The Microbiological Assay of Aflatoxin

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ABSTRACT

A number of possible bases for a biological assay for the aflatoxins were examined, of these two were selected for further study. An inhibition assay using Bacillus megaterium was developed in combination with thin layer chromatographic analysis. This method was capable of detecting 0.1 µg of aflatoxin B_1 and of differentiating between various mycotoxins.

An induction assay using a lysogenic strain of Bacillus megaterium was also developed. This assay has a sensitivity of 2-4 µg/ml aflatoxin B_1 and is claimed to be more specific than the inhibition assay.

A number of other mycotoxins were examined to test the versatility and specificity of the assays. The inhibition assay was able to detect and differentiate between most of the toxins. The induction assay detected only those mycotoxins reported or suspected to be carcinogenic.

PREFACE

It has been known for many years that certain fungi produce substances toxic to human beings and livestock. Toxic Basidiomycetes and ergotism have been responsible for the loss of many lives. It is only comparatively recently however that attention has been directed to the imperfect fungi as sources of toxins. In particular, a dramatic outbreak of an unknown disease among poultry in the United Kingdom, called Turkey X Disease, was eventually traced to a substance produced by Aspergillus parasiticus. This active principle was named 'aflatoxin' after the Aspergillus flavus-oryzae group to which Aspergillus parasiticus belongs.

'Aflatoxin' was soon shown to be in fact a number of related compounds which were designated B_1, B_2, G_1 and G_2 due to the blue or green colour of their fluorescence under ultra-violet light and the relative positions they reach on thin layer chromatographic plates. Not all the aflatoxins are of equal potency, B_1 being the most potent and G_2 the least.

The aflatoxins are of especial interest since they have been shown to be carcinogenic in very low concentrations in addition to their hepatotoxic effect at higher concentrations. Aflatoxins have been reported from a wide variety of fungi, chiefly Aspergillus and Penicillium species though other fungi, including a Rhizopus species, have been implicated. It was also shown that the toxins could be formed on a wide range of substrates and could easily occur in human foodstuffs if the fungus was allowed to grow on them.

Chemicophysical methods of detection and quantitation have been developed, based mainly on extraction by an organic solvent followed by concentration, separation on thin layer chromatographic plates and fluorodensitometric estimations of the spots.

Chemical assays are rapid, sensitive and very precise but may not always accurately reflect the biological activity of the substance under test. This is particularly the case where very small chemical differences

can make very large changes in activity. There have been shown to be at least nine different aflatoxins with biological activities varying from extreme toxicity to practically no effect. It is therefore desirable to have a biological assay to complement the results of the chemical assay.

The ideal bioassay would be simple, speedy, sensitive, specific and, of course, reproducible. Early bioassay methods were based on the toxicity of aflatoxin to vertebrates; the most sensitive and specific assay being that using ducklings. Fish larvae and fertile eggs have also been used. Later an inhibition-zone microbiological bioassay using Bacillus megaterium was developed and put forward as a standard method in the Journal of the Association of Official Analytical Chemists. There have been reports in the literature of other effects of aflatoxin which might be suitable for a bioassay system. Among these are tissue-culture abnormalities, induction of lysogenic bacteria and suppression of chloroplast development in germinating cress seeds.

The assay based on ducklings is relatively specific but requires skill to perform and is not nearly as rapid or sensitive as chemical methods. The inhibition assay using B. megaterium is more rapid and easier to perform but is not very specific and is also less sensitive than chemical assays though it requires much less material than does the duckling assay.

For these reasons it was considered worthwhile to look for an assay as specific as the duckling assay but with the advantages of a microbiological assay. A number of possibilities had already been suggested by various authors and it was these that I proposed to investigate.

ACKNOWLEDGEMENTS

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INTRODUCTION

Mycotoxins: The word 'mycotoxin' is formed from the two roots 'myco' from the Greek 'mykes', a mushroom, and 'toxicon', a poison, originally as applied to arrow heads. Poisons and intoxicants derived from a variety of fungi have been known and used for many centuries. Ergotism caused frequent epidemics in Europe and is still not unknown, and doubtless other local outbreaks of unexplained disease were also due to toxins of mycological origin. It was not until the last two decades, however, that serious attention has been directed toward the fungi as a source of substances likely to be harmful to man and domestic animals.

History of Aflatoxins:

Interest in aflatoxins was first aroused by an outbreak of a mysterious disease affecting turkey poults in Great Britain. Tens of thousands of birds died as a result of the disease. Study of the problem revealed that all the affected birds had been fed on rations containing peanut meal and that other animals fed the same meal could be made to show the same symptoms. (Blount 1961, Asplin and Carnaghan 1961). In the same year outbreaks of a disease of pigs and calves were also shown to be associated with the presence of peanut meal in the ration. (Loosmore and Harding 1961, Loosmore and Markson 1961). A biological assay using ducklings was devised (Sargeant et al. 1961a) to test samples of peanut meal and compounded feeds and later Sargeant and his co-workers (1961a) were able to show that the toxic factor was formed by the common mould, Aspergillus flavus, a member of the Aspergillus flavus-oryzae group. In view of its origin the toxic factor was given the name of 'aflatoxin'. Not long afterwards the structure of aflatoxin was elucidated by Asao et al. (1963). The toxin was shown to consist of a group of similar compounds rather than a single compound. As information on the toxic effects of the aflatoxins accumulated it became apparent that previous reports of unexplained disease, such as those in laboratory animals (Shand 1957) and in rainbow trout hatcheries (Wales and Sinnhuber 1966) were also, almost certainly, due to the presence of aflatoxins in the feed.

Aflatoxins have not, as yet, been found to be of great economic importance in New Zealand. This is probably due to the relatively small

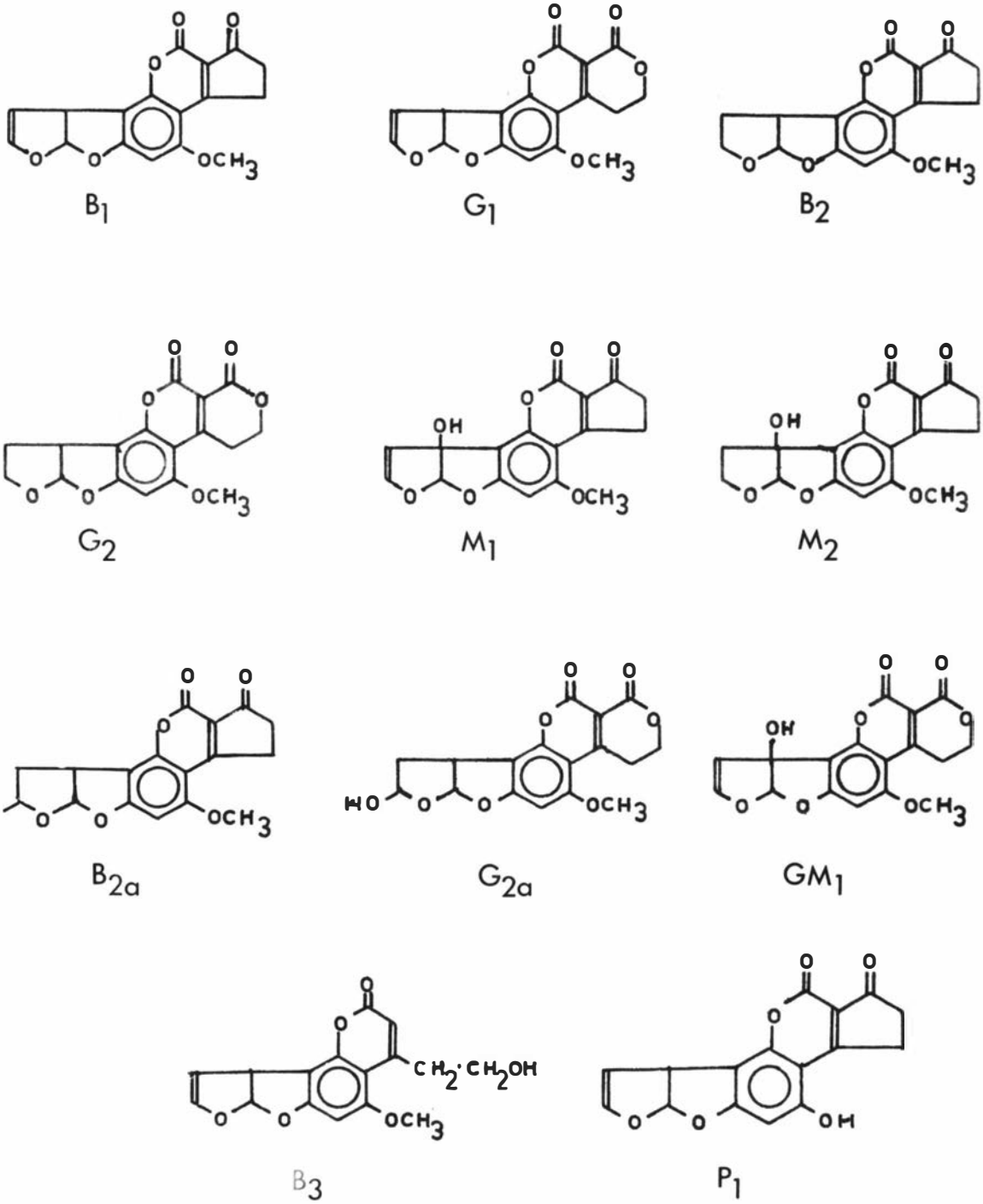
size of the poultry industry in this country and the fact that few pig or dairy farmers rely very heavily on stored feed for their animals. Nevertheless as farming becomes more intensified the possibility of mould damage to stored animal feeds will increase. In addition the bulk of our dairy produce is exported, some of it to countries which have been deeply concerned about the possible damage to health caused by mycotoxins. Such countries may well consider imposing a requirement that our exports be tested and declared free of particular mycotoxins, especially aflatoxin, and for this reason a brief examination of the problem was earlier made by the author. Toxigenic strains of Aspergillus flavus were isolated during a survey (Freke and Richardson, unpublished) and thus a potential problem was revealed. The present work was prompted by these isolations and the reasons outlined above.

The Structure of Aflatoxin:

The structures of the four aflatoxins formed by Aspergillus flavus are given in Figure 0.1. In 1962 Allcroft and Carnaghan found that the milk from cows fed aflatoxin-containing rations contained a toxin with properties similar to the aflatoxins themselves and later (1966) Allcroft et al. suggested the name aflatoxin M (for milk toxin) for this toxic factor. Holzapfel et al. (1966) were able to characterise this factor as consisting of hydroxy derivatives of aflatoxins B_1 and B_2. Aflatoxins M_1 and M_2 can also be isolated from the urine of animals fed aflatoxin-containing feeds and from cultures of the mould itself. Two further aflatoxins (B_{2a} and G_{2a}) were described by Dutton and Heathcote (1966 and 1968). These are very much less toxic than other aflatoxins and are hydroxy derivatives of B_2 and G_2. Later work by Dutton and Heathcote (1969) suggested that the B_{2a} and G_{2a} aflatoxins may be derived from B_1 and G_1 during extraction as they are very easily formed in the presence of alcohols under acidic conditions. They also described two further aflatoxins, B_3 and GM_1. In 1971 Dalezios et al. reported yet another derivative of aflatoxin which they had isolated from the urine of monkeys injected with aflatoxin B_1 and called aflatoxin P_1. The structures of these various aflatoxin derivatives are also given in Figure 0.1.

Figure 0·1

STRUCTURES OF THE AFLATOXINS



Formation of aflatoxins:

A number of other fungi apart from A. flavus were reported as being able to produce aflatoxin, for example other Aspergilli, some Penicillium species (Kulik and Holaday 1967) and Rhizopus species (Van Walbeck et al. 1968). Other workers (Mislivec et al. 1968, Wilson et al. 1967, Parrish et al. 1966) including the present author (unpublished 1969) have been unable to substantiate these reports, however, and it seems probable that aflatoxins are only produced by members of the Aspergillus flavus-oryzae group. Not all members of the A. flavus-oryzae group produce aflatoxin nor do all individuals of the toxigenic species produce it (Parrish et al. 1966). Those strains which do produce the toxin vary in the amount and in the number of different aflatoxins which they produce (Diener and Davis 1966).

The substrate and conditions of growth also effect aflatoxin production. In synthetic media carbohydrates were reported as being necessary as a carbon source for the production of aflatoxin. The most productive media were those containing sucrose, glucose, fructose, xylose, ribose and glycerol (Mateles and Adye 1965). Krebs cycle intermediates were not able to act as carbon sources according to Davis and Diener (1968) but the incorporation of radioactively labelled acetate into aflatoxin by A. parasiticus in the presence of glucose has been reported by Hsieh and Mateles (1971). Various organic and inorganic nitrogen sources have been shown to be capable of supporting aflatoxin production but highest yields were obtained when yeast extract was used as the nitrogen source (Davis et al. 1967). The only metal shown to be of importance for optimal aflatoxin production is zinc. Concentrations of up to 2 µg/ml have been reported by Davis et al. (1967) as necessary for maximum yields.

Schindler et al. (1967) and Diener and Davis (1966) both came to the conclusion that optimum aflatoxin yields are obtained by incubation at 25°C and that peak production occurs at 7 - 9 days.

The requirement for aeration seems to vary in different strains. With strain ATCC 15517 (V - 3734/10) Mateles and Adye (1965) obtained better aflatoxin yields in shake flasks than in stationary submerged culture but Diener and Davis (1969) found that this was one of few strains that did produce aflatoxin in shake flasks.

Biosynthesis of aflatoxin:

The complete biosynthetic pathway of the aflatoxins is not yet known. Donkersloot et al. (1968) conclude that acetate is the major primary precursor of aflatoxin. Schemes such as those suggested by Holker and Underwood (1964) and Thomas (1965) seem most probable. Acetate condenses to form an anthroquinone of some kind from which sterigmatocystin is formed. Aflatoxin is then synthesised from sterigmatocystin. Elsworthy et al. (1970) demonstrated the incorporation of radioactively labelled 5-hydroxy-sterigmatocystin into aflatoxins B_1 and G_1 by Aspergillus flavus, which lends weight to this suggestion.

Toxicity of aflatoxins:

The toxic effects of aflatoxins were first noticed in turkey poults and very shortly afterwards in ducklings and young pheasants (Allcroft 1969). The order of susceptibility in birds was reported as being duckling > turkey and pheasant chicks > domestic chicken and quail chicks. In domestic mammals the order is 3-12 week-old pigs and pregnant sows > calves > fattening pigs and mature cattle > sheep. Sheep seem to be the most resistant farm animals examined.

A similar range of susceptibility is shown by laboratory animals. Guinea pigs and ferrets are very susceptible, rats and dogs less so, monkeys and hampsters somewhat less again and mice are quite resistant (Butler 1969). Trout (Halver 1965) and zebra fish larvae (Abedi and Scott 1969) have also been shown to be affected by aflatoxins. Carcinogenic effects were reported for most of the species examined, the organ generally affected being the liver.

Reports of effects in other organisms are more sparse but mutagenic effects have been reported in bacteria (Ames et al. 1973) and fungi (Ong 1971). Lilly (1965) reported chromosomal abberations in the roots of Vicia faba. Schoental and White (1965) reported inhibition of chlorophyll production in watercress seedlings but Ries (1969) was not able to substantiate this claim. Crison (1973) reported that the growth of developing lettuce seedlings was inhibited by aflatoxin B_1 but could find no effect on germination or chlorophyll formation.

Effects of aflatoxin in cell cultures have been examined and inhibition of a wide range of cell types has been reported (Togan 1969). Legator and Withrow (1964) reported reduction of the mitotic rate in human embryonic lung cells by aflatoxin B_1. The incorporation of thymidine and uridine was shown to be inhibited by aflatoxins B_1 and G_1 and by sterigmatocystin and

some of its analogues. (Engelbrecht and Altenkirk 1972). Portman and Campbell (1970) reported inhibition of RNA polymerase transcription by aflatoxin B_1 and demonstrated that histone protein must be present for the inhibition to be effective *in vitro*.

There are not many reports of the relative toxicity of the different aflatoxins. Those of aflatoxins B_1, B_2, G_1 and G_2 were reported by Carnaghan *et al.* (1963) and further reports on B_1, M_1, M_2 and sterigmatocystin were made by Holtzapfel *et al.* (1966) and on B_{2a}, G_{2a} by Dutton and Heathcote (1969). All these reports dealt with toxicity to ducklings. Engelbrecht and Altenkirk (1972) compared the effects of aflatoxins B_1 and G_1 and a number of sterigmatocystin analogues on cell cultures. *Bacillus megaterium* spores were used by Buckelew *et al.* (1972) to compare the toxicity of aflatoxins B_1, B_2, G_1 and G_2 and a wide variety of other compounds. Abedi and Scott (1969) also examined a range of mycotoxins using zebra fish larvae as the test organism. The results of these studies are given in Table 0.2.

Table 0.2 Relative toxicity of aflatoxins in various biological systems.

	Ducklings[1]	B.megaterium[2]	Cell cultures[3]	Zebra fish[4]
aflatoxin B_1	11.2	144	29	0.5
aflatoxin B_2	34	49	116	1.0
aflatoxin B_3	not stated			
aflatoxin G_1	15.7	20		0.8
aflatoxin G_2	69	4		4.2
aflatoxin M_1	16.6			
aflatoxin M_2	62			
aflatoxin GM_1	not stated			
aflatoxin B_{2a}	non-toxic			
aflatoxin G_{2a}	non-toxic			
aflatoxin P_1	not stated			
sterigmatocystin	toxic but less than B_1		9	0.24

Notes:
1. L.D. 50 (µg/bird)
2. Area of inhibition zone caused by 2 µg (mm^2)
3. Thymidine incorporation in arbitary units. control = 145
4. Lethal concentration 50 (µg/ml)

Although the data are incomplete it can be seen from Table 0.2 that aflatoxin B_1 is the most toxic of the aflatoxins with M_1 and G_1 next, followed by B_2, G_2 and M_2. Sterigmatocystin, a probable precursor of the aflatoxins, is of the same order of toxicity as aflatoxin B_1. Aflatoxins B_{2a} and G_{2a} are reported as being non-toxic to ducklings and have not been examined in other systems.

Methods of detection of aflatoxins:

Clearly the reports of aflatoxin toxicity in a wide variety of organisms and its ability to be carcinogenic in a number of domestic and laboratory animals make it imperative that foodstuffs likely to support the growth of the toxigenic mould should be examined for toxin. To this end a number of assays have been devised. These fall into two types: physicochemical and biological.

The earliest methods, soon adopted as official by the Association of Official Analytical Chemists, were of the physicochemical type. They consist of extraction, purification and concentration procedures followed by thin layer chromatography. Since the aflatoxins are all fluorescent they can be located under long wave ultra-violet light. The method was rapidly improved and the optimum conditions defined by a host of workers including Pons et al. (1968), Engstrom (1969), Nesheim (1969), Pohland et al. (1970). The limit of detection claimed for this method is 0.01 µg/spot (Van Duuren et al. 1968) though 0.1 to 0.2 µg/spot is probably the practical limit.

The first biological assay was that developed by Asplin and Carnaghan (1961) using ducklings. This is still probably the most widely used and accepted method of bioassay. The method has the advantages of a degree of specificity when the livers are examined. Bile duct proliferation is not an entirely specific response to aflatoxin damage but is strongly linked to hepatocarcinogenicity (Legator 1969). The bioassay is capable of detecting aflatoxin B_1 at levels of 2 µg/bird in five days. It is essentially

a qualitative method however as Legator (1969) considers it questionable that a dose-response curve can be constructed.

Clements (1968) suggested a microbiological assay based on the inhibition of B. megaterium. The method is a simple disk inhibition assay of the type used to screen antibiotics. It is more rapid and easier to perform than the duckling assay but lacks its specificity. The sensitivity claimed for it is 1 µg/disk.

The three assays described above, TLC, ducklings and disk inhibition, are in widespread use. Other assays, particularly bioassays, have been described and some may be in routine use in a few laboratories. These will be examined in the next part of this introduction.

Suggested bases for Bioassay systems:

A summary of suggested bases for bioassay systems is given in Table 0.3. The list is not intended to be exhaustive but only to give an indication of the range of biological effects that might be used.

Table 0.3 Summary of suggested bases for bioassay systems for aflatoxins

	Organism	Effect determined by
mammals	Rat	L.D.50
	Rat	liver changes
birds	Duckling	L.D.50
	Duckling	liver changes
	Chicken	distortion of embryo
	Chicken	mortality of embryo
fish	Trout	liver changes
	Zebra fish larvae	L.C.50
plants	Watercress	chlorosis of seedlings
	Lettuce	inhibition of growth
bacteria	B. megaterium	inhibition, induction of lysogenic strains
	E. coli, Staph. aureus	induction of lysogenic strains
	E. coli	formation of filamentous forms
cell culture		inhibition
		mitotic inhibition

Rats are more commonly available than ducklings and might be used in their stead. The onset of liver disturbances is slower in rats than in ducklings (Legator 1969) and rats are about three times less sensitive to aflatoxin B_1 than ducklings.

As the trout was one of the commercially produced animals affected by aflatoxin it has been considered as a test animal (Legator 1969). It has the disadvantage however that it may take up to a year before any effect of the toxin can be observed.

A potentially more useful organism for assay purposes is the chick embryo. Fertile eggs can be treated with aflatoxin and their incubation continued till hatching. The effect can be measured either by the mortality of the embryos or by an examination of the embryos for various developmental disturbances at a set time after treatment. Jayaraman et al. (1968) reported 100% kill of the embryos in 24 hours with only 0.01 µg/egg. Verrett et al. (1964) as reviewed by Legator (1969), produced a dose-response curve for aflatoxin B_1 in the range 0.01 µg to 0.1 µg/egg. The method is very sensitive and relatively easy to perform but is not very specific. Jayaraman et al. (1968) showed that a number of different compounds could cause mortality in the chick embryo.

Abedi and Scott (1969) described an assay using zebra fish larvae. Because of their small size 20-30 individuals could be treated in only 2 ml of solution. The mortality of the larvae was recorded after 24 hour treatment and a dose-response relationship over the range 0.25 to 1.2 µg/ml aflatoxin B_1 was claimed. The assay iself is not very specific but the authors reported that the appearance of the dead larvae was typical of the toxin under test.

The observation of Schoental and White (1965) that watercress seedlings were bleached of their chlorophyll by concentrations of aflatoxin lower than those causing inhibition of germination offered another possible basis for an assay. The effect appeared more specific than some of the other phenomena but somewhat less sensitive.

A number of authors reviewed by Legator (1969) had observed the formation of filaments by different bacteria in the presence of aflatoxin. This effect is about as sensitive as the disk inhibition assay though more difficult to quantify.

Legator (1966) reported induction by aflatoxin B_1 of bacteriophage lambda in lysogenic E. coli and of a bacteriophage in a lysogenic strain of Staph. aureus. In both organisms he was able to produce a dose-response curve in the range 0.06 to 0.5 µg/ml. This phenomenon is more specific than some of the others discussed and apparently very sensitive. It would appear to provide a sound basis for an assay system.

Induction of a bacteriophage in B. megaterium was reported by Lillehoj and Ciegler (1970) though the concentration of aflatoxin necessary for an effect was higher (5-10 µg/ml). They suggested induction might be used as a qualitative assay to supplement other methods.

Finally there is the possibility of using cell cultures to assay for aflatoxin. Inhibition of rat fibroblasts by concentrations as low as 0.02 µg/ml were reported by Daniel (1965). He was able to construct a dose-response curve over the range 0.02 to 0.25 µg/ml for aflatoxin B_1. Inhibition of cells is not a very specific effect but the sensitivity of the method is very great. Legator (1966) presented a dose-response curve over the range 0.01 to 1.0 µg/ml using inhibition of mitosis in human embryonic lung cells as a criterion. This might be more specific and is certainly very sensitive.

The need for a bioassay method:

Physicochemical methods of assay are rapid, sensitive and precise. Few of the biological phenomena discussed would provide as sensitive an assay and none as rapid or precise. Why then concern oneself with a biological assay? The chemical assays may not always accurately reflect the biological activity of the sample under test. This is especially so when small differences in the structure of the compound can make large differences in its biological activity. Eleven aflatoxins have been described with toxicities ranging from extreme carcinogenicity to no detectable activity at all. These different compounds are rarely likely to occur alone and it is difficult, if not impossible, to predict the biological activity of a mixture even when its components are known. It is therefore desirable to have a bioassay method available, not to replace but to complement the chemical method.

The ideal bioassay would be rapid, easy to perform, specific to the compound or group of compounds under test, preferably reflecting the toxicity of the compounds in mammals, sensitive and reproducible. The ideal bioassay probably does not exist but each aspect may be susceptible of some improvement.

Selection of biological effects of aflatoxin for further study:

The two bioassays in general use have disadvantages. The duckling assay is fairly specific but it is difficult to quantify, time-consuming, far less sensitive than the chemical methods and requires specialised conditions and skills. The B. megaterium disk inhibition assay is much simpler to perform and more rapid than the duckling assay. It is also more sensitive, though less so than chemical methods, but it suffers from a lack of specificity. In an attempt to provide a bioassay closer to the ideal than those mentioned, three phenomena were chosen for further study. The induction of bacteriophage was chosen as being sensitive, hopefully more specific than inhibition and likely to be easily handled in many laboratories. The bleaching of chlorophyll in cress seedlings suggested the possibility of an assay using a less complex green plant, perhaps one of the unicellular algae, which might be reasonably specific, readily quantified and easily handled. The third phenomenon was inhibition and it was hoped to combine this with TLC to improve the sensitivity and specificity.

All these assays, if developed, could readily be performed in most microbiological laboratories. Brief descriptions of chlorosis and induction are given below in the next part of the Introduction.

Chlorosis:

The mechanism of the 'bleaching' of cress seedlings by aflatoxin was not discussed or explored further by its discoverers. The effect of streptomycin in 'bleaching' Euglena is discussed fully by Liang Tong et al. (1965) though they came to no firm conclusion. Their work made it clear that the Euglena cells themselves were not greatly affected by streptomycin except in so far as they lost their chloroplasts. Liang Tong et al. (1965)

suggested that, rather than selecting a mutant, the streptomycin was acting directly on the chloroplast. Stanier et al. (1971) describe the 'bleaching' action of streptomycin as being due to a selective effect on 70S ribosomes. These ribosomes occur only in procaryotes and in the mitochondria and chloroplasts of eucaryotes. In their scheme the protein synthesis of the organelle is blocked so that the cells reproduce without a corresponding increase in the number of chloroplasts. The existing chloroplasts degenerate in time and eventually none are left in any of the cells in the culture. One could surmise that some similar mode of action might be responsible for the chlorosis due to aflatoxin.

Induction:

The DNA-viruses of bacteria may be divided into two main classes, virulent and temperate bacteriophages. A bacterial cell infected by a virulent bacteriophage is committed to multiplication of the virus within the cell. Eventually the cell lyses and releases a large number of progeny phages. This is the so-called lytic response. Infection by a temperate phage may lead to one of two responses, (a) a lytic response comparable with that of a virulent phage, or (b) a reductive response. This latter response is generally a rarer event occurring in perhaps 0.1 to 0.01 per cent of the infected cells. In this case the viral DNA fails to replicate on entering the cell and becomes established as a latent phage or prophage. This state is referred to as lysogeny and bacteria carrying prophage are said to be lysogenic. In many systems the viral genome is inserted as a prophage into the chromosome of the host cell. Lysogenic cells continue to multiply, the prophage being replicated with the bacterial chromosome, giving rise to a clone of cells all carrying the virus as a prophage.

