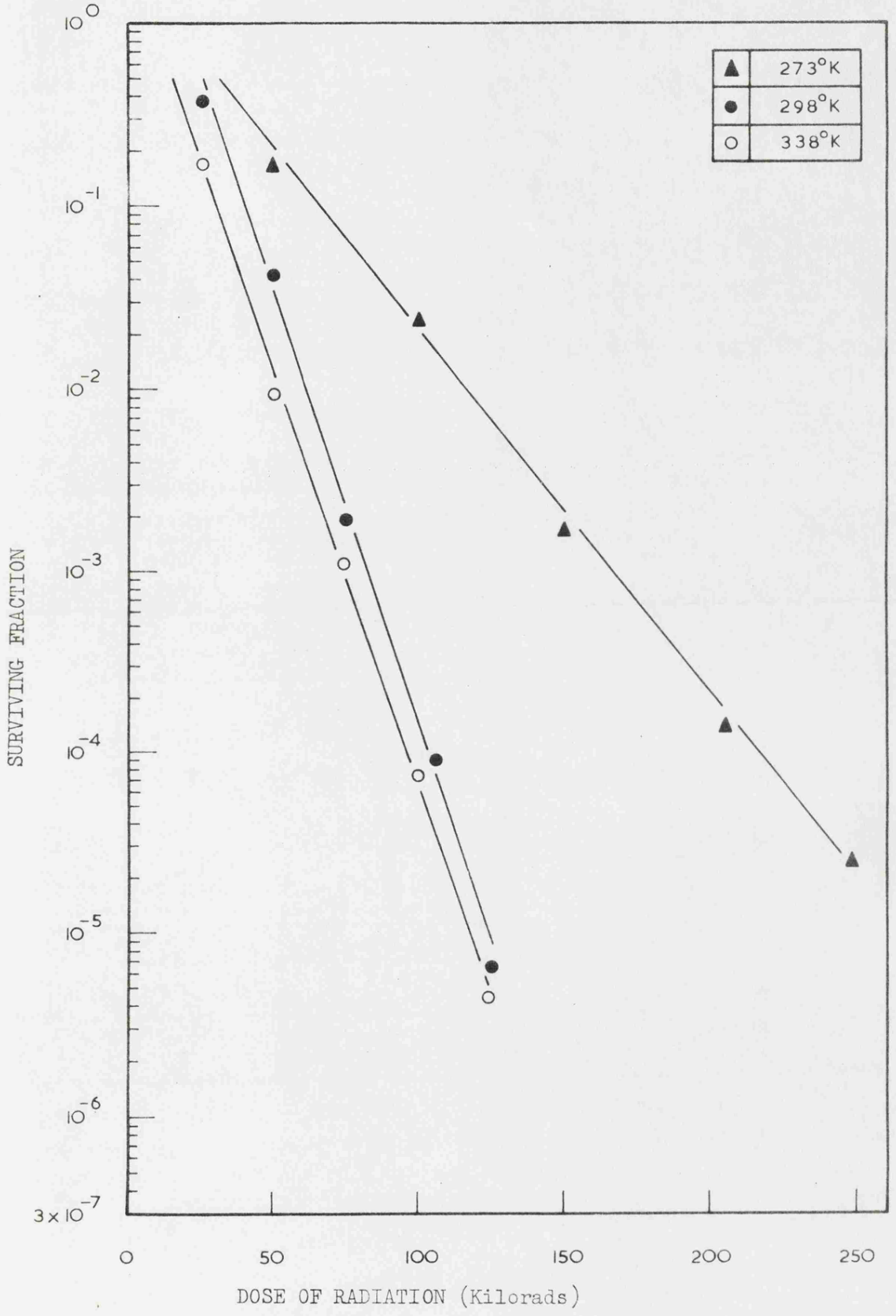


Figure 47. Plot of " surviving fraction" on a log . scale against " dose of radiation " for Bacillus megaterium spores, dried for 6 hours at different temperatures , and irradiated and stored in oxygen.

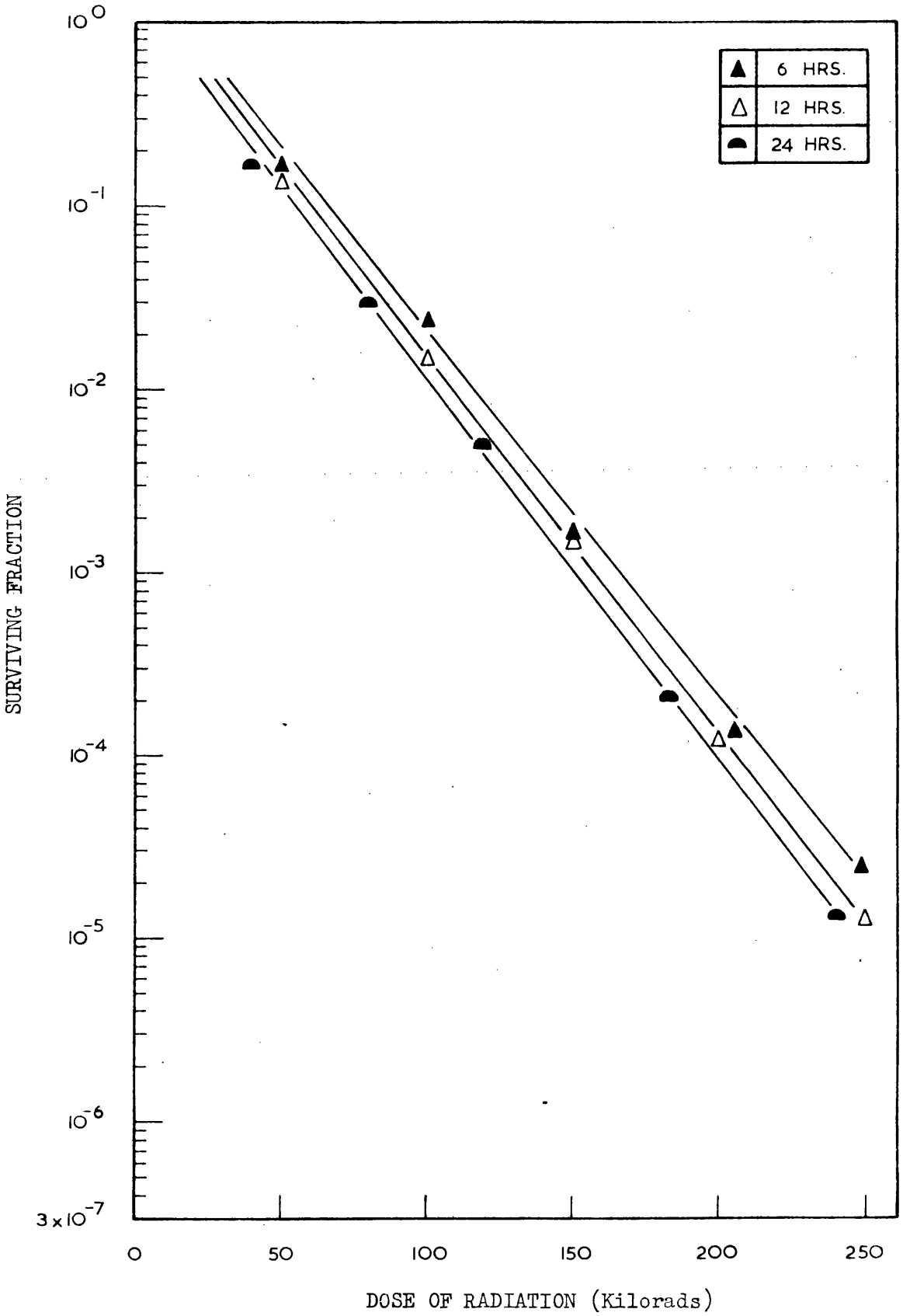


Figure 48. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 273°K and irradiated and stored in oxygen.

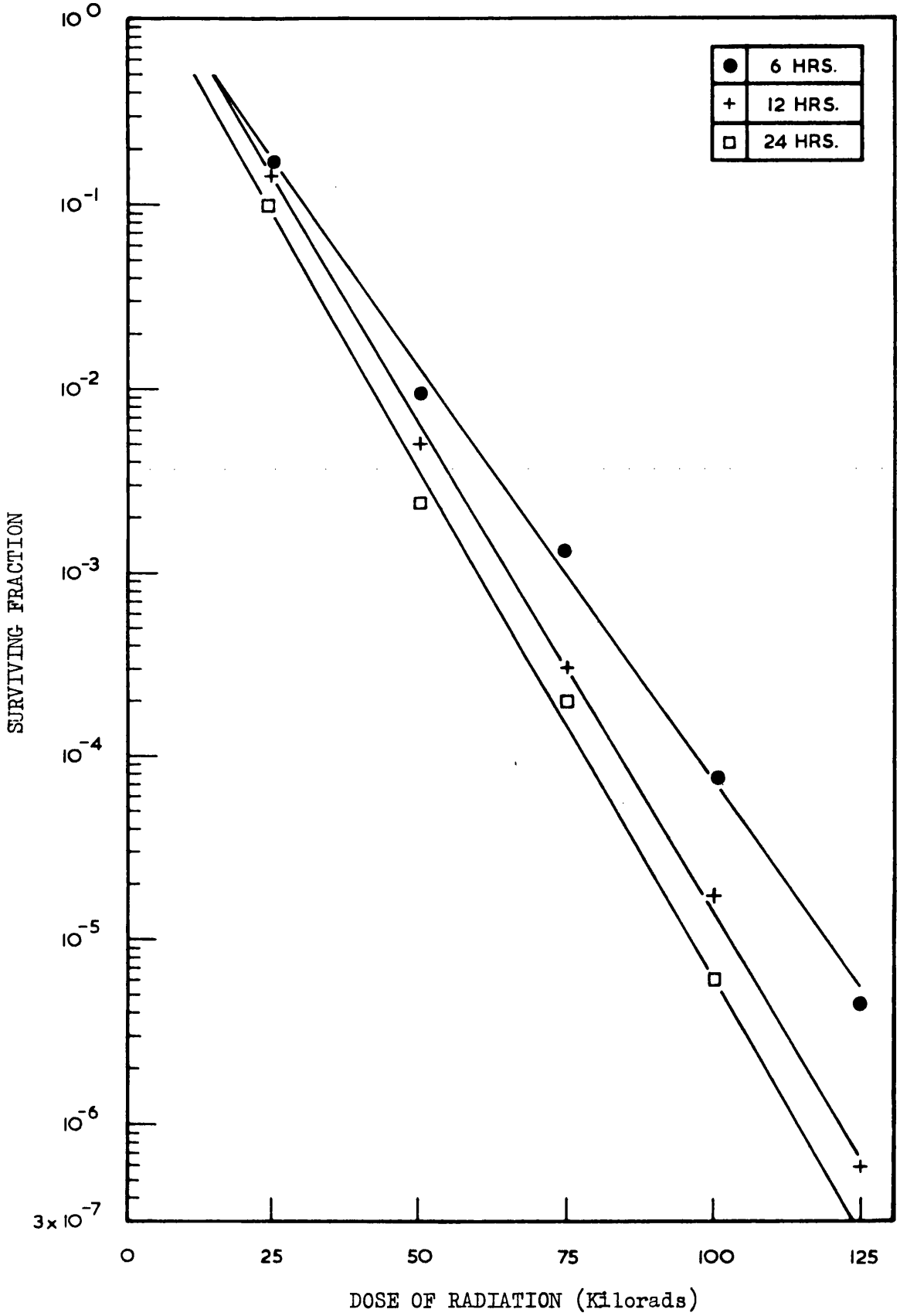


Figure 49. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 298°K and irradiated and stored in oxygen.

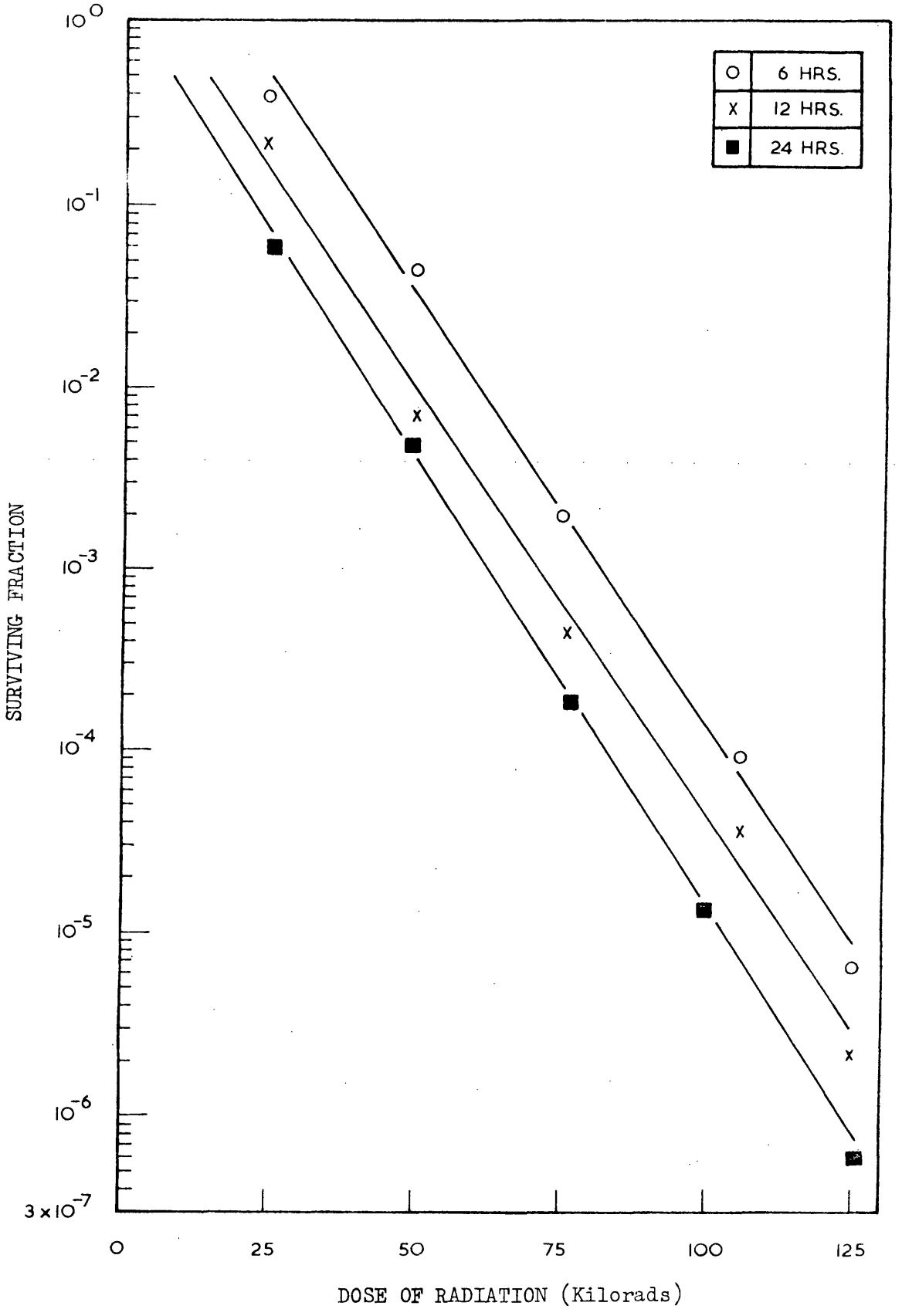


Figure 50. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 338°K and irradiated and stored in oxygen.

TABLE 29

Values of k and n, and associated standard errors, for spores dried for 6 hours, 12 hours and 24 hours at 273°K, 298°K and 338°K, irradiated in oxygen, with 18 hours post irradiation storage, in oxygen, at 323°K.

Drying Time (Hours)	k (kilorad^{-1})	Standard Error of k	n	Standard Error of n	Standard Error of Estimate
A). Drying Temperature 273°K					
6	4.5413×10^{-2}	5.0011×10^{-4}	5.932×10^{-1}	1.554×10^{-1}	1.7840×10^{-1}
12	4.7012×10^{-2}	2.4195×10^{-4}	4.6271×10^{-1}	5.5376×10^{-2}	8.5361×10^{-2}
24	4.7683×10^{-2}	3.2311×10^{-4}	2.8149×10^{-1}	7.1086×10^{-2}	1.1566×10^{-1}
B). Drying Temperature 298°K					
6	1.0390×10^{-1}	9.6081×10^{-4}	7.9352×10^{-1}	1.0999×10^{-1}	1.6952×10^{-1}
12	1.2153×10^{-1}	9.9594×10^{-4}	9.8377×10^{-1}	1.1442×10^{-1}	1.7697×10^{-1}
24	1.2510×10^{-1}	1.5336×10^{-3}	5.4722×10^{-1}	1.7733×10^{-1}	2.7598×10^{-1}
C). Drying Temperature 338°K					
6	1.0869×10^{-1}	1.4990×10^{-3}	1.9754	1.7620×10^{-1}	2.7369×10^{-1}
12	1.0922×10^{-1}	2.0930×10^{-3}	8.5860×10^{-1}	2.4628×10^{-1}	3.8212×10^{-1}
24	1.1484×10^{-1}	9.1965×10^{-4}	2.3901×10^{-1}	1.0603×10^{-1}	1.6345×10^{-1}

The Modifying Effect of Re-equilibration to 10 torr Aqueous Vapour Pressure on the Radiation Resistance of Spores Dried for Different Times at Different Temperatures.

Experiments involving the measurement of heat resistance (see page 141) showed that re-equilibration of dried spores to 10 torr aqueous vapour pressure modifies the heat sensitivity of the spores and also eliminates some of the differences in responses to lethal heat treatments that are demonstrated in spores dried under different defined conditions. The experiments described in this section were designed to determine whether the addition of a measured amount of water, after drying, had a similar effect on the radiation sensitivity of spores dried at different temperatures.

As in the previous series of experiments, the drying temperatures used were 273°K, 298°K and 338°K. Samples were dried, on different occasions, for periods 6, 12 or 24 hours at the chosen temperature. At the termination of the drying process the samples were re-equilibrated to 10 torr aqueous vapour pressure, irradiated in the presence of oxygen, and subsequently given the standard post-irradiation storage treatment. Figures 51, 52 and 53 show the log.survivor/dose curves obtained in these experiments. The values of k and n computed from the experimental data are recorded in Table 30.

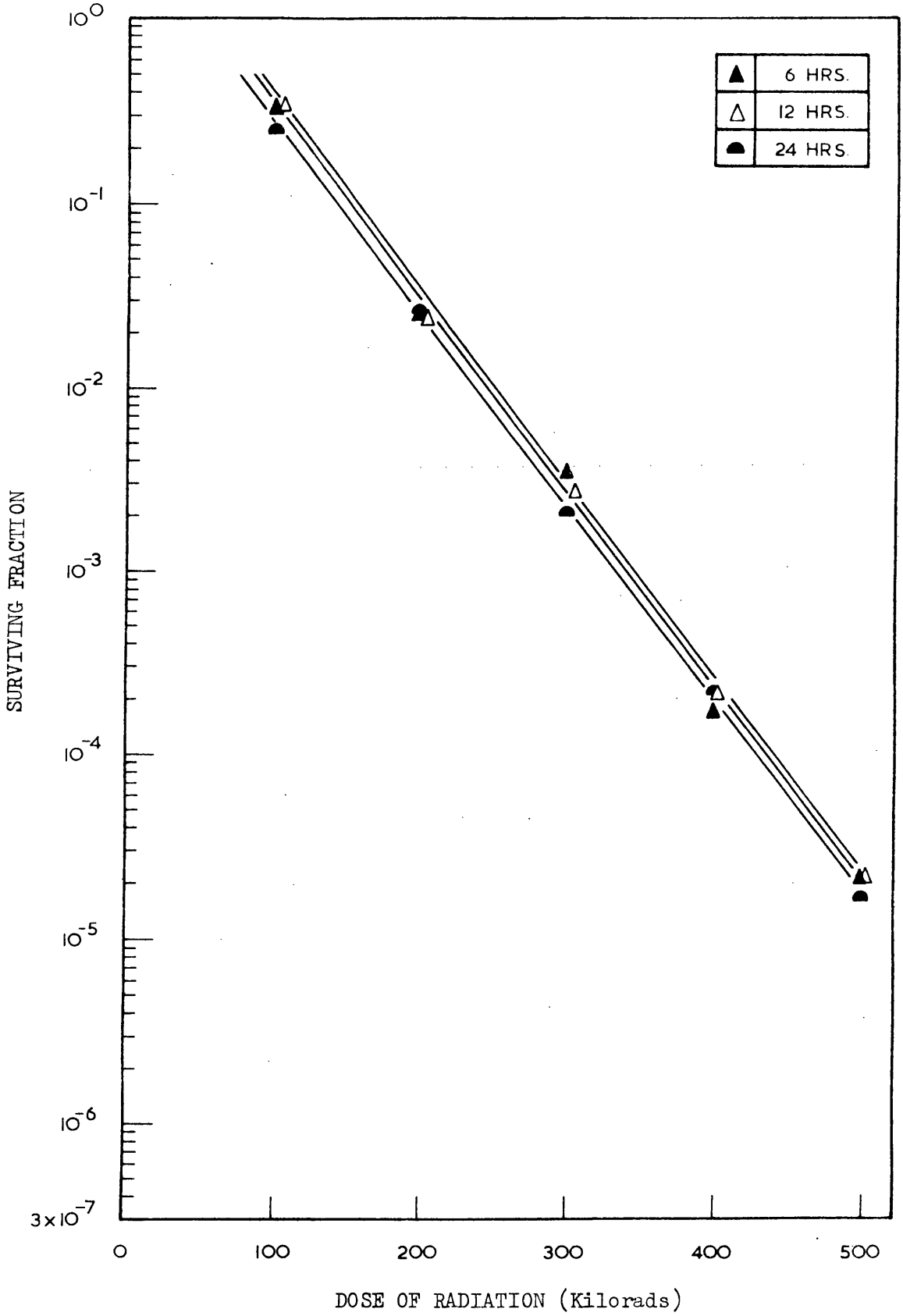


Figure 51. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 273°K, re-equilibrated to 10 torr. aqueous vapour pressure and irradiated and stored in oxygen.

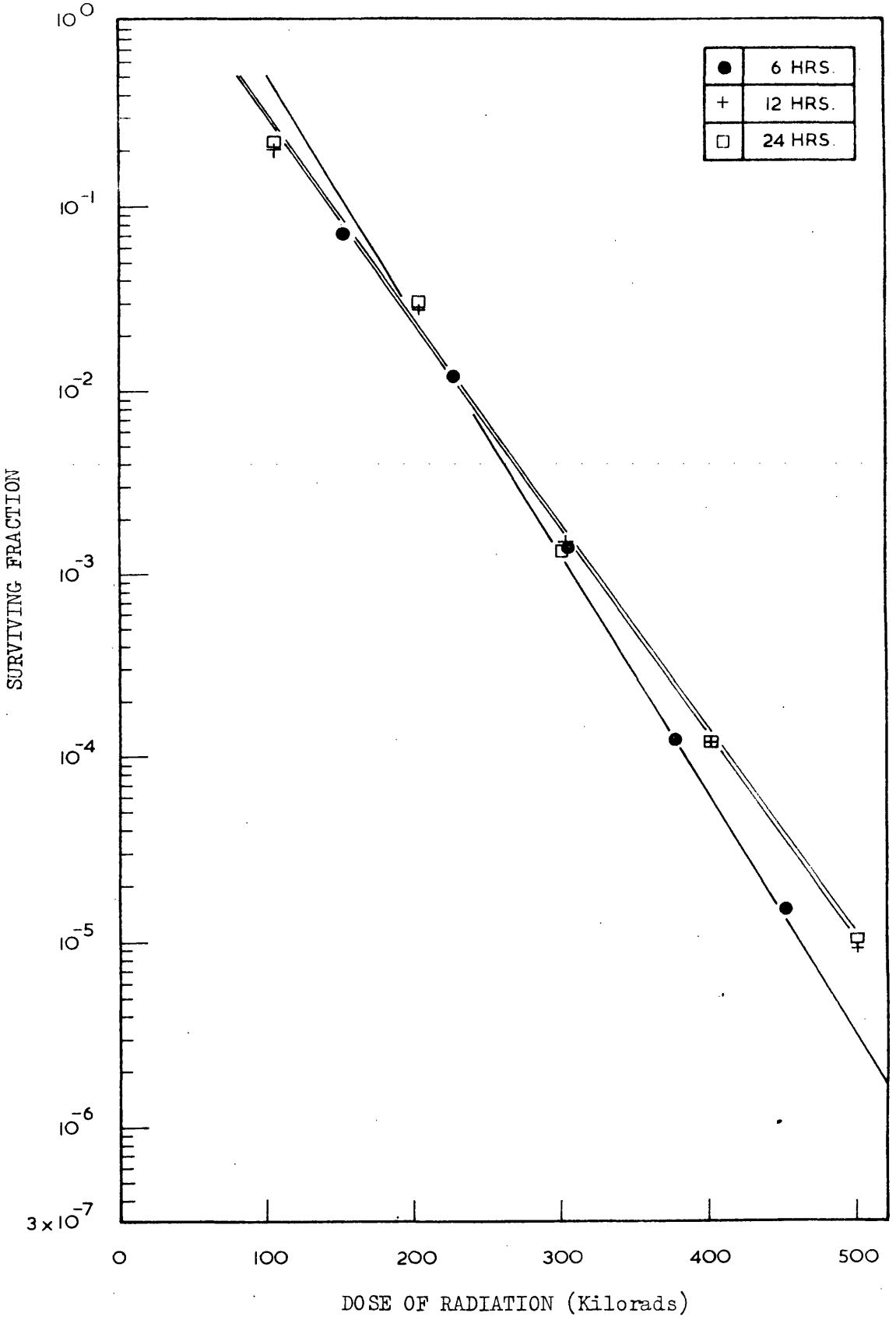


Figure 52. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 298°K, re-equilibrated to 10 torr. aqueous vapour pressure and irradiated and stored in oxygen.

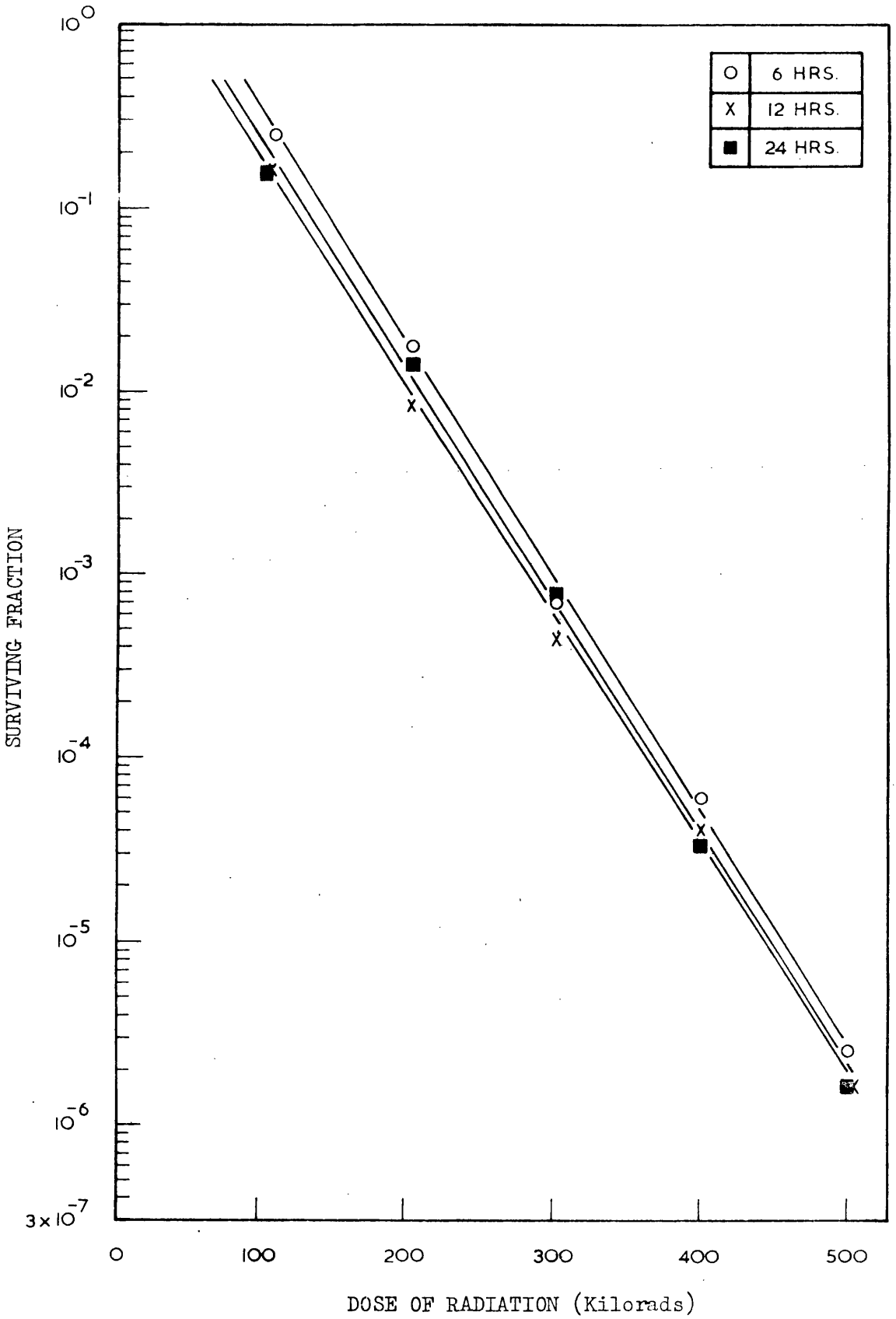


Figure 53. Plot of "surviving fraction" on a log. scale against "dose of radiation" for Bacillus megaterium spores dried for different times at 338°K, re-equilibrated to 10 torr. aqueous vapour pressure, and irradiated and stored in oxygen.

TABLE 30

Values of k and n, and associated standard errors, for spores dried for 6 hours, 12 hours and 24 hours at 273°K, 298°K and 338°K, re-equilibrated to 10 torr aqueous vapour pressure, irradiated in oxygen, with 18 hours post irradiation storage, in oxygen at 323°K

Drying Time (Hours)	k ($kilorad^{-1}$)	Standard Error of k	n	Standard Error of n	Standard Error of Estimate
A). Drying Temperature 273°K					
6	2.4384×10^{-2}	3.0151×10^{-4}	1.3366	1.3813×10^{-1}	2.1307×10^{-2}
12	2.4414×10^{-2}	1.7162×10^{-4}	1.4394	7.8703×10^{-2}	1.2005×10^{-1}
24	2.4084×10^{-2}	1.9294×10^{-4}	1.0950	8.8530×10^{-2}	1.3654×10^{-1}
B). Drying Temperature 298°K					
6	2.9628×10^{-2}	3.6853×10^{-4}	2.0856	1.7706×10^{-1}	2.6015×10^{-1}
12	2.5642×10^{-2}	3.2147×10^{-4}	1.2392	1.4747×10^{-1}	2.2757×10^{-1}
24	2.5324×10^{-2}	2.7247×10^{-4}	1.1625	1.2484×10^{-1}	1.9271×10^{-1}
C). Drying Temperature 338°K					
6	2.8985×10^{-2}	2.2334×10^{-4}	1.6976	1.095×10^{-1}	1.5628×10^{-1}
12	2.8311×10^{-2}	2.3401×10^{-4}	9.8190×10^{-1}	1.0756×10^{-1}	1.6572×10^{-1}
24	2.8869×10^{-2}	3.4007×10^{-4}	1.2867	1.5607×10^{-1}	2.4098×10^{-1}

ELECTRON PARAMAGNETIC RESONANCE STUDIES

Introduction

Previous work in this school has shown that secondary drying causes damage to Bacillus megaterium spores, which was recognised as variable responses to a given heat treatment being shown by different samples (135) The observation that this variability only becomes apparent after heating in the presence of oxygen, and that the induced damage could be reduced by water is analagous to the findings obtained in studies of radiation damage in spores. Electron paramagnetic resonance (E.P.R) studies on irradiated spores have shown that oxygen-dependent damage is mediated through free radicals induced by the radiation (161,162,163,164) and it seemed possible that drying might also cause the formation of free radicals. Some support for this theory was found in work in which a gaseous free radical scavenger, nitric oxide, and a solid free radical scavenger, thiourea, was added to the test system, since both substances were shown to ameliorate the damage caused by the drying process (135).