The lysogenic state is a fairly stable one but, in the course of growth, occasional prophage are converted from the latent state to one of active replication by excision from the host chromosome. Phage multiplication ensues followed by lysis of the cell and release of progeny phages. This change of state from a latent prophage to active phage multiplication is known as induction. Spontaneous induction occurs fairly infrequently,

one cell in a hundred or fewer, but various treatments may increase the frequency. In some lysogenic strains of bacteria the frequency of induction can be increased by a variety of treatments. This is a property of the bacteriophage with which they are lysogenised. Such bacteriophage are said to be inducible. The frequency of induction may be increased by treatment with ionising radiations or with various chemical compounds including nitrogen mustard, mitomycin C, organic peroxides and fluorodeoxyuridine. Many of these treatments may also be mutagenic or carcinogenic. There is, therefore, reason to believe that inducing substances may also be both mutagenic and carcinogenic. Aflatoxins have been reported to induce and to be carcinogenic and mutagenic (Legator 1966, Lillehoj and Ciegler 1970, Ames et al. 1973). E. coli, Staph. aureus, S. typhimurium and B. megaterium may all carry inducible prophages and might be used to demonstrate the inducing properties of aflatoxins.

Summary:

Aflatoxin is a substance produced by the common fungus Aspergillus flavus and thought to be carcinogenic. It is highly desirable to be able to monitor the production and storage of susceptible foodstuffs to ensure the absence of the toxin from them. To do this it is necessary to have analytical methods available to detect and quantify the toxin both chemically and biologically. This work describes an attempt to develop further microbiological assays for aflatoxin. The first section deals with an examination of several reported effects of aflatoxin on micro-organisms which might form the basis of a bioassay. Inhibition of growth and induction of lysogenic B. megaterium were chosen for further study. Section II describes the development of an assay based on inhibition, Sections III and IV describe development of an induction assay system and Section V deals with the application of the two systems.

MATERIALS AND METHODS

1. CULTURES

Euglena gracilis strain Z was obtained from Professor E.F. Carell, University of Pittsburgh, Pens. 15213, U.S.A.

Chlorella pyrenoidosa was obtained from the Forestry Research Institute, Rotorua, New Zealand.

Salmonella typhimurium lysogenised with phage P22 and the wild type *Salmonella typhimurium* were obtained from the Culture Collection, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

Escherichia coli strains P4,λ6 and W1485 were obtained from Dr V.W. Mayer, Genetic Toxicity Branch, Division of Toxicology, Food and Drug Administration, Washington D.C., U.S.A.

Staphylococcus aureus strains carrying various phages were obtained from Mrs A. Jarvis, Dairy Research Institute, Palmerston North, New Zealand.

Bacillus megaterium strains 1368, 3694 and 3695 were obtained from Dr W.C. Haynes, United States Department of Agriculture, Peoria, Illinois, U.S.A.

Strain 1368 is the strain used by Clements (1968)

Strain 3694 is an asporogenous strain isolated by Krueger. This strain was called by him KM and is phage sensitive.

Strain 3695 is a sporing lysogenic strain isolated by den Dooren de Jong (1931) and called by him 899a.

Strains 3694 and 3695 were deposited in the American collection by J.T. Wachsman in 1969.

Aspergillus flavus. Several strains of *A. flavus* were examined for aflatoxin production. Some had been isolated during a survey made for the Dairy Division of the Department of Agriculture in 1970. Others were obtained from the National Health Institute, Wellington, New Zealand and had been isolated by Dr Richardson. Reference cultures of *Aspergillus parasiticus* (IMI91019b and NRRL 2999) were also obtained from the Commonwealth Mycological Institute, Kew, Surrey, England and from the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory, Unites States Department of Agriculture, Peoria, Illinois, U.S.A.

Penicillium citrinum strain IMI24306 was also obtained from the Commonwealth Mycological Institute.

2. TOXINS

Aflatoxins B_1, B_2, G_1 and G_2 and Ochratoxin A were obtained from Makor Chemicals Ltd., Box 6570, Jerusalem, Israel.

Sterigmatocystin was obtained from Prof. R. Hodges, Dept. Chemistry, Biochemistry and Biophysics, Massey University.

Radicinin was obtained from Dr M. Chick, Dept. Chemistry, Biochemistry and Biophysics, Massey University.

Dothistromin and its analogue were obtained from Mr J. Shaw, Dept. Chemistry, Biochemistry and Biophysics, Massey University.

Citrinin was extracted from a culture of P. citrinum strain IMI24306. The mould was grown in 25 ml malt extract, 0.5%, yeast extract 0.25%, glucose 5% broth for eight days at 25°C. The broth was extracted twice by shaking in a separating funnel with 50 ml of chloroform. The chloroform extracts were pooled and washed by shaking with 20 ml distilled water. The chloroform layer was then filtered through activated sodium sulphate to dry it and the chloroform distilled off. The residue was redissolved in 5 ml chloroform and the concentration of citrinin found by measuring the absorption peak at 331 nm in a spectrophotometer.

The Citrinin, and the Dothistromin analogue samples were kindly checked for purity using mass spectroscopy by Prof. R. Hodges, Department of Chemistry, Biochemistry and Biophysics, Massey University.

3. THIN LAYER CHROMATOGRAPHY (TLC)

a) Method: TLC of mycotoxins and extracts of mould cultures was performed on 1/4" plate glass plates 5 x 20 cm, 10 x 20 cm or 20 x 20 cm.

Layers of Merk Silica gel G 250 micrometers thick were made using a Quickfit plate spreading system.

Before use, the plates were activated at 100°C for two hours and allowed to cool in a closed container over activated silica gel.

Solutions of toxins or extracts in chloroform were applied to the

plates with a microlitre syringe. The plates were developed in an unlined unequilibrated tank.

b) TLC Solvents: The following solvent systems were used -

i Chloroform 97 : Methanol 3

ii Chloroform 90 : Acetone 10

iii Benzene 46 : Ethanol 35 : Water 19. The mixture was shaken in a separating funnel and let stand overnight at room temperature to separate into two phases. 50 ml of the lower layer were placed in the bottom of the developing tank and 50 ml of the upper layer in a trough. The TLC plate was placed in the trough for development.

4. FLUORODENSITOMETRY

a) Instruments: Densitometric measurements were made with a Photovolt densitometer consisting of a Photovolt light unit model 52C with a plate-scanning attachment, a Multiplier-Photometer model 520A, an Integrater model 49 and a Varicord recorder model 42B.

b) Method: The equipment was assembled according to the manufacturer's instructions with an ultra-violet light source and the appropriate filter. A filter was placed on the detector head allowing transmission in the 465 nm region. The Photomultiplier was used on sensitivity 3 and the recorder on response setting 1. The plate to be examined was placed on the plate-scanning stage with the silica gel layer on the underside. The entire light unit was covered with a velvet cloth in order to exclude stray light. A standard aflatoxin B_1 spot was found and the recorder adjusted to give 80% full scale deflection for the largest standard spot. The plate was then moved to a region unoccupied by any spots and the recorder adjusted to give a base line at 5% of full scale deflection. At the same time the integrator was adjusted to give less than one count in twenty seconds. Areas containing standard and unknown spots were scanned and the recordings marked to identify the peaks obtained from each sample. The amounts of aflatoxin in the unknown spots were calculated by comparing the areas under the peaks of unknown samples with the areas under the peaks of standards.

5. INCORPORATION OF AFLATOXINS INTO MEDIA

Aflatoxins are only very slightly soluble in water. Extracts and pure standards were dissolved in chloroform for chromatographic work and some method had to be devised to add the toxins to media for induction studies. Five methods were tried; these are enumerated below. All methods were evaluated by TLC followed by an inhibition assay to compare the amount taken into solution with the amount added.

a) Small amounts of concentrated chloroform solutions were added to broth and the mixture shaken well. The chloroform was then evaporated by passing air or nitrogen through the broth. This method gave variable results and often deposited much of the material on the glass.

b) Concentrated chloroform solutions of aflatoxins were placed in the bottom of a tube and the chloroform evaporated off by a stream of nitrogen. Broth was added to the tube and sonicated to disperse the aflatoxin. Again the results were variable and often only 20-50% of the aflatoxin could be found in the broth.

c) A modification of the method described in b) in which the toxin was sonicated in 0.05% Tween 80 instead of broth. This method recovered 60-80% of the toxin.

d) Concentrated solutions of aflatoxin were evaporated and redissolved in ethanol. This solution was then added to the medium. This method was generally fairly successful but could not achieve concentrations of toxin higher than about 150 µg/ml.

e) Concentrated chloroform solutions were evaporated and redissolved in acetone. The acetone solutions were then added to the medium to give the final concentrations desired. This method was preferred to that involving ethanol, partly because the maximum concentrations obtained were somewhat higher, (200 µg/ml) but mainly because ethanol has been shown to react with aflatoxins under acid conditions to give relatively non-toxic compounds (Dutton and Heathcote 1969).

6. ISOLATION OF A STREPTOMYCIN-RESISTANT STRAIN OF BACILLUS MEGATERIUM STRAIN KM (KM/Sr)

B. megaterium strain KM was grown in a shaking water bath at 32°C to mid-exponential phase (Klett 200) in 10 ml minimal salts medium supplemented with 0.1 µg/ml nicotinic acid, 0.25% glucose and 0.1% tryptone. The culture was centrifuged at 3000 x g for 15 minutes at room temperature and the cells resuspended in 2 ml diluent peptone. This concentrated suspension was exposed to short wave ultra-violet light (Mineralite lamp) for 30 seconds at 20 cm and then added to 100 ml tryptone yeast extract glucose (TYG) medium supplemented with 10 µg streptomycin/ml. This sub-culture was incubated at 32°C for five days and then streaked on to a plate of TYG agar containing 10 µg streptomycin/ml. An isolated colony was restreaked on to the same medium and an isolated colony from this plate was selected for propagation as the streptomycin-resistant strain.

7. MEDIA AND REAGENTS

Lennox Agar

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	12 g
Distilled water	1000 ml

Adjust to pH 7.0 before autoclaving at 15 lbs/in^2 for 15 minutes. After autoclaving add 2 ml 50% sterile glucose solution.

Soft Agar

Beef extract	3 g
Peptone	5 g
Sodium chloride	5 g
Agar	5 g
Distilled water	1000 ml

Dissolve by boiling and distribute 3.5 ml amounts in bijou bottles. Sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Minimal Salts Base (MSM)

di Potassium hydrogen phosphate	7.0 g
Potassium dihydrogen phosphate	3.0 g
Ammonium sulphate	1.0 g
Sodium citrate (trihydrate)	0.5 g
Magnesium sulphate (heptahydrate)	0.1 g
Distilled water	1000 ml

Dissolve at room temperature and sterilise by autoclaving at 15 lb/in^2 for 15 minutes. Add sterile glucose, 1 g/l, before use.

Peptone Acetate Agar (PAA)

Peptone	2.0 g
Sodium acetate	0.1 g
Agar	8.0 g
Distilled water	1000 ml

Dissolve by boiling and sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Euglena Agar (EA)

This is PAA with an addition of 2.5 g/l glucose.

Euglena Culture Medium (ECM)

Peptone	5 g
Yeast extract	2 g
Glucose	1 g
Distilled water	1000 ml

Dissolve and sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Maintenance Medium (MM)

Sodium acetate	10.0 g
Magnesium chloride (hexahydrate)	1.02 g
diSodium hydrogen phosphate ($12.H_2O$)	1.32 g
Sodium dihydrogen phosphate ($2H_2O$)	0.98 g
Distilled water	1000 ml

Dissolve and sterilise by filtration.

Basal Medium (BM)

diPotassium hydrogen phosphate	7.0 g
Potassium di-hydrogen phosphate	3.0 g
Ammonium sulphate	1.0 g
Magnesium sulphate (heptahydrate)	0.1 g

Dissolve and sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

B. Megaterium Growth Medium (BMM)

Basal Medium	7.5 ml
5% tryptone solution	1.0 ml
2.5% yeast extract solution	1.0 ml
5% glucose solution	0.5 ml

Aseptically add the sterile solutions to the basal medium before use.

Semi-defined Medium (SDM)

Basal Medium	9.2 ml
5% tryptone solution	0.2 ml
50% glucose solution	0.5 ml
50 µg/ml nicotinic acid solution	0.1 ml

Aseptically add the sterile solutions before use.

Tryptone Yeast Agar (TYA)

Tryptone	5.0 g
Yeast extract	2.5 g
Agar	12.0 g
Distilled water	1 l

Add ingredients to water and let stand 15 minutes. Boil to dissolve the agar, distribute into containers and sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Tryptone Yeast Soft Agar (STYA)

Tryptone	5.0 g
Yeast extract	2.5 g
Agar	5.0 g
Distilled water	1 l

Add the ingredients to water and let stand 15 minutes. Boil to dissolve and distribute 3.5 ml amounts in bijou bottles. Sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Dilution Medium

Peptone	1 g
Distilled water	1 l

Dissolve peptone and distribute in appropriate amounts (9.9, 9.0 or 4.5 ml) in screw-capped bottles. Sterilise by autoclaving at 15 lb/in^2 for 15 minutes.

Triphenyl Tetrazolium Chloride Solution (TTC)

Triphenyl tetrazolium chloride	0.3 g
Sodium chloride	0.8 g
Potassium chloride	0.04 g
Distilled water	100 ml

Dissolve and store in a dark bottle.

SECTION I

THE EXAMINATION OF A NUMBER OF BIOLOGICAL SYSTEMS AS POSSIBLE BASES FOR A BIOASSAY

INTRODUCTION

Of the possible biological effects of aflatoxin that might be used for an assay system, two were selected for further study. The first was the observation of Schoental and White (1965) that treatment of germinating cress seeds with low levels of aflatoxin produced seedlings deficient in chlorophyll. The second was the induction by aflatoxin of lysogenic cultures of bacteria reported by Legator (1966) for E. coli and Staph. aureus and by Lillehoj and Ciegler (1970) for B. megaterium.

The reported inhibition of chlorophyll by aflatoxin was studied to see whether a similar effect could be reproduced in a microbiological system. This might lead to the development of a system which was more rapid and more easily controlled than the use of vascular plants. Euglena gracilis strain Z was chosen as the test organism and the work is described below.

The inducing property of aflatoxin was studied in some detail in Salmonella typhimurium lysogenised with phage P22 and in Bacillus megaterium strain 899a. Lysogenic strains of Escherichia coli and Staphylococcus aureus were also examined. The work on Bacillus megaterium forms the major part of the thesis and is only mentioned briefly in this section.

a. Effect of aflatoxin on greening of Euglena gracilis.

The 'Z' strain of E. gracilis has the property of being able to grow actively in the absence of light if it is maintained in a suitable medium. Under these conditions it does not form chloroplasts, only proplastids. These proplastids can be organised into fully functional chloroplasts under the influence of light (Wolken and Palade 1953). If Euglena cells are irradiated with ultra-violet light or are treated with streptomycin they may become permanently 'bleached', i.e. they lose their ability to form chloroplasts. The resulting bleached cells continue to grow in suitable media however. (Liang Tong et al. 1965)

The reported 'bleaching' of cress seedlings by aflatoxin might be analogous to the bleaching effect seen in Euglena treated by streptomycin, especially since streptomycin had also been shown in the same study, to cause partial bleaching of the cress plants.

Experimental.

A clone of Euglena gracilis strain Z was obtained as described below. Cells were washed from an agar slope with diluent peptone water. The suspension was serially diluted in diluent peptone and 0.2 ml of 10^{-3} and 10^{-4} dilutions placed on the surface of Euglena agar (EA) plates. The 0.2 ml was spread evenly until the surface of the plates appeared dry; the plates were then incubated at 25°C under a fluorescent lamp for six days. By this time small, round, dark green colonies had formed on the plates; a number of colonies were picked from the plates on to EA slopes and the slopes were incubated at 25°C under a fluorescent light.

A single clone was used in subsequent investigations. This clone was sub-cultured in Euglena culture medium (ECM) and incubated in the dark at 25°C for five days. At this time no green colour could be seen in the culture and no chloroplasts were visible when the cells were examined microscopically. The culture was centrifuged at 1000 g for 15 minutes and resuspended in maintenance medium (MM). This suspension was exposed to light at 25°C and examined for chlorophyll content and cell numbers at time 0, 2.5, 19 and 24 hours. Chlorophyll was estimated by the method of Arnon (1949). Two millilitre samples were centrifuged at 1000 g for 15 minutes, resuspended in 4 ml 80% acetone and let stand 15-20 minutes. At the end of this time the sample was again centrifuged at 1000 g and the absorbance of the supernatant at 652 nm measured in a Beckman spectrophotometer. The absorbance figure was converted to concentration of chlorophyll by the formula

$$\text{Chlorophyll g/l} = \frac{\text{Absorbance at 652} \times 1000}{34.5}$$

This is the total concentration of chlorophylls a + b.

Cell counts were made using a haemocytometer. The cells were fixed by adding one drop of 40% formaldehyde to 1 ml and shaking to ensure even distribution of organisms before counting.

Table 1.1 Formation of chlorophyll in E. gracilis

Time (h)	Cell numbers ($X10^{-4}$/ml)	Chlorophyll µg/l	Chlorophyll µg/cell $X10^{7}$
0	271	59	0.21
2.5	318	174	0.54
19	233	4300	18.5
24	372	4600	12.5

This confirmed the ability of this strain to produce chlorophyll in the light.

A dark grown culture was centrifuged at 1000 g for 15 minutes and resuspended in MM at twice the cell concentration. The suspension was divided into eight 5 ml aliquots and sufficient of a 1000 µg/ml solution of streptomycin sulphate added to give final concentrations of 0, 20, 40 and 80 µg/ml in duplicate. The aliquots were mixed well and exposed to light at 25°C. Samples were taken at 0, 24 and 48 hours and examined for cell count, viable count and chlorophyll. Viable counts were made by plating on EA and incubating the plates at 25°C for five days in the light. Differential counts were made, where appropriate, of white and green colonies. White colonies are derived from those cells in which chlorophyll production has been permanently affected by the streptomycin treatment.

Table 1.2 Effect of streptomycin on E. gracilis after 48 hours

Streptomycin µg/ml	Cell count ($X10^{-4}$)	Viable count ($X10^{-4}$)	'Bleached' Colonies %	Chlorophyll/cell (µg $X10^{7}$)
0	152	118	0	40.4
20	205	145	85	19.2
40	188	219	99	20.0
80	152	208	99.9	18.5
initial values	94	N.D.	N.D.	1.2

N.D. = not determined.

There was an increase in chlorophyll in all aliquots but the streptomycin had obviously had an effect as can be seen from the number of bleached colonies. The results seem to be consistent with the greening of existing proplastids but no further formation of new proplastids. This

confirmed the ability of streptomycin to produce permanently 'bleached' cultures of E. gracilis.

Since aflatoxin is not very soluble in water, ethanol was chosen as a vehicle for the addition of the toxin. This would mean that the culture media might contain amounts of ethanol up to 5% so the effect of ethanol on E. gracilis was examined.

Dark grown cells were washed and sub-cultured in Euglena culture medium (ECM) containing 0, 1 and 2% ethanol. The sub-cultureswere incubated in the light, sampled at 24 and 48 hours and examined for cell count and chlorophyll.

Table 1.3 Effect of ethanol on E. gracilis

	24 hours		48 hours	
Ethanol conc. %	Cells $X10^{-4}$	Chlorophyll µg/cell $X10^{7}$	Cells $X10^{-4}$	Chlorophyll µg/cell $X10^{7}$
0	29	26	44	126
1	20	20	59	62
2	25	12	62	41

Cultures grown in the presence of ethanol had clearly grown better than the control culture though the amount of chlorophyll present in the ethanol grown cells was less. Hutner et al. (1966) have shown that ethanol can be used without prior adaptation by E. gracilis. It appears from these results that when ethanol is present it will be used, to some extent, at the expense of photosynthesis. Since chlorophyll production was not completely suppressed, however, it was possible to proceed.

Dark grown cells were washed and sub-cultured in ECM containing 4% ethanol and 0, 1, 2 and 4 µg/ml aflatoxin B_1. The cultures were incubated in the light and sampled at 0, 24 and 48 hours.

Table 1.4 Effect of aflatoxin B_1 on E. gracilis

Aflatoxin B_1 µg/ml	0 hours Count X10^{-4}	24 hours Count X10^{-4}	24 hours Chlorophyll µg/cell X10^{7}	48 hours Count X10^{-4}	48 hours Chlorophyll µg/cell X10^{7}
0	8.5	25	25	59	88
1	9.3	20	32	60	85
2	8.3	19	30	51	61
4	6.6	21	19	65	71

No 'bleached' colonies were found in any of the cultures. There may have been a slight drop in chlorophyll levels at the higher concentrations of aflatoxin but any effect of the toxin was slight and transient. No effect on growth was shown.

Further experiments using 10 and 50 µg/ml aflatoxin failed to show any effect on chlorophyll production or to produce any bleached colonies.

Finally Euglena was grown in EM containing 10 µg/ml aflatoxin B_1 and 2% acetone for ten days at 25°C under artificial light. No differences could be found between the control and aflatoxin-containing cultures.

Euglena gracilis was grown in the presence of varying amounts of aflatoxin in a respirometer both in the light and in the dark to see whether any effects on respiration or photosynthesis could be shown. There were no differences between the controls and aflatoxin up to levels of 4 µg/ml.

b. Effect of aflatoxin on induction of Salmonella typhimurium carrying bacteriophage P22

Since the chloroplast bleaching phenomenon could not be reproduced in Euglena, attention was redirected to the phage induction phenomenon. Legator (1966) had implied that all inducible lysogenic bacteria could be induced by aflatoxin. It was decided to investigate the effects of the toxin on Salmonella typhimurium lysogenised with phage P22 since this was available in the department, was easily handled and gave easily readable plaques. Two kinds of P22 phage were available, one was the normal temperate phage giving centred plaques while the other was a clear plaque mutant.

Lysogenic cultures of both phage types in S. typhimurium were isolated by growing cultures of the organism in MSM to late log phage, inoculating them with large numbers of phage and continuing incubation for a further few hours. The cultures were then plated and colonies picked off on to slopes of Lennox agar. Lysogenic clones were selected on the basis of their ability to produce phage; the clones were designated Stm (P22T) and Stm (P22V) for the temperate and clear plaque phage, respectively.

For convenience in estimating the numbers of cells used in various investigations the viable counts in a logarithmic phase culture were correlated with optical density measurements. A culture of S. typhimurium was grown from 90 $X10^6$ to 480 $X10^6$ cells/ml in MSM at 37°C in a shaking water bath. Viable counts and optical density measurements were made at intervals. Optical density (OD) was measured in a Klett-Summerson colorimeter using the blue filter.

Table 1.5 Optical Density and Viable Count in S. typhimurium

Time (minutes)	OD (Klett units)	Viable Count $X10^{-6}$/ml
0	17	N.D.
20	22	104
40	32	155
90	43	223
110	52	280
150	62	333
175	76	410
195	82	482

See also Figures 1.1 and 1.2.

The correlation between OD and viable count was fairly good; 10 Klett units represented approximately 5.5 $X10^7$ cells.

Before examining the effect of aflatoxin on the cultures it was necessary to establish that the cells were inducible. Their inducibility by ultra-violet light was tested.

Figure 1·1

A COMPARISON OF OPTICAL DENSITY AND VIABLE COUNT DURING GROWTH OF SALMONELLA TYPHIMURIUM (P22T)

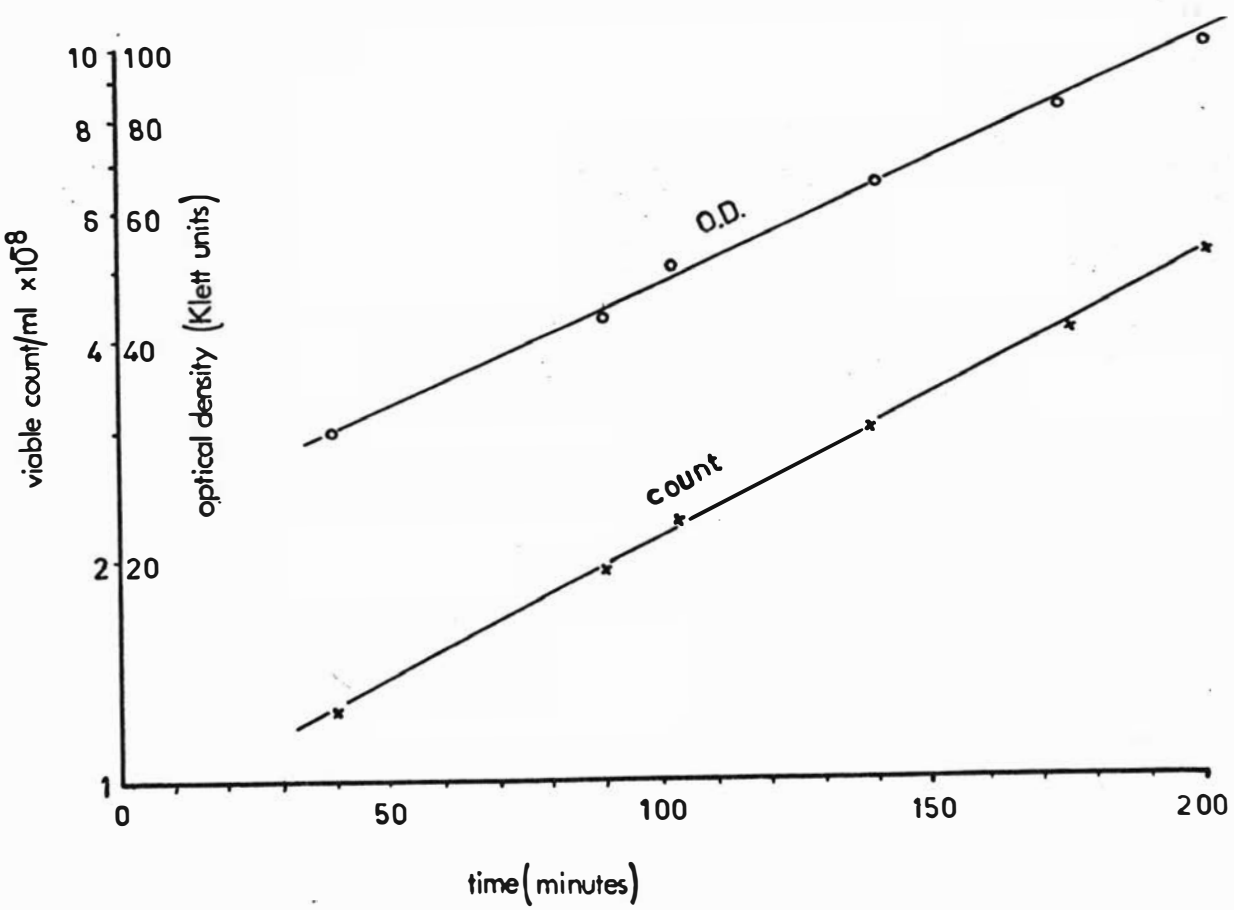
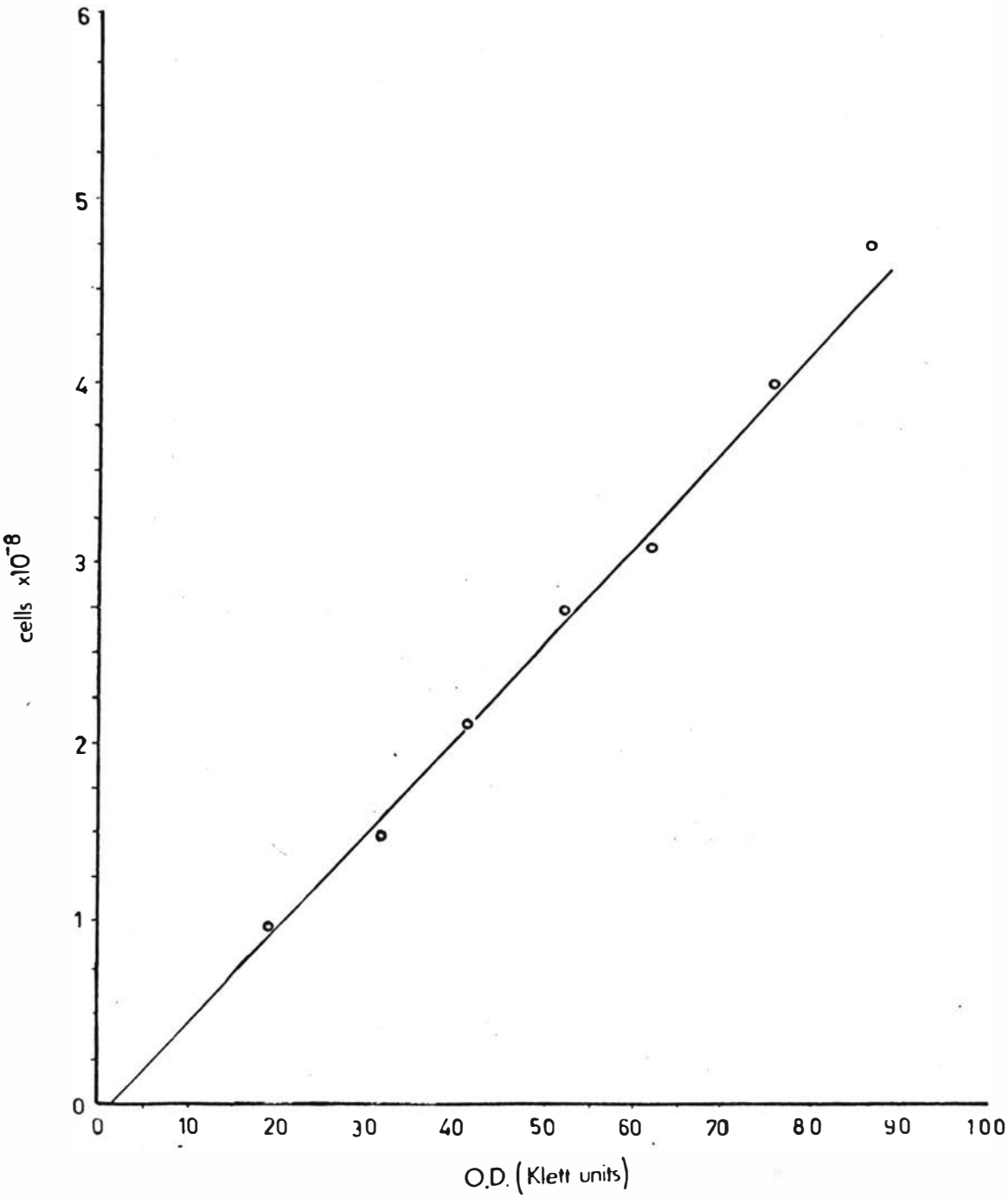


Figure 1.1 The culture was grown in a shaking water bath at 37°C in Minimal Salts Medium. OD measured in a Klett-Summerson colorimeter using a blue filter.

Figure 1·2

CELL NUMBERS vs KLETT UNITS FOR SALMONELLA TYPHIMURIUM P22T.



Cultures of both lysogenic strains were grown to mid-logarithmic phase, centrifuged at 3000 g for 10 minutes and washed twice in quarter-strength Ringer's solution. The cells were resuspended in quarter-strength Ringer's solution and diluted to give about 10^7 cells/millilitre. Two ml aliquots of these suspensions were exposed to short wave ultra-violet light (Mineralite) for 0, 30, 60 and 90 seconds. Samples were plated for infectious centres(IC) and viable count. Viable counts were made by plating 1 ml of an appropriate dilution of the sample in NA. IC counts were made by adding 0.1 ml of an appropriate dilution of the sample to 3.5 ml molten soft agar in a bijou bottle and adding about 0.2 ml of a well-grown culture of sensitive organisms. The bijou bottle was inverted three or four times to mix its contents and the agar then poured on to the surface of a plate of Lennox agar. Both viable and IC count plates were allowed to set and incubated inverted overnight at 37°C.

Table 1.6 Effect of ultra-violet light on lysogenised S. typhimurium

	Viable Count/ml		IC Count/ml	
Time (seconds)	Stm(P22T)	Stm(P22V)	Stm(P22T)	Stm(P22V)
0	10^7	10^7	5×10^6	7×10^5
30	4×10^4	3×10^3	10^6	3×10^5
60	$<10^3$	$<10^3$	$<10^3$	$<10^3$
90	$<10^3$	$<10^3$	$<10^3$	13×10^6

This experiment showed that the selected clones were lysogenic but was inconclusive as to the ability of ultra-violet light to induce phage production.

The experiment was repeated using S. typhimurium carrying P22 temperate (Stm P22T) and exposing the cells for 0, 10, 20 and 30 seconds. Viable and IC counts were made at each time interval.

Table 1.7 Effect of ultra-violet light on Stm (P22T)

Time (seconds)	Viable Count $X10^{-6}$/ml	IC Count $X10^{-6}$/ml
0	690	49
10	510	62
20	400	72
30	390	101

The same experiment was repeated using S. typhimurium carrying P22 'virulent' (Stm(P22V)).

Table 1.8 Effect of ultra-violet light on Stm (P22V)

Time (seconds)	Viable Count $X10^{-6}$/ml	IC Count $X10^{-6}$/ml
0	550	8
10	440	44
20	200	71
30	170	94

Both lysogenic bacteria were inducible by ultra-violet light and Stm (P22V) seemed somewhat more sensitive with respect to both kill and induction.

To confirm the inducibility of the lysogenic cultures the effect of a radiomimetic compound, mitomycin C, was tried on Stm (P22T). Otsuji et al. (1959) reported that lysogenic cultures of E. coli could be induced by mitomycin C and the compound has been widely used as an inducing agent since their report. The organism was grown in MSM for 4½ hours and divided into two aliquots to one of which mitomycin C was added to give a final concentration of 1 µg/ml. Phage counts were made at 0 and 40 minutes.

Table 1.9 Effect of mitomycin C on Stm (P22T)

	IC Counts $X10^{-4}$/ml	
Time (minutes)	Control	1 µg/ml Mitomycin C
0	22	22
40	65	5,200

Mytomycin C was clearly very effective in inducing Stm (P22T) having produced about a hundred times as many infectious centres as are produced spontaneously.

The effect of aflatoxin B_1 on Stm (P22T) was examined; mitomycin C and blank controls were included for comparison.

Stm (P22T) was grown to about 6 X 10^8 cells/ml and diluted one tenth into MSM and into MSM containing 1 µg/ml aflatoxin B_1 or 1 µg/ml mitomycin. The cultures were incubated at 37°C in a water bath for one hour. Samples were then plated for viable count and IC count.

Table 1.10 Effect of Aflatoxin B_1 on Stm (P22T)

	Viable count X 10^{-6}	IC Count X 10^{-6}
Control	187	<1
1 µg/ml mitomycin	42	17
1 µg/ml aflatoxin B_1	186	<1

The original number of cells/ml was about 60 X 10^6 which is nearly the same as the sum of viable cells and infectious centres for the mitomycin sample. Mitomycin had prevented growth of the organism and induced lysis in a proportion of the cells. The other two samples had grown through well over one generation and had not lysed. Aflatoxin B_1 had not had any apparent effect.

This experiment was repeated using 0, 5, 10 and 20 µg/ml aflatoxin but omitting the mitomycin control. Samples were taken at 30, 60, 90 and 120 minutes. Viable counts and IC counts were done for each concentration at each time interval.

Table 1.11 Viable counts/ml X 10^{-6} of Stm (P22T) treated with aflatoxin

	aflatoxin conc. (µg/ml)			
Time (minutes)	0	5	10	20
30	91	109	95	101
60	127	161	152	138
90	112	165	132	185
120	234	166	178	258

Table 1.12 IC counts/ml $X10^{-6}$ of Stm (P22T) treated with aflatoxin

	aflatoxin conc. (µg/ml)			
Time (minutes)	0	5	10	20
30	<1	2	1	<1
60	1	6	1	<1
90	2	4	14	6
120	5	<1	2	<1

The results of the viable counts were rather erratic but growth had occurred in all samples over the period studied. The IC counts were all low but there is no indication of any consistent difference between the treated and untreated cells.

Since no obvious effect of aflatoxin could be shown using Salmonella it was decided to examine those species of bacteria on which the toxin was originally reported to have an effect and work on Salmonella typhimurium was stopped.

c. Effect of aflatoxin on the induction of lysogenic strains of Staphylococcus aureus.

The strains of Staph. aureus received from Mrs A. Jarvis were examined for plaque appearance and ease of counting. The combination chosen was lysogenic strain 22 and sensitive strain 29. Their NTCC strain numbers were 8356 and 8329 respectively.

Before treating the bacteria with aflatoxin it was established that the ratio of infectious centres to viable cell count was 1:100. Legator (1966) had claimed a 350 fold increase in infectious centre count in the presence of 0.48 µg/ml aflatoxin B_1. Assuming that the viable count remained the same, the ratio of IC to viable count would change to 3.5:1.

Strains 8356 and 8329 were grown for 24 hours at 37°C in Brain Heart Infusion Broth (BHI). Strain 22 was sub-inoculated into minimal salts medium (MSM) containing 8% BHI and grown for 4 hours in a water bath at 37°C. This culture was then distributed into aliquots of MSM + BHI medium containing 0, 2 and 10 µg/ml aflatoxin and 1 µg/ml mitomycin. These four aliquots were incubated at 37°C for 30 minutes, then sampled for viable and IC counts. The IC counts were performed as already described except that

the lower layer was Nutrient Agar and the upper layer contained 500 µg/ml calcium. Strain 8329 was used as the phage-sensitive indicator strain.

Table 1.13 Effect of aflatoxin and mitomycin on Staph. aureus

	Control	1 µg/ml mitomycin	aflatoxin B 2 µg/ml	aflatoxin B 10 µg/ml
Viable Count $X10^{-5}$	190	160	190	195
IC Count $X10^{-5}$	<1	5	<1	<1

Aflatoxin did not effect viability and there is no evidence of any induction in contrast with the report of Legator (1966) that very high induction rates could be obtained using only 0.48 µg/ml aflatoxin. Even mitomycin gave only a low number of infectious centres. In view of this result and of other difficulties in working with this system such as clumping of the cells and relatively poor definition of the plaques the investigation was pursued no further.

d. Escherichia coli and bacteriophage λ

Legator (1966) had also reported that E. coli lysogenic for phage λ could be induced by aflatoxin. Culture of E. coli lysogenic for λ and a λ-sensitive culture were both available in the department so an attempt was made to reproduce his results using these cultures. The lysogenic strain was numbered PB240 and the sensitive strain T186.

PB240 was grown in MSM at 37°C in a shaking water bath for four hours. Two millilitre amounts of the culture were exposed to ultra-violet light under standard conditions for 0, 5, 10 and 15 seconds and plated for viable count and IC count using the technique described previously for S. typhimurium.

Table 1.14 Effect of ultra-violet light on E. coli PB240

	Time (seconds) 0	5	10	15
Viable count $X10^{-7}$	146	84	49	15
IC count $X10^{-5}$	26	274	2,000	3,000

Figure 13 EFFECT OF ULTRA-VIOLET LIGHT ON ESCHERICHIA COLI PB240

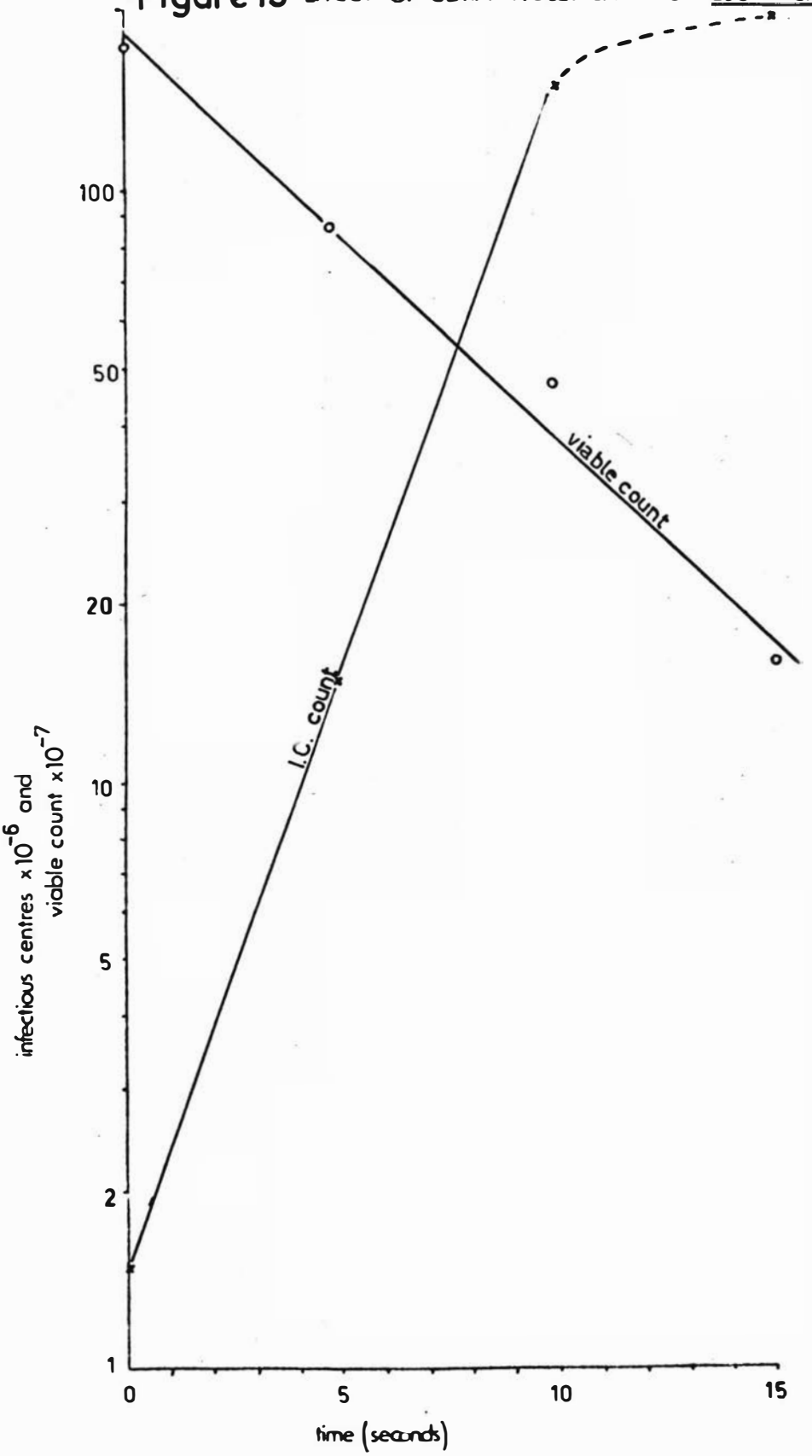


Figure 1.3 2 ml/log culture in MSM exposed to short wave ultra-violet lamp at 2 ft. Counted in Lennox agar.

The results are expressed graphically in Figure 1.3. Both the kill and the induction appear to be logarithmic, though induction shows some levelling off at 15 seconds. This strain quite clearly can be readily induced.

The effect of chemical induction was examined using mitomycin C. An aflatoxin treated sample was also included in the experiment.

A culture of PB240 was grown in MSM for 7 hours in a shaking water bath at 37°C. The culture was distributed into MSM containing 7 µg/ml aflatoxin or 1 µg/ml mitomycin. A control was included. The sub-cultures were incubated for 30 minutes at 37°C in a shaking water bath. Samples were then taken for viable and IC counts.

Table 1.15 Chemical Induction of E. coli PB240

	Control	1 µg/ml mitomycin	7 µg/ml aflatoxin
Viable count $X10^{-7}$	53	42	27
IC count $X10^{-5}$	41	365	11

Mitomycin had increased the number of infectious centres ninefold and reduced the viable count by about 20%. Aflatoxin had caused a greater drop in viability (50%) but a decrease rather than an increase in plaque count. Since the concentration of aflatoxin in this experiment was much higher than those used by Legator it seemed possible that inhibition of the cells might have masked induction. A lower concentration of aflatoxin was tried in the following experiment.

PB240 was grown in MSM at 37°C for 5 hours and diluted 1/10 into MSM with and without 1 µg/ml aflatoxin. The sub-cultures were incubated in a shaking water bath at 37°C and sampled at intervals to follow the growth of the cultures. The 0.5 ml samples were shaken with 0.1 ml chloroform to kill the cells. After 15 minutes the chloroform was evaporated off by bubbling air through the samples at 37°C. Free phage were assayed by the method described previously for infectious centres.

Table 1.16 Growth of PB240 in the presence of 1 µg/ml aflatoxin

	OD (Klett units)		Phage counts X10^{-3}	
Time (minutes)	Control	1 µg/ml aflatoxin	Control	1 µg/ml aflatoxin
0	15	15	1	9
30	21	21	2	6
60	28	30	7	8
100	34	36	4	7
135	45	48	9	5
165	58	59	6	6

Had induction occurred one might have expected to find a drop in OD after a generation or so and a marked increase in the number of phages. This did not occur. No induction by aflatoxin B_1 could be demonstrated.

The above experiment was repeated using a higher concentration of aflatoxin introduced into the system as an acetone solution (see Materials and Methods Section). PB240 was grown in MSM + 0.1% glucose and 1% acetone to an OD of 120 Klett units. The culture was diluted 1/10 into MSM + 0.1% glucose and 1% acetone with and without 10 µg/ml aflatoxin B_1. The sub-cultures were incubated in a shaking water bath at 37°C. OD readings were recorded at fifteen minute intervals and samples were removed and chloroformed for phage counts. The results are shown in Table 1.17 and Figure 1.4

Table 1.17 Growth of PB240 in the presence of 10 µg/ml aflatoxin B_1

	Optical Density (Klett units)		Phage Count X10^{-3}	
Time (minutes)	Control	aflatoxin B_1	Control	aflatoxin B_1
0	17	14	23	9
15	18	16	13	16
30	22	20	27	19
45	28	24	84	93
60	36	32	107	102
75	44	36	127	194
90	49	39	290	232
105	55	42		
120	62	44	1600	610

Figure 1.4 GROWTH OF E. COLI PB 240 IN THE PRESENCE OF 10µg/ml AFLATOXIN B_1

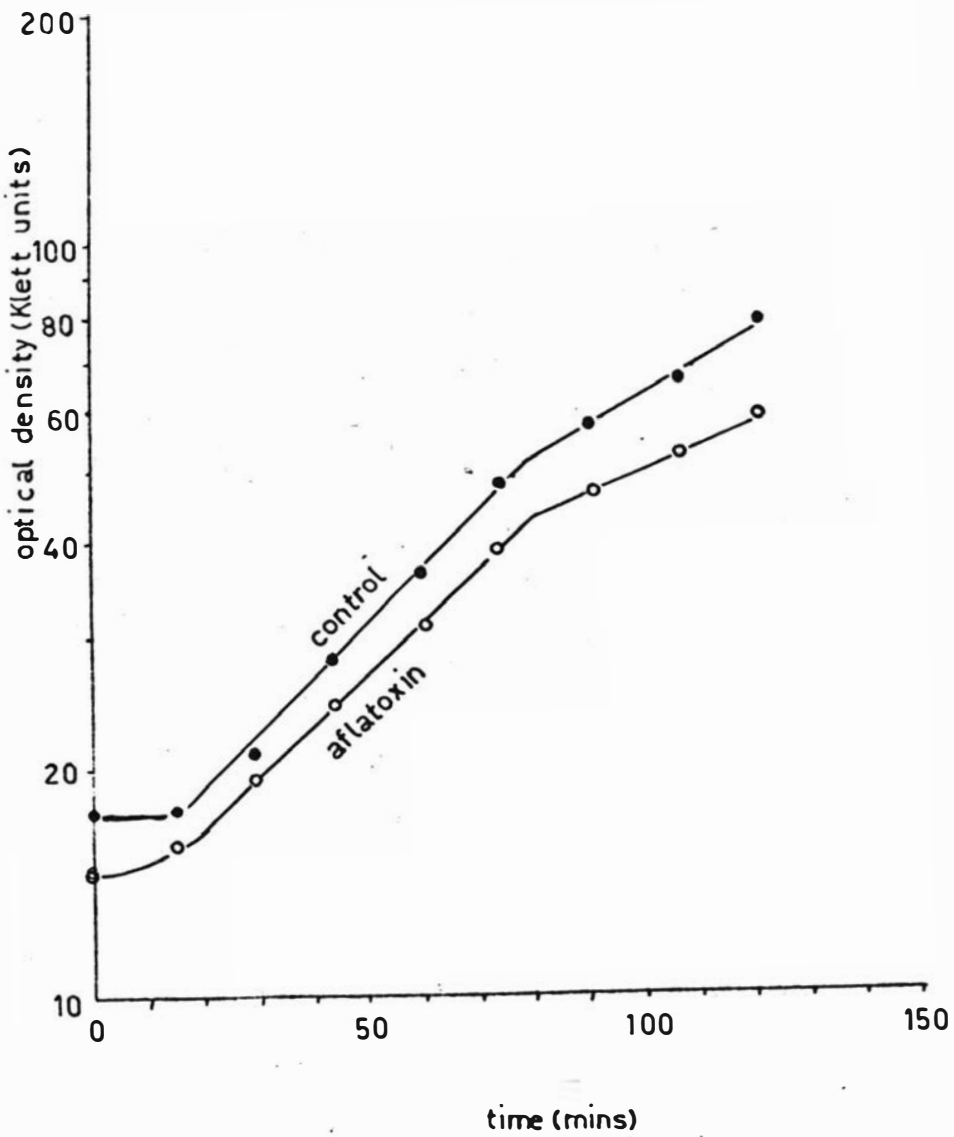


Figure 1.4 Culture subbed into MSM supplemented with aflatoxin in a shaking water bath at 37°C.

Figure 1.4 GROWTH OF E. COLI PB 240 IN THE PRESENCE OF 10µg/ml AFLATOXIN B_1

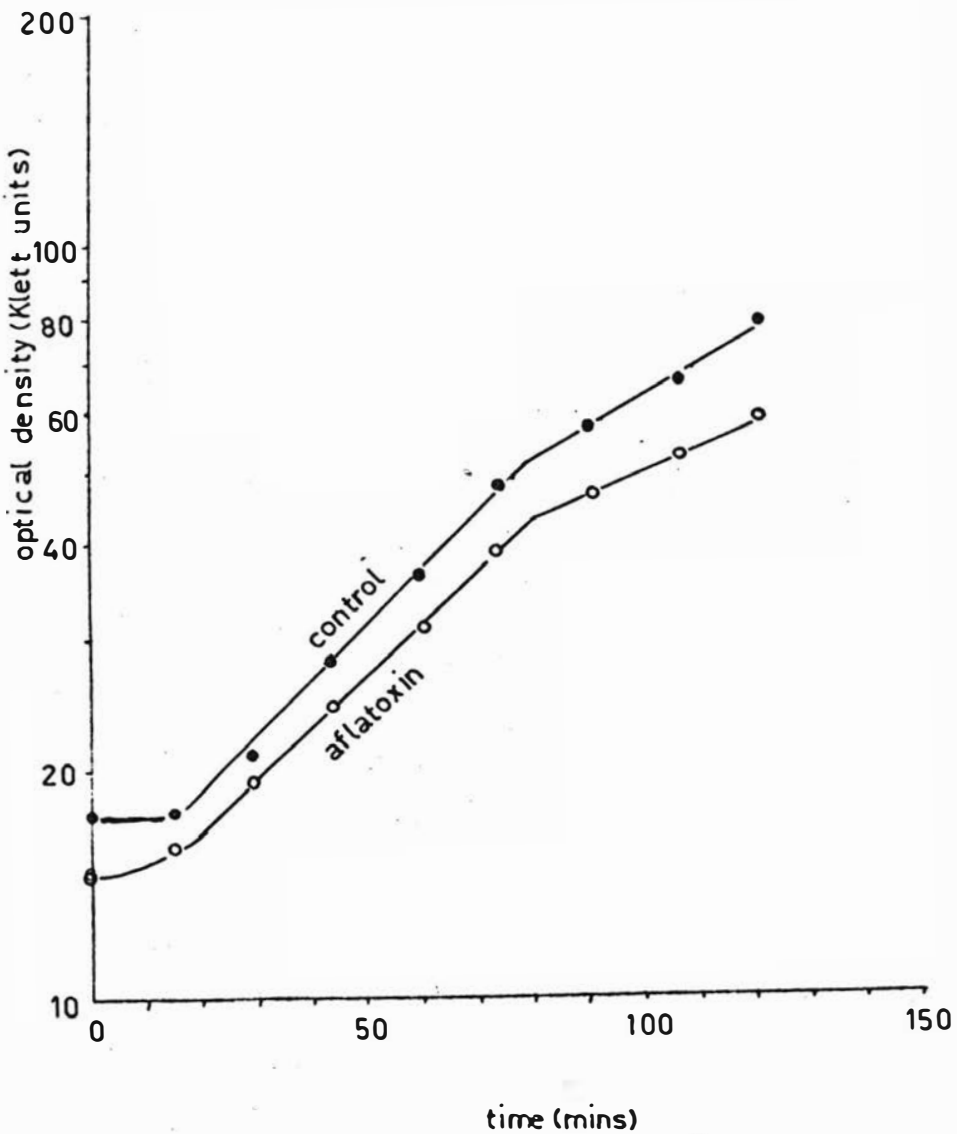


Figure 1.4 Culture subbed into MSM supplemented with aflatoxin in a shaking water bath at 37°C.

The presence of aflatoxin appears to have inhibited growth slightly but there is no evidence to suggest any increase in phage numbers due to induction.

It seemed possible that aflatoxin might not be able to enter the cell. If the integrity of the permeability barrier could be modified without killing the cell it might be possible for the toxin to enter and cause induction. Lieve (1968) described the effect of EDTA on the permeability of various Enterobacteriaceae. She compared the sensitivity of EDTA-treated and untreated cells to actinomycin and found that the cells of most strains of E. coli could be made sensitive to actinomycin by EDTA treatment without any fall in their viability.

A PB240 culture was grown overnight in MSM, diluted 1/10 in MSM and shaken at 37°C for four hours. 10 ml of this culture was centrifuged at 2000 g for 15 minutes and washed twice in pH 8.0 Tris buffer and finally resuspended in 20 ml of the Tris buffer. One millilitre amounts of this suspension were diluted 1/10 in buffer alone, buffer containing 0.15 mM EDTA and buffer containing 0.15 mM EDTA and 5 μg/ml aflatoxin B_1. These suspensions were incubated in a shaking water bath at 37°C for 20 minutes and plated for viable and IC counts.

Table 1.18 Effect of EDTA on PB240

	Control	EDTA only	EDTA + aflatoxin
Viable count $\times 10^{-7}$	130	45	50
IC count $\times 10^{-3}$	140	20	120

EDTA appears to have affected the viability of the culture. The IC counts were ambiguous. EDTA seemed to have reduced spontaneous lysis below the normal level and aflatoxin to have restored it to a little above the normal level. There was a sixfold difference between the two EDTA-containing cultures which may have been due to the presence of aflatoxin.

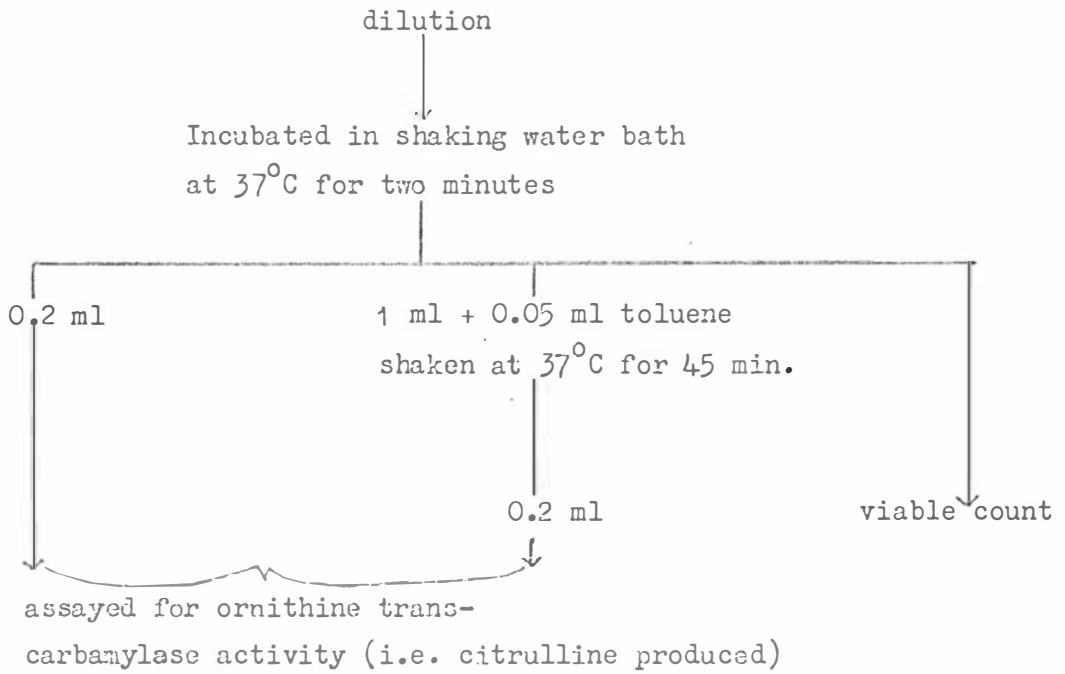
Since this result was ambiguous it was decided to examine the changes in permeability of the cells after treatment with EDTA. Cells were treated with EDTA and then presented with dilithium carbamyl phosphate which does not normally cross the cell membrane. Together with ornithine,

in the presence of ornithine transcarbamylase, citrulline is formed and may be estimated. The formation of citrulline can be used as a measure of permeability of the cells to dilithium carbamyl phosphate.