The involvement of free radicals in the death of vegetative cells both during lyophilisation and on subsequent storage has been demonstrated by a number of workers (see page 25) and good correlation between free radical formation and viability has been found. In contrast, the evidence for the existence of free radicals in freeze-dried spores is conflicting; some workers having reported measurable E.P.R. signals from the dried spores (116) while others have claimed to show a total absence of free radicals species (117).

In the present investigation it was decided to utilise Electron Paramagnetic Resonance techniques to study two parameters. Firstly, to confirm the presence or absence of free radicals in Bacillus megaterium spores both during lyophilisation and after subsequent heat treatment of the dried spores. Secondly, if free radicals could be demonstrated it was hoped to measure their development as a function of the physical

conditions that are known to produce biological damage.

Theory

Electron paramagnetic resonance measurement is based on the fact that atoms, ions, molecules, or molecular fragments that have an odd number of electrons exhibit characteristic magnetic properties which arise from the orbiting action, or the spinning action, or both, of unpaired electrons about the nucleus. These unpaired electrons occur in some molecules, in free radicals, triplet electronic states and transition element ions. In this context a free radical is usually defined as 'a molecule, or part of a molecule, in which the normal chemical binding has been modified so that an unpaired electron is left associated with the system',(161).

The fundamental properties of an electron are its mass, charge, intrinsic angular momentum (spin) and its magnetic moment. If free radicals, in which the unpaired electrons are not coupled to any nuclei, are in a zero magnetic field, the spins and magnetic moments of the unpaired electrons will be pointing in random directions and will all have equal energy. If, however, a strong d.c. magnetic field H_o is applied across the specimen the electrons will orientate themselves either with their spins and moments aligned with the applied field or opposed to it - no intermediate positions being allowed by the quantum conditions - and will precess about the field axis at a frequency which is proportional to both the applied magnetic field and the electron magnetic moment. The electrons will therefore fall into two groups each of which will have different energies. The electrons with their spins aligned with the field will be in the stable orientation i.e. the lowest energy position and will have an energy E, of $\frac{1}{2} g \beta H_o$ less than the zero field value, while those with their spins opposed to the

field will be in the unstable (highest energy) orientation and will have an energy E_2 of $\frac{1}{2}g\beta H_o$ greater than the zero field value. The "spectroscopic splitting factor" or "g" value is a measure of the contribution of the spin and orbital motion of the electron to its total angular momentum and has a value of 2.00229 for a completely free spin. The Bohr magnetron β is a constant that serves to convert the angular momentum to magnetic moment in electromagnetic c.g.s. units and has a value $\frac{eh}{4\pi m.c}$ where m is the mass of the electron, having a charge e, and velocity c, and h is Planck's constant. This splitting of an energy level into two or more components when placed in an external magnetic field is more commonly known as the Zeeman effect.

The splitting between the two energy levels increases with increasing magnetic field and for a given field H_o is equal to $g\beta H_o$ i.e. $E_2 - E_1$. For any system in thermal equilibrium the lower state of the split energy levels is clearly more populated than the higher energy state, and the technique of electron paramagnetic resonance is to make electronic transitions from the lower level or ground state to the higher level. The energy required to produce one such transition is

$$g\beta H_o = h\nu \qquad \ldots\ldots\ldots\ldots\ldots\ldots \quad (22)$$

hv being the quantum of energy of frequency v and h Planck's constant.

In most cases the distribution of electrons between the two energy states is given by the Maxwell-Boltzmann expression, in which the ratio of the number in the upper state n_1, to that in the lower state n_2 is given by

$$\frac{n_1}{n_2} = e^{-\frac{h\nu}{kT}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots \quad (23)$$

where k is the Boltzmann constant and T is the absolute temperature.

If the resonance absorption of energy is to continue there must be some other mechanism, apart from simulated emission, which allows electrons in the upper energy state to lose energy and return to the

lower state. If this were not so, then the larger number absorbing energy in the ground state would rapidly neutralise n_1 and n_2 with the result that no further absorption could occur. The mechanism must allow energy transfer of hv by interaction with some system other than the incident radiation. The ways in which electrons may lose energy and fulfil this condition are termed relaxation processes and are measured in terms of a relaxation time which is the time in which an initial excess of energy given to the spins will fall to $\frac{1}{e}$ of its value.

The experimental approach consists in placing the sample in a steady magnetic field H_o and arranging a second alternating magnetic field at right angles to the first. Resonance will then be observed when H_o and v satisfy the equation $g\beta H_o = hv$. In theory, electron paramagnetic resonance can occur at any frequency, provided the value of the magnetic field is adjusted to satisfy the resonance equation. In practice, however, greater sensitivity is achieved by the use of high magnetic field strength, and a high resonant frequency (in the microwave region), and by working at low temperatures, since the difference between n_1 and n_2 is then increased and a larger net absorption occurs. The upper limit of field strength and resonant frequency is set by purely practical considerations, and as a result most E.P.R. spectrometers that have been employed for free radical investigations have used a field strength of about 3,000 gauss, and a wavelength of 3 cm. equivalent to a frequency of 9000 Mc.sec.$^{-1}$ which occurs in a region commonly known as X-band. However, the klystron oscillator ordinarily employed to attain the high frequencies is more effective when used at a fixed frequency, and thus of the two parameters v and H_o it is customary to fix the value of the former and then vary the value of H_o in order to satisfy the resonance condition.

A further practical difficulty arises in the presentation of the

absorption data, since the E.P.R. signal represents a very small change in the overall absorption of microwave power in the sample cavity. Amplification is therefore necessary, and since it is experimentally more convenient to amplify alternating currents and voltages, part of the d.c. output due to the spins is converted to a.c. by modulation of the magnetic field. As a consequence, the E.P.R. spectrometer gives its data in the form of the derivative of the E.P.R. absorption rather than as the absorption line itself.

Materials

E.P.R. sample vessel - Figure 54 is a diagram of the type of vessel used to hold spores during the determination of E.P.R. signals. It was constructed from quartz tube 2.2 mm. internal diameter and 4 mm. external diameter. The sample of spore suspension was measured into a tube T of length 120 mm. closed at one end by fusion of the glass. The open end of the tube was then fused onto a 4 mm. "Lynx" graded glass seal, quartz to "Pyrex", manufactured by Jencons Ltd. The "Pyrex" part of the seal was fused onto a 1-2 mm. bore high vacuum stopcock leading to a B10 ground glass socket S. The E.P.R. sample vessel was then connected by means of B10 ground glass joints to the manifold M on the vacuum apparatus, via another 1-2 mm. bore high vacuum stopcock. This arrangement of the sample vessel enabled the E.P.R. signal of the contained spores to be measured at intervals during the drying process without breaking the vacuum in the vessel or in the vacuum apparatus.

The E.P.R. sample vessel was cleaned by dipping in hydrofluoric acid for a few seconds, followed by ten rinses in glass-distilled water. The use of tap water for rinsing was avoided and care was taken to prevent the cleaned vessels from coming into contact with metal ions.

E.P.R. Spectrometer - measurements of the E.P.R. signals in

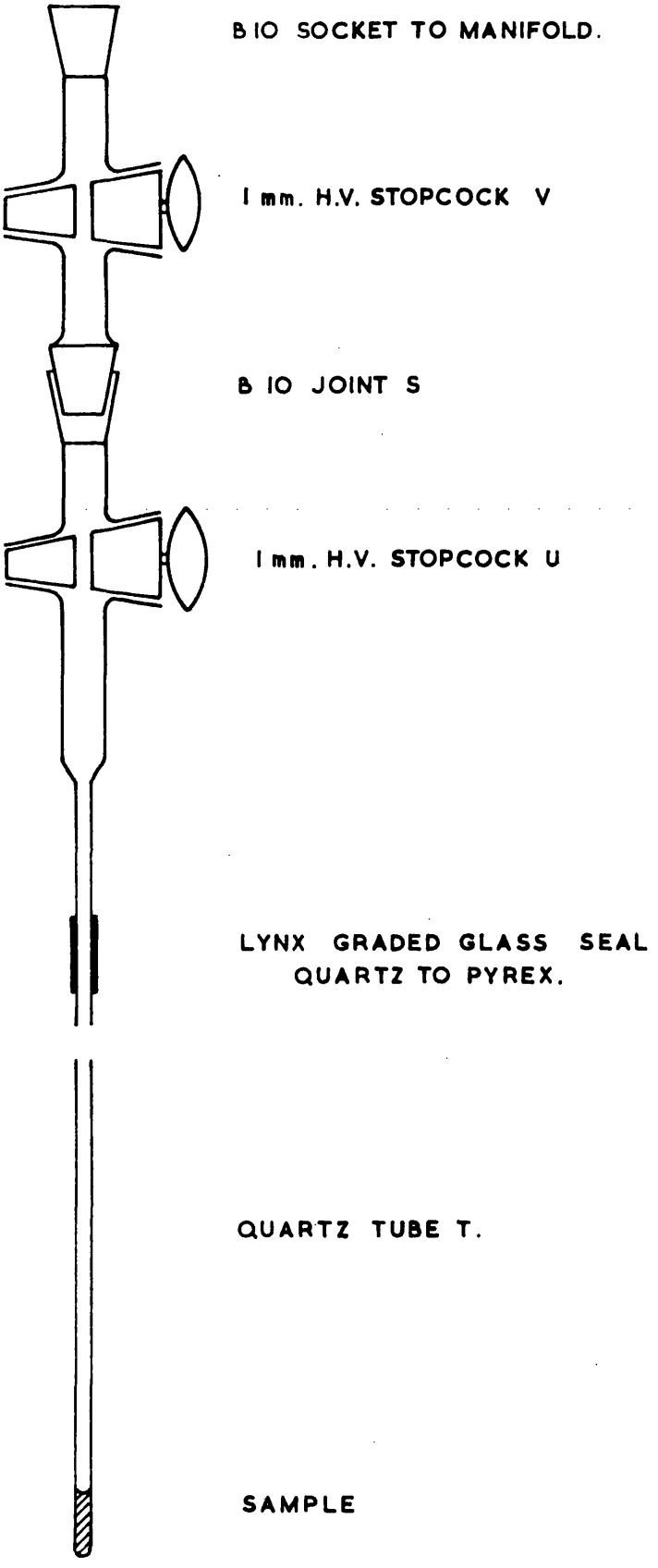


FIGURE.54 THE EPR SAMPLE VESSEL

samples of dried spores were made using a Varian E-3 Electron Paramagnetic Resonance Spectrometer. The block diagram of Figure 55 identifies the principal components of the Spectrometer.

The various components and their functions are:

(i) The electromagnet A provides a homogeneous magnetic field (H_o) which can be varied from near zero to over 5000 gauss.

(ii) The magnet power supply B provides a stable, controlled current to the energising coils of the electromagnet.

(iii) The scanning unit C supplies a control voltage to the input of the current regulated magnet power supply. This control voltage can be varied linearly with respect to time, thus resulting in a linear sweep of the magnetic field.

(iv) The 100 kc sweep oscillator D provides a small modulation of the static magnetic field at the sample location within the resonant cavity. It also supplies a phase reference voltage to the signal phase detector E. The audio sweep generator F supplies an additional magnetic field modulation to the cavity as well as a sweep for the oscilloscope X-axis.

(v) The klystron oscillator G produces microwave energy which is used to irradiate the sample. Although not included in the diagram, a variable alternator is required between the klystron and the hybrid tee in order to control the amount of microwave power applied to the sample.

(vi) The hybrid tee H is a device which will not allow microwave power to pass in a straight line from one arm to the arm opposite. Thus, power emitted from arm 1 is equally divided between arms 2 and 3. If all of the power is absorbed, and none reflected, then no power enters arm 4, and thus the crystal detector receives no energy. The hybrid tee can therefore be considered similar to a balanced bridge,

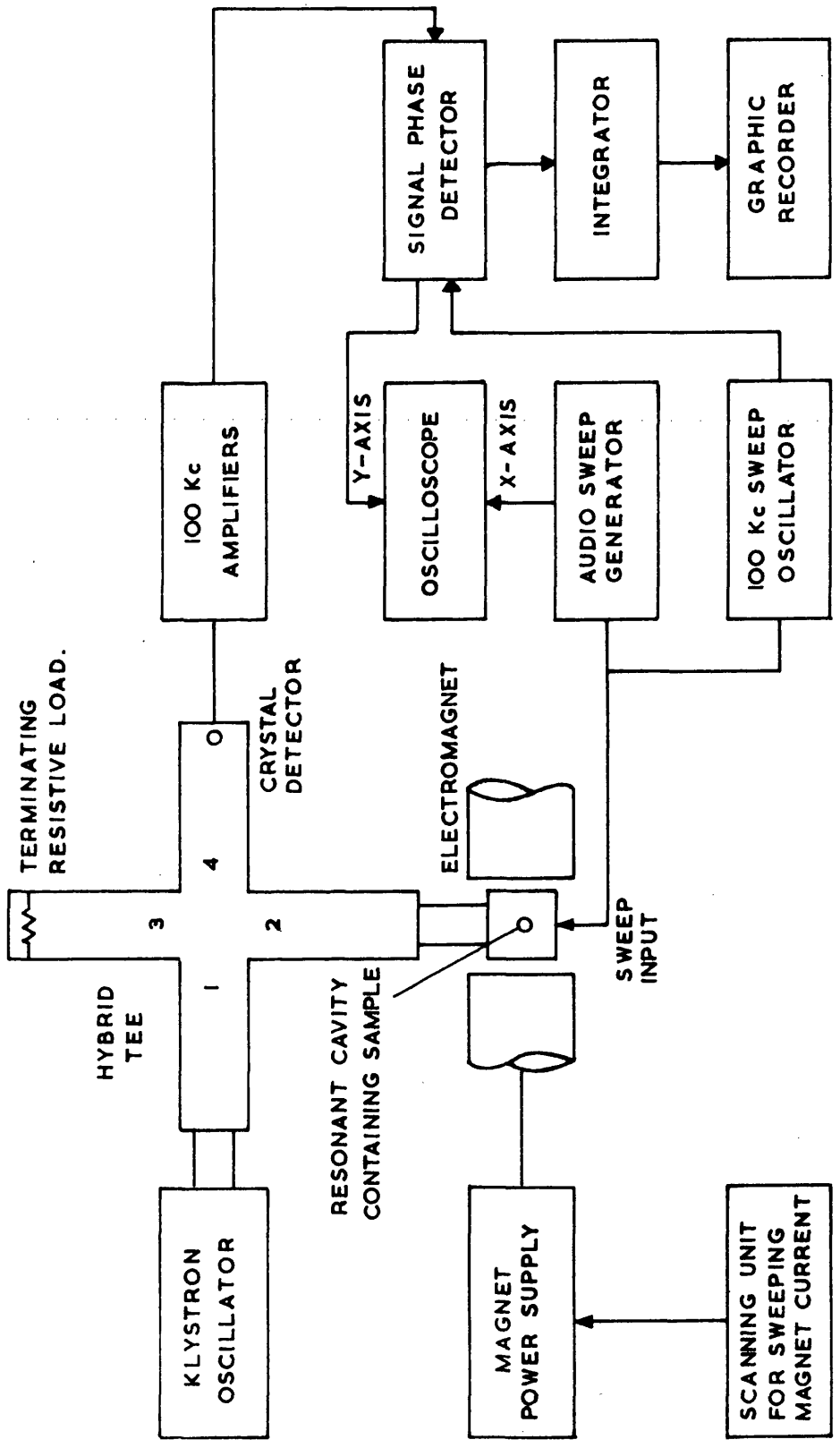


FIGURE.55 BLOCK DIAGRAM OF VARIAN E-3 ELECTRON PARAMAGNETIC RESONANCE SPECTROMETER

since any change in the terminating impedence of arms 2 or 3 will unbalance it and result in energy reaching the crystal detector.

(vii) The resonant sample cavity I acts like a tuned circuit which has a very high Q, where Q is defined as w (energy stored/ power lost) and w = 2π x frequency When a resonance is obtained from the sample the impedence of the cavity is changed and a signal is reflected to the crystal detector in the hybrid tee.

(viii) The crystal detector demodulates the microwave energy. The resulting 100 kc. signal voltage contains the E.P.R. information. The frequency of the voltage corresponds to that of the sweep voltage used to modulate the magnetic field, and to provide a phase reference for the signal phase detector.

(ix) The amplifier J amplifies the signal information from the crystal detector.

(x) The oscilloscope K is a means for the rapid visual display of E.P.R. signals.

(xi) The signal phase detector, combined with an integrator (L) and a graphic recorder M provides a means of displaying the first harmonic presentation of the E.P.R. signal. Bandwidth limiting circuits at the input to the signal amplifier afford a higher signal-to-noise ratio to be obtained on the recorder than that which is observed on the oscilloscope.

Method

It was expected that the E.P.R.signal obtained from the small sample of dried spores would be of low intensity. From the temperature dependence of the population ratio shown in the Maxwell-Boltzmann expression (see page 203) it can be seen that as the temperature decreases the signal intensity increases. Hence to increase the E.P.R. signal of the sample

all measurements were made with the sample at liquid nitrogen temperature ($77^{o}K$). For this purpose, a specially designed long-tailed quartz dewar vessel was fitted into the sample cavity and filled with liquid nitrogen. Dry nitrogen gas was passed through the sample cavity to prevent condensation occurring within the cavity.

A sample of spore suspension was dried in an E.P.R. sample vessel for the requisite period of time. The sample vessel was sealed off by means of taps U and V and isolated from the vacuum apparatus by breaking the vacuum at joint S. The outside of the vessel was cleaned with acetone and the vessel was placed in liquid nitrogen in the quartz dewar vessel. To obtain consistent readings care was taken to maintain constant orientation of the sample vessel within the cavity by aligning the vessel with a particular part of the sample cavity. The spectrometer was adjusted to the required control settings (see page 211) and the first derivative of the E.P.R. signal was measured directly on the graphic recorder.

Preliminary Experiments

Determination of E.P.R. Signal for Bacillus megaterium Spores

A sample of suspension SS6S was centrifuged for 20 minutes at 3000 r.p.m., and the supernatant removed with a Pasteur pipette. The packed spores were then transferred to an E.P.R. sample vessel, and the open vessel was stored in a desiccater over P_2O_5 for 24 hours. The spectrometer was adjusted to the control settings recommended by the manufacturers for the measurement of a low intensity signal. These are: Time Constant 1 second, Scan Time 1 minute, Receiver Gain 8×10^5, Microwave Power 300 mW, Modulation Amplitude 10 gauss. By suitable adjustment of Field Set and Scan Range, signals were recorded over a

range of Field Settings from 200 gauss to 5000 gauss. Figure 56 illustrates the signal recorded over the Field Setting range 2000 gauss to 4000 gauss. This trace and similar ones recorded at the other Field Settings failed to show the presence of any distinct absorption peaks. Paramagnetic species were thus not detectable, under the experimental conditions employed, in spores not subjected to high vacuum drying.

A sample of the spore suspension was dried in an E.P.R. sample vessel for 6 hours at 298^{o}K. At the end of the drying period the E.P.R. signal of the spores was measured under the conditions defined above. The sample was then heated at 393^{o}K for 1 hour and the signal determination repeated on the heated spores. The signals recorded over the range of Field Settings from 2000 gauss to 4000 gauss for the spores after drying and after subsequent heating are shown in Figure 56 superimposed upon the corresponding signal for untreated spores. This scanning experiment revealed the presence of a single absorption peak at a Field Set of approximately 3260 gauss for dried spores. After the dried spores had been heated the intensity of the signal increased, although its position remained unchanged.

Adjustment of Experimental Parameters to Obtain the Optimum E.P.R. Signal from a Sample of Dried Spores

Although an E.P.R. signal had been obtained from a sample of dried spores using the control settings on the spectrometer recommended by the manufacturers it was decided to investigate the effects of various control settings on the presentation of the spectrum in order to obtain the optimum signal under the experimental conditions employed. Because only a single absorption peak had been demonstrated, the Field Set was fixed at 3260 gauss, with a Scan Range of $\pm$ 50 gauss. The control settings investigated were Time Constant, Scan Time, Modulation Frequency,

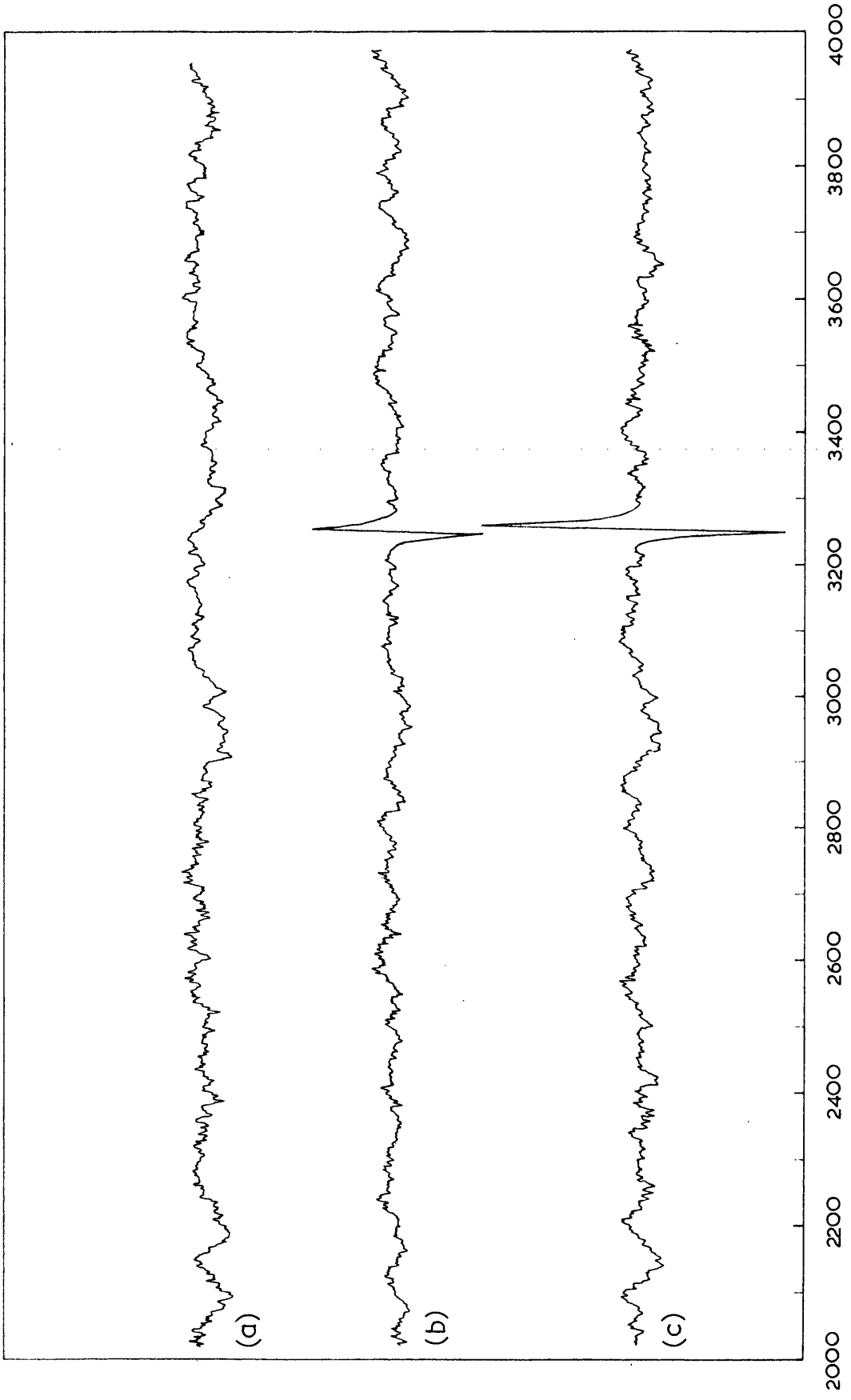


Figure 56. E.P.R. spectra of samples of Bacillus megaterium spores a) stored for 24 hours over P_2O_5, b) dried for 6 hours at 298°K, and sealed in anoxia, c) dried for 6 hours at 298°K, and heated for 60 minutes at 393°K, in anoxia.

(Microwave Frequency 9.156 GHz., Receiver Gain 8×10^5).

and Receiver Gain. A graphic presentation of the effects of varying these settings is shown in Figures 57,58,59 & 60. From these results it was decided to use the following control settings for the determination of E.P.R. signals from all samples of dried spores used in this work.

Field Set	3260 gauss
Scan Range ±	50 gauss
Time Constant	3 seconds
Scan Time	4 minutes
Microwave Power	300 mW
Modulation Amplitude	10 gauss

It was also apparent that the E.P.R. signals were of insufficiently high intensity to be detected at room temperature, and thus all determinations were made with the sample maintained at liquid nitrogen temperature ($77^{o}K$).

Treatment of Results

Characterisation of the E.P.R. Spectrum

The preliminary experiment described above revealed the presence of paramagnetic species in the spores after drying and after subsequent heating. In order to attempt identification of these species and to study their development under experimental conditions it was necessary to characterise the resonance spectrum obtained. Five basic parameters have been used to characterise such a spectrum a) the "g" value, b) the line width, c) the line shape, d) the electronic splitting and e) the hyperfine splitting (161, 189)

(a) The "g" value or "spectroscopic splitting factor"

This constant, the ratio of the frequency to the field at resonance, is used to denote the position of the absorption line and can be defined

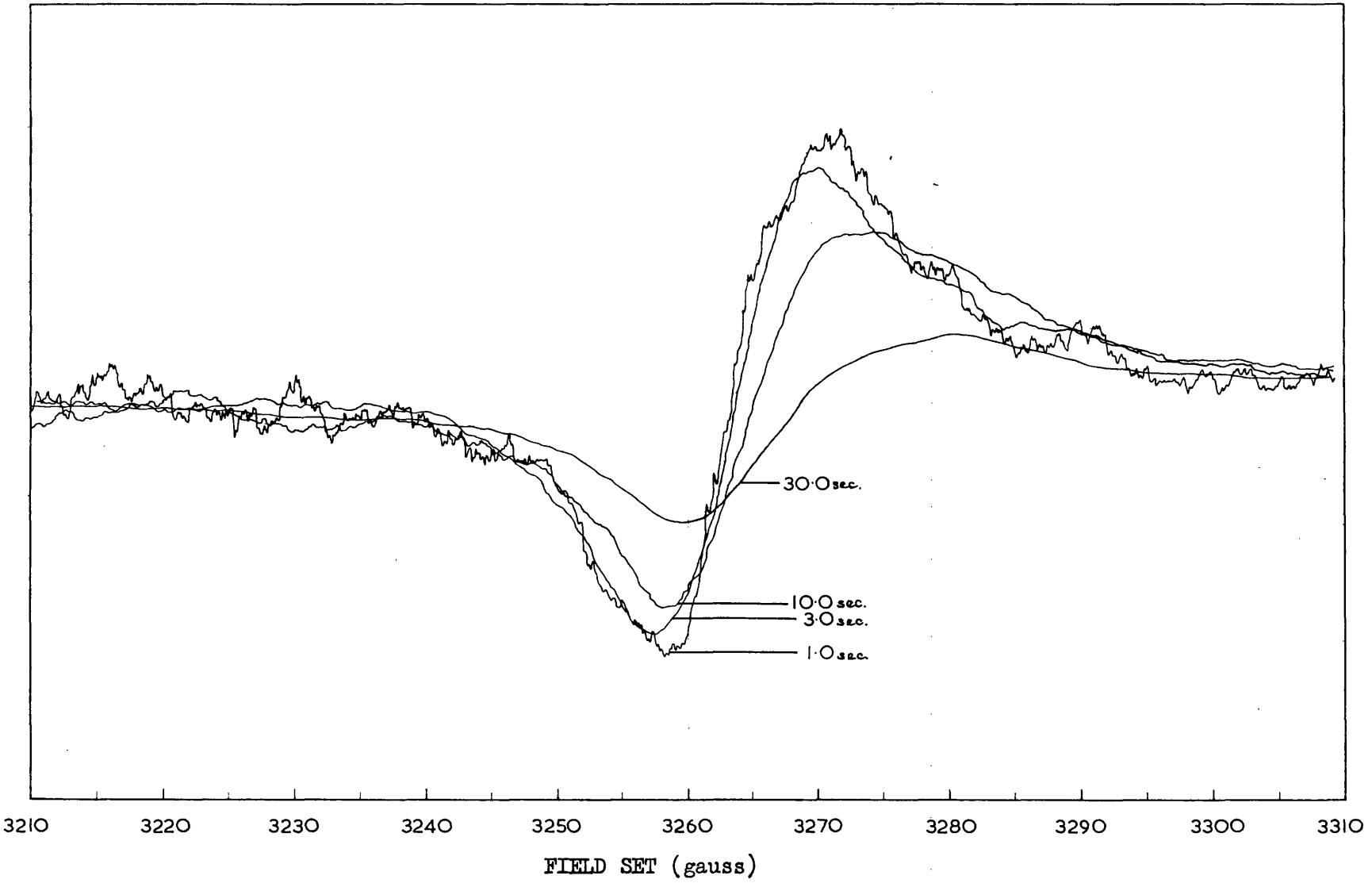


Figure 57. The effect of different Time Constant settings on the E.P.R. spectra of samples of Bacillus megaterium spores dried for 6 hours at 298°K, and sealed in anoxia.

(Microwave Frequency 9.156 GHz., Scan Time 1 minute, Modulation Amplitude 10 gauss, Receiver Gain 8×10^5).