PB240 cells were inoculated into MSM and grown to an OD of about 50 Klett units. The culture was centrifuged at 3000 g, washed and resuspended. This final suspension was diluted as follows:

C	diluted 1/3 in Tris buffer
0.5	diluted 1/3 in buffer + EDTA to give 0.5 mM EDTA
1.0	diluted 1/3 in buffer + EDTA to give 1.0 mM EDTA

Each dilution was treated as follows:



The ornithine transcarbamylase assay was that of Jones (1962) modified as described below:

Reagents
- M Tris buffer pH 8.5
- 0.1 M dilithium carbamyl phosphate (DLC)
- 0.1 M L-ornithine hydrochloride
- 5% Trichloracetic acid (TCA)
- 10% arsenic trioxide in concentrated hydrochloric acid
- 1% 2:3 Butanedione -2 oxime in 5% glacial acetic acid

Notes DLC is unstable in solution and must be stored at $0^{o}C$ when it has a half life of 18 hours.
The arsenic trioxide solution should be kept in a glass-stoppered bottle in a fume cupboard.
The Butane oxime solution should be stored at $4^{o}C$.

Method: 0.05 ml each of buffer, DLC and ornithine were made up to 0.5 ml with 0.2 ml cell suspension and 0.15 ml water. The mixture was incubated in a water bath at $37^{o}C$ for 15 minutes at the end of this time 1 ml TCA was added to each tube to stop the reaction. The samples were centrifuged to remove cells and analysed for citrulline.

Five parts of the arsenic trioxide solution and three parts of the Butane oxime solution were mixed just before use. Two millilitres of this mixture were added to each sample and the samples were boiled for 90 minutes in the dark. The samples were cooled in the dark and 2.5 ml distilled water added to each. After standing for a further 30 minutes the optical density of the samples was measured in a Klett colorimeter using filter number 47.

A standard curve was prepared using known concentrations of citrulline and a blank control was included in each set of determinations.

The toluene-treated cells were included as a control representing 100% permeability. The degree of permeability achieved by EDTA treatment was expressed as a percentage of the toluene-treated cells. The results of this and a number of similar experiments are given in Table 1.19.

Table 1.19 The effect of EDTA treatment on the permeability and viability of PB240

	EDTA concentration (mM)					Toluene-treated
	0	0.4	0.5	0.6	1.0	Cells
µM Citrulline	0	0.27	0.32	0.31	0.25	0.36
% permeability	0	74	86	85	69	100
% kill	0	82	78	79	89	100

The proportion of cells killed was similar to the increase in permeability which suggests that EDTA treatment of the culture affects not only the permeability of the cells but also their ability to survive the

treatment. Induction can only be manifested in metabolically active cells and cells as drastically modified as these were considered unlikely to show any effects of aflatoxin. For this reason attempts to show aflatoxin induction by EDTA treatment were abandoned.

Legator's strains P4X6 and W1483 were obtained from his laboratory. These strains were treated with aflatoxin B_1 under the conditions outlined in his report. No induction could be demonstrated even when the concentrations of aflatoxin were increased. It was not possible to reproduce Legator's work with either Staph. aureus or E. coli and attention was turned to the work on B. megaterium.

e. Bacillus megaterium

Lillehoj and Ciegler (1970) reported that a lysogenic strain of B. megaterium could be induced by aflatoxin and that the numbers of bacteriophage produced were roughly proportional to the concentration of aflatoxin in the medium. Cultures of the lysogenic strain and of a phage sensitive strain were obtained from their laboratory.

Both strains were grown for four hours in nutrient broth. The lysogenic strain (899a) was diluted one tenth in NSM with and without 10 µg/ml aflatoxin B_1. These sub-cultures were incubated in a shaking water bath at 37°C for 90 minutes. At the end of this time the aflatoxin-containing culture had cleared while the control had not. Infectious centre counts, using strain KM as the phage-sensitive organism, and viable counts were made on both subcultures. The results are given in Table 1.20.

Table 1.20 Effect of aflatoxin B_1 on B. megaterium 899a

	Control	10 µg/ml aflatoxin B_1
Mean viable cell count/ml	$4.4X10^6$	$0.03X10^6$
Mean IC count/ml	$3.3X10^7$	$9.CX10^7$

A decrease in the viable cell count was asscciated with an increase in infectious centres though this increase was smaller than might have been expected. Nevertheless, a significant increase in the number of infectious centres suggested that some at least of the decrease in viable cell count was caused by phage induction rather than simple cell death.

Bacillus megaterium had also been used in a disk inhibition assay. Though less specific the method was more sensitive than many other biological assays. This biological response was also examined using three strains of B. megaterium, Strain 1368, the strain used by Clements (1968) in her proposed microbiological assay for aflatoxin B_1, and the strains 899a and KM already referred to.

1, 5 and 10 µg aflatoxin B_1 in chloroform were applied to disks and the solvent evaporated off. Thick suspensions of each of the three organisms were used to seed plates of nutrient agar. When the agar had set disks of each concentration of aflatoxin B_1 were placed on the agar and the plates incubated at 30°C overnight.

Zones were formed round each of the disks though the zone round the 1 µg disk was very small on plates containing 899a and 1368; the zones on the plate seeded with KM were larger and clearer.

These two sets of observations became the basis of a detailed study and their further development is dealt with in the sections which follow.

SECTION II

THE INHIBITION ASSAY

INTRODUCTION

The inhibition of the growth of B. megaterium by aflatoxin has been suggested as the basis of a microbiological assay by Clements (1968). The assay method involves the measurement of zones of inhibition surrounding disks impregnated with aflatoxin placed on plates seeded with B. megaterium.

This inhibition was confirmed in the preliminary experiments described in the previous section. The main limitations of the method are its lack of specificity and its low sensitivity. In the preliminary experiments the limit of detection was 2 µg aflatoxin B_1. Clements (1968) claimed 1 µg as the lower limit but even this is not sufficiently sensitive.

It would be an advantage to be able to check the biological potency of a sample on the same aliquot that was used for the physicochemical assay. This is theoretically possible as the physicochemical method is non-destructive. The amounts involved, however, must be less than 1.0 µg to achieve reasonable resolution so that an increase in the sensitivity of the bioassay would be necessary. This section describes attempts to improve the sensitivity of the inhibition assay.

Various methods of application of aflatoxin in wells:

1. The first attempt to improve the sensitivity was made by using the punched plate technique often used for antibiotic assays. Thin layers of tryptone yeast glucose agar (TYG-Agar) were poured in petri dishes and allowed to set. A pattern of sterile rubber bungs (6 mm diam.) was arranged on this layer and a further amount of TYG-Agar was poured and allowed to set. The bungs were removed leaving small wells in the agar. Aflatoxin was added to these wells either in aqueous solution or in chloroform. In those instances in which chloroform was used, the solvent was allowed to evaporate and the wells filled with sterile water. When the water in the wells had been absorbed by the agar a layer of TYG-Agar seeded with a culture of B. megaterium was poured over the plates filling the wells and forming a second layer. The plates were incubated at 37°C overnight and examined for zones of inhibition.

Table 2.1 shows the results of an inhibition assay in which the two methods of adding aflatoxin were compared using two different strains of B. megaterium.

Table 2.1 Bioassay of aflatoxin in agar wells

		Water Solution				Choroform Solution			
µg aflatoxin B_1		0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8
Zone diam. (mm)	899a	11.6	18.3	15.4	21.1	13.3	15.8	14.5	14.9
	1368	14.8	13.5	10.5	15.5	19.0	15.3	18.4	18.7

Although there is no consistent relationship between the amount of aflatoxin added and the diameter of inhibition zone, inhibition is clearly demonstrable with much smaller amounts of aflatoxin than are necessary for the disk bioassay. Addition of aflatoxin in chloroform was just as effective as addition in water. This suggested an alternative possibility for improving sensitivity which is described below.

Application of chloroform solutions to plates

A solution of aflatoxin in chloroform was applied by a micro-syringe directly to the surface of the pre-poured and dried minimal salts medium agar (MSM agar) plate. A grid placed under the plate was used to ensure that the spots were evenly spaced. The chloroform was evaporated off in a stream of warm air. The plate was then overlayered with soft MSM agar containing B. megaterium strain 899a. The plate was incubated overnight at 30°C and examined for zones of inhibition.

Table 2.2 Bioassay of aflatoxin by direct spots on agar

µg B_1	0.125	0.25	0.5	1.0
Zone diam. (mm)	0	5.3	11.9	16.1

The results show a definite relationship between zone size and amount of aflatoxin. Further experiments were planned to determine the most suitable medium, and the most sensitive organism.

Medium for inhibition assay

To determine whether the inhibition bioassay could be improved by altering the medium, plates were poured with tryptone yeast extract (TYG)-Agar

and MSM agar. These plates were dried at 37°C for one hour and aliquots of aflatoxin B_1, B_2, G_1 and G_2 were spotted on the agar surfaces as described. The plates were overlaid with seeded agar, either soft MSM agar or soft TYG agar being used as the top layer. The plates were incubated at 37°C overnight. The results are shown in Table 2.3.

Table 2.3 Effect of medium on agar plate bioassay

Zone diam. (mm)

Base		TYGA overlay		MSMA overlay	
		TYGA	MSMA	TYGA	MSMA
aflatoxin B_1 µg	1.0	16.5	11.2	6.0	0.0
	0.5	11.2	13.0	0.0	0.0
	0.25	2.0	0.0	0.0	0.0
	0.125	0.0	0.0	0.0	0.0
aflatoxin G_1 µg	20	17.5	10.0	6.0	0.0
aflatoxin B_2 µg	20	13.0	0.0	2.0	0.0
aflatoxin G_2 µg	20	0.0	0.0	0.0	0.0

Faint zones of inhibition could be seen on the MSMA plates overlaid with MSMA after further incubation at 30°C for a further 24 hours, but clearly the richer medium gave much better results.

The relationship between inhibition zone size and amount of aflatoxin added was similar to that in the previous experiment. Much higher amounts of aflatoxin G_1 and B_2 were needed to give inhibition zones similar in size to B_1, while aflatoxin G_2 gave no inhibition zone at 20 µg. This agrees with their toxicities in animal experiments.

Seed organism for the inhibition assay

Attempts to find the best seed organism to use gave rather inconsistent results by the method of chloroform spots on agar plates. Strain KM, appeared to give the clearest and most easily read zones of inhibition. Strain 1368, the strain used by Clements for inhibition assays, gave zones of similar diameter but these were less clearly defined, while strain 899a gave smaller zones.

It was during this series of experiments that the main disadvantages of the chloroform spot method became obvious. While it was possible to improve the sensitivity of inhibition assay, the precision of the test was

reduced due to the behaviour of the chloroform spots. Even very small spots of chloroform tended to move about on the surface of the agar evaporating as they moved. The resulting inhibition zones were very irregular in shape and size making their measurement very difficult or impossible.

The method had shown that amounts of aflatoxin less than one microgram could give zones of inhibition. This suggested that the measurement of inhibition directly on TLC plates might be possible.

Inhibition on TLC plates

A TLC plate coated with a 250 μ layer of Merk silica gel G was activated at 120°C for 2 hours. After cooling in a closed container over silica gel, 1 μg of aflatoxin B_1 in 10 μl chloroform was applied to the plate and the chloroform evaporated off under a stream of warm air. The plate was developed, using 10% acetone in chloroform as solvent until the solvent front was 2 cm from the end of the plate. The plate was removed from the developing tank and the solvent allowed to evaporate. The aflatoxin B_1 spot was located by viewing the plate under an ultra-violet lamp and the outlining of the area of fluorescence. The plate was overlaid with soft TYGA seeded with strain 899a and when the agar had set the plate was incubated overnight in a moist chamber at 37°C.

A zone of inhibition could be seen over the position of the aflatoxin B_1 spot but due to the opaque white background the zone was very difficult to see.

In a second experiment two millilitres of triphenyl tetrazolium chloride solution (TTC) were spread over the surface of the agar after overnight incubation, and the plate reincubated for an hour. Areas where the bacteria had grown turned bright red due to the reduction of the TTC and the zone of inhibition stood out clearly as a white circular patch against a red background. In an attempt to shorten the procedure, TTC was incorporated in the agar overlay but, although growth occurred, no zones were visible. In a control plate treated with TTC after overnight incubation, zones were visible. TTC therefore seems to interfere with the inhibition of growth by aflatoxin possibly by protecting the organisms in some way.

Further experiments suggested that TTC inactivated aflatoxin only in the presence of growing organisms. From this time on TTC was applied only after the overnight incubation when the zone was already fully developed.

Seed organism for the TLC inhibition assay

The next investigation was to find the organism most suited to the TLC method. B. megaterium strains KM, 899a and 1368, Salmonella typhimurium strains P22T and wild type and E. coli strains P4X6 and T183 were grown in TYG broth to an OD of 125 Klett units. All were diluted $5X10^{-1}$ and 0.1 ml was added to 10 ml TYG agar. TLC plates were spotted with 0.2, 0.4, 0.6 and 0.8 µg aflatoxin B_1 and developed in chloroform containing 10% acetone. The plates were layered with TYG-Agar seeded with the various organisms and incubated overnight at 37°C.

Table 2.4 Inhibition of various organisms by aflatoxin B_1

	aflatoxin B_1 (µg)			
	0.2	0.4	0.6	0.8
B. mega KM				
Zone (mm)	7.6	13.7	17.5	22.6
r^2	14	46	77	128
B. mega 899a				
Zone (mm)	2	4.4	8.5	12.2
r^2	1	4.8	18	38
B. mega 1368				
Zone (mm)	0	4.4	7.6	11
r^2	0	4.8	14	30

See Figure 2.1 for graph.

Of the organisms tested only B. megaterium showed zones of inhibition with less than 1 µg aflatoxin. Of the Bacillus strains, KM was much the most sensitive. In all subsequent work KM was used as the seed organism.

Seeding density for TLC inhibition assay

To find the optimum level of seeding the layer the following experiment was performed.

Figure 2·1

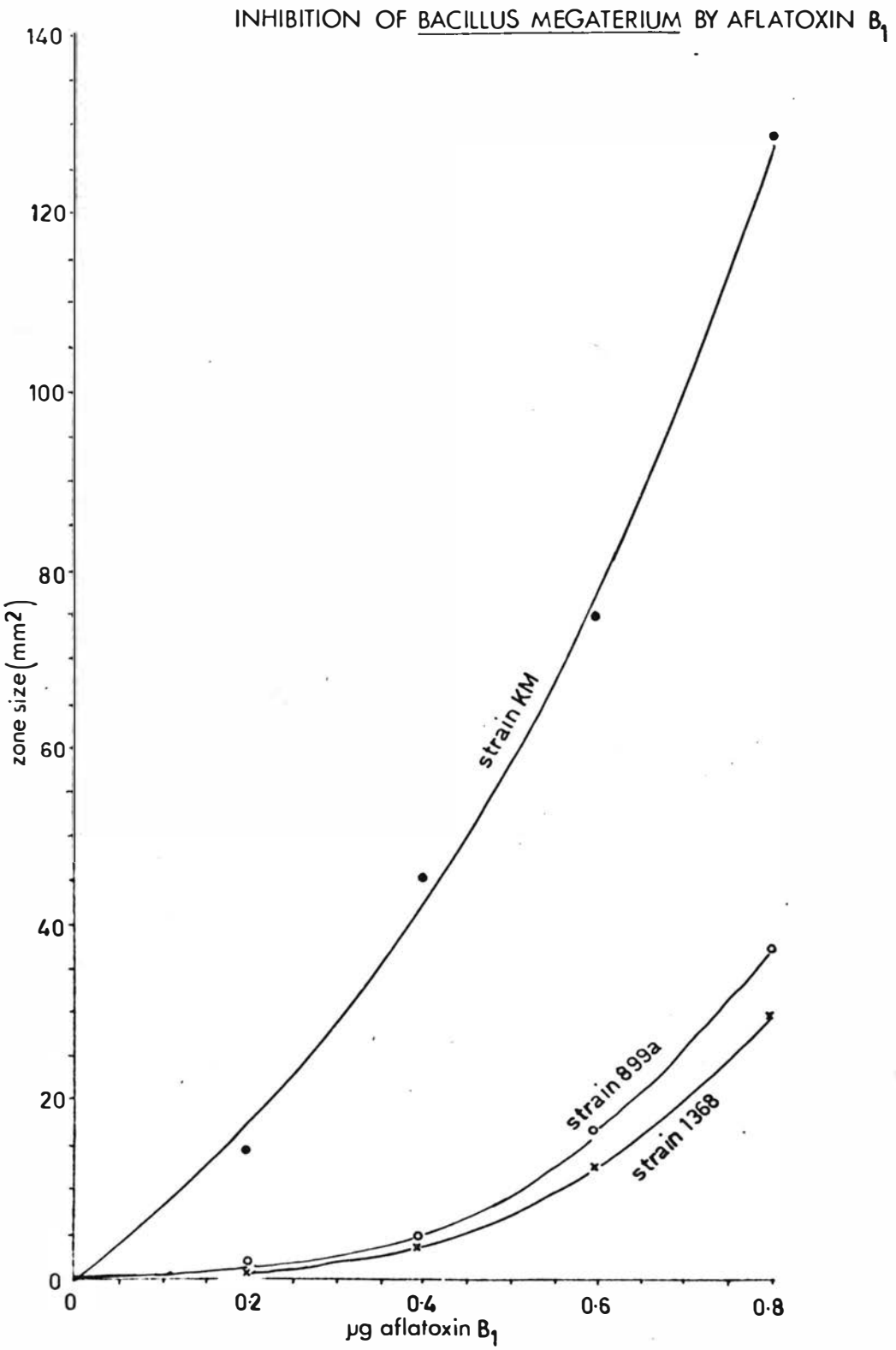


Figure 2.1 Aflatoxin B_1 spots of stated amounts on TLC plates over-layered with TYG agar seeded with the three organisms. Plates incubated overnight at 37°C.

Four TLC plates were spotted with 1 and 0.5 µg aflatoxin B_1. The plates were developed and layered with agar seeded with B. megaterium strain KM. The organism was grown in TYG broth to an OD of 222 Klett units and diluted 10^{-1} 10^{-2} 10^{-3} and 10^{-4} in TYGA. 10 ml of each dilution was used to layer the plates. The plates were incubated overnight and treated with TTC. The zones were measured and the results are recorded in Table 2.5 below.

Table 2.5 Effect of seed density on zone size

dilution		1 µg B_1	0.5 µg B_1	log viable count/ml
10^{-1}	diam. zone (mm)	14.2	11	8.3
	r^2	50	30	
10^{-2}	diam. zone (mm)	16.2	12.9	7.3
	r^2	66	42	
10^{-3}	diam. zone (mm)	19.8	14.3	6.3
	r^2	90	51	
10^{-4}	diam. zone (mm)	22	18.5	5.3
	r^2	121	85	

See Figure 2.2 for graph.

At the lower seeding densities the colonies became rather sparsely distributed and individual colonies could be seen quite clearly. Any further dilution created difficulty in determining the limits of the zones of inhibition. The optimum dilution was one which gave approximately $2X10^5$ viable counts/ml.

Further work showed that spotting aflatoxin solutions on to the TLC plate without subsequent development gave small concentrated spots and the resulting zones of inhibition were more regular and reproducible. Not developing the plates also meant that many more samples could be spotted over its surface.

The results of a number of determinations comparing fluoro-densitometric measurements and inhibition zone sizes of aflatoxin B_1 are shown in Figure 2.3.

Figure 2.4 gives the results of several experiments showing the relationship between known aflatoxin concentration and inhibition zone size on undeveloped plates.

Figure 2·2

EFFECT OF SEED DENSITY ON ZONE SIZE

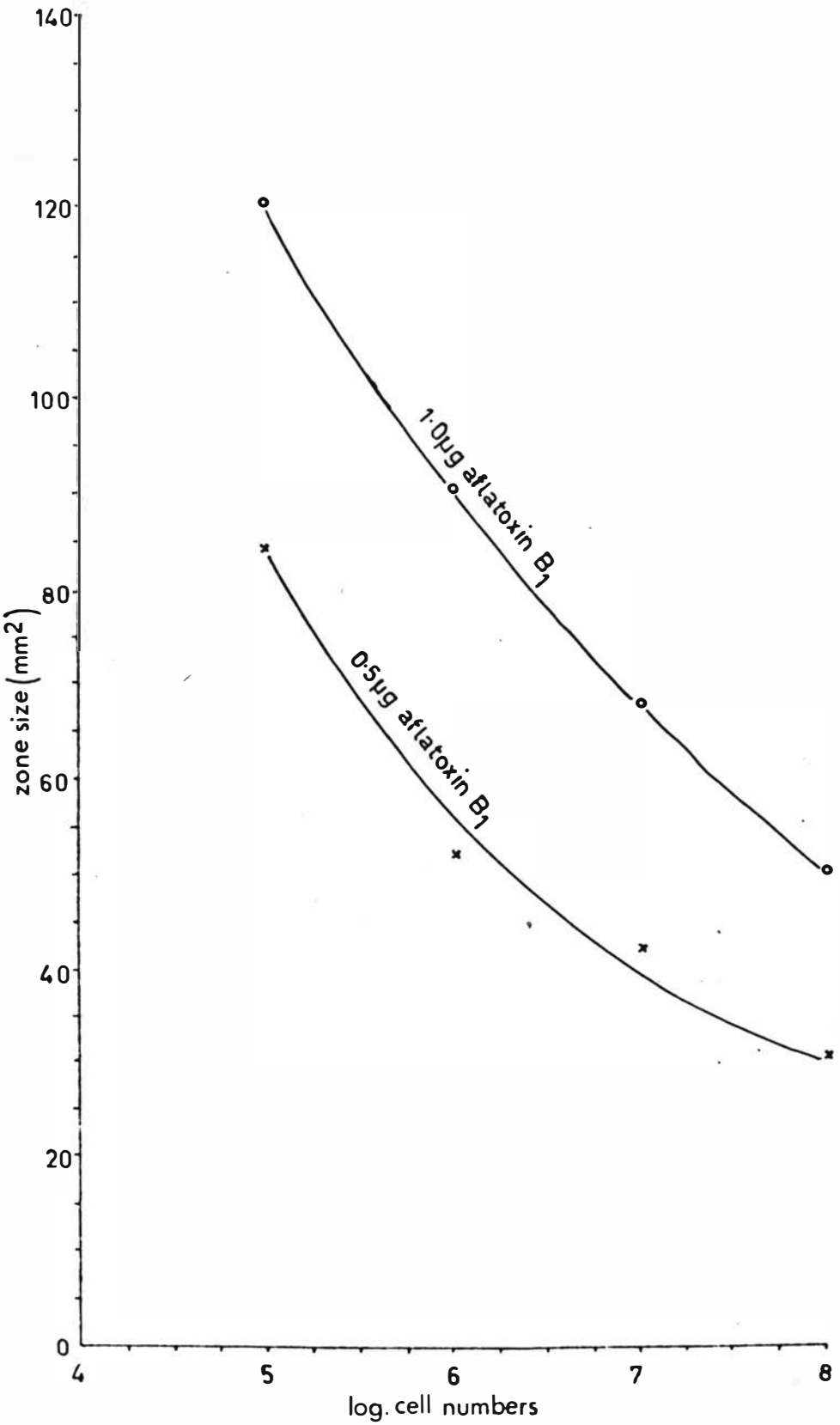


Figure 2·3

RELATIONSHIP BETWEEN DENSITOMETRIC AND INHIBITION ASSAYS

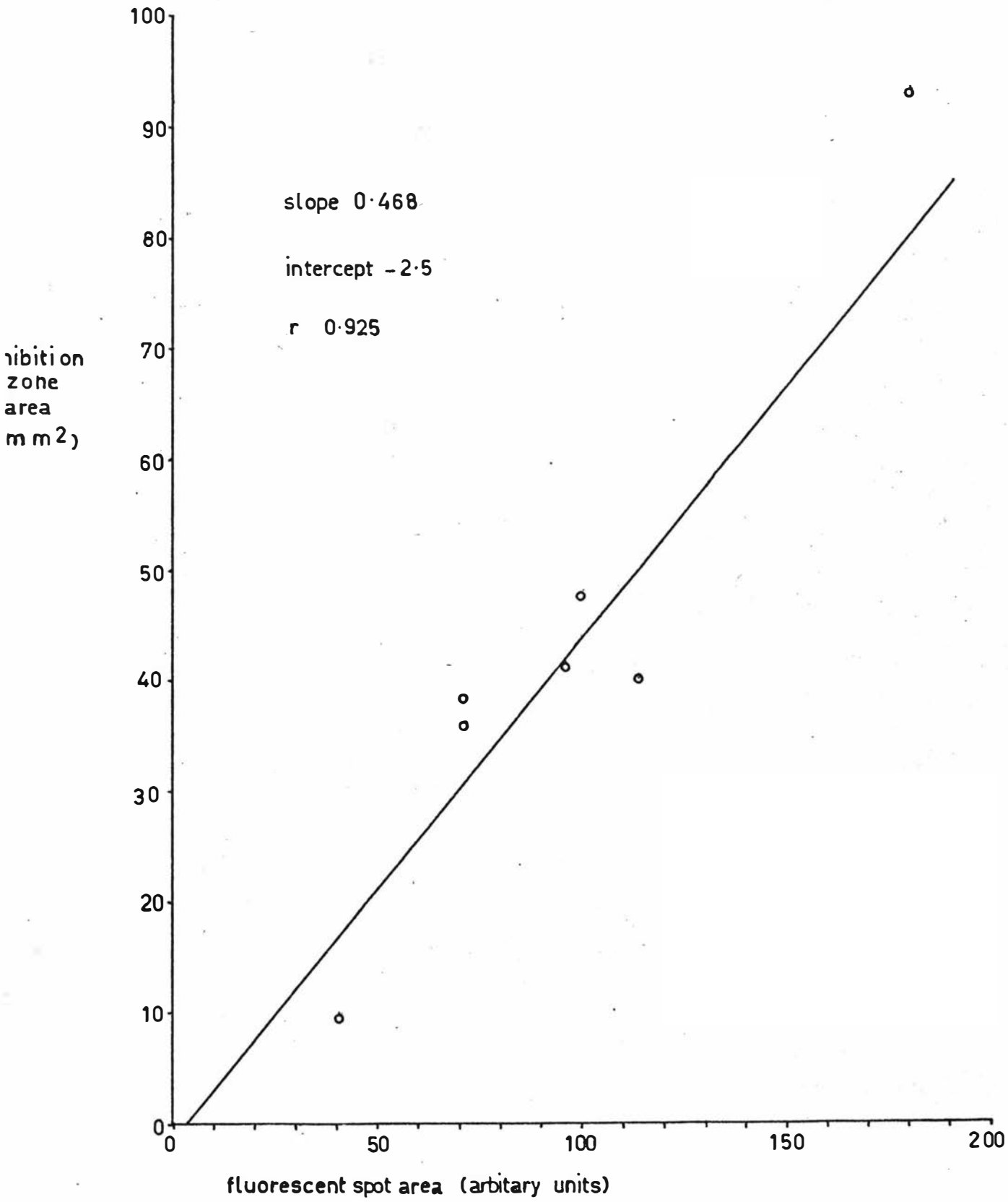
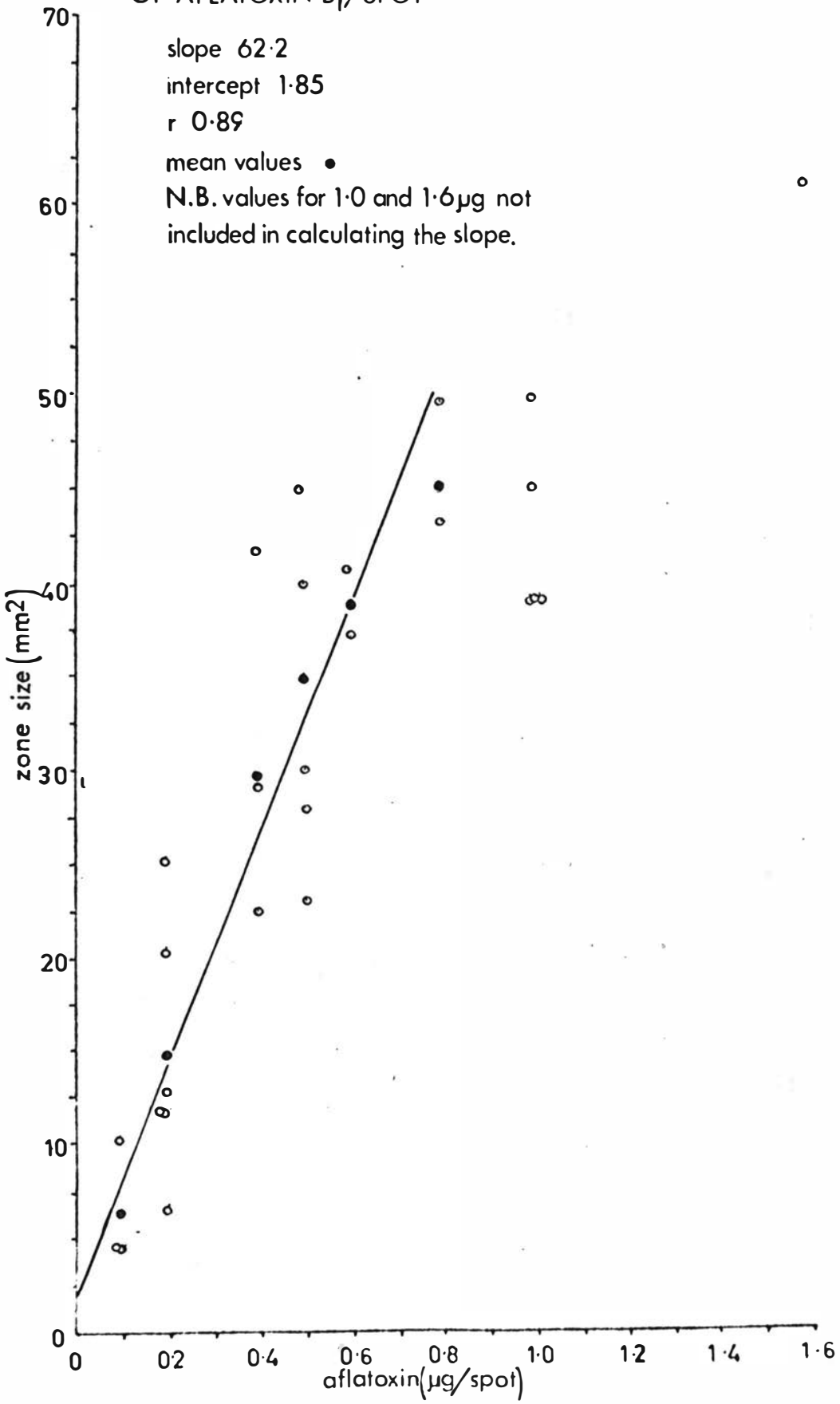


Figure 2·4

RELATIONSHIP BETWEEN INHIBITION ZONE SIZE AND AMOUNT OF AFLATOXIN B_1/SPOT



It is clear from Figure 2.4 that the dose-response relationship is not linear throughout its length. Above approximately 0.8 µg the zones are smaller relative to concentration and these points were omitted when the regression line was calculated.

The assay of aflatoxin on TLC plates after development is less precise and less sensitive than that on undeveloped plates but has the advantage of some degree of specificity. This specificity derives from the comparison of the Rf of the compounds causing inhibition zones with standards of known toxins. The assay on undeveloped plates is less specific but enables replication of samples and standards. The procedure set out below is applicable to both developed and undeveloped plates.

Recommended assay procedure.

Prepare thin layer chromatography plates using silica gel containing binder (Merk silica gel G is suitable) as a layer 250 µ thick. Allow the plates to dry and treat them at 120°C for two hours. Store over silica gel.

Apply spots of unknowns and standards dissolved in acetone or chloroform to the surface of the plate ensuring that the centres of the spots are at least three centimetres apart. Where possible each spot should contain between 0.2 and 0.8 µg aflatoxin B_1. Evaporate the solvent in a stream of warm air.

N.B. For other toxins refer to Section V.

Grow B. megaterium strain KM in yeast tryptone glucose in a shaking water bath at 32°C to an optical density of 125 Klett units. Dilute the culture 1/10 in diluent peptone and add 0.1 ml to 10 ml molten yeast tryptone glucose agar, mixing thoroughly. Using a sterile 10 ml pipette distribute the molten agar evenly over the surface of the TLC plate. Allow the agar to set and incubate the plate in a closed air-tight container at 30°C overnight. After incubation spread 2 ml Triphenyl Tetrazolium Chloride solution evenly over the surface of the agar using a glass spreader. Reincubate the plate at 30°C for one to two hours. Measure the diameter of the zones of inhibition using needle calipers. Express results as $\left(\frac{\text{zone diameter}}{2}\right)^2$.

SECTION III

INVESTIGATION OF BACILLUS MEGATERIUM

AND ITS BACTERIOPHAGE

INTRODUCTION.

The report of Lillehoj and Ciegler (1970) that bacteriophage of B. megaterium strain 899a could be induced by aflatoxin B_1 was confirmed as reported in Section I. The specificity of phage induction makes it attractive as the basis of a bioassay system.

It seemed desirable that the study of bacteriophage induction should take place in a defined medium since unknown substances present in a complex medium may modify the effect of an inducing agent. Lillehoj et al. (1967) have reported reversal of aflatoxin inhibition of an Aspergillus species by yeast extract although yeast extract was included in the medium used in the experiments on induction of the B. megaterium bacteriophage by aflatoxin. In a defined medium the effectiveness of the aflatoxin might be increased.

The B. megaterium system was first isolated for study in 1931 by den Dooren de Jong and was used by Lwoff and his colleagues in their now classical studies of lysogeny in the early 1950's. Despite the attention that this system has received there are inconsistencies in the literature regarding the requirements of the organism for growth and for the propagation of phage after induction.

Lwoff (1950) used a complex mineral salts base containing ammonium sulphate, potassium dihydrogen phosphate, and magnesium sulphate as the main ingredients and iron, manganese, cobalt, zinc, molybdate, borate and copper as minor ingredients. This was supplemented with glucose, yeast extract and further amounts of calcium and magnesium. Tryptone was sometimes added as well. By contrast, Northrop (1956) used a medium containing only ammonium sulphate, potassium dihydrogen phosphate, magnesium sulphate, glucose and traces of iron.

The requirements stated to be necessary for induction and phage propagation are also confusing. Gratia (1936) claimed that calcium was required when plating phage on the B. megaterium 'mutilat' strain of den Dooren de Jong, a strain which is very similar to the KM strain used in the present study. Northrop (1951) reported a requirement of magnesium

and phosphate ions for the production of bacteriophage in a culture of the lysogenic strain. Huybers in 1953 reported that magnesium ions were necessary in nutrient broth for induction to occur after ultra-violet light irradiation and Freidman and Cowles (1953) reported a drastic reduction in the titres of phage when counted in agar containing citrate. On the other hand Gaal et al. (1970) induced B. megaterium 899a bacteriophage with mitomycin C in a medium containing only yeast extract, tryptone, potassium dihydrogen phosphate and sodium chloride.

As there was little consistency in the previous reports on conditions for growth of B. megaterium and for propagation of its bacteriophage, a study was made of the strains that were to be used in the induction assay.

Defined medium for B. megaterium.

All cultures for growth response experiments were incubated in flasks with side arms for optical density measurements. These flasks were incubated shaken in a water bath at 37°C. In later experiments the temperature was lowered to 32°C as 37°C was thought to be too close to the maximum growth temperature of B. megaterium. Growth was followed by measuring the optical density in a Klett-Summerson colorimeter.

Attempts to grow B. megaterium strain 899a in basal medium (BM) supplemented with 0.1% glucose (BMG) were unsuccessful and additional supplements were incorporated into the medium. It was found that the addition of 0.1 µg/ml nicotinic acid was necessary for the growth not only of strain 899a but also of strains 1368 and KM.

Oxygenation of the medium

Shaken cultures grew more rapidly than unshaken cultures. This was assumed to be due to the increased availability of oxygen as reported by Lwoff (1950). To standardise the growth conditions cultures were grown in BMG + nicotinic acid at shake rates varying between 50 and 110 cycles/minute. Growth, as measured by generation time, was unaffected between 70 and 110 cycles/minute and all further experiments were performed at a rate of 90 cycles/minute.

Glucose requirements of B. megaterium

Strain 899a was examined for the effect of glucose concentration on yield. A culture was grown in BM + 0.5 µg/ml nicotinic acid and 0.1% glucose until it was in the exponential phase of growth. The culture was centrifuged; the cells washed in buffer and resuspended in BM. This suspension was used to inoculate aliquots of BM + nicotinic acid containing various levels of glucose. Growth of these cultures at 37°C was followed by taking optical density measurements until no further increase occurred. The maximum growth at various levels of glucose for strains 899a, 1368 and KM were determined and are presented in Figure 3.1. The regression line was calculated only on the results from experiments using strain 899a, but all three strains behaved similarly. The maximum growth is reached at approximately 0.3% glucose beyond which there may be a decline in yield.

Optical density measurement

During the series of experiments described above it was observed that the relationship between cell mass and optical density departed from linearity above a Klett value of 110. Further investigation was undertaken to establish the form of this departure from linearity. By comparing the actual measurements with the slope extrapolated from the linear part of the curve it was established that the relationship was a square function. The departure from linearity was eventually found to be expressed by the term

$$R_t = R + \left[1.6 + 0.57 \left(\frac{R - 110}{10}\right)\right]^2$$

where R_t is the 'true' reading and R is the actual reading. A correction curve was constructed (Figure 3.2) so that optical density readings could be recorded directly without the necessity of diluting aliquots to below a reading of 110. This was desirable if readings were to be made over an extended period on the same culture. Either the correction curve or the correction factor can be used to correct the Klett values. All OD values given in the text are corrected values.

Figure 3·1

MAXIMUM GROWTH OF BACILLUS MEGATERIUM IN VARYING CONCENTRATIONS OF GLUCOSE

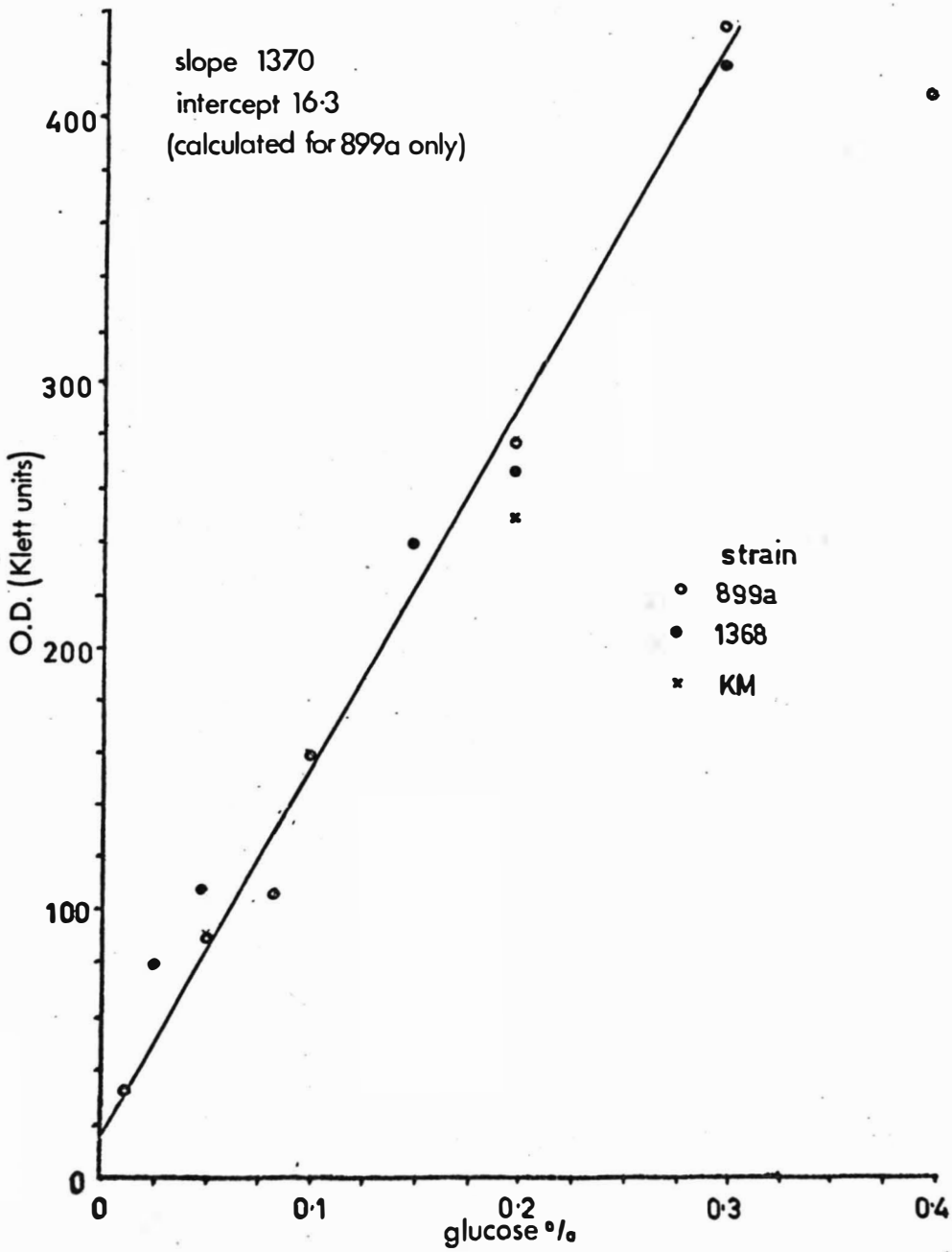
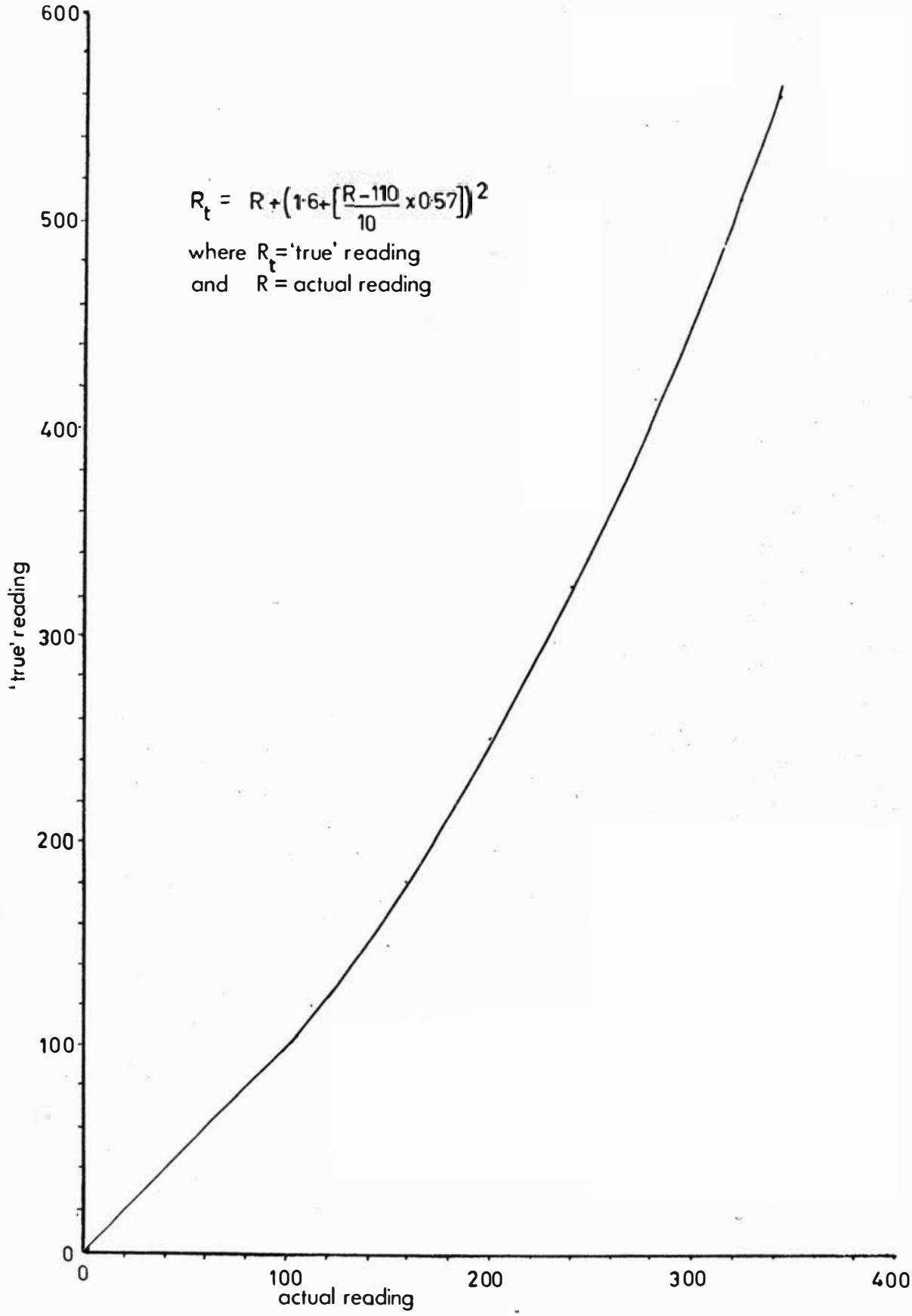


Figure 3.1 Basal medium was supplemented with the stated levels of glucose. All cultures were incubated in a shaking water bath at 37°C until no further increase in optical density was observed.

Figure 3·2

CORRECTION CURVE FOR OPTICAL DENSITY MEASUREMENTS



To confirm the relationship between cell mass and optical density a number of protein estimations were made by the method of Lowry et al. (1951) at various Klett values. The results are shown in Figure 3.3.

The generation time of B. megaterium growing in BM + glucose and nicotinic acid (BMGN) at 37°C is approximately 100 minutes. This slow growth made experiments rather protracted. To increase the growth rate it was decided to sacrifice some of the advantages of a defined medium by adding 0.1% tryptone to BMGN. This had the effect of reducing the generation time without affecting the final yield. A typical experiment using strain 899a is shown in Figure 3.4, in which the generation time was reduced from 105 minutes in BMGN to 55 minutes in BMGN + tryptone. The medium used in all subsequent experiments was basal medium containing 0.1% tryptone, 0.1 µg/ml nicotinic acid and 0.25% glucose unless otherwise stated. This is referred to as semi-defined medium (SDM).

Effect of acetone on the growth of B. megaterium

Since acetone was to be the vehicle for the addition of aflatoxin to the assay system its affect on the growth of B. megaterium strain 899a was examined. 0, 2 and 5% acetone was added to cultures of the organism and growth followed by determining the optical density.

The organism grew normally in 2% acetone and with a slower growth rate in 5% acetone. There was a delay of 100 minutes before growth started in 5% acetone.

Infection of strain KM by phage in broth culture

Work described in Section IV shows that there is a delay of approximately 60 minutes before the induction of bacteriophage 899a by aflatoxin is expressed by a decrease in optical density and a sudden increase in phage numbers. In the E. coli phage lambda system the time elapsing after infection of the cells with bacteriophage lambda and the release of phage (burst time) is shorter than the time elapsing after treatment with an inducing agent and eventual lysis (latent time). (Jacob and Wollman 1953). It was of interest to discover whether the burst time and latent time also differed in the B. megaterium system. Neither a lysogenic KM strain nor a phage

Figure 33

RELATIONSHIP BETWEEN OPTICAL DENSITY AND BACTERIAL PROTEIN

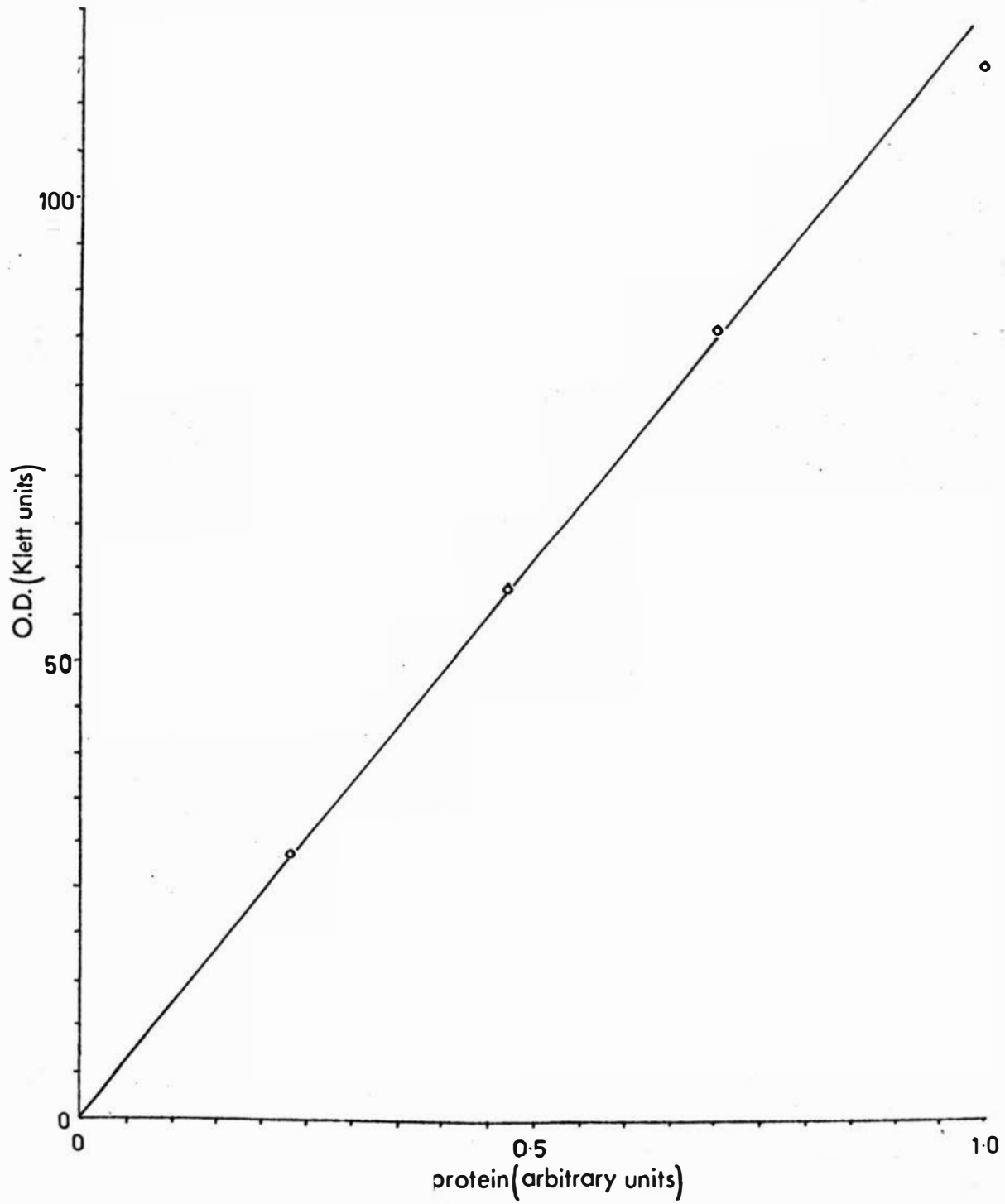


Figure 3·4

EFFECT OF TRYPTONE ON GROWTH RATE OF BACILLUS MEGATERIUM

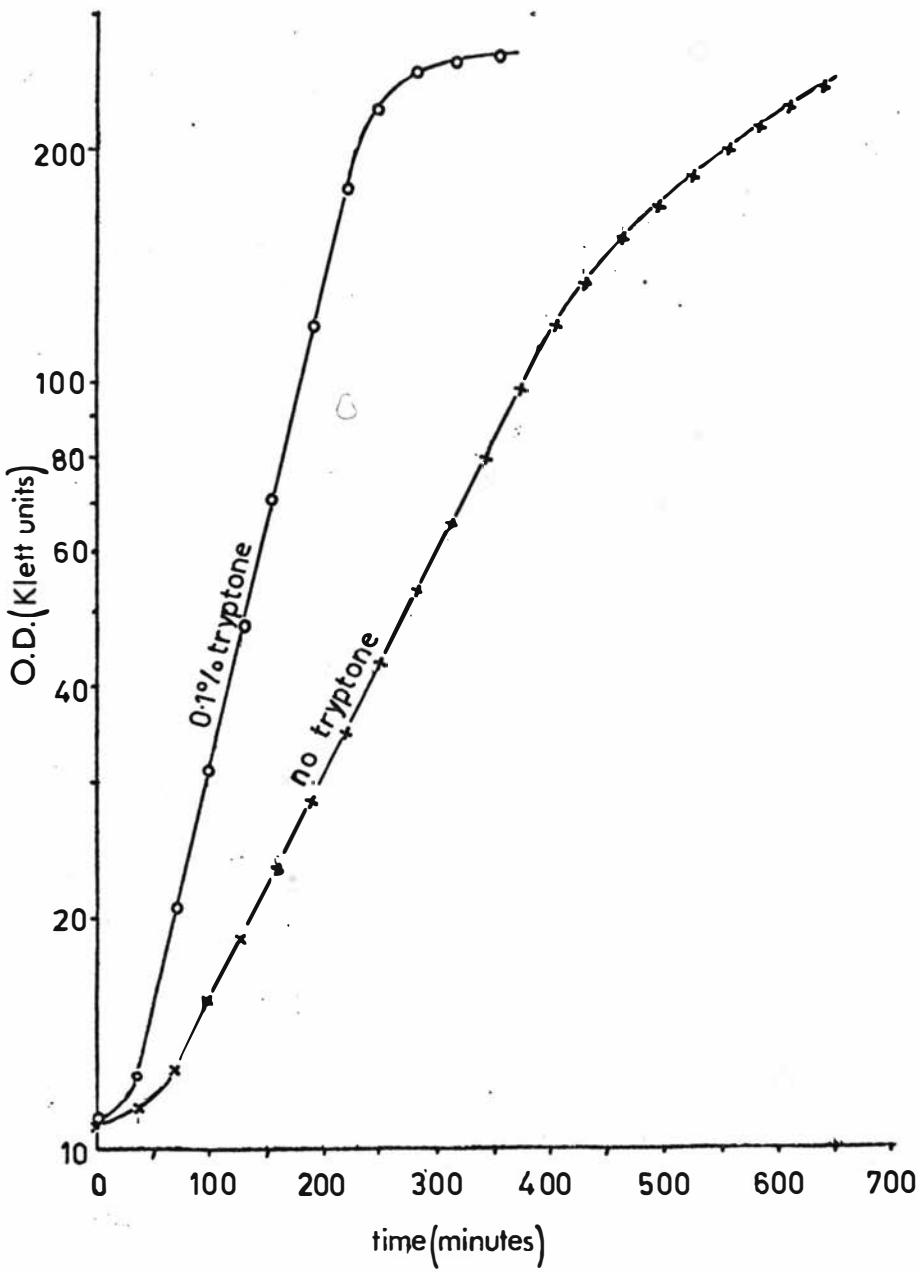


Figure 3.4 Cultures were incubated in a shaking water bath at 37°C in BMGN with and without 0.1% tryptone.

free 899a strain were available so comparisons had to be made between the two strains 899a and KM.