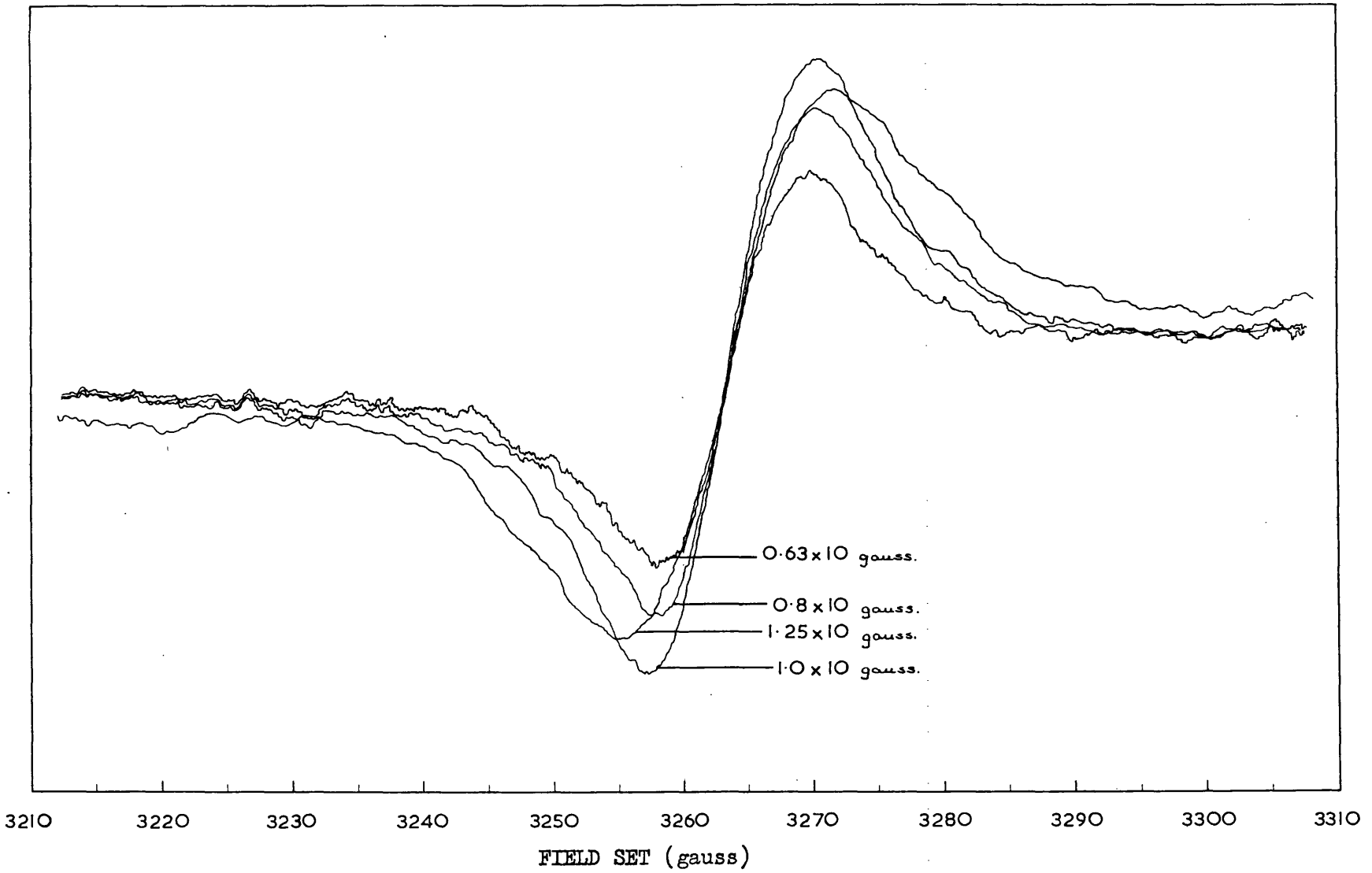


Figure 58. The effect of changing the Modulation Amplitude on the E.P.R. spectra of samples of Bacillus megaterium spores dried for 6 hours at 298°K, and sealed in anoxia.

(Microwave Frequency 9.156 GHz., Time Constant 3 seconds, Scan Time 4 minutes, Receiver Gain 8x105).

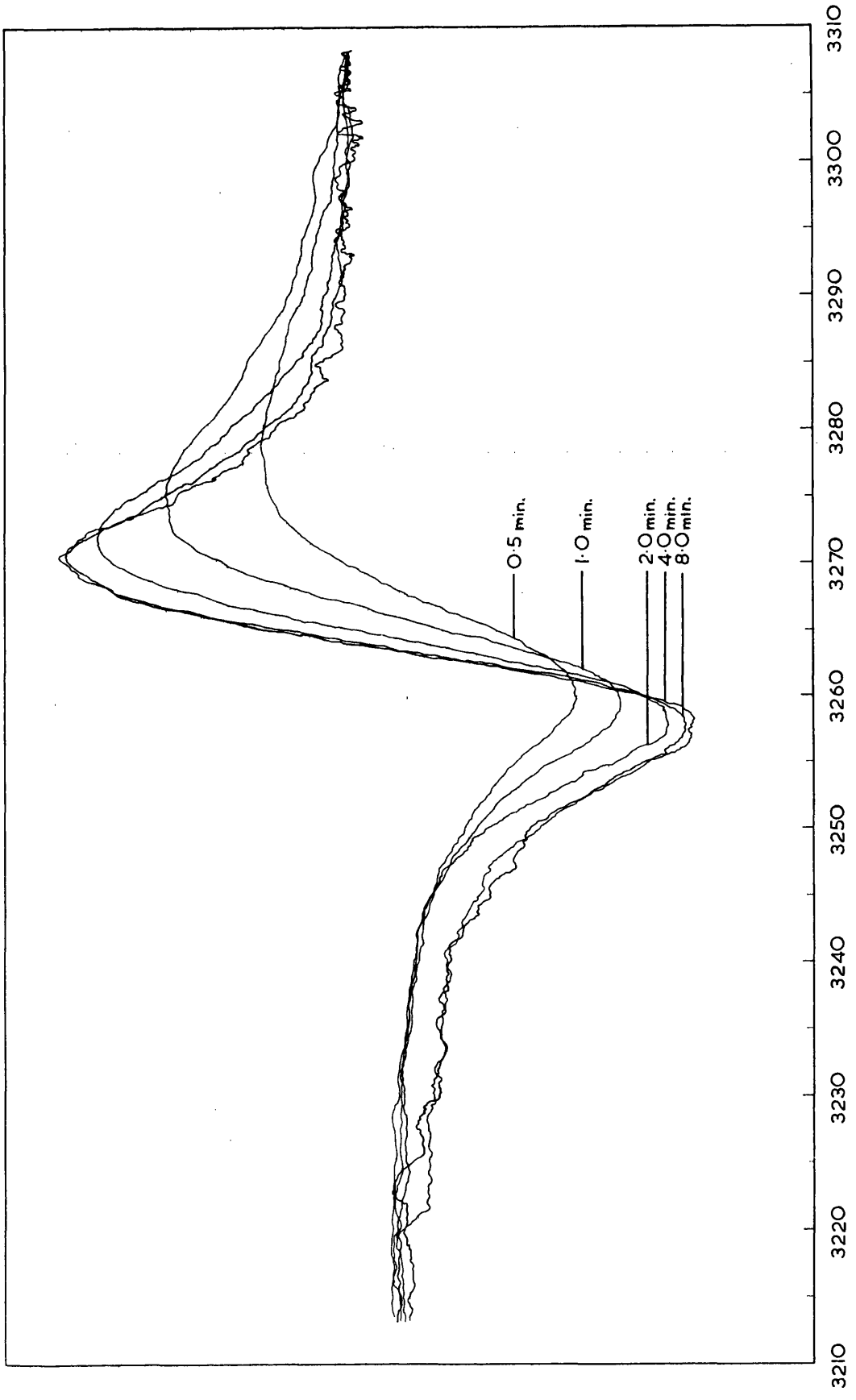


Figure 59. The effect of varying the Scan Time on the E.P.R. spectra of samples of Bacillus megaterium spores dried for 6 hours at 298°K, and sealed in anoxia.
(Microwave Frequency 9.156 GHz., Time Constant 3 seconds, Modulation Amplitude 10 gauss, Receiver Gain 8×10^5).

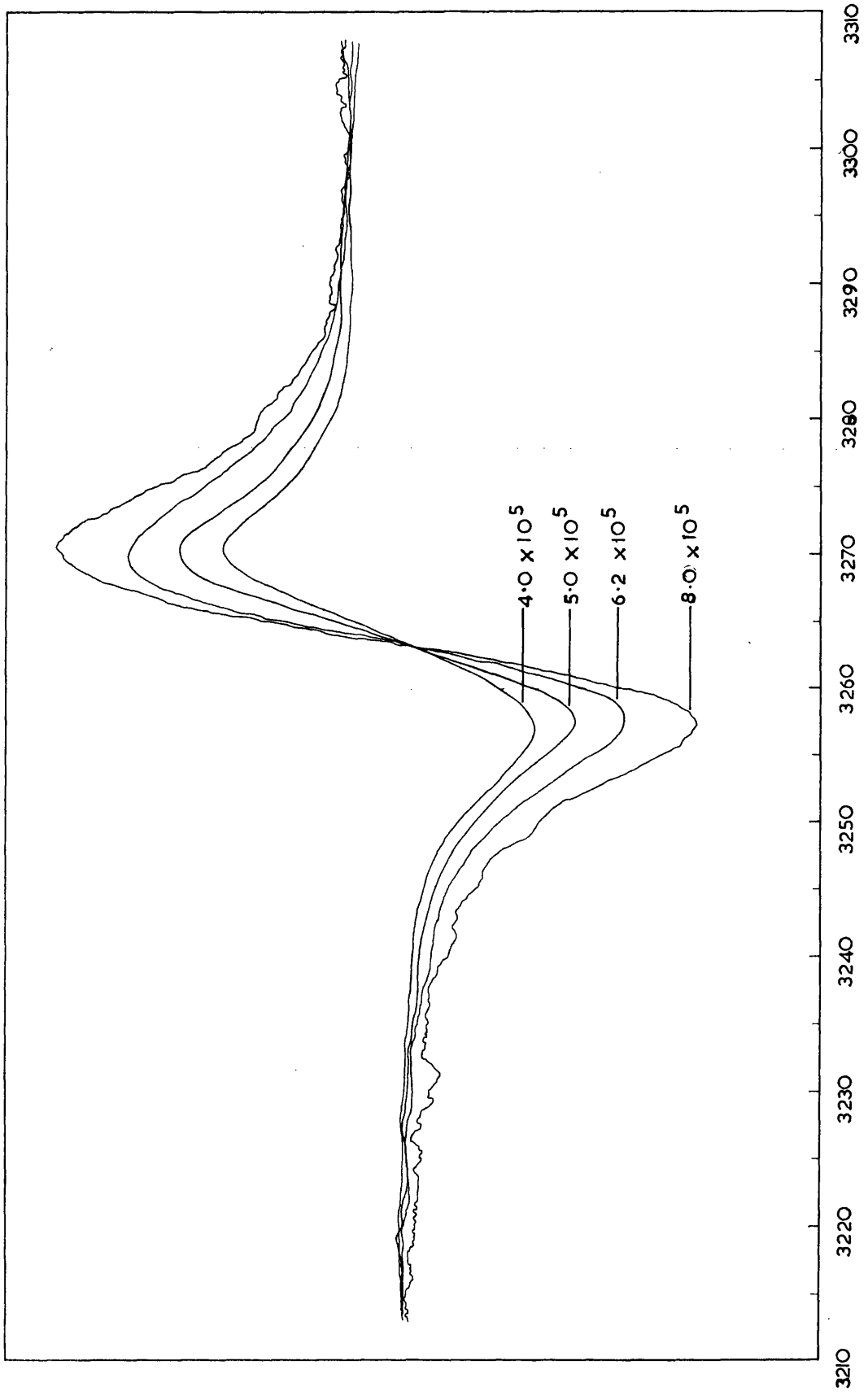


Figure 60. The effect of changing the Receiver Gain on the E.P.R. spectra of samples of Bacillus megaterium spores dried for 6 hours at 298°K, and sealed in anoxia.

(Microwave Frequency 9.156 GHz., Time Constant 3 seconds, Scan Time 4 minutes, Modulation Amplitude 10 gauss).

from the equation for E.P.R. (see page 203) as $g = \frac{h\nu}{\beta H_o}$ where ν is the frequency and H_o the field at which resonance occurs, h is Planck's constant and β is the Bohr magnetron. For the completely free spin this constant will have the value 2.00229. In the case of paramagnetic atoms or molecules the "g" value may vary over a wide range from 1 to 6 or more, depending upon the amount of magnetic interaction between the orbital angular momentum of the unpaired electrons and the electron spin i.e. spin-orbit coupling. The unpaired electron associated with the free radical is, however, moving in a molecular orbit which is delocalised over the whole molecule, and thus strong spin-orbit interaction with any one atom does not take place. The "g" value for a free radical is therefore very close to that for the completely free spin.

For the first derivative of the absorption curve shown in Figure 60 H_o is the field at the mid-point between the two peaks (i.e. at the peak to peak half line width) and has a value 3.263×10^3 gauss. The resonant frequency is 9.146×10^9 cm.sec.$^{-1}$ and the constants h and β have values of 6.62491×10^{-27} erg. sec. and 9.27267×10^{-21} erg. gauss^{-1} respectively. The "g" value is therefore equal to $\frac{6.62491 \times 10^{-27} \times 9.146 \times 10^9}{9.27267 \times 10^{-21} \times 3.263 \times 10^3}$ = 2.00258. This value is within $\pm$ 0.02% of that for the free spin of 2.00229, and indicates that the absorption signal given by the dried spores is more likely to result from free radicals than from any other paramagnetic species.

(b) The line width.

Resonance absorption occurs not at one precise value of the applied magnetic field but rather over a range of field values resulting in E.P.R. lines that always have a finite width. Observations on the variation of this width will give information about the spin environment since the line width is due to magnetic interactions of the electron spin with the environment of the sample. These environmental effects are related to

the relaxation times (see page 204) and examples are those between neighbouring molecules having spin (dipolar spin-spin interactions), between the paramagnetic ions and the thermal vibrations of the crystal lattice (spin-lattice interaction), electron spin exchange between identical and non-identical molecules, and chemical exchange between the paramagnetic molecule and its surroundings.

In addition, certain aspects of the experimental determination of the resonance absorption spectrum can cause line width broadening. If the incident power is so large that normal relaxation processes can no longer restore thermal equilibrium, the peak absorption will start to fall and the lines become wider. This is termed power saturation broadening. The use of magnetic field modulation for the detection and display of the resonance signal (see page 204) gives rise to a further type of broadening of the absorption line - modulation broadening. This will produce a noticeable additional width to the line if the frequency of modulation is greater than, or of the same order of magnitude as the width of the line without modulation.

For the E.P.R. absorption peak the line width 2δ is defined as the separation between the points of half maximum intensity. However, this parameter is not easily derived from the first derivative of the absorption peak as displayed by the spectrometer without recourse to integration techniques. In practice, therefore, a second line width parameter is usually measured from the derivative curve. This is the peak to peak line width 2Δ and is equivalent to the line width between points of maximum slope on the absorption peak.

The first derivative curve for the E.P.R. absorption of dried spores illustrated in Figure 60 has a peak to peak line width 2Δ of 12.5 gauss. The significance of this value is discussed on page 294.

(c) The line shape.

In addition to causing variation in line width, the environmental factors described above also affect the shape of the E.P.R. absorption curve. The two most commonly encountered line shapes are the Gaussian and Lorentzian. Where collision broadening i.e. electron spin exchange is the main factor that determines the line width, the resulting lines are Lorentzian. On the other hand, rigid systems in which the relative orientations and positions of randomly distributed and interacting species do not change with time as in dipolar spin-spin interaction and spin-lattice interaction give rise to Gaussian lines (161,190). The equations which describe these two line shapes are as follows:

$$\text{Gaussian} \quad F(H-H_o) = \frac{1}{\sqrt{2\pi}\Delta} \exp.\left[\frac{-(H-H_o)^2}{2\Delta^2}\right] \quad \ldots\ldots\ldots (24)$$

$$\text{Lorentzian} \quad F(H-H_o) = \frac{\sqrt{3}\Delta}{\pi} \cdot \frac{1}{3\Delta^2 + (H-H_o)^2} \quad \ldots\ldots\ldots (25)$$

where $F(H-H_o)$ is the amplitude of the function, Δ is one half of the width of the function at maximum slope and $(H-H_o)$ is the value of the field measured from the centre of the resonance. Normalised plots of these two functions when Δ is the same for both are shown in Figure 61.

Significant differences between these line shapes are evident. The Lorentzian is a sharper narrower function towards the centre, also it falls off in the wings less rapidly than the Gaussian. At a distance from the centre equal to three half line widths the amplitude of the Gaussian function is essentially zero while at the same point the amplitude of the Lorentzian is approximately 10% of its maximum amplitude. Furthermore, examination of these curves indicates that for the Gaussian the maximum slope of the absorption is very close to the half intensity position while for the Lorentzian the maximum slope occurs at about three-quarters of the maximum intensity.

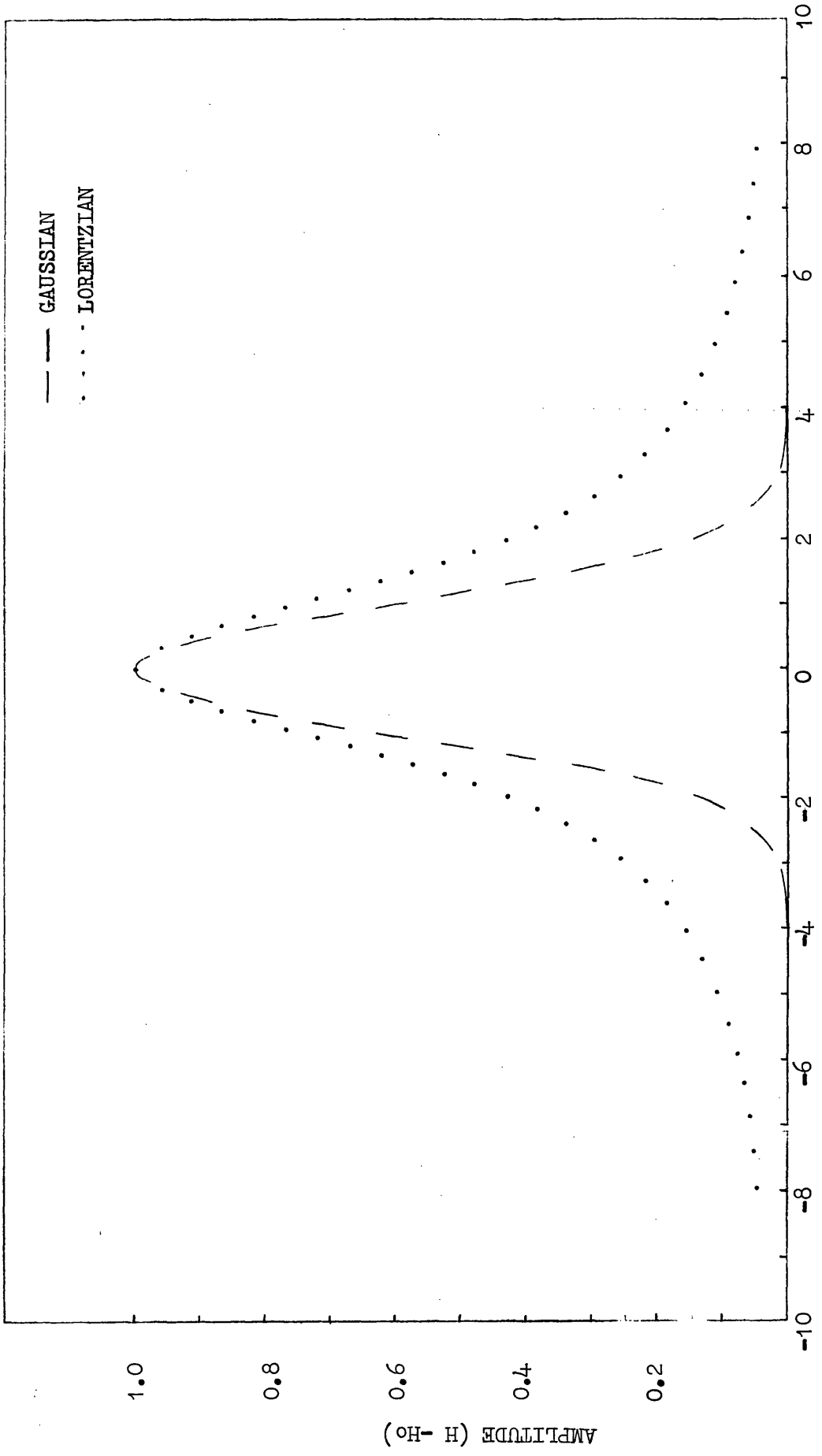


Figure 61. Zero derivative curves for Gaussian and Lorentzian line shape functions. (Curves have the same peak-to-peak line width).

Of more direct practical application are the derivative curves of these functions. The equations describing the two first derivative curves are as follows:

$$\text{Gaussian} \quad F'(H-H_o) = \frac{-(H-H_o)}{\sqrt{2\pi\Delta^3}} \exp.\left[\frac{-(H-H_o)^2}{2\Delta^2}\right] \quad \ldots\ldots\ldots (26)$$

$$\text{Lorentzian} \quad F'(H-H_o) = \frac{-2\sqrt{3}\,\Delta\,(H-H_o)}{\pi}\left[\frac{1}{3\Delta^2 + (H-H_o)^2}\right]^2 \quad \ldots (27)$$

where $F'(H-H_o)$ is the amplitude of the first derivative of the function. The normalised plots of the two first derivative curves, when Δ is the same for both are shown in Figure 62.

These curves exhibit their maximum (or minimum) value at $(H-H_o)$ equal to $\pm\Delta$, and fall off in the wings in a similar manner to the absorption curves. The difference in the rate of decrease of slope in the wings of the two curves can be used to advantage in determining the analytical expression that a given recorded curve fits. In this context the quantity calculated is the ratio of the maximum negative slope of the curve to the maximum positive slope. This ratio has a value of 2.241 for a Gaussian line and 4.0 for a Lorentzian line, and provides a rapid and easy test for the line shape (191).

The experimentally determined first derivative curve for the E.P.R. absorption of dried spores was first inverted to enable comparison to be made with the theoretical curves, and was then normalised by making the maximum intensity of the peak i.e. $(H-H_o)$ at $\pm\Delta$ equal to 1, and expressing the field strength as a fraction of Δ i.e. $\frac{(H-H_o)}{\Delta}$ and is illustrated in Figure 62 together with the Gaussian and Lorentzian first derivative curves, for comparative purposes.

Examination of Figure 62 shows that the first derivative curve obtained with the dried spores is neither Gaussian nor Lorentzian in shape but follows a curve intermediate between the two. The negative

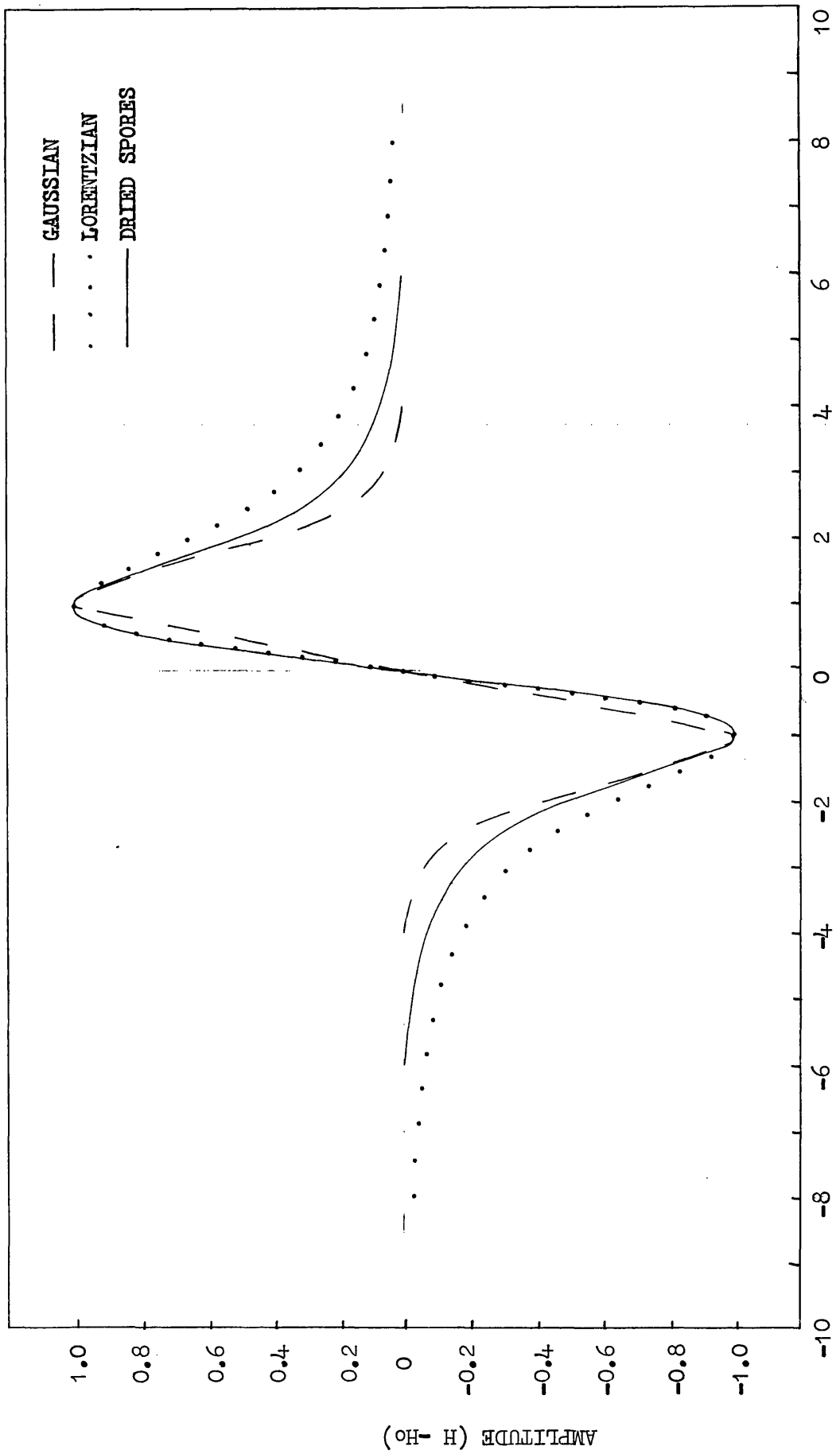


Figure 62. First derivative curves for Gaussian and Lorentzian line shape functions, and for the E.P.R. spectra from Bacillus megaterium spores dried for 6 hours at 298°K and sealed in anoxia, (Curves have the same peak-to-peak line width).

slope of the curve follows the Lorentzian shape while the positive slope approximates more closely to the Gaussian pattern. Further confirmation is found in the calculation of the ratio of the maximum negative slope to the maximum positive slope which for the curve obtained with dried spores gives a value of 3.1 interjacent between the values for Gaussian and Lorentzian functions of 2.241 and 4.0 respectively. The E.P.R. absorption curve obtained with dried spores therefore has a shape which is probably a mixture of both Gaussian and Lorentzian functions, the significance of which will be discussed later.

(d) The electronic splitting.

This may occur in paramagnetic atoms or molecules that have more than one unpaired electron associated with them. However, since no free radicals apart from triplet states possess more than one unpaired electron per molecule, no electron splitting is possible in free radical spectra. Examination of Figure 60 reveals the absence of electronic splitting in the E.P.R. spectra obtained with dried spores, and this is taken as further evidence that the absorption spectra are due to free radicals.

(e) The hyperfine splitting.

This is produced by the interaction of the magnetic field due to the moment of the nucleus of the paramagnetic ion with the electron spin undergoing transition. If the nucleus has spin I, its (2I + 1) possible orientations will produce (2I + 1) component levels in each electronic state, the splitting between these being equal and independent of the external field. It is often possible, therefore, to identify the paramagnetic species involved, by analysis of characteristic hyperfine structure of the E.P.R. spectra. The spectrum obtained from dried spores (Figure 60) does not show a hyperfine pattern under the experimental conditions employed for its measurement, and thus it is not possible, from the E.P.R. data available, to attempt a positive identification of the

free radicals involved.

Measurement and Comparison of the Intensity of the E.P.R. Signals

Preliminary experiments had shown that the intensity of the E.P.R. signal given by dried spores increases after the spores are heated. In order to study this effect in detail it was necessary to determine a method of measuring the intensity of the recorded absorption peaks such that the E.P.R. signals obtained after different experimental treatments of the spores could be compared.

Measurement of the intensity of the signal can also provide information regarding the number of free radicals present in the sample, since in magnetic resonance the area under a particular absorption curve is directly proportional to the number of unpaired spins responsible for that signal. The area under an absorption curve is therefore a more useful parameter for comparison than the peak height, and has been used as the measure of signal intensity throughout the experiments described in this section.

For the first derivative curve of the absorption, the area is proportional to the product of maximum amplitude $F'(H-H_o)_{max}$ and the square of the line width between slope extremes (2Δ) i.e. area = $k'2F'(H-H_o)_{max}\ (2\Delta)^2$. Substitution of the appropriate values for the normalised curves yields the area proportionality constants (k') 0.517 for the Gaussian function and 1.812 for the Lorentzian function respectively (190).

If area measurements are to be used to compare absorption spectra it is essential that all the factors, other than spin concentration that can affect signal intensity must be the same for all the spectral determinations. These factors include the rate of sweep of the magnetic field, the microwave frequency, and the temperature, and each of these experimental parameters was optimised (see page 211) and kept constant

throughout the E.P.R. determinations reported in this section.

In addition, caution must be exercised in comparing the areas of curves having different line shapes, or where a curve changes progressively from one function to another during the course of the experiment (190). It is apparent from the area proportionality constants quoted above that if comparison is made between the first derivative of two curves of the same peak to peak line width, and of the same maximum amplitude, the area under the Lorentzian will be 3.51 times that under the Gaussian.

It was therefore considered necessary to ascertain that the E.P.R. spectra obtained for spores both during drying and during subsequent heating were comparable with respect to the "g" value, the peak to peak line width, and the line shape.

A sample of spore suspension SS6S was dried for 6 hours at 308^{o}K, and subsequently heated at 393^{o}K for 24 hours in anoxia. At intervals during the drying and heating of the sample, E.P.R. determinations were carried out under the standardised recording conditions. The "g" value and peak to peak line width (2Δ) were determined for each of the first derivative spectra obtained. The line shape also was estimated by calculating the ratio of the maximum negative slope to the maximum positive slope. The data from this experiment are recorded in Table 31

It can be seen from Table 31 that the coefficients of variation for the "g" values, line widths and ratio of maximum negative slope to maximum positive slope are each less than 5%, and it was therefore concluded that under the experimental conditions employed, no significant change occurred in the basic parameters of the E.P.R. spectra. Since the line shape of the spectra did not change during the course of the experiment it was decided to estimate the area under the curve without reference to an area proportionality constant k. This area, calculated as $2F'(H-H_o)_{max}(2\Delta)^2$ has been used to characterise all E.P.R. spectra obtained in experiments reported in this section and is referred to as the "relative E.P.R. signal".

The absolute spin concentration can be determined by calibration of the spectrometer with standard samples having known spin concentrations such as α,α-diphenyl-β-picryl hydrazyl (D.P.P.H.). However, the practical difficulties involved in this technique result in low degrees of accuracy. In addition, the method requires that the line shape of the sample under test should be identical to that for the hydrazyl. Since the line shape for the absorption spectrum obtained with dried spores was shown to be intermediate between Gaussian and Lorentzian, it was considered that comparison of the spectrum with that of a standard hydrazyl sample would be of little value, and hence no attempt was made to determine the absolute number of spins in any of the spore samples.

TABLE 31

Measurement of spectral parameters of E.P.R. absorption spectra given by Bacillus megaterium spores dried at 308°K and subsequently heated at 393°K in anoxia.

Time of Treatment (Hours)		Receiver Gain	$2F'x \sim (H-H_o)_{max}$	"g" value	2Δ	Max. Neg. Slope / Max. Pos. Slope
Drying at 308°K	Heating at 393°K					
2	-	1.25×10^6	80.5	2.00262	13.0	2.92
4	-	1.25×10^6	92	2.00261	13.25	3.15
6	-	8×10^5	87.5	2.00308	12.75	3.21
6	0.5	6.2×10^5	86	2.00296	12.5	2.97
6	1	5×10^5	79	2.00308	12.25	3.26
6	3	5×10^5	86	2.00283	12.5	2.96
6	6	5×10^5	93.5	2.00298	12.75	3.05
6	12	5×10^5	106	2.00252	12.5	2.89
6	18	4×10^5	90.5	2.00283	12.25	2.93
6	24	4×10^5	98	2.00264	12.75	3.24
	Mean			2.002815	12.65	3.058
	Standard Deviation			0.000207	0.31629	0.14398
	Coefficient of Variation			0.01%	2.5%	4.71%

Subsequent Experiments

Development of the E.P.R. Signal in Spores Subjected to High Vacuum Drying

Experiments were performed to study the development of the E.P.R. signal during different drying procedures. The drying temperatures investigated were 273°K, 298°K, and 338°K. On different occasions samples were dried in E.P.R. vessels, at each temperature, for a period of 24 hours. At intervals during the drying process the sample vessels were removed from the manifold, placed in liquid nitrogen, with the samples held in anoxia, and the E.P.R. signal given by the contained spores was measured at that temperature. From the first derivative curves obtained, the value of the "relative E.P.R. signal" at each interval was calculated. It was not experimentally convenient to carry out measurements on the samples immediately after the commencement of drying, and thus the first reading on each sample was taken after 10 minutes drying. Furthermore, it was not possible with the technique employed, to control precisely the mass of spores in each sample that was in the microwave beam of the instrument, and hence variations occurred in the initial signal obtained from different samples dried under similar conditions. For comparative purposes therefore, the development of the E.P.R. signal is given in terms of percentage increase in the initial "relative E.P.R. signal", i.e. percentage increase in "relative E.P.R. signal" at time t = $\frac{\text{"relative E.P.R. signal" at time t}}{\text{"relative E.P.R. signal" at 10 minutes}} \times 100$. The results obtained in these experiments are given in detail in Appendix III and are illustrated graphically in Figure 63.

Inspection of Figure 63 reveals that at each of the drying temperatures investigated, an increase in the "relative E.P.R. signal" is observed with increase in the duration of drying. The magnitude of the

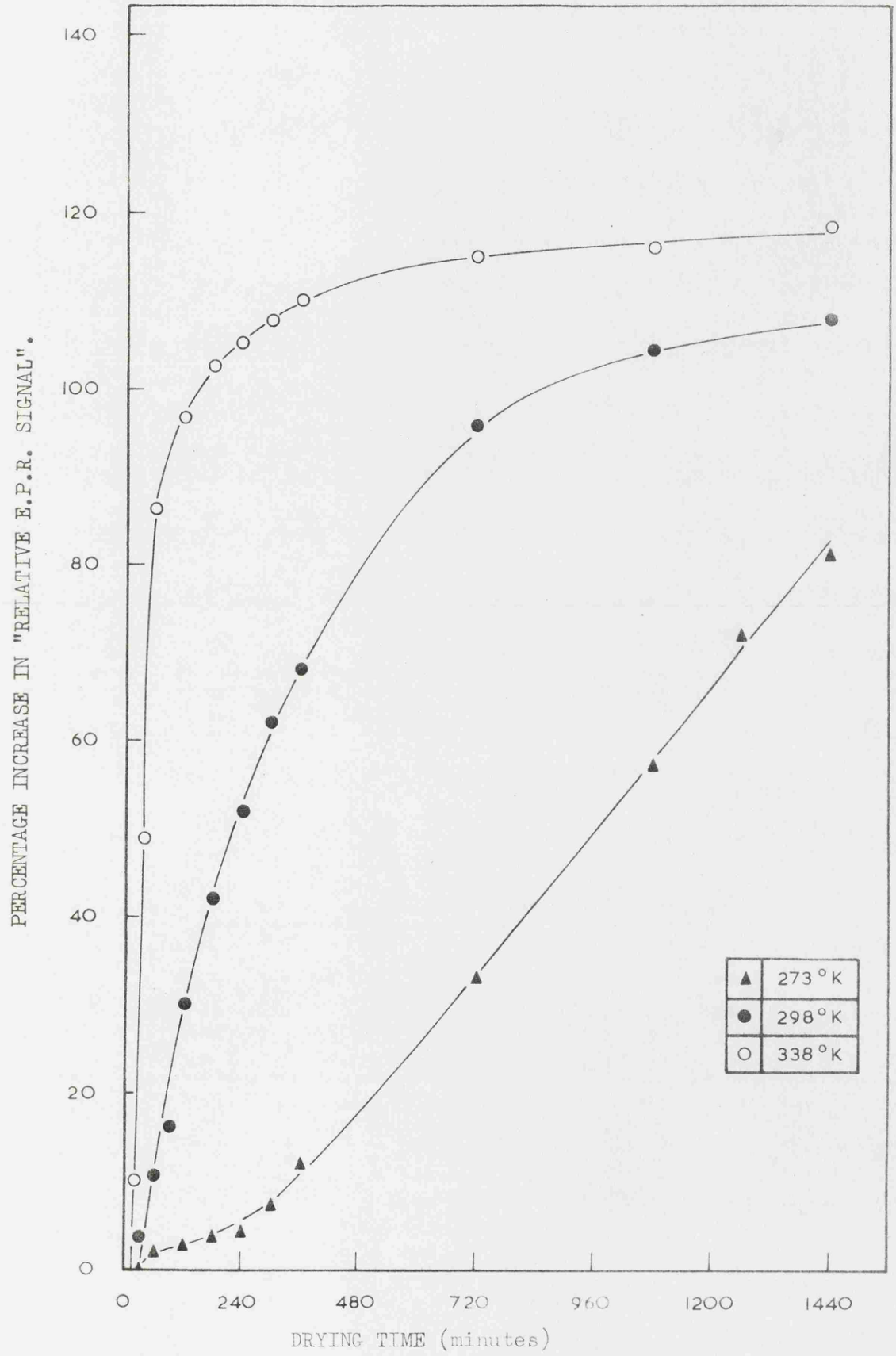


Figure 63. Plot of "percentage increase in relative E.P.R. signal" against "drying time" for Bacillus megaterium spores dried at different temperatures.

percentage increase in "relative E.P.R. signal" at the end of a 24 hour drying period is, however, different at each drying temperature, being greatest in spores dried at 338°K. Furthermore, although the percentage increase observed after 24 hours drying at 338°K appears to be maximal, it is likely that the magnitude after 24 hours drying at 273°K, and to a lesser extent at 298°K, does not represent the maximum development of the E.P.R. signal that can occur at these drying temperatures. The rate at which the greatest observed increase in "relative E.P.R. signal" is attained is also a function of the drying temperature, the fastest rate being obtained at the highest drying temperature (338°K).

At all drying temperatures investigated, the magnitude, shape and position of the E.P.R. signal were not measurably changed by re-equilibration of the samples to 10 torr aqueous vapour pressure, or by subsequent admission of oxygen to the spores.

The Effect of Different Drying Conditions on the Development of the E.P.R. Signal During Subsequent Heating of the Dried Spores.

The drying temperatures investigated were 273°K, 298°K and 338°K. On different occasions, samples of spore suspension were dried in E.P.R. vessels for 6 hours and 24 hours at each of the temperatures, and also for 10 minutes at 338°K. On each occasion, half of the samples were sealed in anoxia, and the remainder in oxygen.

E.P.R. measurements were made on the spores after drying and where necessary, after admission of oxygen. The sample vessels were then heated at 393°K and the E.P.R. determinations on the contained spores were repeated at intervals during the heating. From the E.P.R. measurements, the percentage increase in "relative E.P.R. signal" that occurred after the various heating periods was calculated.

Under all drying conditions investigated, the development of the E.P.R. signal on subsequent heating of the dried spores, follows a similar pattern. Data obtained from duplicate samples of spores dried for 6 hours at 298°K, and heated in anoxia and in oxygen, are shown in Figure 64. It can be seen from Figure 64 that both in oxygen and under anoxic conditions the "relative E.P.R. signal" increases to a maximum value after 120 minutes, this value being lower in oxygen than in anoxia. The rates of development of the signals appear similar under all conditions, showing an initial rapid rise and a subsequent levelling off. Spores heated in oxygen exhibit a decrease in the magnitude of the maximum "relative E.P.R. signal" with subsequent heating over a period of 600 minutes, unlike spores heated in anoxia where prolonged heating has no measurable effect on the maximum signal.

On separate occasions samples were re-equilibrated to 10 torr aqueous vapour pressure after 6 hours and 24 hours drying at 298°K and were heated at 393°K in oxygen and in anoxia. The effect of re-equilibration

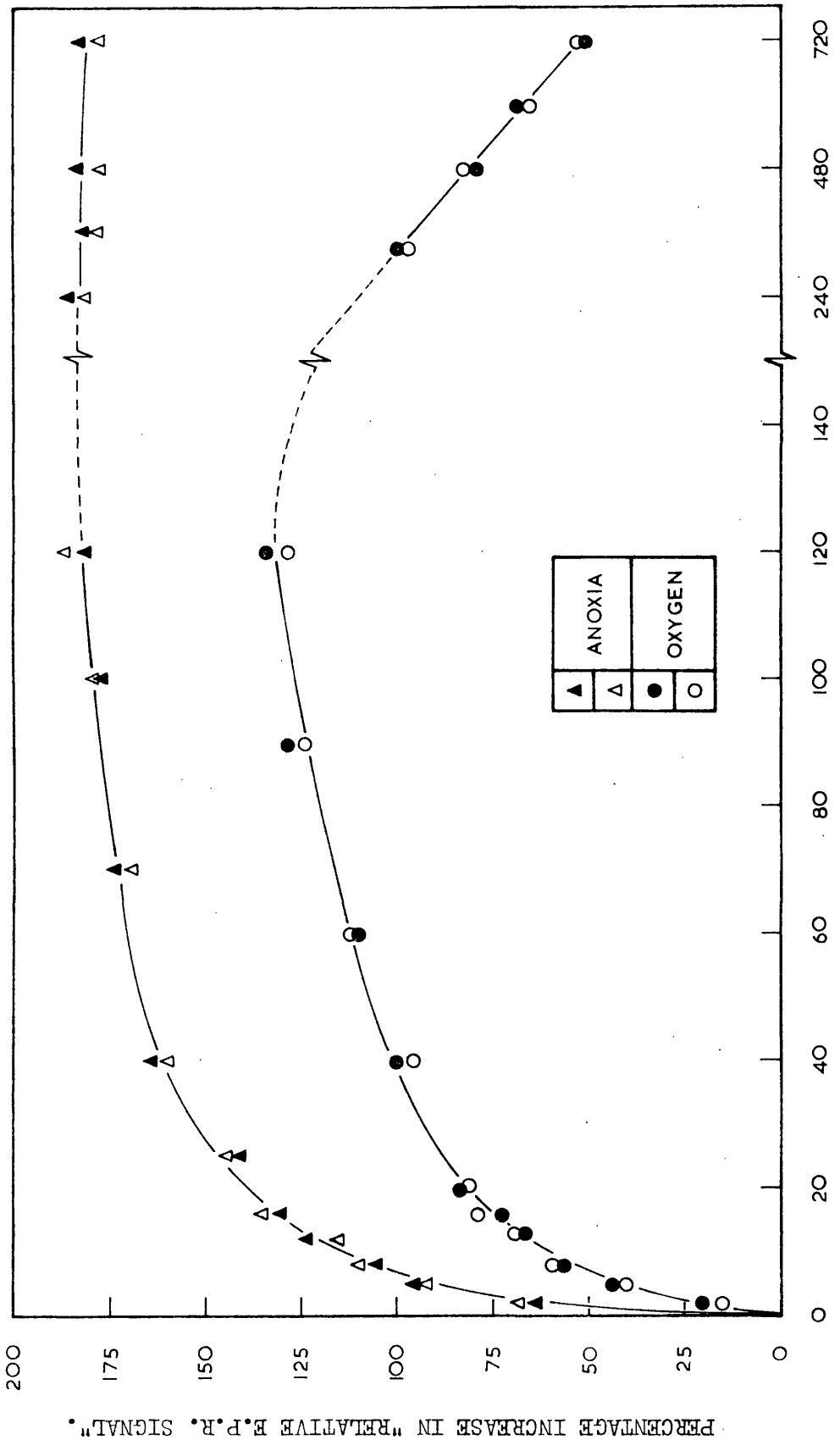


Figure 64. Plot of "percentage increase in relative E.P.R. signal" against "period of heating" at 393°K, for duplicate samples of Bacillus megaterium spores dried for 6 hours at 298°K, and heated in anoxia and in oxygen.

on the development of the E.P.R. signal during subsequent heating of the spores is illustrated in Figure 65. The curves shown in Figure 65 are similar to those obtained for non-equilibrated spores (Figure 64) in that the "relative E.P.R. signal" increases to a maximum after 120 minutes heating. In contrast to the results demonstrated with non-equilibrated dried spores, the maximum signal obtained with re-equilibrated spores was greater in oxygen than in anoxia. Furthermore, prolonged heating of the re-equilibrated spores in both anoxia and in oxygen resulted in a decrease in the "relative E.P.R. signal" whereas this effect was only observed in non-equilibrated spores when oxygen was present, and was not detected under anoxic conditions.

The effect of different drying conditions on the development of the E.P.R. signal during subsequent heating of the dried spores was assessed firstly on the basis of the maximum percentage increases in "relative E.P.R. signal" obtained, and secondly on the rate at which these maxima were attained.

(a) The maximum percentage increase in "relative E.P.R. signal".

It has been shown (page 228) that spores dried under different conditions contain different numbers of paramagnetic species. These concentrations, expressed as percentage increase in "relative E.P.R. signal" are given in Table 32 and are termed "E.P.R. values". The increase in signal intensity with subsequent heating of the dried spores is then expressed in terms of the corresponding increase in the "E.P.R. value".

The maximum "E.P.R. values" observed after 120 minutes heating at 393^{o}K, in anoxia and in oxygen, are also listed in Table 32 for non-equilibrated spores and for spores re-equilibrated to 10 torr aqueous vapour pressure.

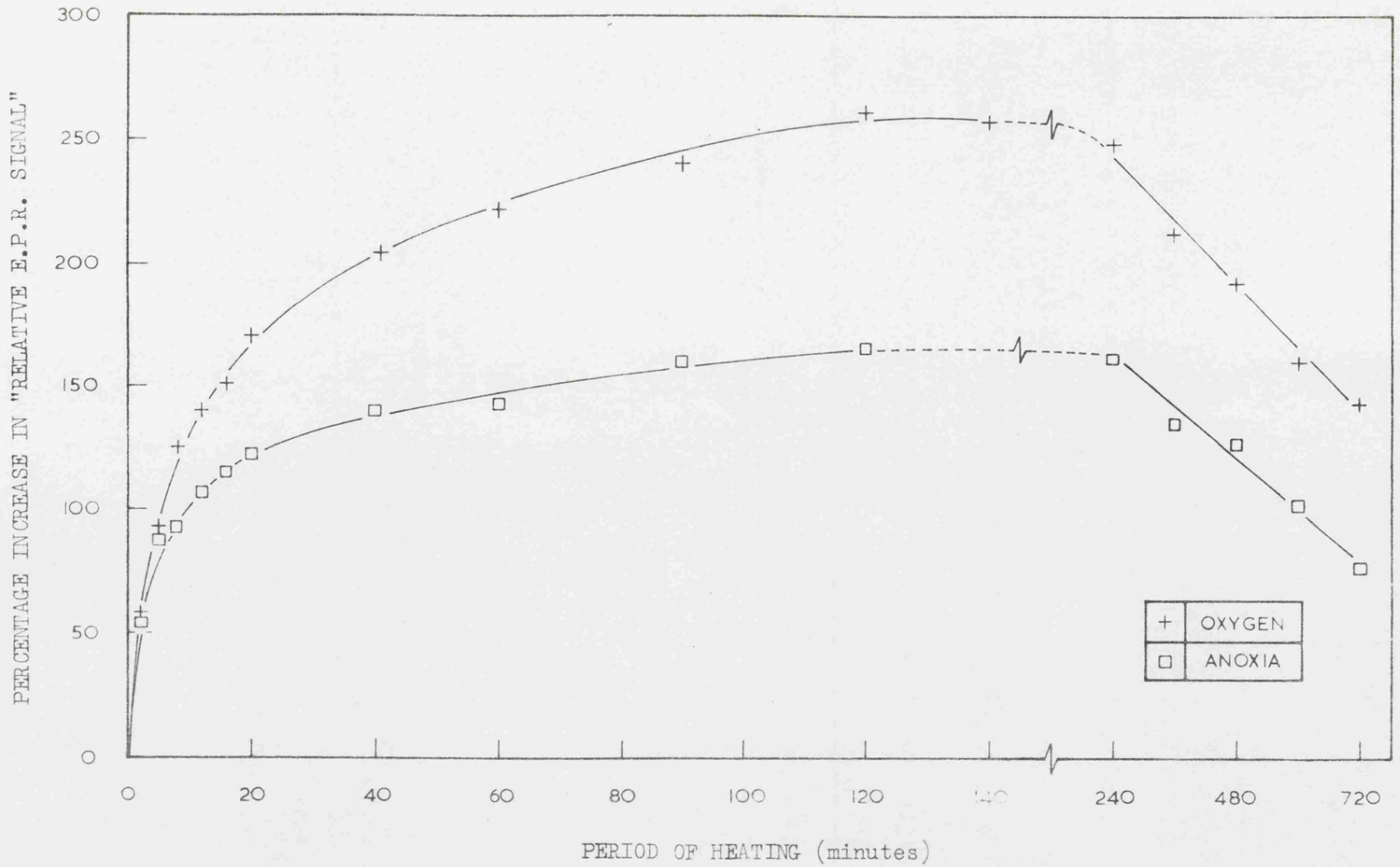


Figure 65. Plot of "percentage increase in relative E.P.R. signal" against "period of heating" at 393°K for Bacillus megaterium spores dried for 6 hours at 298°K, re-equilibrated to 10 torr. aqueous vapour pressure, and heated in anoxia and in oxygen.

TABLE 32

"E.P.R. values" for Bacillus megaterium spores dried under different conditions, with and without re-equilibration to 10 torr aqueous vapour pressure, and heated for 120 minutes at 393°K, in anoxia, and in oxygen.

Drying Time	Drying Temperature (°K)	Initial "E.P.R. value"	Maximum "E.P.R. value"	
			Non-equilibrated	Re-equilibrated
a) Spores heated in anoxia				
6 hrs	273	11.11	42.44	-
24 hrs	273	81.48	243.51	-
6 hrs	298	68.27	196.18	147.86
24 hrs	298	107.69	412.26	284.93
10 mins	338	1	372.94	-
6 hrs	338	110.57	290.94	-
24 hrs	338	118.55	246.17	-
b) Spores heated in oxygen				
6 hrs	273	11.11	29.16	-
24 hrs	273	81.48	188.56	-
6 hrs	298	68.27	160.36	197.29
24 hrs	298	107.69	226.5	390.07
10 mins	338	1	369.94	-
6 hrs	338	110.57	249.16	-
24 hrs	338	118.55	231.14	-

It can be seen from Table 32 that for spores dried under all the experimental conditions investigated, without re-equilibration, the maximum "E.P.R. value" observed is greater when the spores are heated in anoxia than in oxygen. The maximum is also dependent upon the drying time at any given drying temperature. With spores dried at 273°K, the maximum value obtained under anoxic heating conditions is increased from 42.44 to 243.51, a factor of approximately 6, by extending the drying time from 6 hours to 24 hours. A similar increase (by a factor of 6) from 29.16 to 188.56 is also observed when heating is carried out in oxygen. Spores dried at 298°K also show a marked increase in the maximum "E.P.R. value" for a similar prolongation of the drying period, although in this case the difference observed is only a factor of approximately 2 for spores heated in anoxia, and 1.4 for spores heated in oxygen. In contrast, extension of the drying time at 338°K from 10 minutes to 6 hours and then to 24 hours results in a decrease in the maximum signal that is obtained on subsequent heating of the dried spores. This decrease, by a factor of approximately 0.6 is independent of the presence of oxygen during the heat treatment.

When spores dried at 298°K are re-equilibrated to 10 torr aqueous vapour pressure, the maximum "E.P.R. value" observed is again a function of the gaseous conditions at the time of heating, but in contrast to the results obtained with non-equilibrated spores, the maximum is higher in oxygen than in anoxia. The maximum is also increased by extension of the drying time from 6 hours to 24 hours, but this increase, by a factor of approximately 2, unlike the corresponding effect observed in non-equilibrated spores, is independent of the presence of oxygen during the heating.

(b) The development of the maximum "E.P.R. value".

This is illustrated in Figure 66 for spores dried for 6 hours

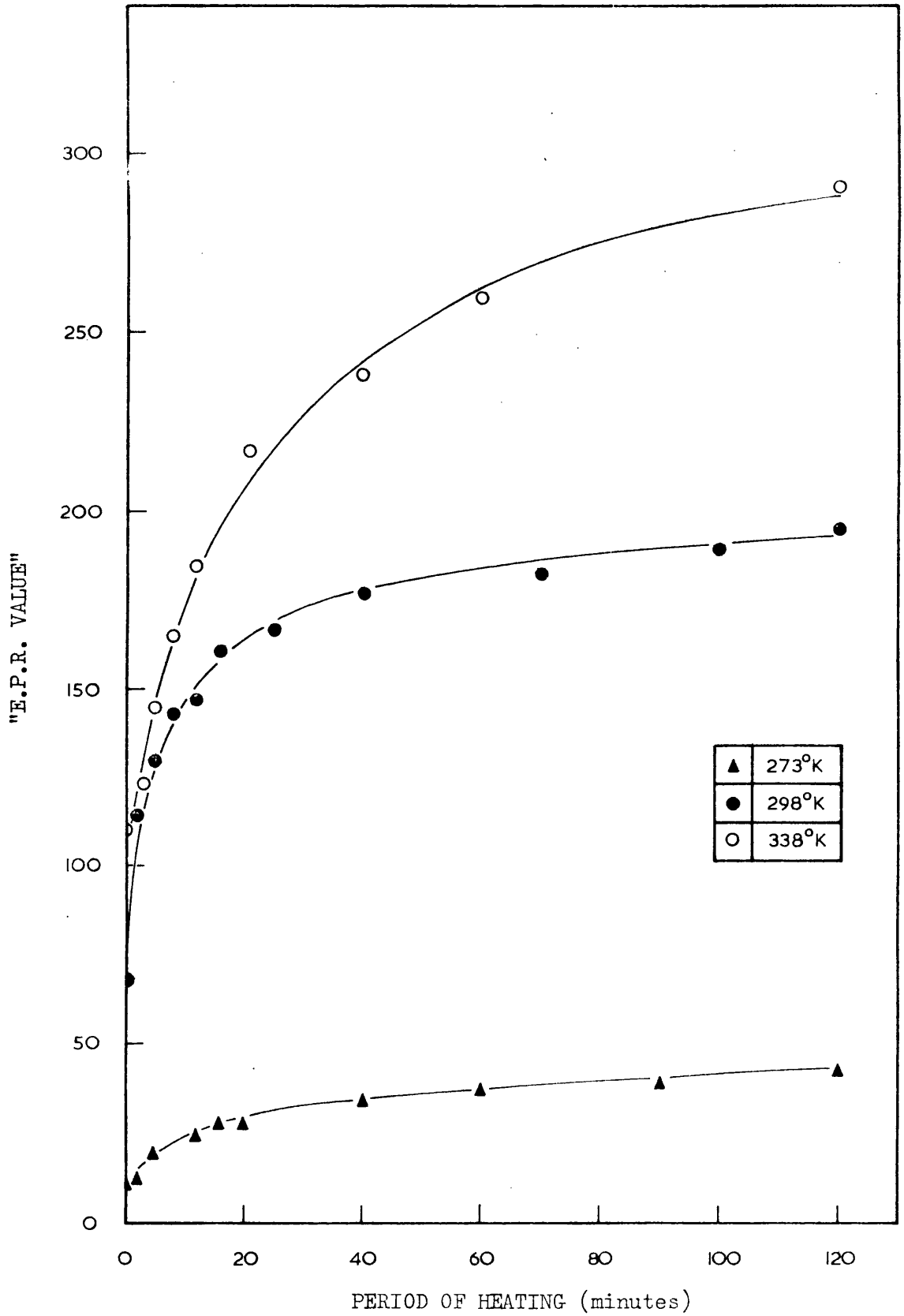


Figure 66. Plot of "E.P.R. value" against "period of heating" at 393°K, for Bacillus megaterium spores dried for 6 hours at different temperatures and heated in anoxia.

at 273°K, 298°K and 338°K, and heated in anoxia. The development follows a similar curvilinear pattern in all cases.

To enable a comparison to be made of the rate of development of the maximum "E.P.R. value" during heating of spores dried under different conditions, the fraction of the maximum observed

$$\frac{\text{"E.P.R. value"} - \text{initial "E.P.R. value"}}{\text{maximum "E.P.R. value"} - \text{initial "E.P.R. value"}}$$ was calculated.

Plots of these values against heating time for spores dried for different periods at 338°K are shown in Figures 67a and 67b for samples heated in anoxia and in oxygen respectively. The effect of different drying temperatures is illustrated in Figures 68a and 68b for spores dried for 6 hours and 12 hours respectively, and heated in anoxia.

It is apparent from these plots that the rate of development of the maximum "E.P.R. value" under the influence of heat follows a similar curvilinear pattern in spores dried under all conditions investigated. It did not appear reasonable to fit all the data obtained in these experiments to a single standard curve. Examination of the data, however, failed to reveal any distinct pattern or indication of a direct relationship between the rate of development and the experimental treatments involved.

Measurements of E.P.R. signals taken from recorder traces are necessarily imprecise and subject to a certain experimental error. During subsequent computation possible differences will be magnified. For this reason and also in view of the fact that the graphs are obtained from wide ranges of signal intensity, i.e. 1 to 372.94, 11.11 to 42.44, and 107.69 to 412.26, it is concluded that there is a reasonable equality between the rates of development of the maximum "E.P.R. value" under the influence of heat, in spores subjected to different drying treatments. It would certainly seem unreasonable, on the basis of the data obtained in these experiments to postulate a significant difference in these rates of

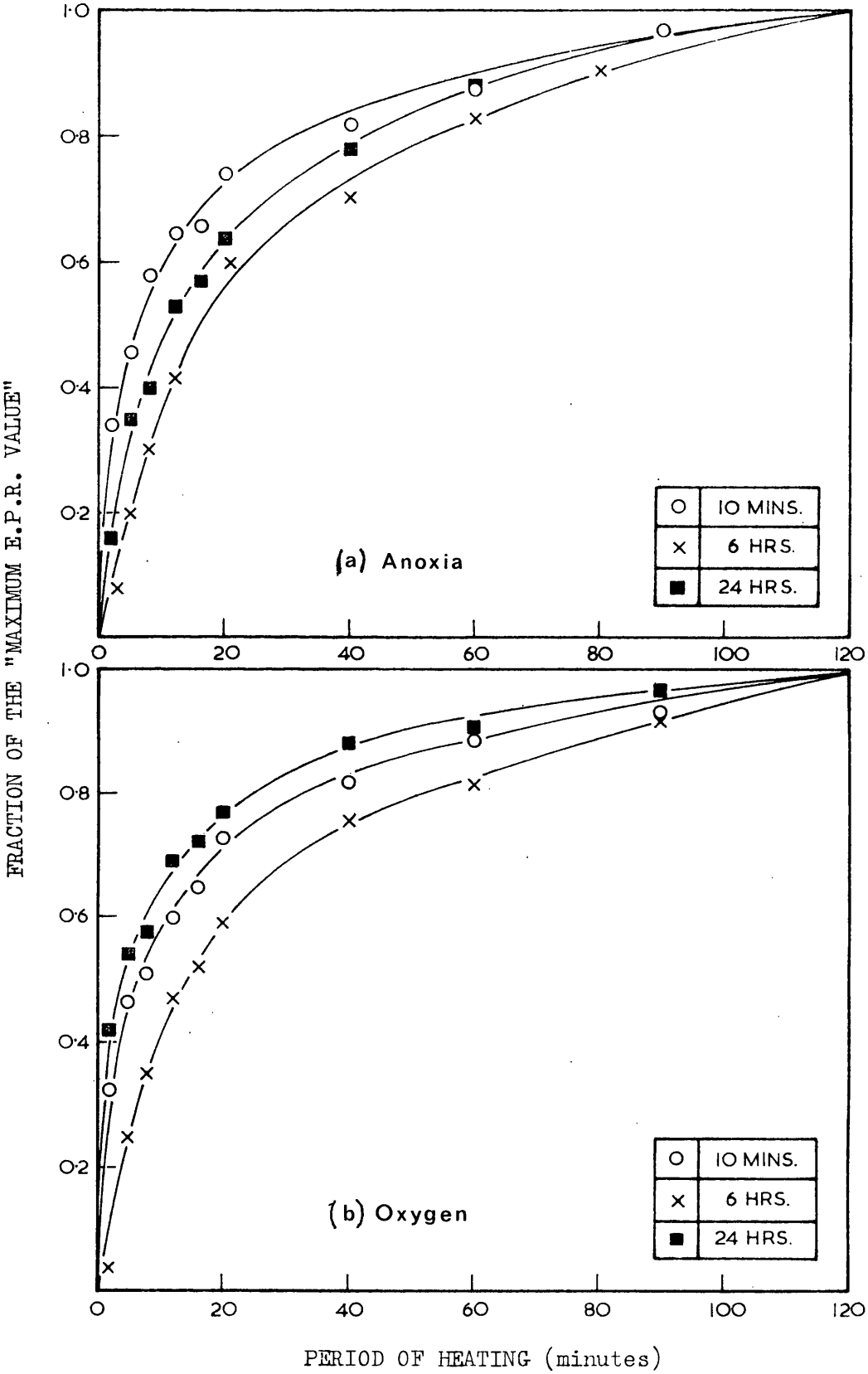


Figure 67. Plot of "fraction of the maximum E.P.R. value" against "period of heating" at 393°K, for Bacillus megaterium spores dried for different times at 338°K and heated (a) in anoxia and (b) in oxygen.