A phage suspension was prepared by plating a culture filtrate of strain 899a on four plates of TYA seeded with strain KM so as to give confluent lysis. Three millilitres of 0.1% peptone diluent were added to each plate and the plates allowed to stand at room temperature for four hours. The diluent was pipetted off and pooled. The suspension was centrifuged at 10,000 x g and the supernatant chloroformed. The suspension was counted and found to contain approximately 10^8 phage/ml.

A culture of strain KM was grown in SDM to exponential phase (Klett value 50) and phage particles were introduced into the culture to give approximately 100 phage/ml. Optical density measurements and plaque counts were made at intervals for 80 minutes after the addition of the phage. There was no effect on growth and no increase in the numbers of plaques observed over this eighty minute period.

Since phage were able to infect and lyse cells on TY agar plates it was clear that the conditions in the agar plate cultures differed in some way from those in the broth cultures. Yeast extract was added to the SDM medium but this still did not permit phage propagation. One possible explanation was that adsorption required the presence of a co-factor which was absent, too dilute or unavailable in the liquid medium.

Effect of chloroform on phage counts

To differentiate between plaques due to free phage and plaques due to phage already adsorbed to indicator bacteria the bacteria must be removed or killed. One standard method of killing the bacteria is to saturate the suspension medium with chloroform and incubate for a few minutes. It was shown that 899a phage was unaffected by chloroform and the method of chloroform killing was adopted. However, despite the standardisation of the plating medium there were still irregularities in the counts from cultures of induced lysogenic bacteria. Counts on cultures which had not been treated with chloroform were regular and predictable but counts on cultures treated with chloroform varied considerably.

The method in use involved removing a 0.1 ml aliquot of the culture into 9.9 ml 0.1% peptone diluent containing 0.1 ml chloroform. The diluent was shaken by hand and incubated at 37°C for ten minutes after which the chloroform was removed by bubbling a stream of filtered air through the diluent.

When phage suspensions were treated by this procedure a marked reduction in count was observed compared with phage suspensions plated untreated. If the phage were placed in diluent containing chloroform but not shaken the counts were not reduced. Since chloroform alone had no effect on numbers it seemed possible that the phage were being inactivated at the interface between the chloroform and water when the diluent was shaken. Table 3.1 shows the effect on the phage count of shaking in the presence and absence of an excess of chloroform.

Table 3.1 Effect of chloroform on phage counts

Treatment*	diluent alone	diluent saturated with an excess of chloroform	diluent saturated with chloroform but no excess
plaque count	120	7	150

* N.B. All treatments were shaken.

All dilutions for free phage counts were subsequently made in diluent saturated with chloroform but in the absence of any excess of free chloroform.

Adsorption of bacteriophage 899a on to strain KM

Cells of strain KM were grown to exponential phase in SDM medium and diluted to a Klett value of 10. Phage particles were added to give approximately 10 phage/cell. Samples were removed into diluent saturated with chloroform at intervals and appropriate dilutions assayed for free phage. Infected and uninfected cells were killed by the chloroform and dilution prevented further adsorption so that only free phage remained as plaque forming units. There was a decline in the numbers of free phage as is shown in Figure 3.5. Clearly phage were able to adsorb to KM cells but were not able to infect them. (See page 66)

The experiment was repeated to confirm the result and to confirm that despite strong adsorption there was no resulting burst.

Figure 3·5

ADSORPTION OF BACTERIOPHAGE 899a TO B. MEGATERIUM STRAIN KM

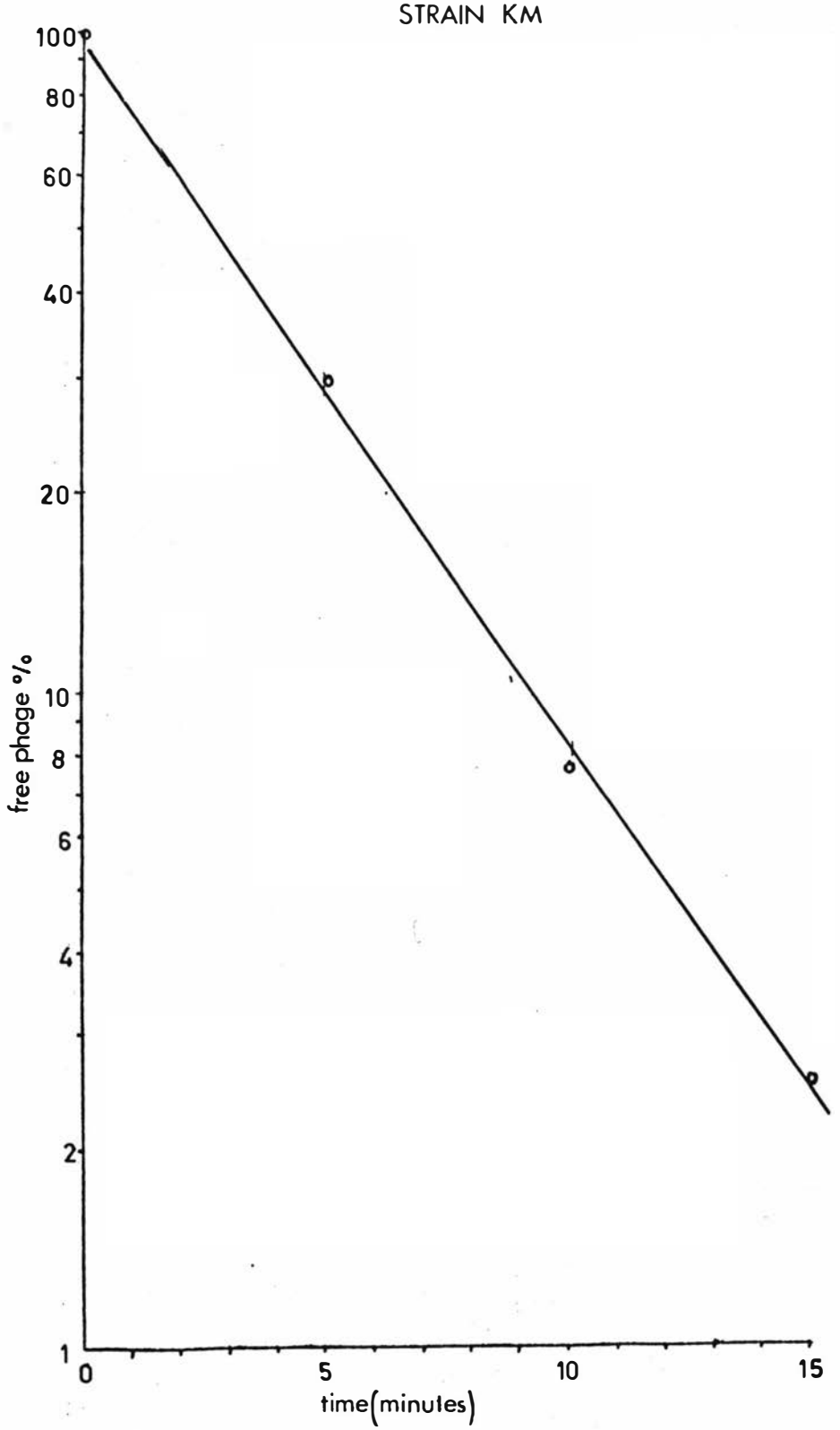


Figure 3.5 B. megaterium at a density of 270 Klett units was incubated in SDM in a shaking water bath at 37°C. Phage were added to give approximately 10 phage/cell.

Effect of substances in the agar on infection of strain KM

MSM supplemented with the same concentrations of yeast extract and tryptone as agar medium contained, failed to permit propagation of the phage on the indicator strain so it was concluded that something in the agar itself must be necessary for lysis. An extract of agar was prepared by soaking Davis New Zealand agar in ten times its own weight of water at 50°C for one hour. The aqueous extract was expressed by squeezing the wet agar in a muslin cloth. This liquor was filtered through No. 1 filter paper and sterilised by autoclaving.

Friedman and Cowles (1953) suggested that divalent ions might be necessary for infection and lysis of indicator cells by bacteriophage 899a. To test this suggestion medium was made up containing tryptone and yeast extract to which was added agar extract, agar extract + 0.1 mM EDTA and EDTA alone. These media were inoculated with strain KM and phage suspension and incubated in a shaking water bath at 37°C. The growth was followed by optical density measurements.

Figure 3.6 shows the effects of the various additions.

The addition of agar extract led to a slight inflexion in the growth curve after 75-80 minutes and a drop in optical density between 130 and 150 minutes. EDTA had no effect on growth but reduced the effect of the agar extract so that the drop in OD at 150 minutes was replaced by a flattening of the curve. This is consistent with suggestion that the active ingredient in the agar is a divalent ion.

To show that the drop in OD was due to lysis and phage production the experiment was repeated using a tryptone, yeast extract medium buffered with phosphate. Duplicate flasks were set up, one with and one without addition of agar extract equivalent to 10% agar. Optical density measurements and phage counts were made at intervals on both flasks. The results given in Figure 3.7 show an even more marked decrease in OD in the flask containing agar extract and a corresponding massive increase in the number of free phage.

To test further the possibility that the effect of the agar extract was due to metal ions in the agar, the effect of agar ash was examined.

Figure 3·6

EFFECT OF AGAR EXTRACT ON THE PHAGE LYSIS OF B. MEGATERIUM STRAIN KM

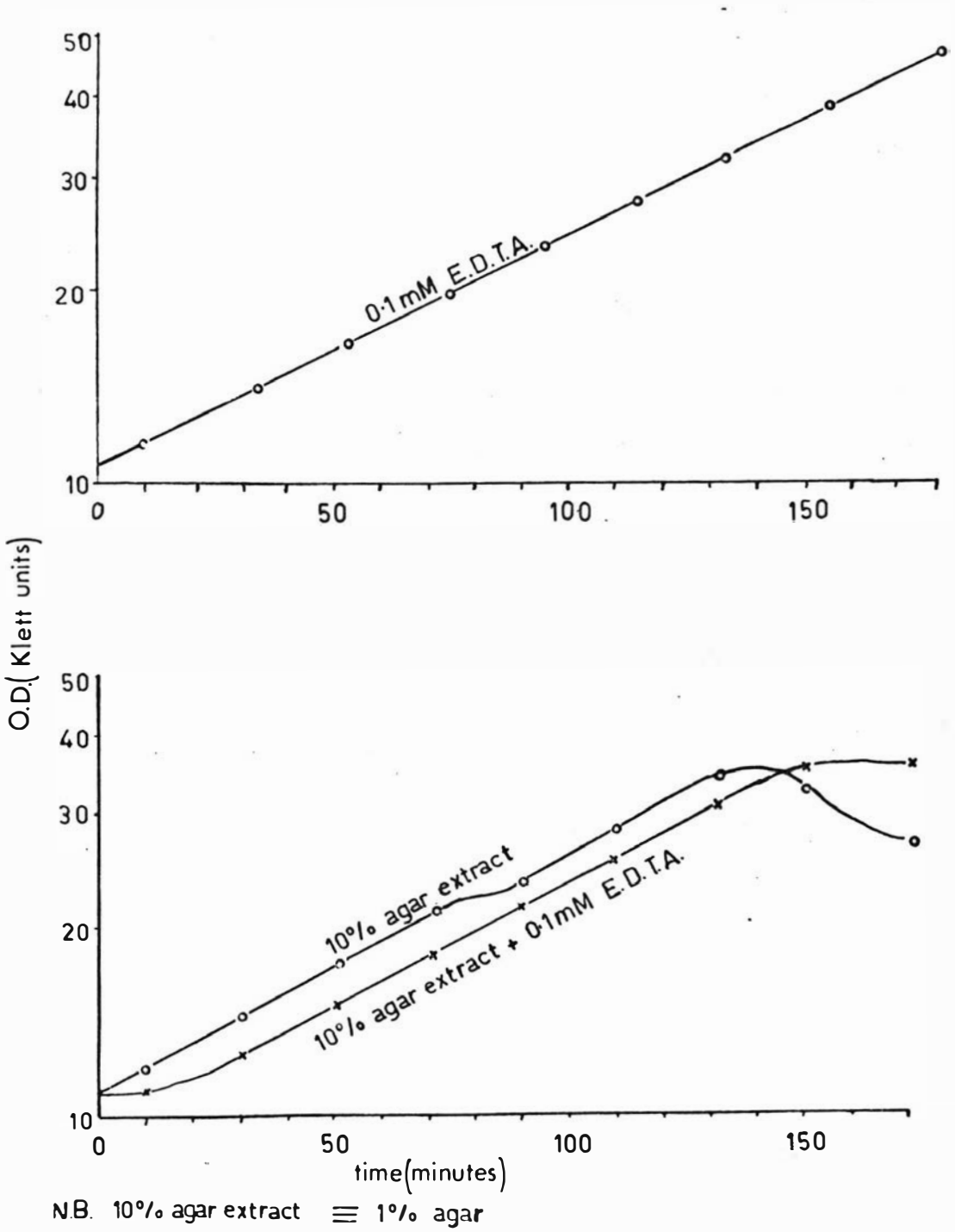


Figure 3.6 TYGP supplemented with various additions inoculated and incubated at 37°C in a shaking water bath.

Figure 3·7

EFFECT OF AGAR EXTRACT ON PHAGE PRODUCTION BY B. MEGATERIUM STRAIN KM

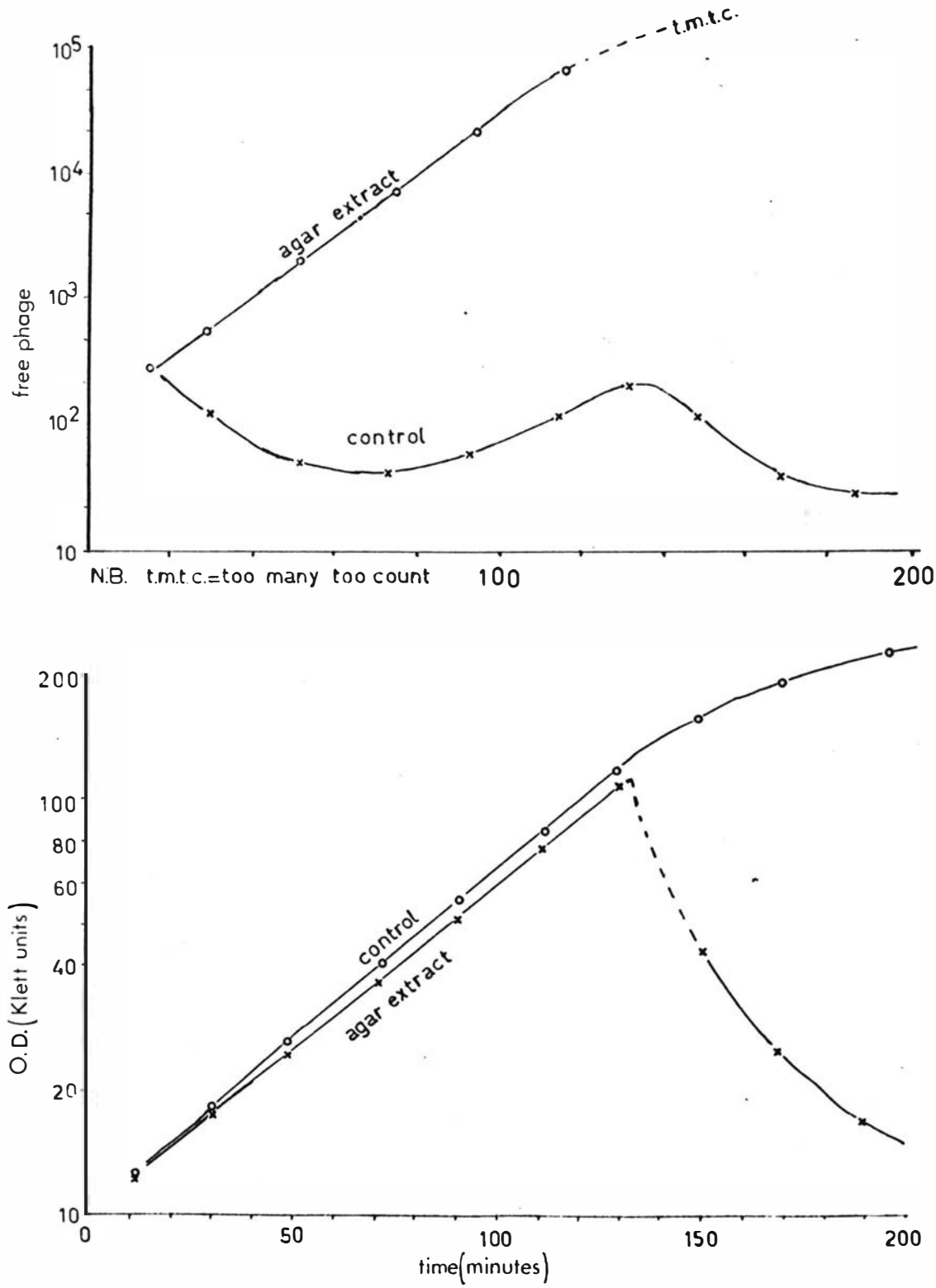


Figure 3.7 TYGP with and without agar extract inoculated and incubated at 37°C in a shaking water bath. Phage counts made in TYA.

One gram of agar was reduced to ash by heating in a furnace at 550°C. The ash was digested in 3 ml 1.0 N hydrochloric acid and reduced to dryness again. The residue was taken up in 0.5 ml distilled water. This solution was added to tryptone yeast glucose phosphate broth (TYGP) and compared with extract from an equivalent amount of agar. The ash allowed the same degree of lysis and the same increase in the numbers of free phage.

Effect of agar ash extract on relative plating efficiency

Ash extract was added to TYGP broth at different levels and the broths were inoculated with log phase cells of strain KM and bacteriophage as described previously. OD measurements and free phage counts were made at intervals. The results are shown in Table 3.2 and Figure 3.8.

Table 3.2 Effect of different levels of agar ash extract on phage propagation in strain KM

% ash extract in TYGP broth[X]	free phage counts at	
	50 minutes	100 minutes
0	30	20
0.5	100	0.9×10^4
1.0	840	7.8×10^4
2.0	1.23×10^3	8.4×10^4
4.0	2.06×10^3	12×10^4
6.0	670	15×10^4

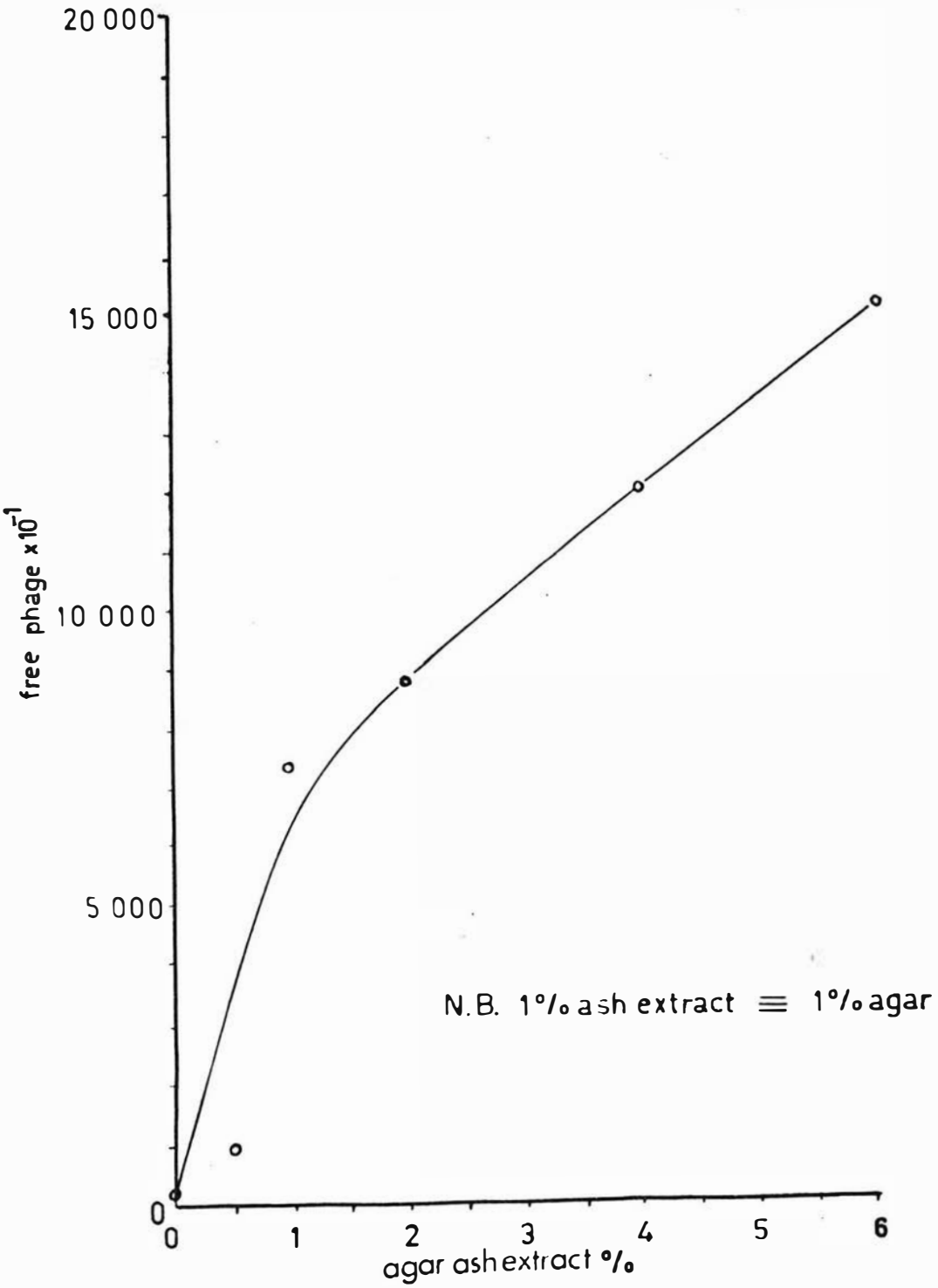
[X] Ash extract contains the extractable material from 1 g agar.

Growth in the broth containing 6% ash was slower than in the other test cultures. This may account for the reduced number of free phage at 50 minutes in the broth supplemented with 6% ash extract. The infection cycle may have been extended and the cells only just beginning to burst. The burst size increased with increasing ash extract concentration up to a level equivalent to 6% agar.

Tryptoneyeast glucose agar plates were normally solidified with 1.2% agar whereas the ash extract gave approximately twice as many free phage at

Figure 3·8

EFFECT OF AGAR ASH EXTRACT ON PHAGE PRODUCTION



a level equivalent to 4% agar. This indicated that the level of the necessary factor was sub-optimal in the agar medium.

The hypothesis that agar plates contained sub-optimal amounts of the factor needed for phage lysis was tested as described below. New Zealand Davis agar was washed three times by shaking 50 g agar in 5 l distilled water for 12 hours and filtering off the agar. Tryptone yeast extract agar was prepared using this washed agar. Plates of this washed agar medium containing various levels of added agar ash extract were poured. The soft agar upper layers were prepared with the washed agar and seeded with cells of strain KM and 899a phage. The plates were incubated at 30°C overnight and the plaques counted. The results are given in Table 3.3 below and Figure 3.9.

Table 3.3 Effect of agar ash extract on the relative plating efficiency of bacteriophage 899a

Agar ash extract %*	Plaques
0	82
2	197
4	252
6	305
8	333
10	417

* 1 ml agar ash extract contains the extractable material from 1 g agar.

Increases in relative plating efficiency were still being observed to the point where the equivalent of eight times as much agar as is usually used in solidifying medium had been incorporated as agar ash extract. An attempt was made to identify the active ingredient by analysis.

Analysis of the agar ash was kindly undertaken by Dr R. Brookes of the Department of Chemistry, Biochemistry and Biophysics. The ash was examined in a Hilger and Watts Emission Spectrograph and the relative concentrations of the elements giving the more important spectral lines were determined. These are given in Table 3.4.

Figure 3·9

EFFECT OF AGAR ASH ON PLATING EFFICIENCY

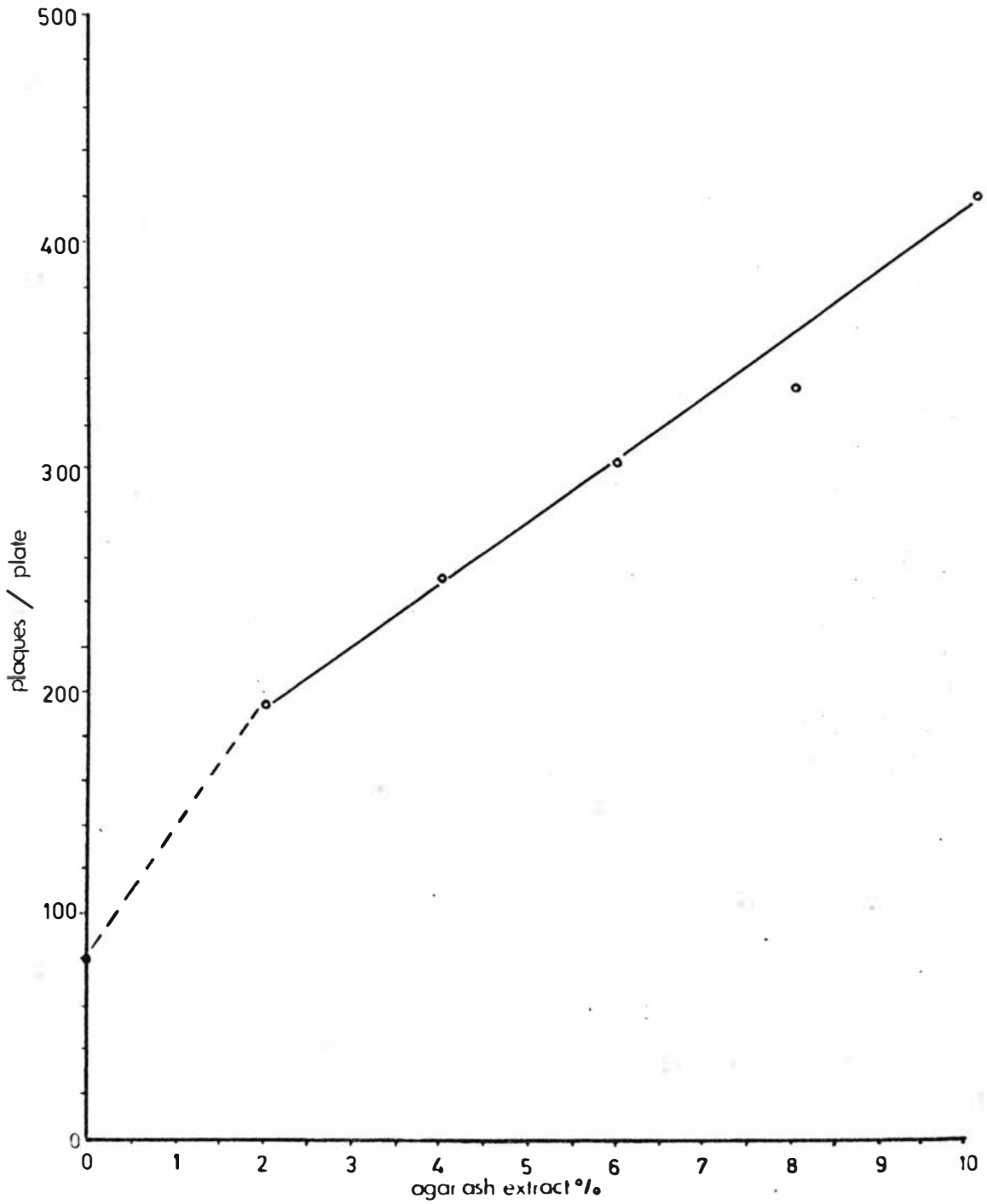


Table 3.4 Analysis of agar ash

Major elements (>100ppm)		Intermediate elements (1-100ppm)		Minor elements (<1ppm)	
Calcium	approx. 1000ppm	Strontium	100 ppm	Molybdenum	1 ppm
Sodium	" 1000 "	Iron	90 "	Vanadium	1 "
Potassium	" 1000 "	Zinc	23 "	Cobalt	0.2 "
Magnesium	" 1000 "	Barium	10 "	Chromium	1 "
Aluminium	" 1000 "	Manganese	3.5 "		
		Nickel	1.5 "		
		Copper	1.5 "		

Batches of tryptone yeast extract medium solidified with washed agar were prepared containing chlorides of the elements in Table 3.4 at the levels shown. Molybdenum, Vanadium and Chromium ions were included at 0.5 ppm. Eighteen batches of medium were prepared, one with none of the elements, one with all of them and sixteen with a different element omitted in each case. Bacteriophage 899a was plated on to each medium and the plates incubated and counted. The results are given in Table 3.5 below.