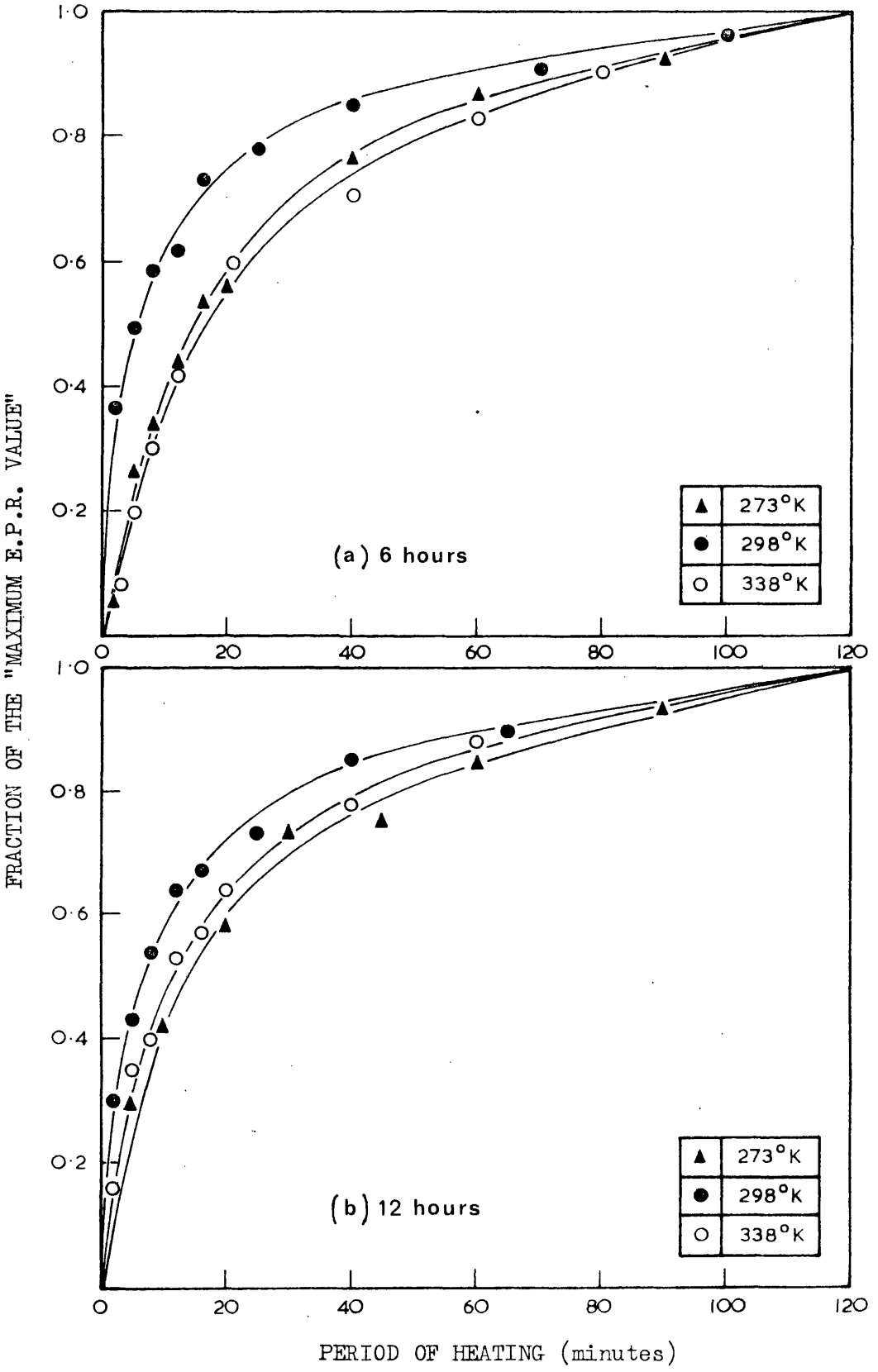


Figure 68. Plot of "fraction of the maximum E.P.R. value" against "period of heating" at 393°K, in anoxia, for Bacillus megaterium spores dried at different temperatures for (a) 6 hours and (b) 12 hours.

development, although definite conclusions regarding the real effect of various drying procedures on the development of paramagnetic species in spores heated in both oxygen and anoxia will have to await the results of further investigation.

The effect of re-equilibration of the spores to 10 torr aqueous vapour pressure on the rate of development of the maximum "E.P.R. value" is illustrated in the plot of the fraction of the maximum observed against heating time at 393^{o}K, shown in Figures 69a and 69b for samples heated in anoxia and in oxygen respectively.

It was concluded from these graphs, that although re-equilibration had been shown to influence the magnitude of the maximum "E.P.R. value" obtained with subsequent heating, the experimental data showed no indication that the rate of development of the maximum was significantly altered by the addition of 10 torr water vapour to the spores.

The significance of these results, in terms of the behaviour of the paramagnetic species in the spore, and in relation to the observed biological responses of the organism, is considered in the Discussion (page 292).

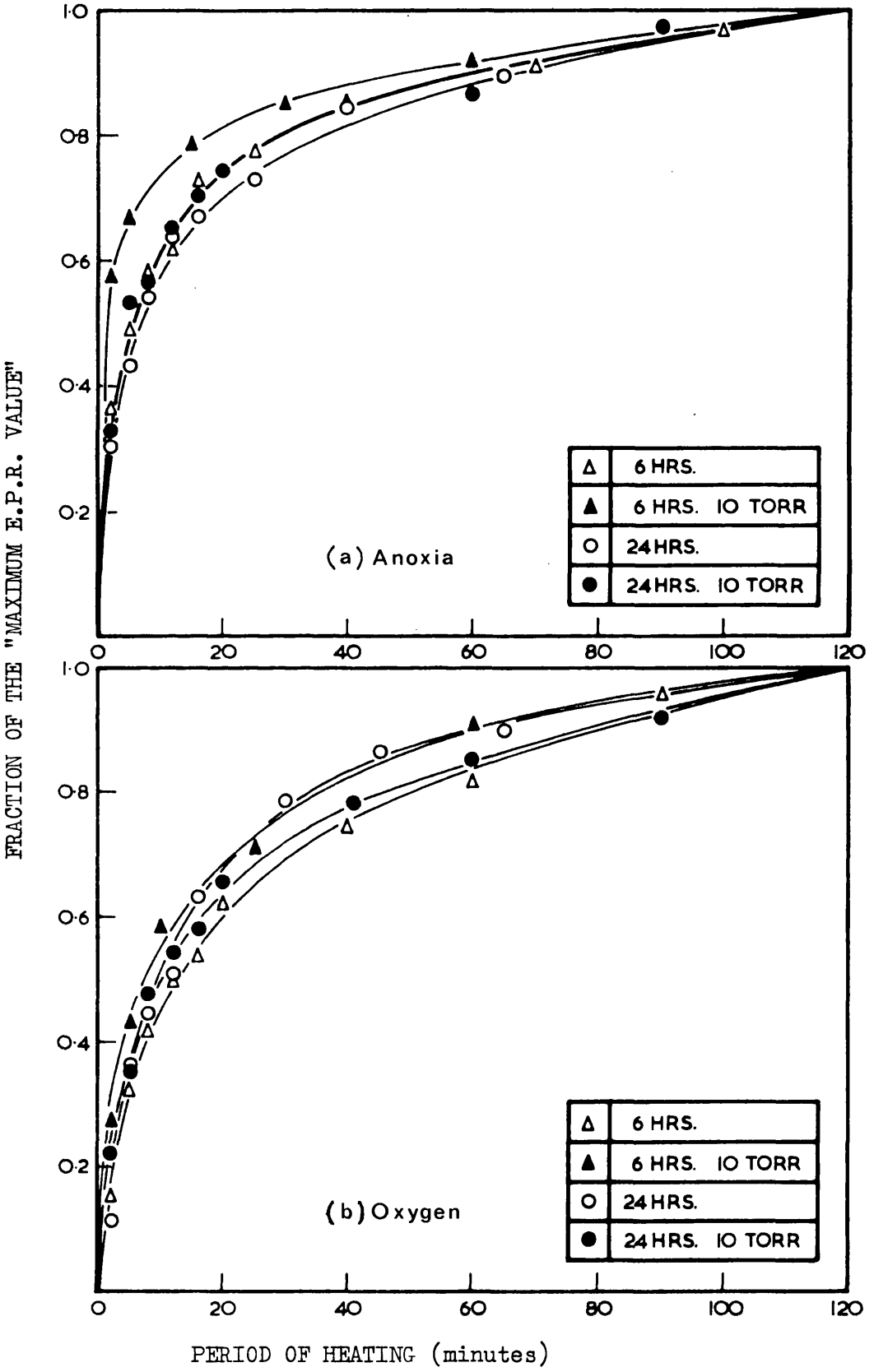


Figure 69. Plot of "fraction of the maximum E.P.R. value" against " period of heating" at 393 °K for Bacillus megaterium spores dried for different times at 298 ° K, re – equilibrated to 10 torr. aqueous vapour pressure, and heated (a) in anoxia and (b) in oxygen.

D I S C U S S I O N

The work presented in this thesis is unique in that it is the first time that the process of freeze-drying has been characterised utilising physical instrumentation to measure weight changes, temperature changes, and free radical production. Since the technique of freeze-drying is widely used in biology, pharmacy and the food industry, the findings obtained from physical measurements are important in themselves as they give an insight into the processes that occur during drying and the mechanisms by which water is removed. This section is therefore in two parts. The first part is confined to a discussion of the physical aspects of the high vacuum drying process, while the second part is concerned with the effects of this process in terms of the biological integrity and responses of the spore.

A description and analysis of the results from physical measurements has already been presented in detail on pages 63 to 104. In the section only those findings for which further interpretation is necessary are considered.

The high vacuum drying technique described on pages 52 to 57 is divisible into four main stages:- 1) the equilibration of the sample to the drying temperature 2) the freezing of the moisture in the sample 3) the removal of water by sublimation from the ice phase i.e. primary drying and 4) dehydration of the non-freezable moisture which remains in the sample after sublimation is complete i.e. secondary drying.

During the initial equilibration of the sample to the drying temperature i.e. to that of the surrounding bath some of the water in the sample is removed. Throughout this equilibration stage the samples are in air, and it would therefore be expected that water loss during this stage would be by simple evaporation. As

indicated on page 92 , this would result in an activation energy for the water removal process at the end of the equilibration period comparable to the heat of vapourisation of pure water, and this is in fact obtained.

The processes occurring during the second stage of the drying i.e. the preliminary freezing of the sample can best be discussed with reference to the initial section of the sample temperature/time profile (Figures 4a, 4b and 5). This section (A ⟶ I in Figure 5) is essentially a typical cooling curve of the type designated a "pseudo-freezing curve" by Luyet (192). The classical shape of such a curve obtained when a sample of material is frozen by placing in a low temperature bath is illustrated in Figure 70.

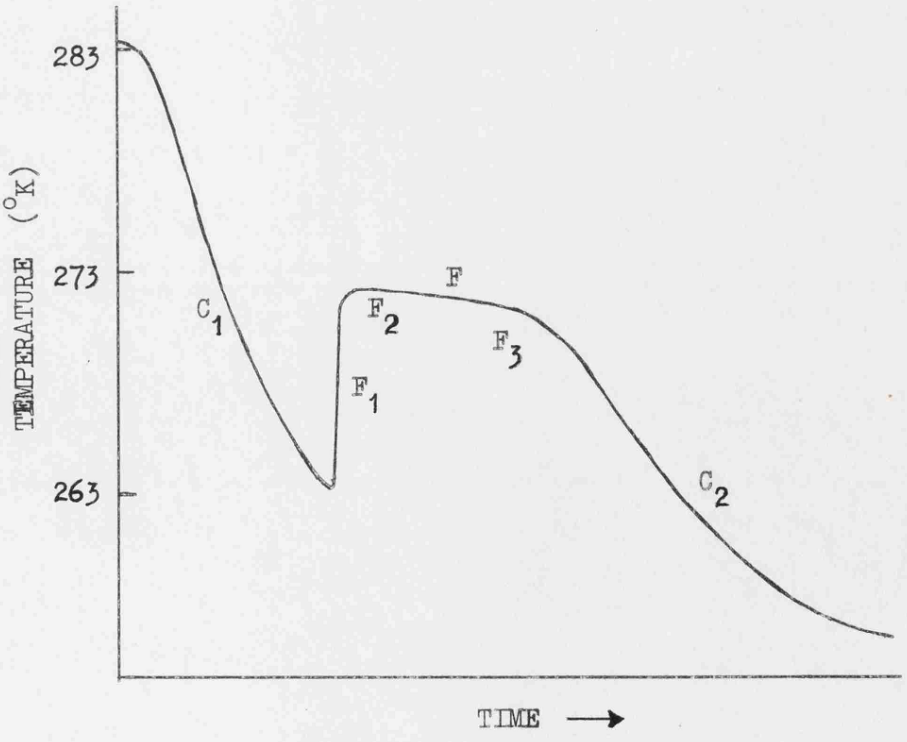


Figure 70. Schematic diagram of the "pseudo-freezing curve" obtained when a sample is frozen by immersion in a low temperature bath. (From Luyet.(192))

The curve consists of three parts. The first part C_1 is a cooling curve of the unfrozen material, the minimum temperature attained being dependent upon the amount of supercooling of the material. The third part C_2 is a cooling curve of the frozen material, and the middle part F represents the temperature changes during freezing and consists of three segments F_1, F_2 and F_3 which correspond to three stages in ice propogation. The stage represented by the almost vertical portion F_1, marks the initial invasion stage of the ice during which unimpeded crystallisation units grow rapidly. The rate of ice formation at this stage will depend on the free energy difference between the supercooled water and ice and increases with increased supercooling. The latent heat of fusion liberated during the development of ice cannot be dissipated as rapidly as it is formed, and a sharp rise in temperature to the freezing point results.

The accumulation of latent heat now acts as a limiting factor on ice formation and brings about the transition from F_1 to F_2. During F_2, invasion of the medium by ice continues at a reduced rate such that little change in temperature occurs, the rate of ice formation and thus the liberation of heat of fusion is limited by the rate of heat withdrawal from the sample by the surroundings. Gradual depletion of available water then results in heat liberation becoming progressively lower, and a gradual drop in temperature occurs (F_3). During this stage, the last fraction of freezable water crystallises.

This classical "pseudo-freezing curve" is obtained under conditions where only freezing is occurring, and thus it is of interest that curves of basically similar shape are obtained during the initial stage of the drying process, where freezing is accompanied by sublimative drying of the frozen water. Differences between the curves obtained during the drying process and the classical curve may

therefore reflect the influence of pressure reduction within the sample vessel, temperature of the surrounding bath, and sublimation of ice, on the freezing process.

At all drying temperatures investigated the final sample temperature at the end of the cooling stage C_1 (A ⟶ F in Figure 5) is $260^{\circ}K \pm 1.2^{\circ}K$, showing that in all cases the spores undergo 12 to 14 degrees of supercooling. At all temperatures apart from $338^{\circ}K$ the time necessary to achieve this minimum is 0.98 ± 0.05 minutes. At $338^{\circ}K$ the time is shorter at 0.78 minutes, presumably because of significant evaporative drying occurring during cooling. The initial rate of ice formation is a function of the degree of supercooling and will thus be independent of the drying temperature over the range investigated. The influence of pressure reduction and drying temperature result only in the curve C_1, being less smooth than the classical curve.

The most marked deviations from the classical curve occur in the freezing portion F (G ⟶ I in Figure 5). At a drying temperature of $273^{\circ}K$ the substages F_2 and F_3 are not observed, and a steady decrease in the sample temperature occurs, as would be expected if the heat of fusion liberated during ice formation was effectively dissipated to the surroundings. At drying temperatures between $288^{\circ}K$ and $308^{\circ}K$ the freezing portion F resembles more closely the classical curve and suggests that at these temperatures the withdrawal of the liberated heat of fusion by the surrounding water bath is less efficient, and thus the F_2 and F_3 stages are observed. By the same argument it would be expected that the stages F_2 and F_3 would be observed at drying temperatures of $323^{\circ}K$ and $338^{\circ}K$. However, this does not occur in practice, the temperature of the frozen sample decreases rapidly at first and then

more slowly. A possible explanation for this observation is that the rate of sublimation of ice is high at these temperatures (see Table 9) and that the heat of fusion liberated at the ice front is effectively dissipated at the rapidly advancing drying front, and thus accumulation of latent heat is not a limiting factor on ice formation at these drying temperatures.

The drying rates observed during the freezing stage of the drying process are significantly higher than those seen during the equilibration stage (Figure 13). This is probably the result initially of increased evaporative drying under the influence of reduced pressure, and also in the latter stages to sublimation of the frozen sample.

The third stage of the drying process i.e. the removal of water by sublimation, commences when the ice phase is first formed (point G in Figure 5). Since the rate of sublimation is dependent upon the absolute temperature drop (Δ T) across the sample (equation 4), the primary drying stage will continue until the temperature of the sample is at equilibrium with that of the surrounding bath and Δ T = 0. The sample temperature will thus increase during the primary drying stage and the resultant sample temperature/time profile will take the form of a classical warming curve (J $\longrightarrow$ M in Figure 5).

Previous workers have suggested that the freezing stage of the drying process may account for a large proportion of the damage induced in cells by freeze-drying (124, 193). In particular it has been demonstrated that a higher proportion of cells in aqueous suspension survive slow freezing in which ice formation is restricted to the extra-cellular space, than survive a rapid rate of freezing where intracellular ice formation is induced (32, 34, 194). The complexity of the sample temperature/time profile obtained during

the drying process does not permit a meaningful value for the freezing rate to be calculated. However, an indication of this rate can be obtained from a consideration of the time taken for the sample to attain the minimum frozen temperature ($t_A \longrightarrow t_I$ in Table 7). A curvilinear relationship exists between this time and the drying temperature (Figure 6), the longest time, 10 minutes, being observed at the lowest temperature ($273^{o}K$). This data would suggest, therefore, that the slowest freezing rate and thus the highest survival of spores would be expected at the lowest drying temperature investigated i.e. $273^{o}K$.

Mazur (194) has also shown that cell survival is dependent upon the rate of thawing of a frozen suspension. However, in freeze-drying this is of no importance since water is removed before thawing can take place. It is therefore difficult to predict the importance, in terms of spore survival, of the time taken for the sample to warm up from the minimum frozen temperature to the drying temperature ($t_I \longrightarrow t_M$ in Table 7), or to suggest the significance of the curvilinear relationship that is shown to exist between this time and the drying temperature (Figure 6).

By using the Micro-Force Balance to determine changes in sample weight during drying it is possible to follow when water is lost and equate this to changes in sample temperature. In the Introduction (pages 5 to 8) a number of equations have been given to characterise the rate of sublimation, in terms of loss of weight, obtained under idealised theoretical conditions. By making certain basic assumptions it is possible to apply these equations to the non-ideal experimental drying system, and to obtain an assessment of certain functions that characterise the rate of sublimation and hence influence the primary stage of the process. In particular it

should be possible to obtain an estimate of the drying factor, f, and thermal conductivity, k_T, for the frozen spore suspension relative to the values obtained for pure ice (pages 6 and 7). and thus to get a measure of the overall efficiency of the drying process.

The basic assumptions that must be made in applying these equations are:-

1) that changes in sample weight with drying time are entirely the consequence of sublimation of ice from the sample and that the corresponding drying rate represents the rate of sublimation $\frac{dm}{dt}$
2) that spores are evenly distributed throughout the sample and remain so during the drying process,
3) that the frozen sample is in the form of a cylinder, and sublimation takes place in a manner such that the drying boundary is parallel to the upper surface of the sample and moves in a direction perpendicular to it,
4) that heat is delivered to the drying boundary by conduction from the heated surface of the sample vessel in contact with the base of the cylindrical frozen sample.

From equation (1) the drying factor f can be calculated as

$$f = \frac{dm}{dt} \quad \frac{2\pi RT_s}{\alpha P_s}^{\frac{1}{2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (28)$$

where $\frac{dm}{dt}$ is the drying rate in g. cm^{-2} sec^{-1}, and $\left(\frac{2\pi RT_s}{\alpha P_s}\right)^{\frac{1}{2}}$ is the maximum rate of sublimation of pure ice at a temperature T_s and vapour pressure P_s. The minimum temperature of the frozen sample (I in Table 7) is used as the temperature of the subliming interface T_s. The drying rates at time t_I taken from the sample weight/drying time profiles (see pages 92 to 100) and all relevant

data for the calculations are given in Table 33, together with the corresponding values of f.

The drying factor is usually expressed in terms of $\frac{1}{f}$, and with this treatment the values obtained lie randomly between 61 and 74. This indicates an inefficient, slow drying rate which results from the combined resistance of the dry shell of the sample and the drying apparatus to the flow of vapour. Using thin samples (10^{-1} mm to 10^{-2} mm) and an efficient drying apparatus in which there was no obstruction to water vapour flow between the sample and the condensing surface, other workers have obtained values of $\frac{1}{f}$ as low as 10, although with thick samples ($>$ 1 mm.) values greater than 100 were frequently observed. (7, 195). It is clear, however, that the lack of efficiency of the drying process is not a function of the drying temperature, over the range investigated.

From equation (2) it can be seen that the drying factor, f, is a composite of two probability functions f_e and f_s. Insufficient data is available concerning the geometry and structure of the dry shell to enable the contribution of the shell to the total resistance to vapour flow, and so f_s, to be calculated. However, the contribution of the drying apparatus to the overall inefficiency of the process can be calculated from f_e. The type of water vapour flow from the dried shell, and thus f_e in equation (2) is dependent upon the mean free path λ of the water vapour molecules. For a given temperature T and pressure P

$$\lambda = \frac{2.331 \times 10^{-20} T}{d^2 P} \quad \ldots\ldots\ldots\ldots (29)$$

where d is the molecular diameter and has a value of 4.6×10^{-8} cm. for water vapour (196). During primary drying the rotary vacuum pump only is operating and the pressure in the system does not fall

TABLE 33

Date obtained with Bacillus megaterium spores during primary drying.

Drying Temperature T (^{o}K)	Sublimation Temperature T_s (^{o}K)	Vapour Pressure at T_s (P_s) (torr)	Drying Time to Attain T_s (min)	ΔT ($T - T_s$) (^{o}K)	$\frac{\alpha P_s}{(2\pi RT_s)^{\frac{1}{2}}}$ ($gm(cm^2)^{-1} sec^{-1}$)	Drying Rate at T_s (observed) ($gm(cm^2)^{-1} sec^{-1}$)	Drying factor f	Thermal Conductivity K_T ($cal.cm^{-1} sec^{-1} cm^{-2} {}^{o}K^{-1}$)
273	239.5	2.208×10^{-1}	10	33.5	4.038×10^{-3}	5.455×10^{-5}	1.35×10^{-2}	1.09×10^{-3}
288	243	2.859×10^{-1}	5.5	45	5.191×10^{-3}	8.527×10^{-5}	1.64×10^{-2}	1.27×10^{-3}
298	246	3.89×10^{-1}	3.5	52	7.019×10^{-3}	9.784×10^{-5}	1.39×10^{-2}	1.26×10^{-3}
308	247.5	4.53×10^{-1}	2.25	60.5	8.149×10^{-3}	1.117×10^{-4}	1.37×10^{-2}	1.24×10^{-3}
323	250	5.8×10^{-1}	1.5	73	1.038×10^{-2}	1.485×10^{-4}	1.43×10^{-2}	1.36×10^{-3}
338	251.5	6.71×10^{-1}	1.04	86.5	1.197×10^{-2}	1.753×10^{-4}	1.46×10^{-2}	1.36×10^{-3}

below 5×10^{-2} torr. At the environmental temperature of 298°K, the mean free path of the water vapour molecules is less than 6.6×10^{-2} cm. Since this distance is smaller than the minimum radius of the drying apparatus (2×10^{-1} cm. at the sample vessel constriction) the flow of water vapour from the surface of the dry shell to the vapour removal surface will be viscous rather than the theoretically ideal molecular type. Thus it is clear that during the primary drying stage of the process, the design of the drying apparatus is a major factor responsible for the slow rates of drying that are observed in practice, compared to those that are theoretically possible.

The thermal conductivity k_T of the frozen spore sample can be derived from equation (4) as

$$k_T = \frac{dm}{dt} \frac{L}{\Delta T} \quad \ldots\ldots\ldots\ldots\ldots\ldots (30)$$

where L is the latent heat of sublimation of ice (6.7×10^2 cal. gm^{-1}). The values of k_T obtained at different drying temperatures are recorded in Table 33, and range from 1.09×10^{-3} cal. $cm.^{-1}$ $sec.^{-1}$ $cm.^{-2}$ $^oK^{-1}$ to 1.36×10^{-3} cal. $cm.^{-1}$ $sec.^{-1}$ $cm.^{-2}$ $^oK^{-1}$. These values are low compared to the value for pure ice of 5.7×10^{-3} cal. $cm.^{-1}$ $sec.^{-1}$ $cm.^{-2}$ $^oK^{-1}$, and are also lower than the value of 2.6×10^{-3} cal. $cm.^{-1}$ $sec.^{-1}$ $cm.^{-2}$ $^oK^{-1}$, recorded by previous workers for frozen tissue (8). Thus, rather surprisingly, increasing the drying temperature does not have a great influence on k_T and therefore cannot markedly influence the overall efficiency of the drying process, even if a more efficient system for removal of water vapour was devised. The low values of k_T obtained indicate that the rate of heat transfer to the drying boundary is an important limiting factor in the drying process, and are unexpected because

of the small contribution of the spores to the total mass of the sample ($<5\%$).

Theoretically, at the end of sublimation the temperature of the sample will return to that of the environment, and there should also be a sudden marked reduction in the rate of water removal as shown by changes in sample weight. The data from sample temperature/time profiles given in Table 7 show that a curvilinear relationship exists between the time from the start of the process until the sample has re-equilibrated to the temperature of the surrounding bath ($t_A \longrightarrow t_M$), and the drying temperature (Figure 6), these times increasing from 5.25 minutes to 18.5 minutes with a decrease in drying temperature from 338°K to 288°K. The drying rate/drying time curves derived from the Micro-Force Balance data show that a curvilinear relationship also exists between the time during which the fast drying rate is maintained, and the drying temperature, but these times are always longer, ranging from 8 minutes at 338°K to 27 minutes at 288°K (Figure 12). The difference between the times taken from the sample temperature/time profiles and those derived from drying rate/drying time curves is considered to be due to the fact that the thermocouple is sited at the centre of the sample, and not at the base. Thus, while the drying time obtained from sample temperature data are reproducible and good for comparison, they do not give a true estimate of the end of sublimation in the total sample. It is therefore considered that the end of the period during which the fast drying rate is maintained, determined from the drying rate/drying time curves in Figure 12, give a true indication of the end of sublimation (and therefore of the primary drying stage) for drying temperatures between 288°K and 338°K. These times are shown to exhibit a direct relationship with the reciprocal of

of the drying temperature (Figure 14). Thus the duration of the primary drying stage is a function of the drying temperature, even though the overall efficiency of the process, as measured by the drying factor and the thermal conductivity of the spore samples is independent of the temperature.

It is also significant that at the end of the fast drying rate period the percentage weight loss from the sample is independent of the drying temperature and is constant at 89.7% $\pm$ 0.9%, which lends support to the suggestion that this point is a realistic indication of the end of sublimation for drying temperatures above 288^{o}K.

At a drying temperature of 273^{o}K the end of the fast drying rate period is not clearly defined, and thus the end of sublimation cannot be accurately determined by this method. However, it is apparent from the sample temperature/time profile data (Table 7) that the duration of the primary drying stage must be in excess of 35 minutes at this temperature.

During the final desorptive stage of the drying process measurable changes in sample temperature are not observed and the sample remains at equilibrium with the surrounding bath. Thus for the period of secondary drying the sample is essentially in storage in anoxia at the drying temperature. Throughout this period the pressure in the vacuum system is maintained below 10^{-5} torr. The mean free path for the water vapour molecules under these conditions is in excess of 3.2×10^{2} cm., and thus the flow of vapour from the sample surface to the vapour removal surface will be molecular. The mass of water vapour flow under these conditions can be calculated from equation 9. For $r = 2 \times 10^{-1}$ cm., $l = 1.1 \times 10^{2}$ cm., and $P_D = 5 \times 10^{-4}$ torr the values of G obtained are 1.75×10^{2} cc. sec.$^{-1}$ and 7.17×10^{3} cc. sec.$^{-1}$, at the extremes of the drying

temperatures used - 273°K and 338°K respectively. The density of water vapour at 298°K (the temperature of the environment) is 2.3×10^{-5} gm. cc.$^{-1}$ (197) and thus the rates of water vapour transfer from sample surface to cold trap are 4 mg. sec.$^{-1}$ at 273°K and 1.65 x 10^{2} mg. sec.$^{-1}$ at 338°K. Secondary drying rates are entremely small in comparison with these vapour transfer rates and will not depend upon the design of the drying apparatus, but will be a function only of the geometry, the internal structure, and the composition of the sample.

It is known that removal of water is taking place during the drying period since changes in sample weight are recorded without a loss in the total number of spores. Loss of weight has been demonstrated at all drying temperatures investigated. The final drying rate at high drying temperatures is so slow that its precise measurement is not possible with the electronic balance. However, even after the most rigorous treatments e.g. 24 hours drying at 338°K, water was still being lost, and in no case was a completely dry sample obtained. This observation casts doubt on the validity of the technique used by some previous workers (59, 60, 61, 175) whereby the residual moisture content of a sample is calculated with reference to the weight after 3 hours drying at 333°K in a vacuum of either 10^{-1} torr (175) or 10^{-5} torr (60), this being considered to be the weight of a completely dry sample.

Following drying under defined conditions spores have on occasions been rehydrated by exposure to water vapour over ice at controlled temperatures. At all re-equilibration aqueous vapour pressures studied between 2×10^{-3} torr and 10 torr rehydration of the spores was followed by measuring increases in the weight of the sample.

Provided that removal of water had taken place to a point below the aqueous vapour of the re-equilibration bath, one would expect that during rehydration, if sufficient time were allowed for equilibrium to be attained, all samples would rehydrate to the same level and would weigh the same. Thus the final water content of the spore would be independent of the drying conditions, and therefore independent of the original weight of the dried sample. The amount of water taken up would therefore be a function of the "dryness" of the spore and thus of the drying treatment. However, experimental results show that for each re-equilibration aqueous vapour pressure the increase in weight of the spore sample at the end of rehydration is constant and independent of the drying conditions. The rehydration process therefore results in a specific weight of water being taken up by the sample, rather than the sample attaining a defined weight level. These findings suggest that the sites onto which water is being re-adsorbed are not necessarily the same sites from which dehydration occurs during the latter part of the drying process.

A possible explanation for these results can be suggested if it is assumed that water is removed from specific sites within the spore, and that these sites are of two types; those at which dehydration is reversible and those at which dehydration is irreversible. If the number of specific sites at which dehydration is reversible is a direct function only of the aqueous vapour pressure of the environment, then during re-equilibration, water would only be taken up at these "rehydratable sites", and thus the increase in weight of the spores would be a function only of the re-equilibration aqueous vapour pressure, and not of the drying conditions. The total number of "rehydratable sites", and thus

the amount of water taken up by the spores will increase with increase in re-equilibration aqueous vapour pressure. A linear relationship is in fact shown to exist between the log. of the re-equilibration aqueous vapour pressure, and the log. of the weight of water adsorbed by the spore sample. The ease with which the sites are rehydrated may be a function of their molecular configuration or alternatively may depend on the ease with which water can diffuse through the dried spore and any inherent diffusion barrier, onto the site. However, the observation that the time taken for the sample to attain constant weight (60 minutes) is independent of the re-equilibration aqueous vapour pressure and of the drying conditions prior to rehydration, would suggest that the number of available sites in the spore is the primary limiting factor, and that the ease with which water can reach these sites is of secondary importance.

Support for the suggestion of "rehydratable sites" in the spore is given by the biological data. These data are considered in detail later, but briefly, it has been shown that spores dried under different defined conditions exhibit different sensitivities in response to a given heat treatment. Following rehydration the heat sensitivity of the spores is the same, and independent of the drying conditions and thus of the water content. This can be explained on the basis that the "rehydratable sites" are important in determining the response of the spore to heat. During re-equilibration these sites are rehydrated to the same extent, and thus the heat sensitivity of the spores is the same. In contrast, during the drying process, differences in water content are important, since water is removed from some of the "rehydratable sites". Removal of water from different numbers of these sites as a consequence of the different

drying treatments results in the large variation in heat sensitivity observed in dried spores.

The results of Marshall and Murrell (198) lend further support to the idea of specific sites for water rehydration within the spore. These authors have shown that the water sorption isotherm for Bacillus megaterium spores exhibits a sigmoidal shape with a steep slope in the region of low water activity (a_w) i.e. the Langmuir adsorption phase. The latter is characteristic of a high affinity for water and of binding at specific sites which the authors attribute to monomolecular adsorption onto -CO-NH- groups and polar side chains in the spore, although the reasons for this are not made clear. The water activity is defined as the relative humidity expressed as a fraction of 1. The re-equilibration aqueous vapour pressures employed in the present experiments (5×10^{-2} torr and 10 torr) correspond to low a_w values (8×10^{-3} and 4×10^{-1}) and thus the findings and interpretation of these authors are applicable to the system under discussion.

Other workers have demonstrated that the heats of adsorption for water molecules adsorbed at these low water activities are in excess of 20 kcal.mole^{-1}. (199,200). These high values suggest locallisation of the adsorbed water molecules by strong interaction with specific groups in the organic cell constituents, and provide further evidence to support the suggestion that water rehydrates specific sites within the spore.

The most direct method of determining changes in biological characteristics induced by drying is to assess the spore viability. In this context the viability of a spore is defined as its ability to germinate and reproduce to the point of macroscopic detection

i.e. the production of a colony, when inoculated into nutrient agar medium. As such it is probably an extremely sensitive assay as the replicating mechanism must be in full working order, since if, for example, only two divisions were possible in the vegetative cells formed after germination the spore would be counted as non-viable.

In the high vacuum treatments employed in previous work (135) aluminium silicate has been used as a carrier to minimise the loss of spores from samples during the drying treatment. The experiments reported on page 57 show that it is possible by a careful control of sample volume and pressure reduction rate to dispense with the use of a carrier and to dry spores directly from distilled water suspension. Differences between the total numbers of spores in dried and undried samples were shown to be small ($<5\%$) and within the limits of error associated with normal sampling under all drying conditions investigated.

When samples are dried for 6 hours at different temperatures spore viability is seen to vary with drying temperature but in a complex and non-systematic manner.(Figure 17). This relationship is further complicated by the fact that changes in viability may be the result of either prolonged holding at the drying temperature or the removal of biological essential water from the spore, or a combination of both. If viability is a function of the number of sites from which water is removed during drying a direct relationship would be expected to exist between viability and drying temperature, if the drying time is constant. This is not observed in practice, and from Figure 17 it would appear that two possible types of relationship could exist. If the values recorded at $288^{o}K$ are interpreted as an artifact, a threshold effect is observed, the drying temperature having no affect on spore viability until a temperature of $308^{o}K$ is

reached. However, the data obtained at 288°K were shown to be real and reproducible and it is therefore unreasonable to ignore these values. If these data are included it would seem that a curvilinear relationship exists between viability and drying temperature with a minimum in the region of 288°K and possible explanations for such a relationship must be found.

One possibility is that the viability differences observed may be a function of the methods of spore water removal, rather than of the total amount removed. However, as no relationship between viability and any function of the drying process such as sample temperature changes, or freezing and drying rates could be found, this would seem to be an unlikely explanation.

A reasonable explanation would be afforded if it is assumed that during drying two processes can be operative, one inactivating and the other "activating". At the lowest drying temperature (273°K) 6 hours drying induces neither "activation" nor inactivation, with the result that the spore viability is unchanged from that observed in aqueous suspension. At 288°K however, a significant loss in viability is observed and is presumed to be the results of harmful effects of the high vacuum treatment. "Activation" processes are assumed to be either non-operative or insignificant at this drying temperature. The harmful effects of high vacuum also occur during drying at higher temperatures, but at these temperatures the 6 hour drying period also induces a process analogous to heat activation. Under these conditions the observed viability of the spores is therefore a summation of the effects of inactivating and "activating" processes. At drying temperatures of 298°K and 308°K the degree of inactivation induced by the high vacuum treatment is balanced by the amount of "activation" taking

place, and the net effect is that the viability of dried spores is apparently unchanged from that of spores in aqueous suspension. After 6 hours drying at the highest temperature investigated (338°K) the amount of "activation" induced is in excess of any inactivation that occurs and a significant increase in spore viability is observed.

Increase in the period of drying at 338°K from 6 hours to 24 hours results in a statistically significant decrease (4 - 5%) in the percentage viability of the dried spores. However, experiments not reported in this thesis failed to show any similar decrease in spore viability when the drying time at the lower temperatures was extended to 24 hours. These findings can be explained on the basis that only a limited amount of "activation" can take place in dried spores. At low drying temperatures this amount of "activation" is still sufficient to nullify the increased inactivation that is presumed to occur with extension of the drying time. This is not the case at 338°K where the degree of inactivation caused by the 24 hour high vacuum treatment exceeds the amount of "activation" that can occur and a resultant decrease in spore viability is observed.

Furthermore, it would be expected that the inactivating and activating processes that are assumed to occur during drying would be unaffected by subsequent rehydration of the dried spores. The experimental data in fact shows that re-equilibration of the spores to 10 torr aqueous vapour pressure has no significant effect on spore viability under any of the investigated drying conditions.

The "activation" phenomenon that occurs during drying is probably different from true heat activation, since Beers (203) and Powell and Hunter (204) have shown that water must be present for heat activation to take place, and that it is not possible to

activate lyophilised spores. Nevertheless, the present work shows that vacuum drying at high temperatures "activates" spores inasmuch as the percentage of dormant spores is decreased duringsubsequent incubation on nutrient medium. At this stage though, the mechanisms of this "activation" cannot be postulated.

The admission of oxygen to the spore sample after drying, or after re-equilibration to 10 torr aqueous vapour pressure, has been shown to have no significant effect on the viability of spores, under the drying conditions investigated. These findings are in contrast to those reported with lyophilised bacteria (see page 24), where it has been shown that survival is lower if oxygen is admitted to the samples after drying than if the samples are maintained in anoxic conditions. However, in view of the long exposure times at high temperature that are required to demonstrate an oxygen effect during subsequent heating experiments, it would be surprising if any change in spore viability were observed, since oxygen is only in contact with the spores for ten minutes, at the relatively low temperature of 298°K, before the samples are reconstituted.

A basic characteristic of the bacterial spore is its apparent lack of metabolic and developmental processes. The change from the dormant spore into a vegetative cell involves three sequential processes, activation, germination,and outgrowth. Spore viability, as determined from colony counts therefore assesses only the final outcome of these sequential processes, and the result obtained may well mask changes that occur in one or more of these processes as a consequence of different pre-treatments.

Previous workers have shown that the extinction of visible light by a spore suspension decreases by up to 60% during germination,

due to excretion and solubilisation of dry matter from the spores with consequent reduction in refractility in individual spores (201, 202). It is therefore possible to study the kinetics of germination by reference to this decrease in optical density. In the present series of experiments an initial incubation period was observed during which the optical density of the spore suspension remained unchanged. Although in theory a decrease in optical density would be expected to follow the initial lag period, this was not demonstrated in practice. This may be an artifact of the experimental system resulting from working at the lower limit of sensitivity of the spectrophotometer. It does mean, however, that the kinetics of spore germination, after different drying treatments, cannot be investigated. The influence of drying conditions must therefore be assessed in terms of the duration of the initial lag period (this being considered to represent the time for outgrowth of the spores) and the growth rate of the vegetative cells formed as a consequence of spore germination. The initial lag period has been shown to be approximately 220 minutes, and within the limitations imposed by the technique employed for its estimation is unaffected by the drying conditions (Figures 22 to 25). Thus if the increase in viability observed during 6 hours drying at $338^{o}K$ is due to an activation process, this process does not speed up the commencement of germination as might be expected, but merely makes spores which would have remained dormant susceptible to the normal germination conditions.

In general, the duration and temperature of drying have been shown to have no significant effect on the growth rate of the vegetative cells germinated from the spore, this in all cases being approximately 2.6×10^{-2} minutes^{-1}. The only observed

deviation from this is shown in spores dried for 6 hours at $308^{o}K$, when the subsequent growth rate of the vegetative cells is significantly lower at 2.27×10^{-2} minutes^{-1}. An explanation for this phenomenon, which does not appear to be related in any way to changes in spore viability, must await the results of further investigation.

In all experiments in which dried spores were heated, a clear relationship is shown to exist between surviving fraction and time, the surviving fraction decreasing with increasing exposure time to a given heat treatment. On these occasions, plots of log. survivors against heating time were linear below a surviving fraction of 0.1. The slopes of these curves give one parameter used to describe the measured response of the spore system to heat, their values are referred to as heat inactivation constants.

However, since the log. survivor/heating time curves obtained in these experiments exhibit a broad shoulder in the region of high surviving fraction, heat inactivation constants on their own do not provide an adequate description of the curves, and other parameters are necessary for complete characterisation of the response of the spore system to heat. A measure of the heating time required to produce a surviving fraction below which the curve is linear (i.e. 10^{-1}) has therefore been calculated and is given by the shoulder constant s. (see page 149). The shoulder constant has been shown to vary in a systematic manner with different drying and heating treatments, and is thus considered to be a suitable parameter for describing the response of the spores to a given heat treatment.

For any experimental condition the constants k and s are therefore numerical values expressing the probability of death

due to lethal mechanisms operative under this experimental condition, The heat inactivation constant k is a measure of the rate at which lethal mechanisms occur, and thus changes in k reflect alterations in the susceptibility of the spores to these mechanisms. The shoulder constant s is a measure of an apparent lag that is necessary before the lethal mechanisms represented by k become operative. The possible reasons for this lag and the changes observed in its value with different treatments are discussed later. During this lag a second mechanism of death, at a rate much slower than k may be operative. However, the existence of such a mechanism cannot be established from the experimental data recorded in thesis, and thus it is not considered further.

An alternative interpretation of this type of survivor curve, based on the kinetics of repair in damaged cells has led to the development of a number of equations to describe such curves (205, 206). These expressions incorporate constants representing the fraction of the total population which has suffered no lethal damage and the fraction which has suffered lethal damage but has a probability of subsequent recovery. An equation of this type has been applied to data obtained in the present heating experiments and has been shown to provide an adequate fit to the log. survivor/heating time curves obtained with primary dried samples. In order for repair to take place during the heating of bacterial spores the factors responsible for such repair must be capable both of withstanding high temperatures without denaturation, and also of carrying out their function under these conditions. Since spores are able to maintain their viability after being subjected to temperatures that are lethal to normal cells, certain spore proteins i.e. those essential for viability must be able to resist thermal denaturation. A large

number of heat stable enzymes have in fact been demonstrated in spores (207), although at present the existence of possible repair enzymes that can resist thermal denaturation has not been established Even if such enzyme systems are present in the spore it is highly unlikely that these would be able to function at the high temperatures in excess of 353°K, at which the spores are heated. If repair is to take place therefore it is most likely to occur during the reconstitution stage prior to plating, or during the incubation period necessary for detection of survivors by the plating technique. Repair of thermal injury has in fact been demonstrated in strains of Staphylococcus aureus (208) and it is possible that similar repair occurs in heat damaged spores under suitable conditions. However, in view of the lack of evidence at the present time for thermal repair in spores this would seem the least likely of the two possible explanations for the shape of the survivor curves. For the purposes of discussion it is therefore assumed that the shoulder of the log. survivor/heating time curves represents a lag necessary before lethal mechanisms become operative. The characteristics of lethality arising from exposure of spores to heat have therefore been investigated by studying the relationship between the heating conditions and the constants k and s that are used to describe the response of the spores to a given heat treatment.

In order to assess the influence of different drying treatments on these characteristics it was first necessary to study the response to heat of spores in aqueous suspension. The data given in Table 21 shows that the heat inactivation constant k for spores heated in air in distilled water increases with increase in exposure temperature T. A plot of log. k against $\frac{1}{T}$ is linear and of negative slope (Figure 30) showing that the quantitative relationship between the temperature

and the rate of inactivation expressed by the Arrhenius equation is obeyed by the spore system, over the range of temperature investigated The energy of activation Ea and the frequency factor A calculated from the Arrhenius equation (see page 150) can thus be used to characterise the lethal reaction. For spores in aqueous suspension Ea has a value of 32.18 K cal.mole^{-1} and A a value of 1.01 x 10^{19} minutes^{-1}. (Table 34)

The values of Ea and A obtained with spores heated after drying under different conditions are recorded in Table 34. The drying conditions investigated are primary drying in which water is removed from the spores by sublimation from the ice phase, and secondary drying for 6 hours at 298^{o}K during which additional water is removed by isothermal desorption processes. In addition, the effect of rehydration is considered by studying the response of spores that are dried for 6 hours at 298^{o}K and re-equilibrated to 10 torr aqueous vapour pressure prior to heating. The presence or absence of oxygen during heating is also taken into account in experiments involving secondary dried spores.

It can be seen from Table 34 that with the possible exception of spores heated in anoxia after 6 hours drying at 298^{o}K, there is little difference in the values of Ea obtained under different experimental conditions, although large differences in A are observed. This data would suggest that the probability of a lethal reaction occurring is affected by the drying treatments, but that the lethal mechanisms involved are probably the same in all cases.

Nowadays the Eyring theory of absolute reaction rates (209) is considered to provide more valuable data with regard to reaction mechanisms, and thus detailed analysis and interpretation is based on the thermal inactivation data treated in accordance with this theory.

TABLE 34

Arrhenius constants and thermodynamic constants for Bacillus megaterium spores dried under different conditions.

Drying Conditions	Heating Atmosphere	Heating Temperature Range (^{o}K)	Ea (K cal.mole^{-1})	A (minutes^{-1})	ΔH^{*} (K cal.mole^{-1})	ΔS^{*} (cal.deg^{-1}mole^{-1})
Aqueous suspension	air	363-393	32.18	1.01×10^{19}	31.43	17.82
Primary dried	air	383-433	35.72	8.41×10^{17}	34.75	12.33
6 hours at 298^{o}K	anoxia	373-403	29.94	5.11×10^{15}	29.19	2.76
6 hours at 298^{o}K	oxygen	373-403	34.04	1.67×10^{18}	33.29	14.25
6 hours at 298^{o}K re-equilibrated to 10 torr aqueous vapour pressure.	oxygen	383-413	36.36	3.84×10^{18}	35.59	15.83

The thermal inactivation of spores approximates a first order reaction over heating periods during which the log.survivor/heating time curve is linear, and the heat inactivation constant k in minutes^{-1} is determined by the free energy of activation (ΔF^*) and the absolute temperature (T) as follows:

$$k = \frac{KT}{h} e^{-\Delta F^*/RT} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (31)$$

where k is Boltzmann's constant, h is Planck's constant and R is the universal gas constant.

The substitution of $\Delta F^* = \Delta H^* - T\Delta S^*$ into equation gives the usual form of the Eyring equation relating reaction rates to thermodynamic parameters:

$$k = \frac{KT}{h} e^{-\Delta H^*/RT} e^{\Delta S^*/R} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (32)$$

where ΔH^* is the enthalpy of activation and ΔS^* the entropy of activation. By rearrangement and substitution of values, equation 32 reduces to:

$$\log.k = 10.3 + \log. T - \frac{0.22\,\Delta H^*}{T} + 0.22\,\Delta S^* \quad \ldots\ldots \quad (33)$$

If the heat inactivation constants are measured at a number of temperatures the entropy of activation and the enthalpy of activation for the lethal process can be calculated from equation 33.

The values of ΔH^* and ΔS^* calculated for spores heated in suspension and after drying under different conditions are recorded in Table 34. (Data obtained using computer program page 327).

A change in the value of ΔH^* with different drying treatments can be considered to indicate a possible change in the mechanism of spore inactivation. A change in the value of ΔS^* would then be indicative of a change in the susceptibility of the spore to lethal mechanisms. Since the Arrhenius constants Ea and A are directly

related to the thermodynamic parameters ΔH^* and ΔS^*, the quantitative changes that occur in the former as a consequence of the drying treatments are also observed in the latter.

At this stage it is not really certain that the differences observed in ΔH^* as a consequence of the drying treatments are real, particularly since, in the majority of cases, data for only four heating temperatures is available for the calculation of the thermodynamic parameters. Inspection of the log.k against $\frac{1}{T}$ plots shown in Figure 30 reveals that except for spores heated in anoxia after drying for 6 hours at 298°K all the curves appear to be parallel, and this would suggest that the mechanism of inactivation is the same under all conditions examined. It is felt, therefore, that more experimental data is required before any real differences in mechanism can be confidently postulated. However, statistical tests carried out on the values of ΔH^* recorded in Table 34 show that certain of the observed differences are in fact significant, and so the data are discussed in the light of the results of these tests.

Statistical analysis shows that there is no significant difference in the value of ΔH^* observed during heating of spores in oxygen after primary drying, secondary drying (6 hours at 298°K), or secondary drying with subsequent re-equilibration to 10 torr aqueous vapour pressure. These values are, however, significantly larger than those obtained with spores heated either in anoxia after secondary drying or in air in aqueous suspension, suggesting that the mechanism of lethal reaction under the latter conditions is different to that operating in spores heated in oxygen after drying. The most sensible reason for these differences in ΔH^* would appear to be that during drying, changes occur within the spore which result in

modifications in the nature of the lethal mechanism. These changes are associated with sublimation, since no additional change is apparent after secondary drying, or after the addition of water during re-equilibration after secondary drying, as long as oxygen is present during heating. If oxygen is not present during heating, the value of ΔH^* is much lower. If further experiments show this to be a real effect, then it is very surprising, as oxygen would be expected e.g. if it acted as a catalyst, to decrease ΔH^*. All the evidence of previous workers in respect of the response of bacteria to radiation and drying (see Introduction) suggests that oxygen sensitises. It would not be surprising if the mechanism of inactivation when spores are heated in aqueous suspension were different from that when spores are heated in the "dry state", with larger values of ΔH^* being observed in the dry state. A number of previous workers have suggested that basic differences exist in the mechanism of death due to moist heat and death due to dry heat (see page 31) and the design of sterilisation procedures has been based on this belief.

The susceptibility of the spore to lethal mechanisms is also affected by the drying treatment as indicated by the significant differences observed in the values of the frequency factor A. As might be expected, the greatest susceptibility is demonstrated in spores heated in aqueous suspension ($A = 1.01 \times 10^{19}$ minutes^{-1}). The differences in A appear to be a function of the spore water content. Removal of water during drying for 6 hours at 298^{o}K causes a reduction in the susceptibility of the spores to lethal mechanisms as shown by a decrease in A to a value of 1.67×10^{18} minutes^{-1}. However, the presence of a certain amount of water in the spores, as in primary dried spores, seems able to confer even greater

protection than removal of water by extensive drying, a further decrease in A to a value of 8.41×10^{17} minutes^{-1} being observed. In contrast, the addition of larger quantities of water, during re-equilibration to 10 torr aqueous vapour pressure, sensitises the spores to the lethal reaction and results in an increase in A to a value of 3.84×10^{18} minutes^{-1}.

Also of significance is the low value of A observed when spores are heated in anoxia (5.11×10^{15} minutes^{-1}) in comparison to the value shown when spores are heated in oxygen (1.67×10^{18} minutes^{-1}) after an identical drying treatment. This data suggests that although a different mechanism of thermal inactivation, associated with a lower enthalpy of activation, appears to be operative when spores are heated in anoxia rather than in oxygen, the susceptibility of the spore, that is, the probability of such a reaction occurring, is so low that the overall observed effect, in the absence of oxygen, is one of enhanced resistance to heat.

If one wishes to gain information on the nature of the lethal mechanisms, in addition to assessing the susceptibility of the spore to these mechanisms, then ΔS^* also has to be considered, since the magnitude of this parameter is characteristic of certain reactions.

Under all environmental conditions investigated, the values of ΔH^* and ΔS^* observed with heated spores are large and positive, and are comparable to those demonstrated in chemical reactions and in the denaturation of proteins and polypeptides (210, 211). In connection with protein denaturation it has been suggested that small values of ΔH^* indicate denaturation by rupture of a very few, very strong and probably co-operative bonds (211). Thermal denaturation of enzymes and proteins is associated, in general, with large positive values of ΔH^* and ΔS^* (212). Because these

large values of ΔH^* cannot be accounted for in terms of breakage of a single chemical bond, it has been suggested that the values obtained are due to the rupture of a large number of weak, non co-operative bonds, most probably of hydrogen. When sufficient numbers of bonds have been broken, the large molecule of protein is no longer held firmly in its native configuration; the collapse of structure resulting in a large increase in entropy.

High ΔH^* and ΔS^* values have been reported also for the thermal inactivation of deoxyribonucleic acid (DNA) and have been interpreted as evidence that the mechanism involved in probably breakage of hydrogen bonds followed by collapse of the DNA structure (213). The thermal degradation of ribonucleic acid (RNA), in contrast to DNA or protein inactivation has been associated with low ΔH^* values and low or negative values for ΔS^* (214).

It is not unreasonable to apply the same arguments to the large positive values of ΔH^* and ΔS^* observed with heat inactivation of spores, and to suggest that these indicate the breakage of a large number of chemical bonds, and subsequent collapse of structure. The nature of these bonds and their location within the spore cannot be deduced from the experimental data. However from the findings of other workers outlined above, it would seem likely that the mechanism of heat inactivation of spores involves denaturation of proteins (including enzymes) and/or DNA, and does not appear to involve degradation of RNA. It is interesting to note that a similar explanation has recently been proposed for the mechanisms of heat inactivation of freeze-dried Lactobacillus and Streptococcus, on the basis of the large ΔH^* and ΔS^* values obtained during exposure of the dried bacteria to elevated

temperatures (215).

The other parameter used to describe the response of the spores to heat, the shoulder constant s is also shown to vary with the heating temperature. Under all conditions a plot of log.s against $\frac{1}{T}$ is linear and of positive slope. For a given drying treatment the value of s therefore approaches zero, with increase in the heating temperature, although the logarithmic relationship that is shown to exist between s and $\frac{1}{T}$ would preclude a zero value being reached.

The plots of log.s against $\frac{1}{T}$ obtained with spores subjected to different drying treatments are seen to be parallel (Figure 31). It is assumed, therefore, that under all drying conditions increase in temperature has a similar modifying effect on s, and thus the lag that is necessary before lethal mechanisms become operative can be assessed by comparing the values of s obtained at any given constant temperature.

On this basis it can be seen that the smallest s value at any given temperature is observed in spores heated in aqueous suspension. However, it is believed that the lethal mechanism in aqueous suspension may be different from that in the dried condition, and the lower value of ΔH^* observed under the former conditions would tend to support this belief. Discussion of the changes in s with temperature and treatment are therefore confined mainly to the results obtained with spores in the "dry state".

A possible explanation for the shoulder constant s is that during the period of heating, structural changes occur within the spore, so that it becomes susceptible to the lethal mechanism. The time required to achieve the necessary degree of structural modification, and so the magnitude of the shoulder on the log.survivor/

heating time curves is dependent upon the heating temperature as shown by the linear plot of log.s against $\frac{1}{T}$ (Figure 31), the higher the temperature the shorter being the time required. It is also presumed that these structural changes are brought about by high vacuum drying, as well as by heat and thus a decrease in the shoulder constant would be expected when spores are subjected to secondary drying prior to heating. It is further postulated that the addition of water to the spores after drying facilitates the recovery of some of these structural changes and a corresponding reversal of the spore structure to its stable state. The consequent increase in the stability of the spore would result in an increase in the magnitude of the shoulder constant s.

In the following discussion changes in mechanism are not considered. It is assumed that the heat inactivation constant k is a measure of the rate of the lethal mechanism, and that the shoulder constant s is indicative of the time required for the occurrence of structural changes within the spore that are necessary in order for this mechanism to become operative. The feasibility of this interpretation will be considered in the light of the effects that defined environmental conditions have on the values of k and s. In this way it is hoped to determine whether the differences that are observed in the heat resistance of spores, after different drying treatments can be adequately explained on the basis of this "model".

393^{o}K was used as the heating temperature in all experiments designed to study the effect of different drying treatments on the heat resistance of spores.

The heat inactivation constant for spores heated in anoxia has been shown to vary significantly with the temperature at which

the spores are dried (for a period of 6 hours). The k values increase from 4.9331 x 10^{-2} minutes^{-1} for spores dried at 273°K to 1.0375 x 10^{-1} minutes^{-1} for spores dried at 298°K and remains constant at this level for spores dried at higher temperatures (Figure 34a). It is apparent, therefore, that water is necessary for the maintenance of heat stability in spores, and its removal by high vacuum drying causes sensitisation to heat and thus larger k values. The degree of sensitisation is independent of the drying temperature above 298°K. At the lower temperatures it would appear that not all the water required for spore heat stability is removed by the 6 hour drying procedure. It is likely that 6 hours drying at high temperatures removes greater amounts of water than the same period at lower temperatures, and therefore it has to be assumed that the water responsible for heat stability is removed first, and that removal of further moisture has no measurable influence on the heat inactivation constant of the spores.

In addition to influencing the proposed lethal reaction that is represented by k, the drying conditions also influence the value of the shoulder constant s. This constant exhibits a steady decrease from a value of 87.31 minutes at a drying temperature of 273°K, to 26.24 minutes at 338°K (Figure 346). Removal of water during high temperature drying therefore continues to affect the shoulder constant even though it has no apparent effect on k. If it is assumed that s represents a period during which necessary structural changes are taking place in the spore, the observed effects of drying temperature on these values can be explained on the basis that the drying treatments themselves induce structural modifications, the extent of such modifications increasing with increase in drying temperature. Since structural changes are

induced by drying, less modification of the structure is required during heating before the spore becomes susceptible to the lethal mechanism. The value of s observed, on subsequent heating of the dried spores thus decreases with increase in the drying temperature.

The relationship shown when spores dried at different temperatures are heated in anoxia is paralleled by that shown when spores are heated in oxygen, the values of k being larger under the latter conditions, ranging from 1.017×10^{-1} minutes^{-1} at 273°K to approximately 1.7×10^{-1} minutes^{-1} at temperatures above 308°K (Figure 34a). Values of the shoulder constant are slightly smaller when spores are heated in oxygen, than when they are heated in anoxia, ranging from 70.73 minutes at a drying temperature of 273°K to 23.96 at 338°K (Figure 346).

The effect of oxygen can be expressed in terms of the ratio of the value of a given parameter obtained in oxygen to the value of the same parameter obtained in anoxia, this ratio being referred to as the oxygen enhancement ratio R. Two such ratios are of interest in these experiments, R_k the ratio with respect to heat inactivation constants, and R_s the ratio with respect to shoulder constants.

The calculated ratio R_k is smaller in spores dried at high temperature than in spores dried at low temperatures, being 2.1 at 273°K and approximately 1.5 above 298°K. Whether these differences are significant and indicate that the ratio R_k is a function of the drying temperature and thus of the spore water content, is not known. What is clear, however, is that oxygen increases the sensitivity of the spore to lethal mechanisms at all water levels equivalent to six hours drying at the different temperatures.

In contrast, the values of s are hardly if at all, affected

by the presence of oxygen during heating, the R_s values ranging from 0.8 to 1 for spores dried at all temperatures investigated. Under these conditions, therefore, oxygen has little or no effect on the structural changes that are postulated to take place in spores.

Changes in the overall heat resistance of spores are also observed after different drying times at 298°K and 338°K. For spores heated in anoxia, an increase in drying time from 6 hours to 24 hours at 298°K results in an increase in the value of k from 1.0375×10^{-1} minutes to 2.3348×10^{-1} minutes^{-1}. Physical measurements show that water is being removed from the spores throughout the 24 hour drying period, and the biological data would therefore suggest that this water is important in maintaining the heat resistance of the spores. These results are in contradiction to those obtained with spores dried for 6 hours at different temperatures, where the removal of greater amounts of water at 338°K than at 298°K had no effect on the observed value of k. It would seem, therefore that at drying temperatures above 298°K it is the drying time rather than the temperature that is important.

Increase in the drying time at 338°K is also shown, from physical experiments, to result in further removal of water from the spore, and is seen from Table 25 to cause an increase in the value of k, from 1.0883×10^{-1} minutes^{-1} after 6 hours drying, to 2.0204×10^{-1} minutes^{-1} after 24 hours drying. If it is assumed that after a defined drying time, more water is removed from the spore at 338°K than at 298°K, the smaller values of k obtained after 12 hours and 24 hours drying at 338°K compared to those observed after identical drying times at 298°K, (Table 25) cannot be explained solely on the basis of the removal of biologically active water. The possibility that exposure to high vacuum per se

may produce changes in the susceptibility of the spore to lethal mechanisms, (which are not related in any way to water removal), cannot be ignored, and may explain changes in the value of k that are not readily explicable in terms of spore water contents.

Values of the shoulder constant s, for spores heated in anoxia, decreased from 41.55 minutes to 20.07 minutes for an increase in drying time from 6 hours to 24 hours at 298°K and from 26.24 minutes to 15.94 minutes for a similar extension of the drying time at 338°K. These results indicate that the extent of changes in spore structure that are assumed to take place during drying is a function of both the drying time and the drying temperature. Again, the structural changes induced by drying result in less modification of structure being required during heating, and thus a decrease in s is observed with increase in drying time at constant temperature.

The results obtained when spores dried for different times at a given temperature are heated in oxygen again parallel those observed under anoxic conditions, but with larger k values, and slightly smaller s values being obtained (Table 25). The value of R_k ranges from 1.9 to 1.2 at 298°K, and from 1.6 to 1.1 at 338°K. It is apparent, therefore, that oxygen always has a sensitising effect on the spore, resulting in increases in the heat inactivation constant. Furthermore, the trend in R_k values suggests that the effect of oxygen is diminished with extension of the drying time, although insufficient experimental data is available to indicate a definite inter-relationship. The value of the oxygen enhancement ratio may be a function of the spore water content. Alternatively, since R_k appears to decrease with increases in the anoxic value of k that result from extended drying, the effect of oxygen may be a function of physical changes induced in the spore as a consequence

of the high vacuum treatment, and not of spore water content. In this context, it would not be unreasonable for the sensitising effect of drying to be similar to that of oxygen.

Again an oxygen effect was not observed in the values of the shoulder constant, the ratio R_s being between 0.8 and 1.1 for spores dried at both 298°K and 338°K, irrespective of the duration of drying. Thus, although extended drying appears to induce increased amounts of structural change in spores, this, not surprisingly, is independent of oxygen.

The differences in heat resistance observed under anoxic conditions as a consequence of drying for 6 hours at different temperatures are not observed if the dried spores are re-equilibrated to 10 torr aqueous vapour pressure prior to heating. For ease of comparison the values of k and s recorded for dried and re-equilibrated spores are given in Table 35. The heat inactivation constants obtained with re-equilibrated spores are smaller than those observed with dried spores, and are independent of the drying temperature over the range 273°K to 323°K, the values remaining constant at approximately 1.03×10^{-2} minutes^{-1}.