Table 3.5 Effect of various elements on relative plating efficiency of phage 899a

Element omitted	Plaque count	Element omitted	Plaque count	Element omitted	Plaque count
All	32	Aluminium	520	Nickel	435
None	410	Strontium	410	Copper	300
Calcium	480	Iron	370	Molybdenum	430
Sodium	430	Zinc	425	Vanadium	430
Potassium	440	Barium	400	Cobalt	410
Magnesium	68	Manganese	465	Chromium	530

Magnesium is obviously necessary for optimum plating efficiency and copper may also be involved. The relatively high counts when calcium, aluminium or chromium were omitted suggest that these elements may be inhibitory. The effectiveness of magnesium and copper in promoting phage propagation and of calcium and aluminium in inhibiting it were examined.

Effect of metal ions on plating efficiency

Plates containing 0.4 ppm Cu Cl_2 $2H_2O$ (0.0024 mM) and varying amounts of magnesium ion were prepared and used to plate 899a phage. Plates were also prepared containing 80 ppm $MgCl_2$ $6H_2O$ (0.4 mM) and varying amounts of copper ion. The results of the two experiments are shown in Figure 3.10. The optimum for magnesium was approximately 0.4 mM. Copper reached a maximum effect at 0.0024 mM and had no inhibitory effect up to three times that value. Magnesium had an increasing inhibitory effect on lysis above 100 ppm Mg^{++} so that addition of the optimum concentration of ion is important in achieving maximum plating efficiency.

Plates containing 0.4 mM Mg^{++} and 0.0024 mM Cu^{++} and varying concentrations of aluminium or calcium were prepared and used to plate 899a phage. The results are shown in Figure 3.11. Aluminium was inhibitory at the levels tested while calcium was less inhibitory. The levels of aluminium and calcium expected in an agar gel are approximately 0.05 mM and 0.025 mM respectively which would be expected to cause less than 5% depression of the plating efficiency. The levels of magnesium and copper to provide optimum plating efficiency are approximately ten times the levels usually present in an agar gel.

The effect of glucose concentration on the relative plating efficiency

The counts of plaques during the titring of phage suspensions for these experiments often were irregular. Occasionally plates would have no plaques at all or the plaques would all appear as tiny pin points. Some of this irregularity can be explained by the effect of sub-optimal magnesium in the media but the glucose concentration was also found to be important.

The medium first used for plaque counting contained tryptone, yeast extract and 0.1% glucose. When yields of B. megaterium in different concentrations of glucose were measured there was an indication of inhibition of growth above 0.3% glucose. This suggested that drying of the plates during incubation might cause some local concentration of

Figure 3·10

EFFECT OF MAGNESIUM AND COPPER IONS ON PLATING EFFICIENCY

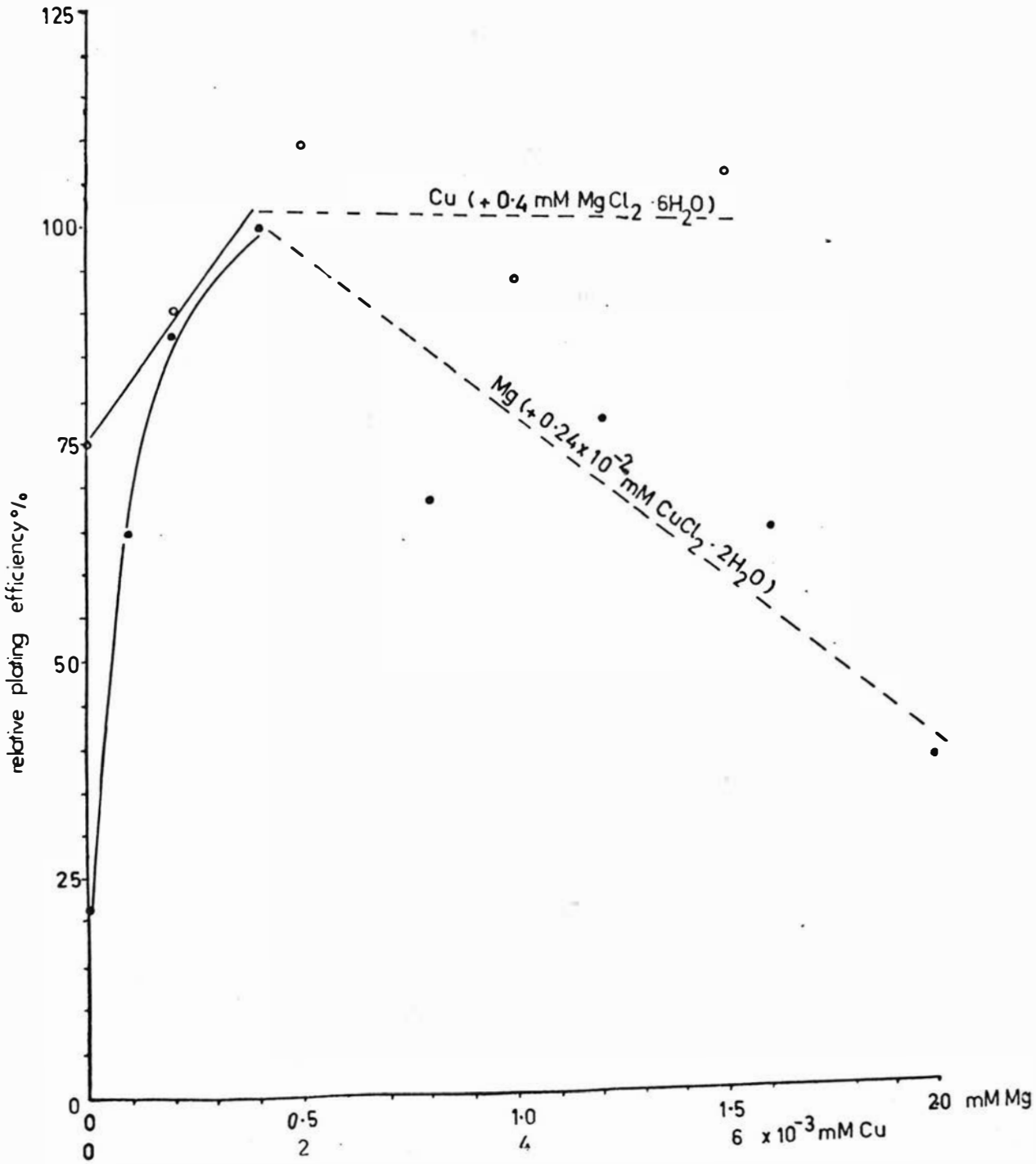
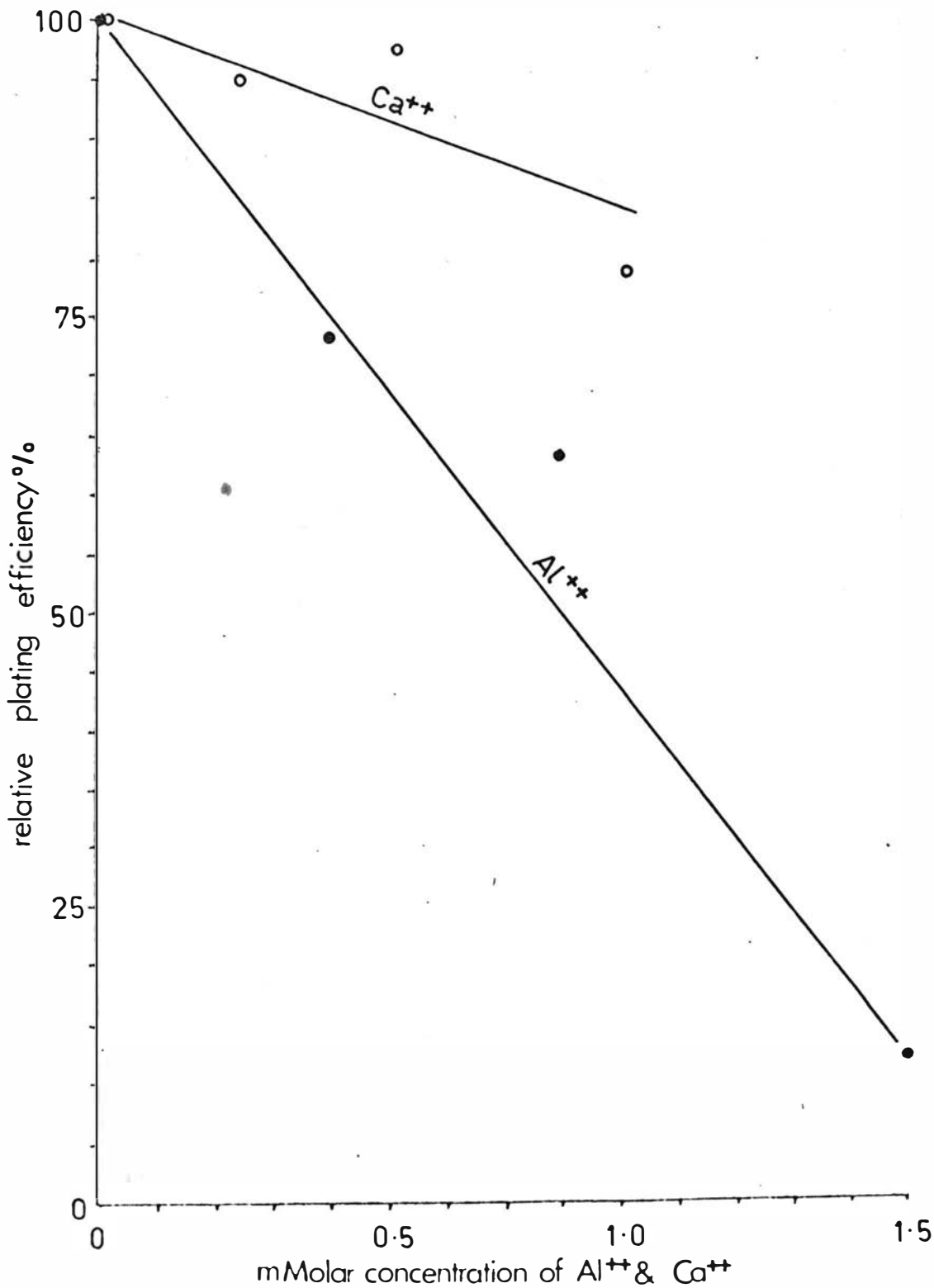


Figure 3·11

INHIBITION OF PLATING EFFICIENCY BY ALUMINIUM AND CALCIUM IONS·



glucose and affect growth of the seed organism. To examine this possibility plates of tryptone yeast extract agar supplemented with Mg^{++} and Cu^{++} were prepared with varying concentrations of glucose in the base and tryptone yeast extract agar with no glucose as the seeded soft agar upper layer. The results are shown in Table 3.6.

Table 3.6 Effect of glucose on relative plating efficiency

Glucose %	0	0.1	0.2	0.4
Relative plating efficiency %	100	86	85	36

The plaques on all plates containing glucose were smaller than those on the control plate. The medium containing 0.4% glucose was particularly striking as the plaques were all pin points and very difficult to count. The density of growth of the seed organism on the media containing glucose was also less, especially in the medium containing 0.4% glucose.

These results, together with those given earlier, led to the formulation of the media for plaque counting given below.

Media for phage counts

Agar Base	Tryptone	5.0 g
	Yeast extract	2.5 g
	Agar	12.0 g
	Water	1 l

Before pouring add 1 ml/100 ml of a sterile solution containing 0.84% $MgCl_2.6H_2O$ and 0.004% $CuCl_2.2H_2O$ (Final concentration = 0.4 mM Mg^{++} and 0.024 mM Cu^{++}).

Soft Agar upper layer	Tryptone	5.0 g
	Yeast extract	2.5 g
	Agar	0.5 g
	Water	1 l

Burst time of megaphage 899a propagated on strain KM

Having established that magnesium and copper ions are necessary for the effective infection of B. megaterium strain KM by megaphage 899a it

was possible to return to the investigation of the burst time of the system.

Tryptone yeast extract broth with and without optimum amounts of magnesium and copper were inoculated with KM and bacteriophage 899a, at a density of approximately 1 phage/10 cells, and phage counts were made at intervals. The results are given in Table 3.7.

Table.3.7 Effect of magnesium and copper on the propagation of phage 899a on strain KM

		Time (minutes)				
		0	20	40	60	80
phage count	Mg Cu absent	22	22	17	9	7
phage count	Mg Cu present	29	58	77	250	6,200

The first big release of phage occurred at about 60 minutes.

Appearance of the bacteriophage

A heavy suspension of B. megaterium strain KM was infected with megaphage 899a at a level of approximately 100 phage/cell. The suspension was incubated for 30 minutes, long enough for adsorption to occur but not long enough to allow lysis, and then one drop of 40% formaldehyde was added. The treated culture was centrifuged and the cells were resuspended in a minimum of distilled water. This suspension was used to prepare grids for electron microscopic examination. Figures 3.12 and 3.13 show the appearance of the bacteriophage free and adsorbed to a cell. The dimensions of the phage are given in Table 3.8.

Table 3.8 Dimensions of megaphage 899a

Head	65 x 58 nm
Tail length	250 nm
Tail width head end	8 nm
Distal end	11 - 12 nm

These measurements and its latent period of 80-90 minutes are similar to the types M1 and M3 of Friedman and Cowles (1953) classification.

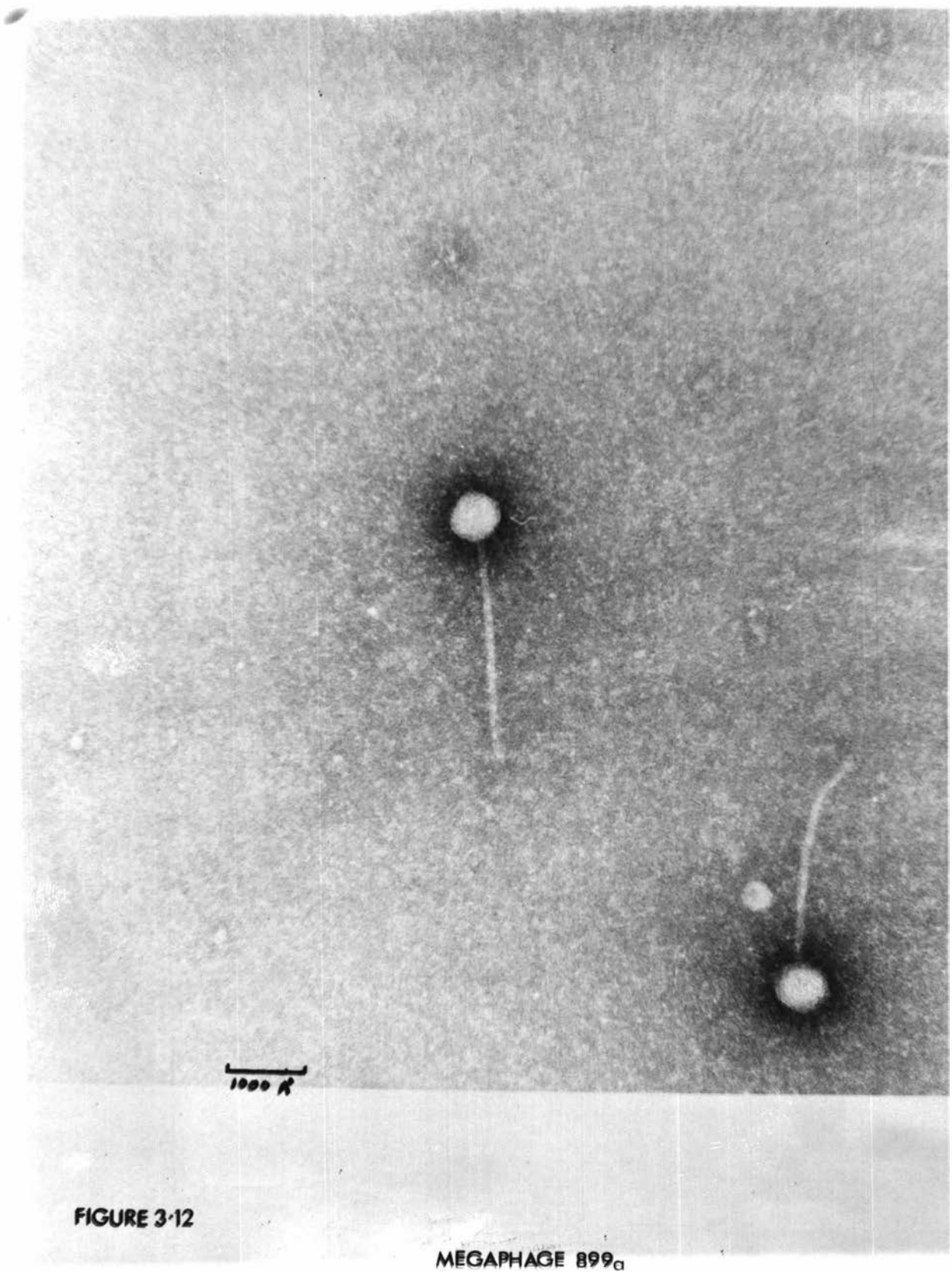


FIGURE 3·12

MEGAPHAGE 899a

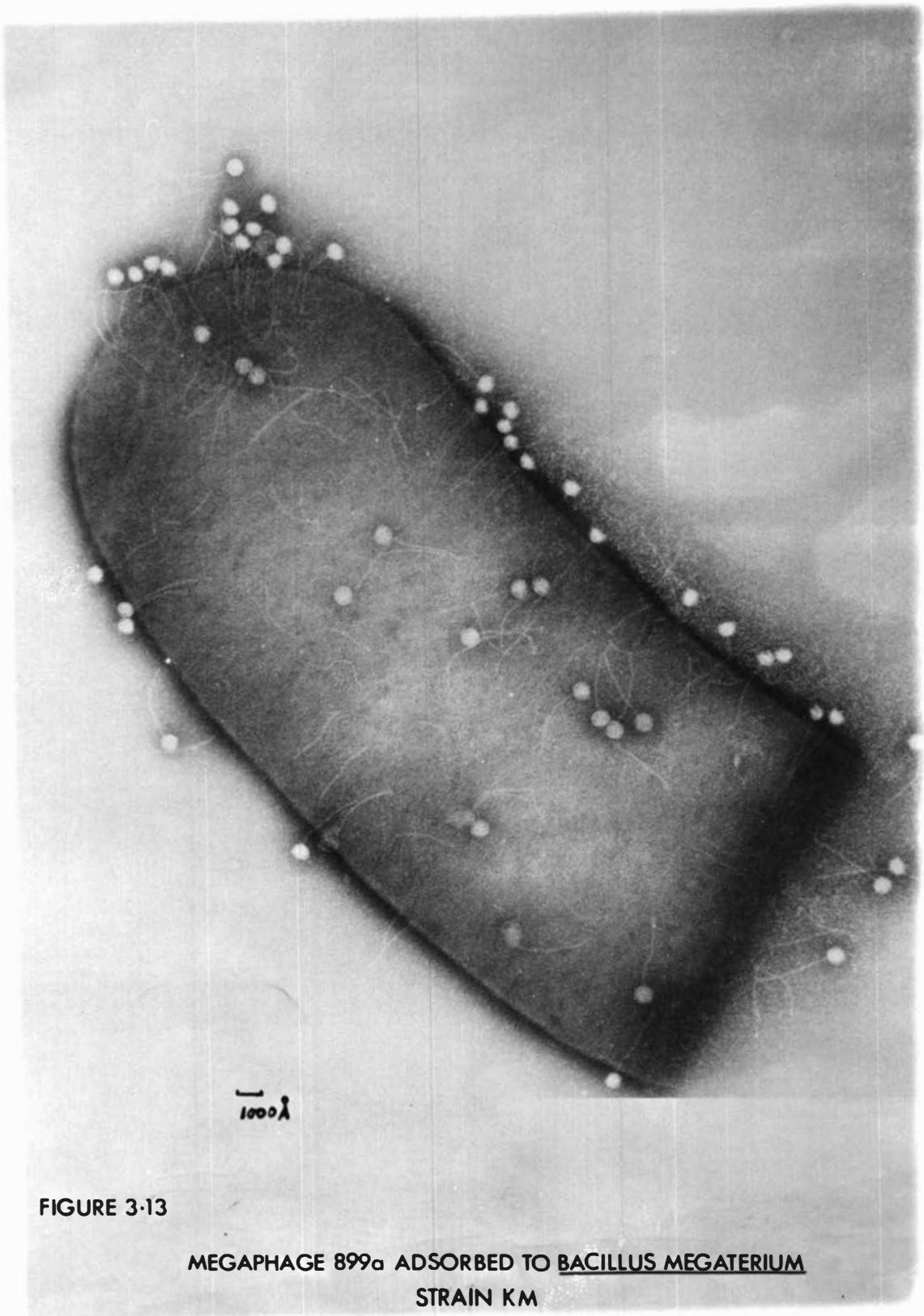


FIGURE 3·13

MEGAPHAGE 899a ADSORBED TO BACILLUS MEGATERIUM
STRAIN KM

Summary

The defined medium for B. megaterium finally adopted differed from those of Lwoff et al. (1950) and Northrop (1951) in that nicotinic acid was found to be necessary for growth. Their cultures required no additional growth factors. Although an additional complex nitrogen source was not necessary for growth, a low level of tryptone increased the rate of growth.

Magnesium was found not to be necessary for induction despite the report of Huybers (1953) but was necessary for the propagation of bacteriophage 899a on strain KM. This was not reported by any of the other workers though Friedman and Cowles (1953) had shown that citrate could reduce the numbers of plaques appearing on plates. This was presumably due to the reduction of available magnesium by the citrate. Calcium was found not to be required for the adsorption or propagation of bacteriophage 899a as reported by Gratia (1936); indeed it had a slightly inhibitory effect on propagation. This inhibitory effect and that of aluminium may be due to competition between metal ions. A comparison of the amounts of magnesium included in media by various workers is made in Table 3.9.

Table 3.9 Millimolar concentrations of magnesium in media for B. megaterium

Lwoff (1950)	Northrop (1951)	Huybers (1953)	others	Basal medium	this study
1.2	0.1	0.1	0	0.4	4.0

Other workers were able to obtain phage propagation at much lower levels of magnesium than were found to be necessary in this study. This suggests either that they had sub-optimal levels of magnesium present or that there has been a change in the characteristics of the phage or the sensitive strain.

The effect of glucose on relative plating efficiency does not seem to have been noted by previous workers. It is a curious phenomenon and merits further investigation.

The burst time of about 60 minutes is similar to that reported by Lwoff (1951). He found that the optical density of the infected cultures

started to decline at 60 minutes and the first detectable increase in phage numbers occurred earlier at 45 minutes. In this study the rapid increase in phage numbers started between 40 and 60 minutes. The relationship between latent time of induction and burst time will be discussed more fully in the next section.

SECTION IV

INDUCTION OF LYSOGENIC

B. MEGATERIUM BY

AFLATOXIN B$_1$

SECTION IV

INDUCTION OF LYSOGENIC B. MEGATERIUM BY AFLATOXIN B$_1$

Induction of lysogenic bacteria by aflatoxin was first reported by Legator (1966) for E. coli and Staph. aureus. He suggested that all inducible lysogenic bacteria would be affected in the same way. Later Lillehoj and Ciegler (1970) reported induction of B. megaterium by aflatoxin though at concentrations a hundred times greater than those reported by Legator. The idea of using induction as the basis for a bioassay is attractive for a variety of reasons. It is a microbiological system, which requires less equipment and skill to handle than a system using higher organisms. It is likely to give a result more quickly than an animal system. The main potential advantage of induction, however, is its reported specificity. Relatively few compounds are able to induce lysogenic cells but, more importantly, there is claimed (Heineman and Howard (1964) and Lein et al. (1962)) to be a correlation between the inducing ability of a compound and its ability to cause cancer. If this is the case then interference by other compounds having the same biological effect as aflatoxin in an assay would be an advantage rather than otherwise since one would be made aware of a potentially dangerous toxin.

The failure to repeat Legator's work with E. coli and Staph. aureus has been reported in Section I. While this result was discouraging the subsequent confirmation of induction of B. megaterium by aflatoxin reopened the possibility of using this system as the basis of a bioassay. It was necessary, however, to repeat and amplify the bare observation of induction and to find out more about the behaviour and requirements of the organisms involved in the system. Some of this work has already been described in Section III. The work described in this section is concerned with the induction system itself and its eventual development into a usable bioassay.

Response of lysogenic B. megaterium to varying aflatoxin concentrations

An essential requirement for a quantitative bioassay is that the response of the system to the compound being assayed shall vary in some

regular relationship with the amount or concentration of the compound. The first experiment which has already been described in Section I had confirmed the occurrence of induction. The second experiment attempted to show a concentration/response relationship.

B. megaterium strains 899a and KM were grown overnight in nutrient broth. Strain 899a was sub-cultured in fresh nutrient broth and incubated in a shaking water bath at 37°C until the culture had reached an OD of 55 Klett units. The culture was centrifuged at 3000 g for 15 minutes and washed twice in saline to minimise the numbers of free phage. The cells were resuspended and added to aliquots of nutrient broth containing various concentrations of aflatoxin B_1 so as to give a density of cells approximately 1/10 the original culture.

(In this early experiment the aflatoxin was added to the medium as a concentrated chloroform solution and the chloroform removed by bubbling nitrogen through the medium. This was subsequently shown to be an unsatisfactory method of adding the toxin to the medium and as a result the concentrations shown in the table may not be reliable).

The broth cultures were incubated in a shaking water bath at 37°C and samples removed after 1½ hours for viable counts and phage counts using strain KM as the indicator strain. The results are given in Table 4.1 below.

Table 4.1 Induction of strain 899a by various amounts of aflatoxin B_1

		aflatoxin concentrations (µg/ml)			
	Time (hours)	0	5	10	20
Viable counts $X10^{-3}$	1½	8900	7500	7600	1
Phage counts $X10^{-4}$	1½	4500	7000	>10,000	12

Although the dilutions chosen for plating were not the most appropriate the results suggest a response proportional to aflatoxin concentration. They also indicate that above a certain level, aflatoxin is inhibitory to the extent that both growth and induction are suppressed.

The two investigations described so far showed an increase in the numbers of phage following aflatoxin treatment, but no corresponding decrease in optical density. A number of attempts were made to demonstrate

lysis by a decrease in optical density without success. In retrospect it is now clear that this was due to the unsatisfactory method of addition of aflatoxin B_1. Later the method of addition using ethanol as a vehicle was tried (see Materials and Methods Section) with the results given below.