It has already been established that re-equilibration of dried spores to 10 torr aqueous vapour pressure results in the addition of a defined weight of water (approximately 4×10^{-1} mg.) to the sample regardless of the drying conditions, and therefore of the residual moisture content. It has also been suggested that this water rehydrates specific sites within the spore. The results of the heating experiments suggest that these specific sites, in addition to being reversibly dehydrated must also be of definite biological importance, since the same amount of water adsorbed onto the spores during re-equilibration is able to modify the large

TABLE 35

Values of k and s for Bacillus megaterium spores dried for 6 hours at different temperatures.

Drying Temperature (^{o}K)	k (minutes^{-1})		s (minutes)	
	unequilibrated	re-equilibrated	unequilibrated	re-equilbrated
Heated in anoxia				
273	4.9331×10^{-2}	1.055×10^{-2}	8.731×10	1.0715×10^{3}
298	1.0375×10^{-1}	1.0114×10^{-2}	4.155×10	9.905×10^{2}
308	1.1794×10^{-1}	1.0473×10^{-2}	3.366×10	9.716×10^{2}
323	1.0981×10^{-1}	1.0309×10^{-2}	2.753×10	8.829×10^{2}
338	1.0883×10^{-1}	1.5471×10^{-2}	2.624×10	7.004×10^{2}
Heated in oxygen				
273	1.017×10^{-1}	2.3019×10^{-2}	7.073×10	4.525×10^{2}
298	1.9742×10^{-1}	2.2966×10^{-2}	3.881×10	4.568×10^{2}
308	1.709×10^{-1}	2.2744×10^{-2}	3.099×10	4.649×10^{2}
323	1.6247×10^{-1}	2.3208×10^{-2}	2.726×10	4.74×10^{2}
338	1.7023×10^{-1}	3.2052×10^{-2}	2.396×10	4.452×10^{2}

variation in values of k that are obtained as a consequence of different drying treatments, with the result that the spores, although containing different amounts of water, exhibit essentially constant and higher heat resistance.

An exception occurs with drying at 338^{o}K, where the k values after re-equilibration is higher than that observed at lower drying temperatures and has a value of 1.5471×10^{-2} minutes^{-1}. Under these conditions, the addition of 10 torr of water vapour is apparently unable to modify completely the effect of the drying treatment.

It would seem that 6 hours drying at 338°K, in addition to removing water from biologically active sites also damages a fraction of these sites in such a way that they are no longer rehydratable during subsequent re-equilibration of the spores. The non-rehydratable sites would account for the apparent increase in sensitivity to heat observed under these conditions.

At all drying temperatures the value of k is greater when re-equilibrated spores are heated in oxygen rather than in anoxia (Table 35), showing that oxygen always has a sensitising effect on the spore. The degree of sensitisation is however independent of the temperature at which spores are dried prior to re-equilibration, the R_k value being constant at between 2.1 and 2.3. These results suggest that under these conditions the effect of oxygen is a function of the sensitivity of the spore to heat as reflected by the anoxic value of k and is independent of spore water content.

It has been suggested that 6 hours drying at 338°K damages some of the biologically important sites in the spore, so that they are no longer rehydratable, and it might be expected that similar damage would result from prolonged drying at lower temperatures, resulting in similar variations in k being observed after subsequent re-equilibration of the dried spores. The heat inactivation constants obtained with spores dried for different times at 298°K prior to re-equilibration to 10 torr aqueous vapour pressure, are recorded in Table 36, and show a steady decrease with increase in drying time.

These results would suggest that water is in fact able to rehydrate all the biologically important sites and therefore that these sites are not apparently damaged by prolonged drying at 298°K. However, it is of interest that the lethal mechanisms

TABLE 36

Values of k and s for Bacillus megaterium spores dried for different times at 298°K and 338°K.

Drying Time (hours)	k (minutes^{-1})		s (minutes)	
	unequilibrated	re-equilibrated	unequilibrated	re-equilibrated
Dried at 298°K, heated in anoxia				
6	1.0375×10^{-1}	1.0114×10^{-2}	4.155×10	9.905×10^{2}
12	2.1123×10^{-1}	5.4207×10^{-3}	2.006×10	1.241×10^{3}
24	2.3348×10^{-1}	4.8544×10^{-3}	2.007×10	1.14×10^{3}
Dried at 298°K, heated in oxygen				
6	1.9742×10^{-1}	2.2966×10^{-2}	3.811×10	4.568×10^{2}
12	2.6014×10^{-1}	1.7243×10^{-2}	1.652×10	7.015×10^{2}
24	2.7257×10^{-1}	1.7564×10^{-2}	1.604×10	7.174×10^{2}
Dried at 338°K, heated in anoxia				
6	1.0883×10^{-1}	1.5471×10^{-2}	2.624×10	7.004×10^{2}
12	1.8621×10^{-1}	9.9672×10^{-3}	1.916×10	4.722×10^{2}
24	2.0204×10^{-1}	7.696×10^{-3}	1.594×10	3.732×10^{2}
Dried at 338°K, heated in oxygen				
6	1.7023×10^{-1}	3.2052×10^{-2}	2.396×10	4.452×10^{2}
12	2.1165×10^{-1}	1.4485×10^{-2}	2.152×10	4.528×10^{2}
24	2.251×10^{-1}	1.1192×10^{-2}	1.504×10	4.253×10^{2}

operative under these conditions show a much greater sensitivity to oxygen, R_k values of 2.1, 3.2 and 3.6 being observed in spores dried for 6 hours, 12 hours, and 24 hours respectively.

When heating is in anoxia, the values of s obtained with re-equilibrated spores are greater by a factor $> 10^2$ than the corresponding values obtained with dried spores (Tables 35 and 36). For a six hour drying period these values also show a smaller decrease with increase in drying temperature, being 1071.5 minutes at $273^{o}K$ and 700.4 minutes at $338^{o}K$. Extension of the drying time at $298^{o}K$ has little effect on the shoulder constants, values of 990.5 minutes and 1140 minutes being obtained after 6 hours and 24 hours drying respectively.

The influence of rehydration on the values of s can be explained in terms of structural changes in the spore if it is postulated that water is able to modify the changes induced by secondary drying, and cause a reversion of the structure to a stable form, comparable to that in primary dried spores. The values of s observed after re-equilibration of secondary dried spores therefore approach, in magnitude, the value obtained with primary dried spores. The effects of drying are therefore nullified by rehydration, and thus a greater amount of structural change must now be carried out during heating, before the spore becomes susceptible to the lethal mechanism, and hence large values of s are observed during subsequent heating of the re-equilibrated spores.

The ratio R_s observed in re-equilibrated spores increases from a value of 0.42 in spores dried for 6 hours at $273^{o}K$, to a value of 0.64 for spores dried for the same period at $338^{o}K$. Extension of the drying time at $298^{o}K$ from 6 hours to 24 hours results in an increase in R_s from 0.46 to 0.63. The significant feature of this data is that in re-equilibrated spores, in contrast to dried spores, oxygen has a marked influence on the value of the shoulder constant. The structural changes that are presumed to take place during

drying, and during subsequent heating have been shown to be unaffected by the presence of oxygen. The modification of these structural changes, with subsequent reversion to the stable state, that is postulated to occur in the presence of water is, however, affected by oxygen. It would seem, therefore, that this reversal to the stable state is not complete at the end of the hydration process, and that oxygen combines with the spore structure in such a way that further stabilisation is prevented. On this basis it could be postulated that the stabilisation of the spore structure by water is a time-dependent process. Thus if spores were re-equilibrated for a longer period prior to the admission of oxygen, it might be expected that more stabilisation would take place, and on R_s ratio closer to 1 would be obtained during subsequent heating. Likewise, re-equilibration of the spores to a higher aqueous vapour pressure e.g. 22 torr, might also be expected to increase R_s. By the same argument it might be expected that if oxygen was added to the spores before re-equilibration, and removed by light pumping, subsequent rehydration would have a much reduced effect, thus resulting in R_s values very much smaller than 1. It is considered that experiments of this type would be useful in testing the feasibility of the proposed "model" for interpretation of the constants k and s, and current work is being directed along these lines.

The log.survivor/ heating time curves obtained with spores dried for different times at 338°K, prior to re-equilibration are complex. The decrease in k observed during anoxic heating would suggest that water is able to modify the differences in sensitivity brought about by extended drying. The values of k obtained after re-equilibration of spores dried at 338°K are larger than those

observed at 298°K (Table 36). It has been suggested that 6 hours drying at the high temperature damages the biologically important sites in the spore, and it would seem likely, therefore, that the increased sensitivity observed after re-equilibration of spores dried for longer periods at this temperature may result from damage induced by the drying process. It is of interest that the lethal mechanisms operative under these conditions are less sensitive to the effect of oxygen than those operative at 298°K, the ratio R_k being 2.1 at 6 hours drying and 1.45 at drying times in excess of 12 hours.

The shoulder constant observed during anoxic heating is seen to decrease from 700.4 minutes to 472.2 minutes to 373.2 minutes with 6 hours, 12 hours and 24 hours drying respecitvely (Table 36). From this data it would seem that some of the structural changes induced during drying at high temperature cannot be modified by the subsequent addition of water to the spores during re-equilibration. The extent of this non-reversible structural change increases with increase in drying time, and its presence would mean that less modification of structure would be necessary during heating, and thus smaller values of s would be observed.

The action of oxygen in preventing the modification by water, of structural changes induced by drying, has a similar observed effect to the presence of non-reversible changes, and results in a further decrease in the value of s obtained after 6 hours drying from 700.4 minutes to 445.2 minutes. Oxygen causes no further change in the value of s observed after longer drying times at 338°K, the R_s ratio being 0.96 and 1.14 after 12 hours and 24 hours drying respectively.

The results of physical measurements carried out on the

drying system have indicated that the water that rehydrates the spore during re-equilibration does not behave in the same manner as that which is removed during drying. Further evidence of this was provided by experiments in which the resistance to heat at 393°K of re-equilibrated spores was compared with the resistance of spores dried directly to the equivalent water (weight) level. In theory, if the external water which rehydrates the spore behaves biologically in the same manner as the original cellular water one would expect that the heat resistance of spores at a given water level would be the same regardless of whether the water level had been reached by drying or by re-equilibration. The data illustrated in Figure 45 shows that under experimental conditions this expectation is not realised.

Spores dried at different temperatures to a weight level equivalent to 5×10^{-2} torr were less resistant to heat at 393°K in anoxia, as shown by larger k values, than spores dried for 6 hours and re-equilibrated to 5×10^{-2} torr aqueous vapour pressure, irrespective of the temperature of drying (Table 27). It would therefore appear that the biologically important water removed from the spore by subsequent drying beyond the point equivalent in spore weight to 5×10^{-2} torr is adequately replaced by the external water rehydrating onto the spore. Furthermore, this water, which is identical in weight to that removed by drying seems able to confer on the spore additional protection from lethal heat damage. It is therefore suggested that the specific sites from which removal of water takes place, in addition to being either reversibly or irreversibly dehydrated can also be considered to be either of primary or secondary biological importance. During re-equilibration water will be preferentially adsorbed at those rehydratable sites

which are of primary biological importance in maintaining the integrity of the spore. This preferential rehydration would result in an overall increase in biological functions, such as heat resistance, being exhibited in re-equilibrated spores when compared to spores dried to an equivalent weight level where the remaining cellular water is randomly distributed at all types of specific site in the spore, and not distributed solely at the biologically important sites.

As in previous experiments, re-equilibration of the dried spores results in an increase in the magnitude of the shoulder constant. As would be expected, the degree of reversal of structural changes, and thus the increase in s, resulting from re-equilibration to 5×10^{-2} torr aqueous vapour pressure, is less than at 10 torr aqueous vapour pressure. However, it must be remembered that the absolute weight of dried spores cannot be determined with the apparatus used in these experiments. It is likely, therefore, that although the spores are dried down to, and re-equilibrated to, an equivalent weight level, this level will be different at each drying temperature and may explain the differences in s that are observed at each temperature.

It can be seen that the majority of the results obtained during the heating experiments can be satisfactorily explained on the basis of the proposed interpretation of the constants k and s, where k is a measure of the rate of the lethal mechanism, and s represents the time required for the occurrence within the spore, of structural changes necessary before the lethal reaction becomes operative. Some indications of the possible mechanisms of the lethal reaction have been obtained from thermodynamic data from these experiments, and it is suggested that this is likely to involve denaturation

of proteins including enzymes, and/or DNA. The experimental data does not, however, allow any specific suggestions of this sort to be made concerning the nature of the structural changes in the spore. Nevertheless, it is pertinent at this stage to consider some of the components of the spore that could possibly undergo such changes.

Since it has been suggested that proteins and enzymes could be involved in the lethal mechanism, it would not seem unreasonable for the changes that have been shown by previous workers (211) to occur in these molecules prior to denaturation to be implicated in the development of the shoulder of the log.survivor/heating time curve.

The conformation of proteins, as well as many of their reactions are determined to a large extent by covalent disulphide bonds and by non-covalent interactions (216). The former play a major part in maintaining the tertiary structure, while the latter, which include hydrogen bonds,and hydrophobic bonds between non-polar side chains, are responsible for the secondary structure. (211). The protein molecule is considered to be made up of one or several polypeptide chains, more or less regularly folded, and thus denaturation appears as an unfolding process, and conversely reversal occurs when re-folding takes place. Water molecules, in addition to assisting in the stabilisation of the protein structure, can play a part in denaturation since their hydrogen bonding properties favour replacement of internal hydrogen bonds with external hydrogen bonds to water (217). The fact that protein denaturation is, to an extent, reversible would suggest that the constant s may well indicate the time required for sufficient bond breakage to occur for reversible denaturation to become irreversible. In globular proteins it has been suggested that the hydrogen bonds of α-helices are protected by the hydrophobic bonds of the non-polar side chains (218) and

thus during heating a lag period may well be expected, when rupture of the "protective" bonds is occurring, before rupture of the hydrogen bonds and concomitant unfolding of the α-helix occurs.

In the present "model" it is postulated that the required structural changes take place during high vacuum drying, as well as during heating. In this context it is of interest that previous workers have demonstrated conformational changes of proteins resulting from freeze-thawing and freeze-drying (219, 220, 221). For example, Hanafusa (219) has shown that freeze-drying causes the unfolding of the helical structure of myosin, a rod like protein, and the dissociation into sub-units of catalase, a globular protein. It has been further suggested that water removal, in addition to modifying protein conformation, also exposes the hydrophilic sites at the protein surface to the gases present in the atmosphere surrounding the dried sample (222). The reduced water combining capacity of dried proteins supports a hypothesis that oxidation of hydrophilic sites follows removal of water by freeze-drying, and could reduce the degree of reversibility of denaturation (223). The hypothesis is in accordance with the explanation that has been suggested for the effects of oxygen and rehydration on the shoulder constant s (pages 284 and 285).

An alternative possibility is that the structural changes taking place before the lethal mechanism becomes operative may be in the DNA molecules. It has been shown that spore DNA is double stranded (224), and the Watson and Crick model for the structure of DNA proposes that the two component strands are joined together by hydrogen bonds between the nucleotide bases (225). It has also been suggested that hydrophobic bonds are involved in maintaining the stability of the DNA helix (226). The rupture of hydrogen bonds

and hydrophobic bonds will thus result in denaturation, which, when complete, will yield separation of the two complementary strands. In addition, heating of DNA has been shown to cause degradation resulting from breakage of covalent glycosyl bonds (227). Denaturation of DNA has been shown to be partially reversible (227) and thus the structural changes proposed in the present work could involve bond rupture of DNA, a certain amount of which could be reversible under the rehydration conditions employed.

A further possibility is that the proposed structural changes may involve not necessarily the internal conformation of proteins or enzymes, but rather the environmental conformation of such molecules within the spore. In particular it has been postulated that complexation of enzymes with Ca^{++} and DPA renders them heat resistant (228). The shoulder of the log.survivor/heating time curve may thus be a measure of the time required to break these complexes before the enzyme can become susceptible to denaturation. In this context the Ca:DPA ratio appears to be important. It is particularly significant that under conditions allowing maximum DPA synthesis, an increase in calcium content increased not only the heat resistance of spores as indicated by the heat inactivation constant but also prolonged the initial shoulder of the survivor curve (229,230). On the other hand factors which retard or prevent calcium accumulation lower the heat resistance and shorten or completely eliminate the shoulder (230,231).

The observation that a fully formed cortex is necessary for a stable resting spore with full heat resistance (232) suggests another possible site for structural changes within the spore prior to thermal inactivation. Murrell (198) has postulated that the heat resistance of spores is determined by the water content of the protoplast, and that the protoplast is mechanically restricted by a structurally

contracted and stable cross-linked cortex. The cross-linking is suggested to involve the amino groups of diaminopimelate, and peptide side chains and may include calcium (232). The proposed structural changes may thus involve breakage of bonds, causing some unfolding in the cortical polymer and coats. The relaxation of the "contractile cortex" may then result in changes occurring within the protoplast and corresponding changes in the susceptibility of protoplasmic constituents to thermal inactivation.

Unfortunately the experimental data do not indicate which, if any of these possibilities is the true explanation. There may well be other alternatives, or the shoulder of the log.survivor/heating time curves may in fact be the result of factors which are non-structural. This theorising does, however, have the advantage that it encourages experiments to be devised to test the validity of the proposed "model".

The reported biological experiments give no conclusive indication of the mechanisms, at the molecular level, by which spores are resistant, or of the ways in which drying, rehydration, and the presence of oxygen change these mechanisms. Although it has been suggested that denaturation of proteins, enzymes or DNA is likely to be implicated in lethal reactions in spores, the denaturing lesions are not known. However, it is almost certain that rupture of chemical bonds is involved, and it is likely that this would result in the production of free radicals. It was hoped, therefore, that a detailed study of the E.P.R. spectra of spores would give some indication of the involvement, if any of free radicals in the maintenance of spore integrity, and of the importance of such radicals in the mechanisms of spore inactivation. In particular, since free radicals have been

shown to be involved in the oxygen-dependent radiation damage in spores (161,162,163,164), and in the death of freeze-dried bacterial cells after storage in oxygen (110,111), it was hoped to show if such radicals are also implicated in the oxygen-dependent damage observed during heating experiments.

Water in its liquid state has a large non-resonant absorption due to the interaction of its large dipole moment with the microwave electric field, and thus aqueous suspensions will cause large damping and a considerable reduction in sensitivity if inserted into the cavity of the E.P.R. spectrometer. For this reason it was not possible to demonstrate the presence or absence of paramagnetic species in the dormant spore in aqueous suspension. A resonance signal was not, however, observed in spores centrifuged from suspension and dried for 24 hours over phosphorus pentoxide. Since paramagnetic species were not detected under these mild drying conditions it is considered highly improbable that such species exist in the spore in aqueous suspension. Previous workers have shown that free radical decay occurs rapidly in conditions of high relative humidity (111, 163), and thus, even if radicals were formed in the dormant spores, their lifetimes would be expected to be extremely short in aqueous conditions, and outside the limits of detection with the equipment employed. Furthermore, the metabolic activity within the spore is so low that the possibility of radicals being formed as a consequence of normal metabolic reactions is considered to be small.

When spores in suspension are subjected to a high vacuum drying treatment a resonance signal is observed after only 10 minutes drying, and persists throughout the drying process. The signal obtained is a sharp single line resonance with a "g" value of 2.00258 and a line width (2Δ) (measured as the peak to peak distance of the first

derivative curve) of approximately 12 gauss.

The "g" value obtained is within $\pm$ 0.02% of that for the free spin (2.00229) and indicates that little spin-orbit interaction is occurring between the unpaired electrons in the paramagnetic species and atoms in the molecules. This suggests that the resonance signal observed in spores is more likely to arise from free radicals than from any other type of paramagnetic species.

Further characterisation of the resonance signal, with reference to its origin can be gained from a study of the line width. Strong spin-lattice interactions are common in paramagnetic species, giving rise to short relaxation time and line broadening. In free radicals, spin-lattice coupling is much smaller and the relaxation times are correspondingly longer so that the spectra of such radicals are very seldom broadened by strong spin-lattice interaction. Such long relaxation times can however produce a saturation broadening because the spins in the excited level cannot return to the ground level sufficiently quickly. Dipolar spin-spin interaction can occur between the electron spins in the free radicals, and can produce line broadening, although this is likely to have a marked effect only at high concentrations of radicals. During the optimisation of the E.P.R. signal (see page 211) the microwave power was adjusted such that saturation broadening was minimal. Under these conditions line broadening can thus be considered as resulting from strong interactions and indicative of paramagnetic species other than free radicals being present. In addition, the other main factor affecting line widths, exchange of spin orientation occurs between electrons in identical energy states in the majority of free radicals and results in narrowing of the line width.

The narrow line width of approximately 12 gauss shows that

line broadening is not present in the spectra from dried spores and again is indicative that the resonance results from free radicals. Resonance lines with similar "g" values and line widths have also been demonstrated in a number of biological products (112) and in lyophilised Escherichia coli (110) and have been attributed to free radicals. In contrast, spectra obtained with paramagnetic species such as manganese and copper in spores showed a single broad curve 460 - 510 gauss wide with the "g" value also displaced away from the value for the free spin (117).

Positive verification of the absence of line broadening is however difficult if the line widths only are considered and thus for more reliable conclusions to be drawn the line shape also must be taken into account. The first derivative curve obtained from dried spores is not one of the two classical shapes i.e. Gaussian or Lorentzian (see page 222) but is an intermediate between the two. The Lorentzian type shape shown by that portion of the spectra (inverted) with negative slope is indicative of spin exchange, probably between electrons in identical energy states, and is typical of free radical behaviour. However, the Gaussian characteristics of that portion of the spectra with positive slope provides evidence of spin-spin interaction, and to a certain extent, spin-lattice interaction. Where mixed spectra, of the type observed with spores, are obtained, the resonance is more likely to arise from free radicals than from other paramagnetic species.

In E.P.R. spectrometry, identification of the paramagnetic species is often possible from a study of the hyperfine splitting of the resonance spectra. In free radicals, however, the unpaired electron occupies the π molecular orbital so as to become delocalised. Since such an orbital has got low density at the position of the

nuclei, no hyperfine splitting of the spectrum would be expected. The single line spectrum lacking hyperfine splitting that is observed in dried spores is therefore in agreement with the suggestion that the spectrum results from free radicals in the spore, but does not permit further identification of the radicals. Miyagawa et al (113) have detected splitting of spectra from rat tissue at a frequency of 23 K Mc sec^{-1}, which was not detected at 9 K Mc sec^{-1}. Since the latter frequency has been used throughout the present investigation the spectra obtained may not present a complete picture. Extension of the present work to study the Q-band spectra of spores may thus provide additional information aiding more positive identification of the free radical component.

On the basis of results from the present investigation it can be concluded that the high vacuum drying process induces the formation of paramagnetic species in Bacillus megaterium spores. From the characteristics of the resultant E.P.R. spectrum, with respect to "g" value, line width, line shape and splitting , it is further concluded that the resonance signal most probably results from free radicals in the spore, but the identification of such radicals is not possible from experimental data obtained. Likewise for reasons given on page 227 the absolute spin concentration per spore cannot be reliably calculated, and thus the "relative E.P.R. signal" only is discussed.

Previous work in this department has shown that the drying process produces damage in spores which in the presence of oxygen and water shows certain similarities to radiation induced damage mediated through free radicals (135). The present work confirms the presence of free radicals in dried spores, but the resonance spectrum obtained is markedly different from that seen in irradiated

Bacillus megaterium spores. In comparison to the single line spectrum exhibited by dried spores, a spectrum interpretable as a singlet and a triplet was obtained during irradiation at low temperature which changed rapidly to a five line spectrum dissociable into a doublet and a triplet at room temperature (163). It must be concluded, therefore, that the free radicals formed during the drying process are not of the same type as those formed during irradiation.

The resonance signal first observed after 10 minutes drying is shown to increase in intensity with drying time, the kinetics of this increase being a function of the drying temperature. After 24 hours drying at 338^{o}K the percentage increase in relative E.P.R. signal appears to be maximal. After similar periods of drying at 273^{o}K and 298^{o}K the magnitude of the relative E.P.R. signal is smaller than that at 338^{o}K and from Figure 63 would appear to be non-maximal. Since the drying procedures were not carried out for long enough periods at the lower temperatures for the maximum signal to be obtained it is not possible to deduce if this maximum is a direct function of spore numbers. It is also not possible to say whether the development of radicals during drying is solely a function of exposure time and temperature, or is the result of withdrawal of water from the spore. However, since free radicals result from homologous breaking of chemical bonds, it would seem unreasonable to assume that sufficient energy is imparted to the system during low temperature drying to enable such bond breaking to be a function only of temperature, and it is therefore suggested that the removal of water from the spore is the primary factor responsible for chemical bond breakage and free radical production. The data in Table 31 illustrate that the shape and position of the resonance signal is the same throughout the drying process, only the amplitude of the signal is

observed to change, and it seems unlikely therefore that changes occur in the nature of radicals formed during drying. It is possible however that more than one type of free radical is formed, since the spectrometric technique would not be able to differentiate between these if their resonance characteristics were identical.

The nature of the radicals produced during drying is not known. Commoner et al (112) have demonstrated that free radicals in tissues are associated with protein moieties, and it is possible that radicals produced during drying may also result from bond breakage in spore proteins brought about by water removal. The suggestion put forward by Heckly et al (111), that free radicals observed in lyophilised cells may result from reactions involving certain labile components of dead cells would not seem to be a feasible explanation for the radicals formed during drying, since spore death has not been detected. A further suggestion of these authors that such radicals may arise as a consequence of reactions involving oxygen is most unlikely in these experiments where high vacuum is employed during the drying process.

A further point of importance in these experiments is that the magnitude, shape and position of the E.P.R. signal are not measurably changed by re-equilibration of the samples to 10 torr aqueous vapour pressure or by subsequent admission of oxygen to the spores. Previous workers have shown that the E.P.R. signal from lyophilised or irradiated spores decays rapidly under conditions of high relative humidity (111,163). The presence of water is considered to increase the mobility of atomic groups, thereby facilitating radical recombination. The absence of signal decay in dried spores after re-equilibration indicates either that the free radicals produced are stable and show no tendency to recombine under the experimental conditions examined, or that the fraction of radicals recombining is equivalent to the

number of new radicals being formed, such that the overall signal intensity remains unchanged. Alternatively, the recombination processes, if they occur, may be so slow as to be undetectable in the absence of suitable storage conditions after re-equilibration. However, it is clear that the modification of structural changes within the spore that is considered to be brought about by re-hydration, does not involve removal of free radicals.

The addition of oxygen has been shown to change the five line spectrum of irradiated spores into a single typical oxyradical signal (163). Data from the present experiments indicate that the shape of the resonance signal of dried spores is unaltered by the admission of oxygen to the spores after drying, and provides additional proof that the free radicals formed during drying are of a different type to those induced by radiation. The findings are in agreement with those of Tanooka (116) who was also unable to demonstrate a change in shape of the E.S.R. spectrum after exposure or storage of dried spores in air. Similarly, admission of oxygen to dried E. coli cells did not influence the signal shape, although increases in signal intensity were noted with increase in time of exposure up to a maximum after four hours storage at room temperature (91). In experiments not reported in this thesis dried spores were stored in oxygen for 6 hours at 77°K and 298°K, and in neither case was any change in signal intensity or shape observed after the storage period. It must be concluded, therefore, that formation of oxyradicals is unlikely during oxygen exposure of dried spores. It also appears unlikely that the radicals combine with bound oxygen molecules, since an electron localised on these molecules would give rise to a small asymmetry in the "g" value of the resonance spectrum, and asymmetry of this type has not been detected in the spectra of dried spores.

When dried spores are heated at 393°K a further development in the resonance spectrum is observed. When heating is carried out in anoxia or in the presence of oxygen, the "relative E.P.R. signal" increases, rapidly at first, and then more slowly to a maximum value after 120 minutes heating, this value being lower in oxygen than in anoxia. Under all conditions no change in the position or shape of the resonance signal is observed. This would suggest either that the radicals produced during heating are identical to those induced by the drying process, or that if different types of free radical are produced their resonance characteristics are so similar as to render them inseparable with the measurement technique employed. The development of the resonance signal during heating is observed with spores dried under all conditions investigated. These results are again in contrast to those obtained in radiation experiments, where it has been shown that heat treatments reduce the intensity of the E.P.R. signal (163).

The development of the resonance signal during heating follows a similar pattern to that observed with radiation induced radicals in amino acids (233). The exponential shape of the dose response curve obtained in the latter experiments could be explained on the basis of recombination of radicals, and it is possible that the radical development observed during heating is the result of both production and recombination of radicals. However, it is difficult to explain the constant maximum signal observed in spores heated in anoxia, on the basis of recombination between radicals unless it is assumed that radical production is also occurring throughout the 12 hour heating period at an equivalent rate. If the latter is the case, this leaves unexplained why a maximum signal is observed at all. Commoner et al (112) have shown that free radicals associated with a number of

natural products are apparently indefinitely stable at room temperature, and they state in addition that a number are resistant to heating, although the conditions of heating are not stated. Their explanation for this radical stability as being the result of trapping of the free radicals in condensed, polymerised ring structures within the product, may well explain also the stability of radicals in the spore.

In contrast to spores heated in anoxia, those heated in oxygen exhibit a decrease in the magnitude of the maximum "E.P.R. value" with prolonged heating over a period of 600 minutes. This decline in free radical concentration may be the result of recombination of stable radicals, under the influence of the oxygen molecules.

When spores are re-equilibrated to 10 torr aqueous vapour pressure prior to heating a similar development of the resonance signal to that in un-equilibrated spores is observed, with the maximum again being attained after 120 minutes heating. With spores dried for 24 hours at 298^{o}K prior to re-equilibration the maximum "E.P.R. value" obtained during anoxic heating is 284.93 compared with the value of 412.26 for non-equilibrated spores. Similar results are obtained with spores dried for 6 hours at 298^{o}K (Table 32). The lower maximum "E.P.R. value" indicates either that the production of stable radicals is hindered by the presence of water, or that the majority of those formed revert immediately to the ground state. The radicals that are formed are less stable in the presence of water than in its absence, as shown by the decay of the anoxic signal, after 12 hours heating, to a value of 190.61.

The maximum "E.P.R. value" observed with re-equilibrated spores is higher when heating is in the presence of oxygen, than when heating is in anoxia, and is also higher than that obtained with non-equilibrated spores heated in oxygen, being 390.07 and 226.5 for re-

equilibrated and dried spores respectively. It is apparent, therefore, that full development of free radicals during heating in oxygen requires the presence of water in the spore. It is however possible that the signal observed after re-equilibration to 10 torr aqueous vapour pressure is not maximal for Bacillus megaterium spores, and that there is an optimum moisture content for maximum free radical accumulation in spores, as has been postulated by Heckly et al for lyophilised yeast (111).

Decay of radicals is still observed in re-equilibrated spores after 12 hours heating in oxygen, but the rate of decay is apparently unaffected by the presence of water, the decrease in the maximum "E.P.R. value" being approximately 30% for both re-equilibrated and dried spores.

The maximum accumulation of free radicals in dried spores during heating, and their subsequent stability is therefore a function of the gaseous environment during heating, and of the presence of water. This maximum is also seen from Table 32 to be a function of the drying conditions to which spores are subjected prior to heating. The accumulation of radicals during heating does not however appear to be directly related to the number of radicals present at the end of the drying process. Furthermore, heating does not seem to result in a constant increase in the maximum "E.P.R. value", since although spores exhibit a higher number of radicals after 24 hours drying at 338^{o}K, when they are subsequently heated, the maximum "E.P.R. value" is observed in spores dried for 24 hours at 298^{o}K. Hence a complex inter-relationship is seen to exist between the E.P.R. signal at the end of the drying (and therefore the drying conditions) and the development of the resonance on subsequent heating of the spores. It is not possible, with the limited experimental

data obtained in the present work to elucidate this relationship. Nevertheless it is difficult to reconcile the observed developments with the production and decay of a single species of radical in the spore. It is likely that a species or number of species of radical are produced during drying, and continue to be formed during the subsequent heating. In addition, other species may be formed only during the heating process, as a result of chemical bond breakage induced by the high exposure temperature. If all these radicals have the same type of resonance characteristics the experimental technique would not be able to differentiate between them. The inter-relationship between production and decay of these different species would probably result in apparently non-systematic changes being observed in the overall maximum resonance signal exhibited by spores under various environmental conditions, as are observed in spores heated under standardised conditions after being subjected to different drying treatments (Table 32). Full resolution and explanation of these findings must therefore await the results of a more extensive study of E.P.R. spectra, aimed at the differentiation of the radical components responsible for such spectra.

The experimental data also show that the rate of development of the maximum E.P.R. signal during heating is not significantly altered by the different drying treatments to which the spores are subjected, or by re-equilibration of the spores to 10 torr aqueous vapour pressure. Likewise the rate is apparently unaffected by the presence of oxygen during heating. These findings would suggest that the rate of development of the maximum resonance signal is a function solely of the heating temperature, although investigations have not yet been conducted at other heating temperatures, to confirm these suggestions.

This study of the E.P.R. spectra of spores was undertaken to

determine the importance of free radicals in the maintenance of spore integrity and in the mechanisms of spore inactivation. Of particular significance therefore is the observation that the development of free radicals in dried spores during heating apparently shows no direct correlation with spore survival. With spores dried under all conditions tested, the maximum resonance signal develops after 120 minutes heating at 393°K. The survival of spores after the same period of heating shows an extremely wide variation ranging from a surviving fraction of below 10^{-6} for spores dried for periods in excess of 9 hours at 298°K to a surviving fraction indistinguishable from 1 in spores re-equilibrated to 10 torr aqueous vapour pressure. It is obvious, therefore, that the rate of development of free radicals during heating at 393°K in no way parallels the rate of inactivation of spores during the heating. Likewise, the maximum "E.P.R. values" observed cannot be directly correlated with the degree of spore inactivation.

As it seems a reasonable possibility that secondary drying alters the structure of proteins or DNA in a way that affects the integrity of the spore when it is exposed to heat, it was decided to see if the radiation sensitivity is also dependent upon the same structural changes. If this were so, then the radiation sensitivity would also be a function of the drying treatment. It was anticipated that if alterations in the response of the spore to radiation did occur, these would be apparent in conditions under which the maximum sensitivity to radiation had been observed. Hence, in the present studies, spores were irradiated in oxygen and subjected to post irradiation storage (in oxygen) under conditions which have been shown to permit the development of the maximum postirradiation oxygen effect (152).

In this way the observed radiation response would represent a summation of the effects of both oxygen dependent and oxygen independent mechanisms.

In all radiation experiments reported in this thesis, the surviving fraction of spores decreased with increase in radiation dose. Log. survivor/dose curves were shown to be linear below a surviving fraction of 0.5, and to be adequately represented by the equation $\frac{N}{N_o} = ne^{-kD}$ (see page 188). The inactivation constant k derived from the slope of the curve, and the extrapolation number n obtained by extrapolation of the linear portion of the curve onto the y axis were therefore regarded as suitable numerical expressions of the radiation sensitivity of the spores. With the standardised irradiation conditions used in these experiments, any changes observed in the values of these constants with different pre-irradiation drying treatments would reflect changes in the radiation response of the spores induced by the drying process.

Previous workers have shown that the constant n for Bacillus megaterium spores has a value, expressed in surviving fraction, of about 1.3 and does not appear to change significantly with different radiation and postirradiation treatments (157). The value of n recorded in Tables 29 and 30 are expressed in terms of ln. surviving fraction, and are therefore transposed into surviving fraction for ease of comparison.

When spores are dried for different periods at 273°K, 298°K, and 338°K, the values of n obtained after irradiation are larger than 1.3, ranging in the majority of cases from 1.3 to 2.7. There is also some indication that n is dependent upon the duration of pre-irradiation drying at any given temperature, the highest values being obtained with spores dried for 6 hours, and values approaching

1.3 being observed in spores dried for 24 hours. Previous workers have shown that n does not change significantly with re-equilibration of the spores to increasing aqueous vapour pressures, i.e. to different water contents (157). However, in the present series of experiments spores have been dried to different moisture levels as a result of the different drying treatments. It is not possible therefore to decide whether the differences in n observed under these experimental conditions are the result of a reduction in the number of sites that have to be hit, or are a function of the different water levels to which the spores are dried.

When spores are re-equilibrated to 10 torr, aqueous vapour pressure, after being dried under different conditions, the n values obtained after irradiation are much larger than those obtained by previous workers, ranging from 2.7 to 8.1. There also appears to be no systematic relationship between the values of n and either the drying time, or the drying temperature, prior to re-equilibration. It is apparent that re-equilibration causes a prolongation of the shoulder of log.survivor/radiation dose curves in the same way that it results in an extension of the shoulder of log.survivor/heating time curves. It would seem therefore that rehydration of the sensitive sites within the spore confers a degree of protection against radiation damage that is manifest as an increase in the value of n, in the same way that it confers protection against heat damage, although the mechanisms of such protection, if it indeed exists, cannot be deduced from the experimental data.

Changes induced in spores by the various drying treatments are also reflected in the values of k, the inactivation constant, obtained during subsequent irradiation of the spores. The k values for spores dried for 6 hours at 298°K and 338°K are 1.039×10^{-1} kilorad^{-1} and 1.0869×10^{-1} kilorad^{-1} and change only slightly to 1.251×10^{-1} kilorad^{-1} and

1.1484×10^{-1} kilorad $^{-1}$ respectively when the drying period is extended in each case to 24 hours. Certainly the small increase in radiation sensitivity resulting from the extension in drying time is insignificant compared with the changes in heat resistance that have been shown to occur under similar conditions. The value of k shown by spores dried for 6 hours at 273^{o}K is, however, smaller by a factor in excess of 2 than that observed with spores dried at higher temperatures, being 4.4513×10^{-1} kilorad $^{-1}$. Furthermore, the decreased sensitivity does not appear to be a function of the relative amounts of water removed from the spore during drying, since extension of the drying time at 273^{o}K to 24 hours does not result in a significant change in the value of k, this being 4.7683×10^{-1} kilorad $^{-1}$ under these conditions. Whether this decreased radiation sensitivity results from exposure of the spores to low temperature prior to irradiation, or whether, even 24 hours drying at this temperature is insufficient to remove enough of the water to allow full manifestation of lethal mechanisms, cannot be deduced from these data. Further experiments with low temperature drying are needed to elucidate the causes of this observed effect.

Spores re-equilibrated to 10 torr aqueous vapour pressure prior to irradiation are more resistant to radiation damage than non-equilibrated spores, as shown by the smaller values of k obtained. These values range from 2.4084×10^{-2} kilorad $^{-1}$ to 2.9628×10^{-2} kilorad $^{-1}$ for spores dried under all investigated conditions, prior to re-equilibration. The increase in radiation resistance with increase in re-equilibration aqueous vapour pressure has been demonstrated by previous workers, and has been shown to result from the action of water reducing the magnitude of Class II and Class III components of radiation damage (157).

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A P P E N D I X I

Statistical Analyses

Equality of Two Estimates of a Parameter

The equality of estimates p_1 and p_2 with respective variances s_1^2 and s_2^2 of a parameter P is assessed by means of the following statistic:

$$t = \frac{p_1 - p_2}{\sqrt{s_1^2 + s_2^2}}$$

The value of t is compared with tabulated values (234) with $n_1 + n_2 - 4$ degrees of freedom, where n_1 and n_2 are the number of observations used in the estimation of p_1 and p_2 respectively. If the value of t does not exceed the tabulated value at the 5 per cent probability level, the parameters are assumed to be indistinguishable at that probability level.

Equality of more than Two Estimates of a Parameter

When more than two estimates of a parameter P are tested for equality, the following statistic is used:

$$B = \frac{(p_i - \bar{p})^2}{\hat{\sigma}^2}$$

If the estimates p_i all come from the same normal distribution $\frac{(p_i - \bar{p})^2}{\hat{\sigma}^2}$ will have a χ^2 distribution with N - 1 degrees of freedom, where N is the number of estimates, and $\hat{\sigma}^2$ is given by the expression.

$$\hat{\sigma}^2 = \frac{n_1 s_1^2 + n_2 s_2^2 + \ldots\ldots\ldots\ldots n_n s_n^2}{n_1 + n_2 + \qquad\qquad n_n}$$

where s_1, s_2 etc. are the standard errors associated with the estimates p_1, p_2 etc. and n_1, n_2 etc. are the number of observations used in determining the estimates.

Regression Analysis

When a linear relationship is assumed to exist between two variables it is usual to fit a straight line by a least squares regression analysis. The simplest statistical model for this assumes that the independent variable x is known without error of measurement, and that the corresponding measured values of the dependent variable y are scattered normally from their true values. Hence each value y_i of the dependent variable is normally distributed with variance σ^2 and mean $\alpha + \beta x_i$

The method of least squares obtains estimates of a and b in the equation $y = a + bx$ such that the sum of squares of the deviations of the observations y_i from their mean $\alpha + \beta x_i$ is a minimum.

These values are

$$b = \frac{n \Sigma x_i y_i - \Sigma x_i \Sigma y_i}{n \Sigma x_i^2 - (\Sigma x_i)^2}$$

$$= \frac{\Sigma (x_i - \bar{x})(y_i - \bar{y})}{\Sigma (x_i - \bar{x})^2}$$

$$a = \frac{\Sigma y_i - b \Sigma x_i}{n}$$

$$= \bar{y} - b\bar{x}$$

Variance of b

This is termed s_b^2 and is given by the equation

$$s_b^2 = \frac{\sigma_e^2}{\Sigma (x_i - \bar{x})^2}$$

where σ_e^2 is the error variance per observation.

Minimisation of s_b^2 is enhanced by bringing $\Sigma (x_i - \bar{x})^2$ to a maximum. This can be achieved by increasing the number of

observations n and by increasing the values of $(x_i - \bar{x})$.

Variance of Q

Q is given as the interval $a - a_o$ where a is the observed value of y at x = o and a_o is the calculated value of y at x = o.

The variance of Q, namely s_Q^2 is given by

$$s_Q^2 = s_a^2 + s_{a_o}^2 = \sigma_e^2 \left[\frac{1}{t} + \frac{\Sigma(x_i)^2}{n\,\Sigma(x_i - \bar{x})^2} \right]$$

where t is the number of observations at x = o.

When applied to survivor curves, the dependent variable y is the ln. number of surviving spores in 0.3 ml. of reconstituted spore suspension. The independent variable x is either the heating period at a given temperature, or the radiation dose. The value of the constant b is the inactivation constant k. a_o represents the calculated value of the ln. number of surviving spores in 0.3 ml. of reconstituted spore suspension at zero time/dose, while a is the observed ln. number of surviving spores at sero time/dose. n is the number of observations used to calculate the regression analysis.

A P P E N D I X II

Computer Programs

PROGRAM 1

Fortran Program for Least Squares Regression Analysis

```
      PROGRAM LSTSQ
      DIMENSION X(100),Y(100),XY(100),XSQ(100),YCALC(100),DSQ(100)
      READ(5,5)NCASES
      DO 30 ICASE=1,NCASES
      WRITE(6,1)ICASE
    1 FORMAT(' CASE NUMBER',I3/T2;14('*')/)
C     FIND OUT HOW MUCH DATA IS TO FOLLOW
    5 FORMAT(I10)
      READ(5,5)N
C     READ X AND Y DATA
      READ(5,10)(X(I),Y(I),I=1,N)
   10 FORMAT(2G10.4)
      DO 20 I=1,N
   20 Y(I)=LOG(Y(I))
C     SUMMATIONS
      SUMX=0
      SUMY=0
      SUMXY=0
      SUMXSQ=0
      SUMYSQ=0
      DO 100 I=1,N
      SUMX=SUMX+X(I)
      SUMY=SUMY+Y(I)
      XY(I)=X(I)*Y(I)
      SUMXY=SUMXY+XY(I)
      XSQ(I)=X(I)*X(I)
      SUMXSQ=SUMXSQ+XSQ(I)
      SUMYSQ=SUMYSQ+Y(I)*Y(I)
  100 CONTINUE
C     CALCULATIONS
      DENOM=(N*SUMXSQ)-(SUMX*SUMX)
      SLOPE=((N*SUMXY)-(SUMX*SUMY))/DENOM
      CEPT=((SUMY*SUMXSQ)-(SUMX*SUMXY))/DENOM
      CORCOF=(N*SUMXY-SUMX*SUMY)/SQRT(DENOM*(N*SUMYSQ-SUMY*SUMY))
      WRITE(6,110)SLOPE,CEPT,CORCOF
  110 FORMAT(' SLOPE       =',E15.7/' INTERCEPT=',E15.7/'
     1CORRELATION COEFFICIENT=',E15.7)
      SUMDSQ=0
      DO 150 I=1,N
      YCALC(I)=SLOPE*X(I)+CEPT
      DSQ(I)=(YCALC(I)-Y(I))*(YCALC(I)-Y(I))
      SUMDSQ=SUMDSQ+DSQ(I)
  150 CONTINUE
      SIGMAM=SQRT(N*SUMDSQ/((N-2)*DENOM))
      SIGMAC=SQRT(SUMXSQ*SUMDSQ/((N-2)*DENOM))
      WRITE(6,160)SIGMAM,SIGMAC
  160 FORMAT(' STANDARD DEVIATION OF SLOPE   =',E15.7/'
     1STANDARD DEVIATION OF INTERCEPT=',E15.7///)
   30 CONTINUE
      STOP
      END
```

PROGRAM 2

Algol Program for Fitting Experimental Data to the Eqations $N/N_o = ne^{-kD}$ or $N/N_o = ne^{-kt}$ and Calculating the Constants

```
'BEGIN'
'INTEGER'L,K,I,O,T,M,N,J,Q,PP,F,S;
'REAL'WW,FF,DD,ZZ,AA,BB,GG,VV;
'ARRAY'E,H(/1:20/),C,U,X,W,Y,D,Z(/1:400/),P(/1:95/);
F:=FORMAT('('-D.7D@+NDC')');
L3:P(/22/):=READ(60);'COMMENT'EXPERIMENT NUMBER;
'IF'P(/22/)=-1'THEN''GOTO'L2;
WRITE TEXT(30,'('%')');WRITE(30,FORMAT('('-N4D.5DCC')'),P(/22/));
L:=READ(60);WRITE(30,FORMAT('('-NDDSS')'),L);
'COMMENT'NUMBER OF COUNTS AT ZERO DOSE;
K:=READ(60);WRITE(30,FORMAT('('-N6DC')'),K);
'COMMENT'DILUTION AT ZERO DOSE;
P(/14/):=P(/20/):=P(/32/):=P(/33/):=P(/35/):=P(/36/):=0.0;
'FOR'I:=1'STEP'1'UNTIL'L'DO'
'BEGIN'
WW:=READ(60);WRITE(30,FORMAT('('-N3DSS')'),WW);
'COMMENT'COUNTS AT ZERO DOSE;
X(/I/):=LN(K*WW);P(/14/):=P(/14/)+X(/I/);
P(/20/):=P(/20/)+X(/I/)*X(/I/);
C(/I/):=LN(WW);P(/32/):=P(/32/)+C(/I/);
P(/33/):=P(/33/)+C(/I/)*C(/I/);
W(/I/):=WW;P(/35/):=P(/35/)+W(/I/);
P(/36/):=P(/36/)+W(/I/)*W(/I/);
'END';
P(/21/):=P(/20/)-P(/14/)*P(/14/)/L;O:=L-1;P(/50/):=P(/21/)/O;
WRITE TEXT(30,'('%P50=')');WRITE(30,F,P(/50/));
'COMMENT'VARIANCE LN. SURVIVORS AT ZERO DOSE;
P(/15/):=P(/14/)/L;WRITE TEXT(30,'('P15=')');WRITE(30,F,P(/15/));
'COMMENT'MEAN LN. SURVIVORS AT ZERO DOSE;
P(/93/):=EXP(P(/15/));WRITE TEXT(30,'('P93=')');WRITE(30,F,P(/93/));
'COMMENT'NUMBER OF SURVIVORS AT ZERO DOSE;
P(/94/):=EXP(P(/15/)-P(/15/));WRITE TEXT(30,'('P94=')');
WRITE(30,F,P(/94/));'COMMENT'SURVIVING-FRACTION AT ZERO DOSE;
P(/37/):=P(/32/)/L;P(/38/):=P(/33/)-P(/32/)*P(32/)/L;
P(/39/):=P(/38/)/O;P(/40/):=P(/38/)/P(/37/);P(/41/):=P(/35/)/L;
P(/42/):=P(/36/)-P(/35/)*P(/35/)/L;P(/43/):=P(/42/)/O;
P(/44/):=P(/42/)/P(/41/);
WRITE TEXT(30,'('%P37=')');WRITE(30,F,P(/37/));
'COMMENT'MEAN LN. COUNT;
WRITE TEXT(30,'('P38=')');WRITE(30,F,P(/38/));
WRITE TEXT(30,'('P39=')');WRITE(30,F,P(/39/));
'COMMENT'VARIANCE OF LN. COUNTS;
WRITE TEXT(30,'('P40=')');WRITE(30,F,P(/40/));
WRITE TEXT(30,'('P41=')');WRITE(30,F,P(/41/));
'COMMENT' MEAN COUNT;
WRITE TEXT(30,'('P42=')');WRITE(30,F,P(/42/));
WRITE TEXT(30,'('P43=')');WRITE(30,F,P(/43/));
'COMMENT'VARIANCE OF COUNTS;
WRITE TEXT(30,'('P44=')');WRITE(30,F,P(/44/));
'COMMENT'INDEX OF DISPERSION OF COUNTS;
T:=1;M:=0;P(/65/):=P(/66/):=0.0;
N:=READ(60);WRITE TEXT(30,'('%')');WRITE(30,FORMAT('('-NDD')'),N);
'COMMENT'NUMBER OF DOSE LEVELS OTHER THAN ZERO DOSE;
'FOR'J:=1'STEP'1'UNTIL'N'DO'
'BEGIN'
```

```
Q:=READ(60);WRITE TEXT(30,'('%')');
WRITE(30,FORMAT('('-NDDSS')'),Q);
'COMMENT'NUMBER OF COUNTS;
FF:=READ(60);WRITE(30,F,FF);'COMMENT'DILUTION;
DD:=READ(60);WRITE(30,FORMAT('('-N4D.5DC')'),DD);
'COMMENT'DOSE;
S:=T+Q-1;
P(/11/):=P(/12/):=P(/52/):=P(/53/):=P(/55/):=P(/56/):=0.0;
"FOR'I:=T'STEP'1'UNTIL'S'DO'
"BEGIN'
M:=M+1;ZZ:=READ(60);Z(/I/):=ZZ;
WRITE(30,FORMAT('('-N3DSS')'),ZZ);
Y(/I/):=LN(ZZ*FF);D(/I/):=DD;
P(/11/):=P(/11/)+Y(/I/);
P(/12/):=P(/12/)+Y(/I/)*Y(/I/);
U(/I/):=LN(ZZ);P(/52/):=P(/52/)+U(/I/);
P(/53/):=P(/53/)+U(/I/)*U(/I/);
Z(/I/):=ZZ;P(/55/):=P(/55/)+Z(/I/);
P(/56/):=P(/56/)+Z(/I/)*Z(/I/);
'END';
P(/13/):=P(/12/)-P(/11/)*P(/11/)/Q;
O:=Q-1;
E(/J/):=P(/13/)/O;H(/J/):=LN(P(/13/)/O);
WRITE TEXT(30,'('%EJ=')');WRITE(30,F,E(/J/));
'COMMENT'VARIANCE OF LN. SURVIVORS;
P(/65/):=P(/65/)+E(/J/);P(/66/):=P(/66/)+H(/J/);
P(/85/):=P(/11/)/Q;P(/83/):=EXP(P(/85/));
P(/84/):=EXP(P(/85/)-P(/15/));
WRITE TEXT(30,'('P85=')');WRITE(30,F,P(/85/));
'COMMENT'MEAN LN. SURVIVORS;
WRITE TEXT(30,'('P83=')');WRITE(30,F,P(/83/));
'COMMENT'NUMBER OF SURVIVORS;
WRITE TEXT(30,'('P84=')');WRITE(30,F,P(/84/));
'COMMENT'SURVIVING-FRACTION;
WRITE TEXT(30,'('%')');
P(/57/):=P(/52/)/Q;P(/58/):=P(/53/)-P(/52/)*P(/52/)/Q;
P(/59/):=P(/58/)/O;P(/60/):=P(/58/)/P(/57/);
P(/61/):=P(/55/)/Q;P(/62/):=P(/56/)-P(/55/)*P(/55/)/Q;
P(/63/):=P(/62/)/O;P(/64/):=P(/62/)/P(/61/);
WRITE TEXT(30,'('P57=')');WRITE(30,F,P(/57/));
'COMMENT'MEAN LN. COUNT;
WRITE TEXT(30,'('P58=')');WRITE(30,F,P(/58/));
WRITE TEXT(30,'('P59=')');WRITE(30,F,P(/59/));
'COMMENT'VARIANCE OF LN.COUNTS;
WRITE TEXT(30,'('P60=')');WRITE(30,F,P(/60/));
WRITE TEXT(30,'('P61=')');WRITE(30,F,P(/61/));
'COMMENT'MEAN COUNT;
WRITE TEXT(30,'('P62=')');WRITE(30,F,P(/62/));
WRITE TEXT(30,'('P63=')');WRITE(30,F,P(/63/));
'COMMENT'VARIANCE OF COUNTS;
WRITE TEXT(30,'('P64=')');WRITE(30,F,P(/64/));
'COMMENT'INDEX OF DISPERSION OF COUNTS;
T:=T+Q;
'END';
P(/1/):=P(/2/):=P(/3/):=P(/4/):=P(/7/):=0.0;
PP:=M-2;
```

```
'FOR'I:=1'STEP'1'UNTIL'M'DO'
'BEGIN'
P(/1/):=P(/1/)+D(/I/)*Y(/I/);
P(/2/):=P(/2/)+D(/I/);P(/3/):=P(/3/)+Y(/I/);
P(/4/):=P(/4/)+D(/I/)*D(/I/);P(/7/):=P(/7/)+Y(/I/)*Y(/I/);
'END';
P(/5/):=P(/1/)-P(/2/)*P(/3/)/M;P(/6/):=P(/4/)-P(/2/)*P(/2/)/M;
P(/8/):=P(/7/)-P(/3/)*P(/3/)/M;P(/9/):=P(/8/)-P(/5/)*P(/5/)/P(/6/);
P(/10/):=P(/9/)/P(/6/);BB:=P(/5/)/P(/6/);AA:=P(/3/)/M-BB*P(/2/)/M;
P(/16/):=P(/9/)/PP;VV:=SQRT(P(/16/));P(/17/):=P(/10/)/PP;
P(/23/):=SQRT(P(/17/));P(/18/):=M*P(/6/);
P(/67/):=P(/9/)/L+P(/9/)*P(/4/)/P(/18/);
P(/19/):=P(/67/)/PP;P(/25/):=SQRT(P(/19/));GG:=AA-P(/15/);
WRITE TEXT(30,'('%B=')');WRITE(30,F,BB);'COMMENT'SLOPE;
WRITE TEXT(30,'('P10=')');WRITE(30,F,P(/10/));
WRITE TEXT(30,'('P17=')');WRITE(30,F,P(/17/));
'COMMENT'VARIANCE OF SLOPE;
WRITE TEXT(30,'('P23=')');WRITE(30,F,P(/23/));
'COMMENT'STANDARD ERROR OF A SLOPE;
WRITE TEXT(30,'('%G=')');WRITE(30,F,GG);
'COMMENT'LN. EXTRAPOLATION NUMBER;
WRITE TEXT(30,'('P67=')');WRITE(30,F,P(/67/));
WRITE TEXT(30,'('P19=')');WRITE(30,F,P(/19/));
'COMMENT'VARIANCE OF LN. EXTRAPOLATION NUMBER;
WRITE TEXT(30,'('P25=')');WRITE(30,F,P(/25/));
'COMMENT'STANDARD ERROR OF LN. EXTRAPOLATION NUMBER;
WRITE TEXT(30,'('%A=')');WRITE(30,F,AA);
'COMMENT'NUMBER OF SURVIVORS AT ZERO DOSE BY EXTRAPOLATION;
WRITE TEXT(30,'('P9=')');WRITE(30,F,P(/9/));
WRITE TEXT(30,'('P16=')');WRITE(30,F,P(/16/));
'COMMENT'VARIANCE OF ESTIMATE OF LN. NUMBER OF SURVIVORS;
WRITE TEXT(30,'('V=')');WRITE(30,F,VV);
'COMMENT'STANDARD ERROR OF ESTIMATE OF LN. NUMBER OF SURVIVORS;
'IF'GG =252*LN(2)'THEN'
'BEGIN'
WRITE TEXT(30,'('%%% G   =  252*LN(2)     CASE TERMINATED')');
'GOTO'L3;
'END';
P(/24/):=EXP(GG);P(/27/):=P(/65/)/N;P(/28/):=LN(P(/27/));
P(/30/):=M/N-1;P(/29/):=N*P(/30/)*P(/28/);P(/31/):=P(/30/)*P(/66/);
P(/45/):=P(/29/)-P(/31/);P(/46/):=1+N;P(/47/):=3*N*P(/30/);
P(/48/):=1+P(/46/)/P(/47/);P(/49/):=P(/45/)/P(/48/);
WRITE TEXT(30,'('P24=')');WRITE(30,F,P(/24/));
'COMMENT'EXTRAPOLATION NUMBER;
WRITE TEXT(30,'('P49=')');WRITE(30,F,P(/49/));
'COMMENT'B/C(BARTLETT S TEST);
'GOTO'L3;
L2;
'END'
```

PROGRAM 3

Fortran Program for Calculating the Entropy of Activation and the Enthalpy of Activation

```
'BEGIN'
'REAL' X,T,R,K,Y,YSUM,Y2SUM,XSUM,X2SUM,XY,B,A,DELTA,ALIMIT,BLIMIT,AA;
'INTEGER' N,I;
START:N:=READ(60); R:=READ(60);
YSUM:=Y2SUM:=XSUM:=X2SUM:=XY:=0;
'FOR' I:=1'STEP'1'UNTIL'N'DO'
'BEGIN'
K:=READ(60); T:=READ(60);
Y:=0.4343*LN(K/(60*T))-10.3187;
X:=-1/(2.303*R*T);
YSUM:=YSUM+Y;
Y2SUM:=Y2SUM+Y*Y;
XSUM:=XSUM+X;
X2SUM:=X2SUM+X*X;
XY:=XY+X*Y;
'END';
B:=(XSUM*YSUM-N*XY)/(XSUM*XSUM-N*X2SUM);
A:=(YSUM-B*XSUM)/N;
AA:=(A)*2.303*R;
DELTA:=SQRT((Y2SUM-YSUM*YSUM/N-B*B*(X2SUM-XSUM*XSUM/N))/(N-2));
BLIMIT:=DELTA/SQRT(X2SUM-XSUM*XSUM/N);
ALIMIT:=DELTA*SQRT(1/N+XSUM*XSUM/(N*N*X2SUM-N*XSUM*XSUM));
WRITE TEXT(30,'('DELTAS=')');
WRITE (30,FORMAT('('-D.DDD')'),AA;
WRITE TEXT(30,'('DELTAH=')');
WRITE (30,FORMAT('('-DDDDD.DD')'),B);
WRITE TEXT(30,'('BLIMIT=')');
WRITE (30,FORMAT('('-DDDD.DD')'),BLIMIT);
WRITE TEXT(30,'('ALIMIT=')');
WRITE (30,FORMAT('('-DD.DD')'),ALIMIT);
WRITE TEXT(30,'('INITIALA=')');
WRITE (30,FORMAT('('-DD.DD')'),A);
'GOTO' START;
'END'
```

A P P E N D I X III

E.P.R. Data for *Bacillus megaterium* Spores

Subjected to High Vacuum Drying

E.P.R. data obtained from a sample of Bacillus megaterium spores after different periods of drying at 273°K

Drying Time	$2F'(H-H_o)_{max}$	2Δ	"Relative E.P.R. signal" (x 10^4)	Percentage increase in "relative E.P.R. signal"
10 mins	54	13.5	0.98415	0
30 mins	54	13.5	0.98415	0
1 hour	55	13.5	1.0024	1.85
2 hours	55.5	13.5	1.0115	2.77
3 hours	56	13.5	1.0206	3.7
4 hours	56.5	13.5	1.0297	4.63
5 hours	58	13.5	1.0571	7.41
6 hours	60	13.5	1.0935	11.11
12 hours	72	13.5	1.3122	33.33
18 hours	85	13.5	1.5491	57.4
21 hours	93	13.5	1.6949	72.22
24 hours	98	13.5	1.7861	81.48

E.P.R. data obtained from a sample of Bacillus megaterium spores after different periods of drying at 298°K

Drying Time	$2F'(H-H_o)_{max}$	2Δ	"Relative E.P.R. signal" (x 10^4)	Percentage increase in "relative E.P.R. signal"
10 mins	52	13.5	0.9477	0
30 mins	54	13.5	0.98415	3.85
1 hour	55	13.5	1.0024	10.58
1.5 hrs.	60.5	13.5	1.1026	16.34
2 hours	67.5	13.5	1.2302	29.81
3 hours	74	13.5	1.3487	42.31
4 hours	79	13.5	1.4398	51.93
5 hours	84.5	13.5	1.54	62.5
6 hours	87.5	13.5	1.5947	68.27
12 hours	102	13.5	1.859	96.16
18 hours	106.5	13.5	1.941	104.81
24 hours	108	13.5	1.9683	107.69

E.P.R. data obtained from a sample of Bacillus megaterium spores after different periods of drying at 338°K

Drying Time	$2F'(H-H_o)_{max}$	2Δ	"Relative E.P.R. signal" (x 10^4)	Percentage increase in "relative E.P.R. signal"
10 mins	60	13.5	1.0935	0
20 mins	66	13.5	1.2029	10.0
40 mins	89.5	13.5	1.6311	49.16
1 hour	112	13.5	2.0412	86.67
2 hours	127.5	13	2.1548	97.06
3 hours	131.25	13	2.2181	102.84
4 hours	133.25	13	2.2498	105.74
5 hours	134.375	13	2.2709	107.67
6 hours	136.25	13	2.3026	110.57
12 hours	139.375	13	2.3554	115.4
18 hours	139.84	13	2.3633	116.12
24 hours	141.41	13	2.3898	118.55