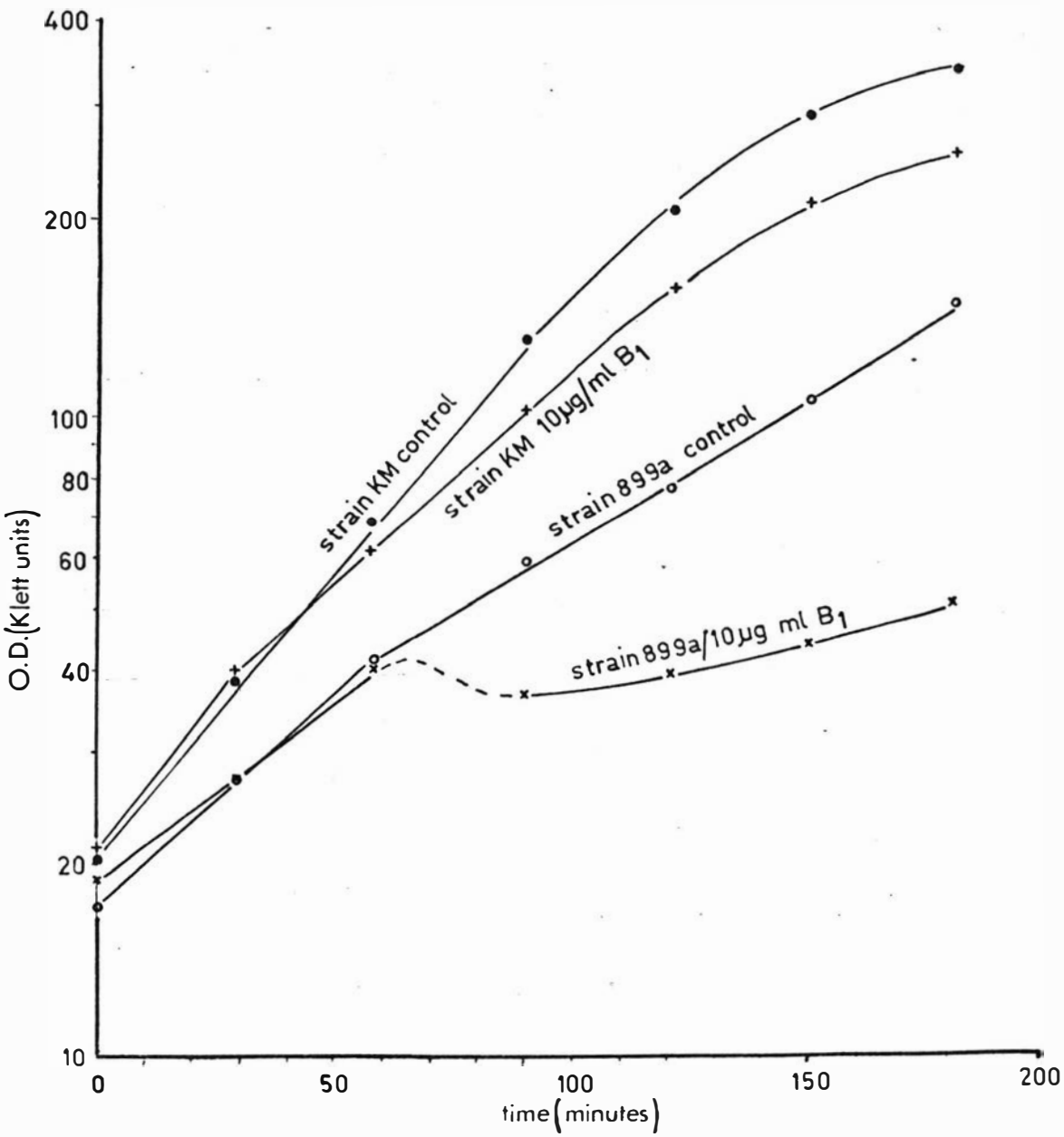
Strains KM and 899a were grown in basal medium supplemented with 0.25% glucose, 0.1 µg/ml nicotinic acid and 0.1% tryptone. Each strain was diluted 1/10 in the same medium containing 10 µg/ml aflatoxin B_1 added as a 1000 µg/ml solution in ethanol. Controls containing only the ethanol were also included. The cultures were incubated in a shaking water bath at 35°C and optical density measurements taken at intervals. Smears were made of the cultures at 150 minutes and stained for microscopic examination. The slides of both control and treated cultures of strain 899a and also of the control culture of strain KM showed normal rods in chains but the slide of strain KM grown in the presence of aflatoxin showed mostly long filaments. This appearance is similar to that reported by Wragg et al. (1967) for E. coli and by Lillehoj et al. (1967) for Flavobacterium aurantiacum.

The results of the optical density measurements are given in Figure 4.1. A drop in optical density occurred between 60 and 90 minutes only in the culture of the lysogenic strain 899a containing aflatoxin. Thereafter growth was resumed but more slowly. This curve has a number of interesting features not reported by Lillehoj and Ciegler in their study on B. megaterium. The initial growth rate in the presence of aflatoxin is little different from that in the control (generation times 55 and 45 minutes respectively). The decrease in OD after 60 minutes is followed after a delay of a further 30 minutes by a resumption of OD increase at a slower rate (equivalent to a generation time 135 minutes). The final OD values after overnight incubation of strain 899a were 293 for the control and 49 for the aflatoxin-treated culture indicating that further lysis had occurred in the latter before exhaustion of the medium.

The inflexion in the OD curve could be interpreted as being due to the lysis of a proportion of the cells in the culture which was induced immediately the cells came into contact with the aflatoxin. Their rate of

Figure 4·1

GROWTH OF B. MEGATERIUM STRAINS 899a AND KM IN THE PRESENCE OF AFLATOXIN B_1



OD increase was unaffected however until they lysed approximately one generation later. It may be assumed that some proportion of cells would be induced throughout the remainder of the time that the culture was under observation and these too would lyse after the elapse of the latent period. This interpretation would account for both the observed fall in OD after an initial period of increase and the subsequent period of increase at a slower rate. The actual rate of OD increase after the initial lysis would depend on the growth rate and the proportion of cells induced.

The time course of phage production after induction

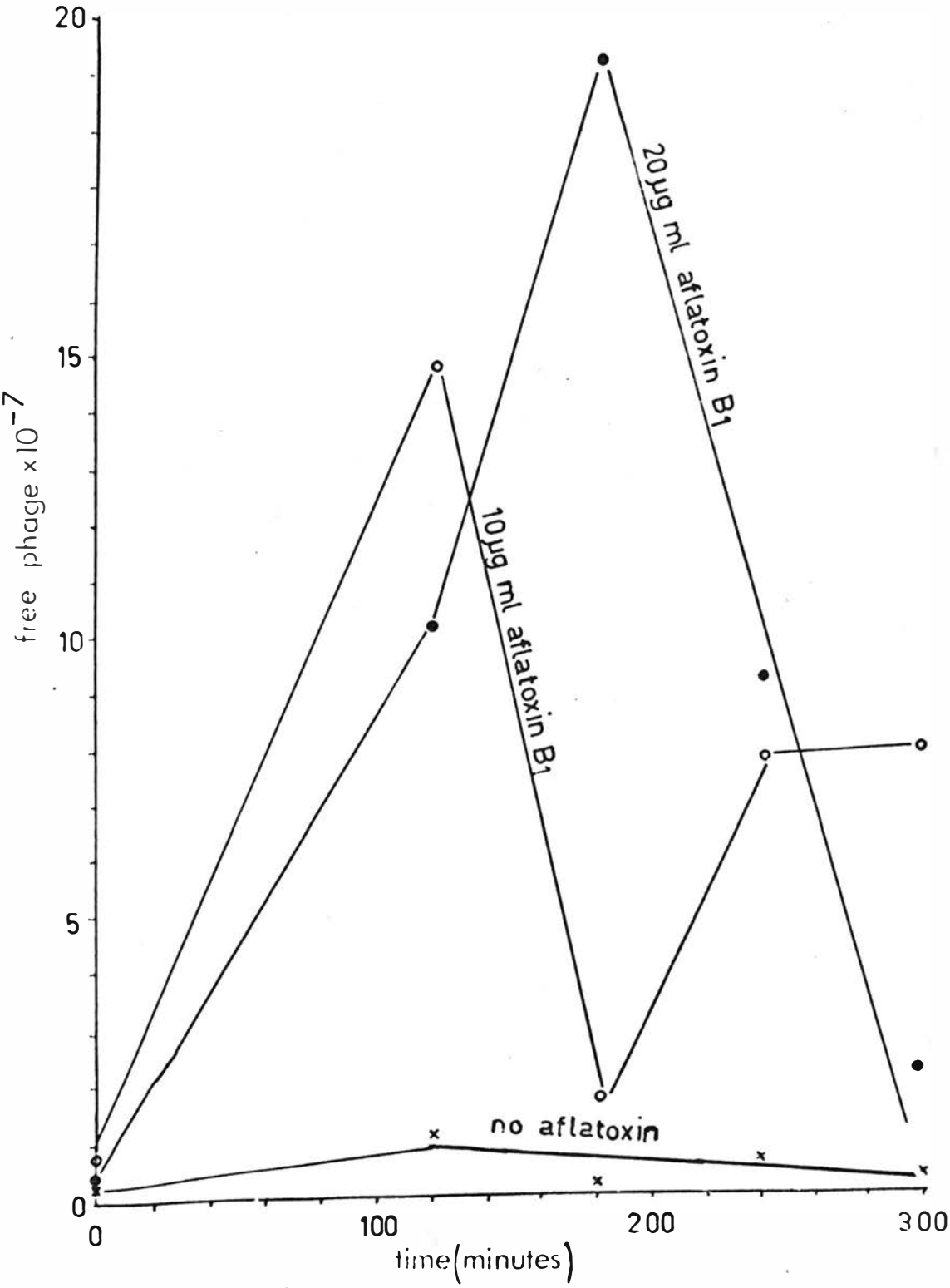
The changes in the optical density curve of strain 899a in the presence of aflatoxin prompted the investigation of the time course of phage production following induction by aflatoxin.

B. megaterium strain 899a was grown to an optical density of 110 Klett units in SDM. Media containing 0, 10, and 20 µg/ml aflatoxin B_1 were prepared by incorporating the appropriate amounts of a 1000 µg/ml solution of aflatoxin to 10 ml SDM using acetone as the vehicle in place of ethanol (see Materials and Methods). Sufficient additional acetone was added to all the flasks to give a concentration of 2% acetone in each medium. 0.2 ml of the exponential culture of strain 899a was added to each medium in a side arm flask. Optical density measurements were made at thirty minute intervals and free phage counts were made at zero time and at 120, 180, 240 and 300 minutes. Results are given in Figure 4.2.

Phage production was greater in the presence of 20 µg/ml than in 10 µg/ml aflatoxin B_1. The effect on optical density was also more marked in the presence of 20 µg/ml aflatoxin B_1. The results show two unexpected features, however. There was a small apparent increase in the free phage numbers in the control culture. Furthermore in all three cultures a decrease in free phage occurred after the first burst. Both observations were subjected to further examination in later experiments. Though there was a marked increase in the numbers of free phage in those cultures containing aflatoxin and a decrease in the optical density, the intervals chosen were not sufficiently close to determine whether the phage burst and OD decrease coincided. This was examined in the next experiment.

Figure 42

INDUCTION OF BACTERIOPHAGE 899a BY AFLATOXIN B_1



Comparison of phage production and OD decrease

B. megaterium strain 899a was grown to an OD of 190 Klett units in SDM and diluted one tenth into flasks containing SDM supplemented with either 1% acetone alone or 1% acetone and 10 µg/ml aflatoxin B_1. The cultures were incubated at 35°C and samples were removed into chloroformed diluent at intervals of 30 minutes when OD measurements were also made. Appropriate dilutions of the samples were assayed for phage. Results are given in Table 4.3 and Figure 4.3.

Table 4.3 Phage counts after induction of 899a by aflatoxin B_1

Time (minutes)	0	30	60	90	120	150	180
Control $X10^{-4}$	26	28	680	260	23	11	19
10 µg/ml aflatoxin B_1 $X10^{-4}$	17	58	7,700	22,000	12,100	3,400	2,800

In both control and treated cultures a peak of phage production was observed. In the control culture this occurred at sixty minutes after dilution of the parent culture and in the aflatoxin-containing culture a much larger peak recurred later between sixty and ninety minutes after dilution. Closer time intervals were used in a further experiment (see Figure 4.4) where the time of sixty minutes for the phage peak in the control culture was confirmed while a time of approximately eighty minutes was found for the aflatoxin-containing culture. The latter peak coincided closely with the fall in OD. The rate of decline in free phage numbers after the peak varied in different experiments but the same pattern was observed in all cases. This decrease in the numbers of free phage after the peak was assumed to be due to their adsorption on to unlysed cells and fragments of cell wall of lysed cells. The number of free phage in the aflatoxin-containing cultures after the peak had subsided was still greater than the numbers in the control.

Phage production after the initial peak

The period after the peak of phage production was investigated to see whether there were further peaks or whether a relatively steady state was reached. Samples from cultures containing 0, 5 and 10 µg/ml aflatoxin B_1

Figure 43

OPTICAL DENSITY AND PHAGE COUNTS AFTER INDUCTION

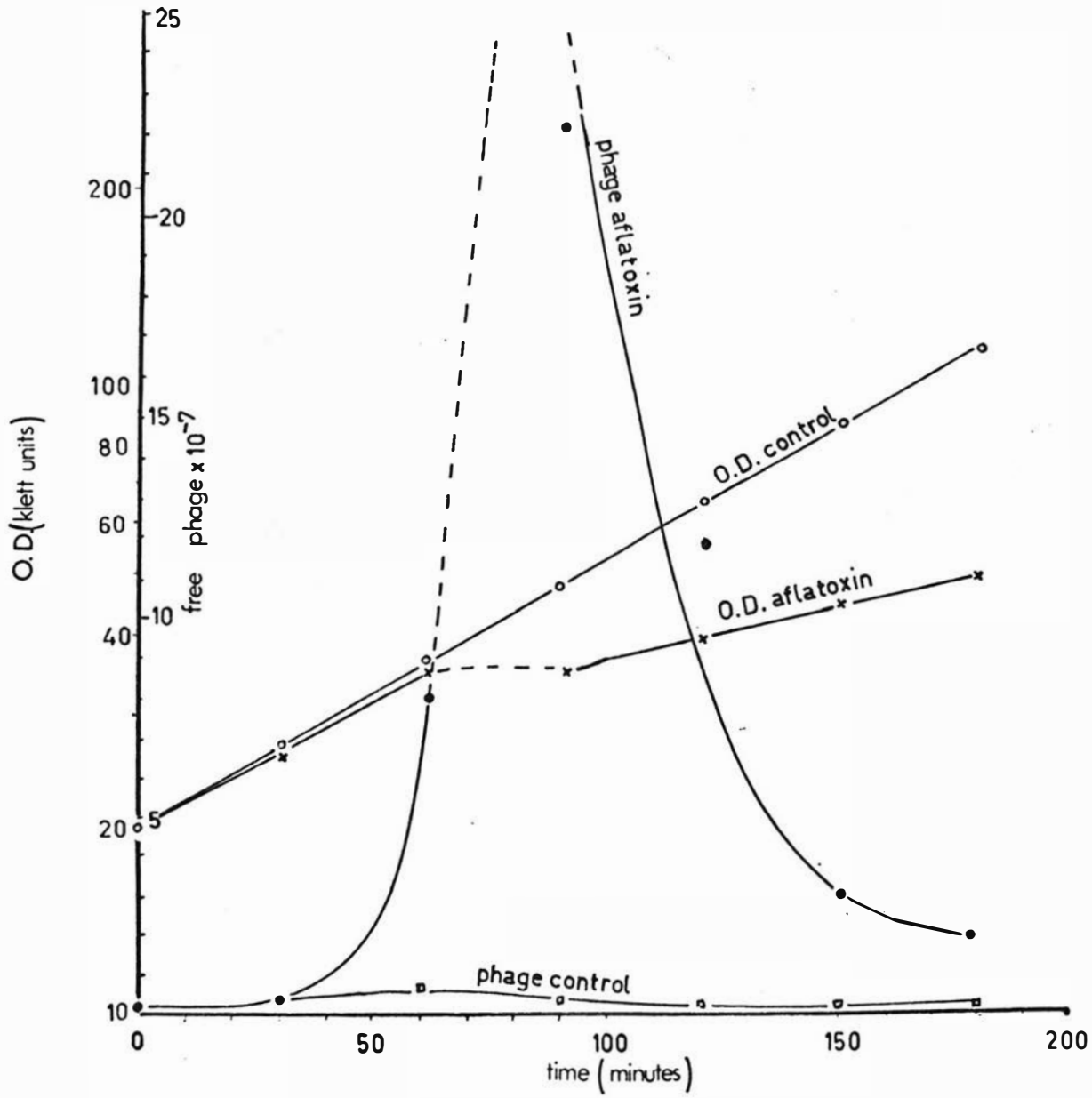


Figure 4·4

TIME COURSE OF PHAGE PRODUCTION AFTER INDUCTION BY AFLATOXIN

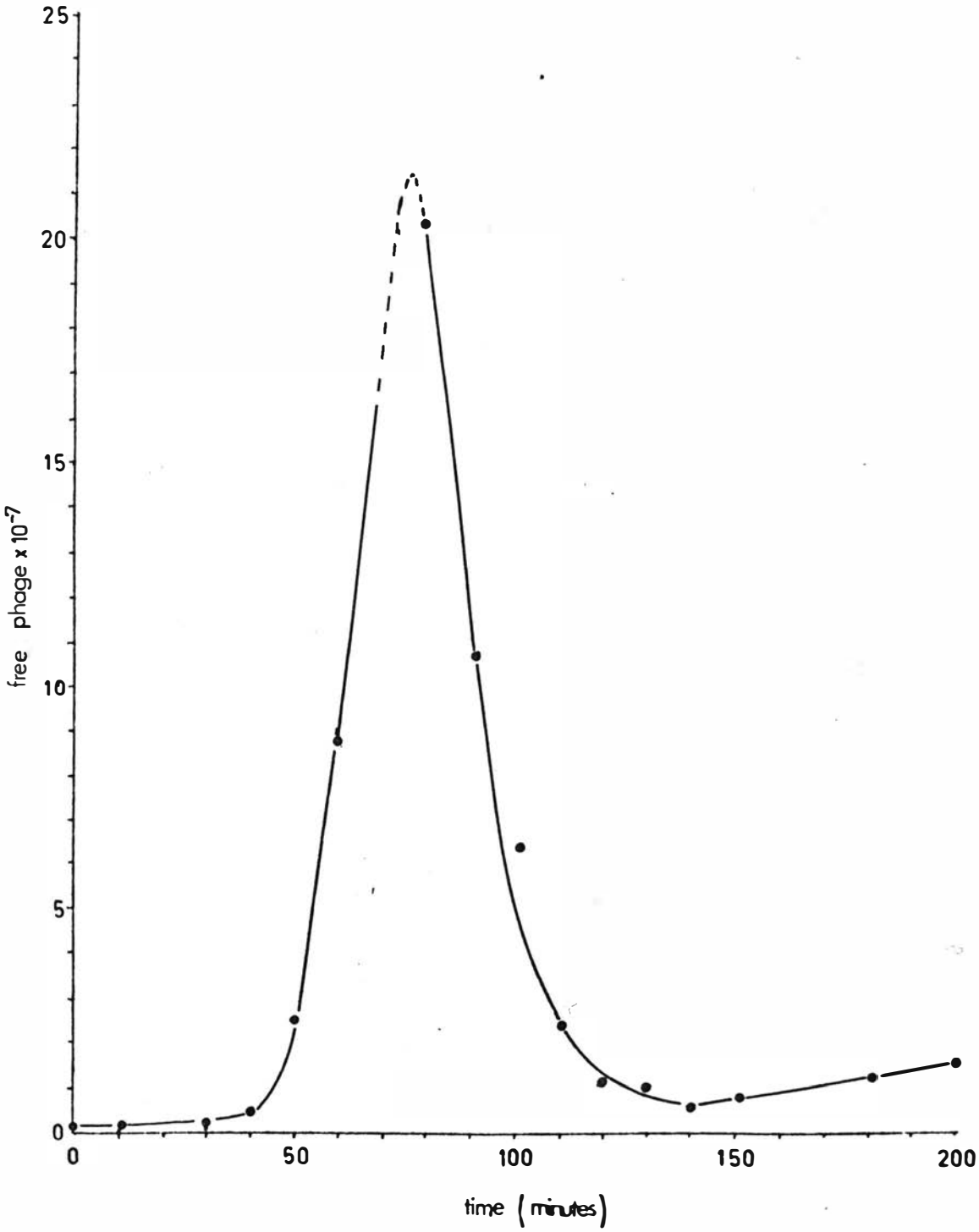


Figure 4.4 SDM + 10 µg/ml aflatoxin B_1 inoculated with exponential phase cells of *B. megaterium* strain 899a. Samples chloroformed and counted in TM + Mg and Cu.

were assayed for free phage at thirty minute intervals between 150 and 330 minutes after dilution. Samples were also taken at 0 and 75 minutes to confirm the presence of the peak. The results are given in Figure 4.5. The decrease in numbers of free phage appeared less rapid than in previous experiments where the rate of decrease was almost as great as the rate of increase.

In each culture there was an increase in free phage again after the period of decline. The rapidity of this second increase was greater when the aflatoxin concentration was higher. This is consistent with the earlier suggestion of continuing lysis after the initial burst. The free phage present in the medium would represent the difference between those being produced by lysis and those being removed by adsorption.

Induction following dilution

Ideally the size of the burst of phage due to induction should be compared with free phage numbers in an uninduced control. The existence of a small peak of phage production in the control cultures distorts this comparison. The effect had also been observed by Lwoff et al. (1950). They reported that as many as 20-30% of bacteria lysed after dilution. The actual proportion varied with the stage of growth and was at a maximum just before stationary phase. They investigated the effect of redox potential and medium conditions but were unable to find any clear cause for the induction although oxygen level appeared to play a role. It seemed possible that the shock of transferring a culture from a temperature of 35°C to medium at room temperature, as had been done in all my previous work, might effect some induction. This was tested by comparing duplicate cultures diluted into medium at room temperature and medium pre-warmed to 35°C. Since no effect on the dilution induction was observed it was concluded that temperature change was not a factor in this phenomenon.

Decline in free phage numbers after the initial burst

The sharp peak in the numbers of free phage of the initial burst makes it very difficult to choose an appropriate time at which to sample.

Figure 4·5

PHAGE PRODUCTION AFTER THE INITIAL BURST

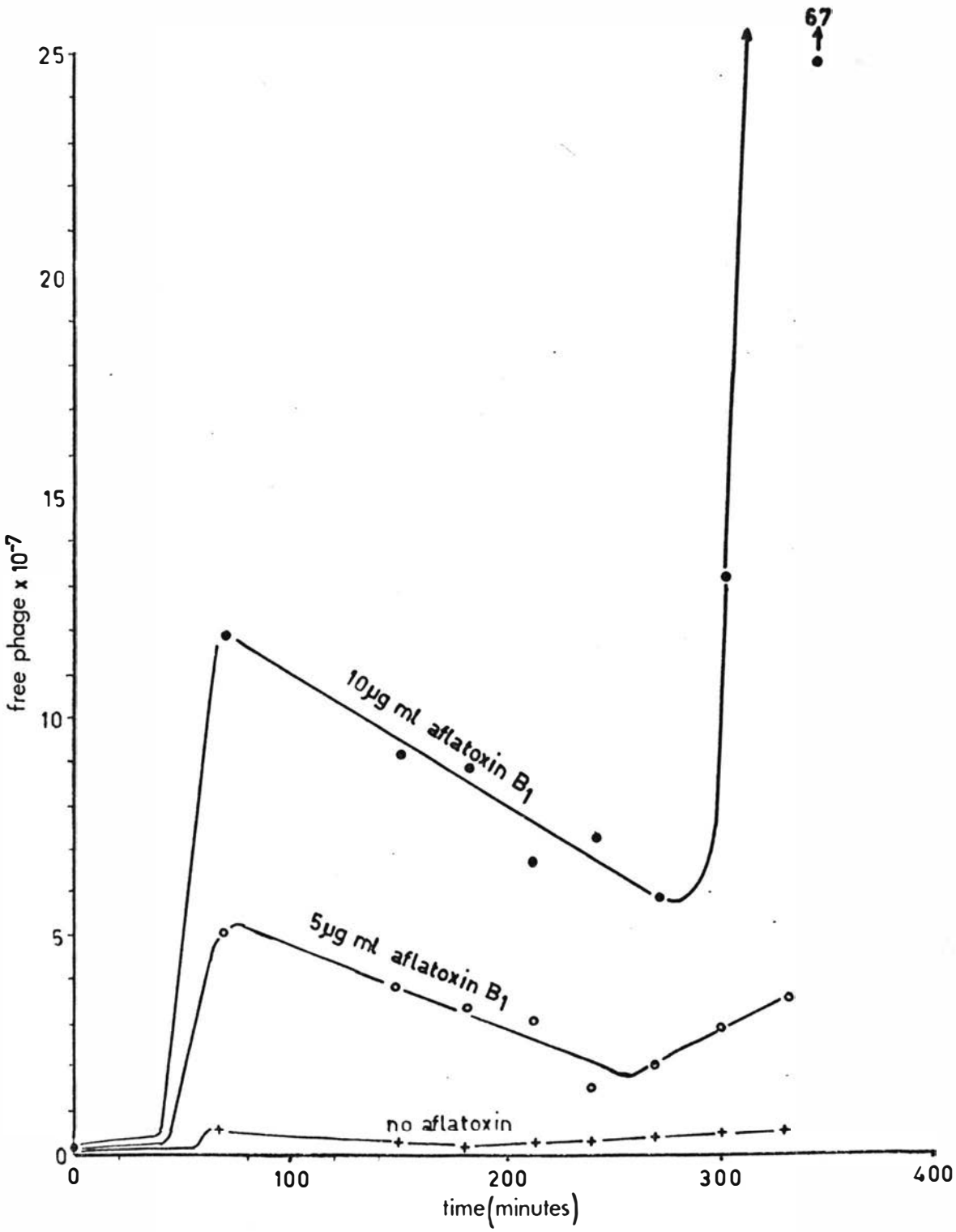


Figure 4.4 shows that the free phage numbers in one experiment rose and fell tenfold within sixty minutes.

If the decrease in free phage numbers after the initial burst is due to adsorption this should be eliminated by diluting the culture after treatment with aflatoxin for sufficient time for induction to have been initiated.

Cultures of strain 899a were held for 20 minutes in SDM with and without 10 μg/ml aflatoxin B_1 and at an OD of 10 Klett units and were then diluted 1/100 into fresh pre-warmed medium and sampled at intervals. The samples were assayed for free phage. This experiment was carried out twice. On both occasions lysis was delayed until approximately 120 minutes after dilution.

In both the control and the aflatoxin-containing culture there was a peak of phage production and a decline.

This dilution was not, therefore, effective in preventing the decline in numbers following the burst.

Effect of magnesium on induction by aflatoxin B_1

The work described so far was done before the effects of magnesium on plating efficiency (Section III) had been investigated. Since magnesium was so important in the infection cycle its effect on induction had to be examined. A culture of *B. megaterium* strain 899a was grown to an OD of 190 Klett units and diluted 1/100 into media containing 2 μg/ml aflatoxin B_1 with and without magnesium and copper. A control containing the metals but no aflatoxin was also included. Free phage in the cultures were assayed at intervals. The results are given in Table 4.4.

Table 4.4 Effect of magnesium and copper on induction of bacteriophage 899a by aflatoxin B_1

	free phage counts $X10^{-3}$				
Time (minutes)	0	40	80	120	160
Control	2	2	3	23	85
2 μg/ml B_1 alone	5	3	1	63	247
2 μg/ml B_1 + metals	3	2	4	64	225

There was no real difference in free phage counts due to the presence of the metal ions.

Counting induced cells

At this point sufficient information had been accumulated to make it clear that aflatoxin B_1 was able to induce bacteriophage 899a and that the degree of induction was dependent on the concentration of toxin. It was difficult however to find a reliable parameter for measurement of the effect. Because the peak of burst is transient it would be difficult to select a sampling time that would coincide with the peak. There are also large variations in the numbers of free phage in the phase after the burst. In the hope that it might prove a more reliable parameter attention was concentrated on finding a way of counting the actual numbers of induced cells rather than the progeny of their lysis. B. megaterium strain 899a was grown in SDM to an OD of 150 Klett units. The culture was then centrifuged at 3000 g for 20 minutes at room temperature, washed once in peptone diluent and resuspended in diluent to its original volume. This washing was done to remove free phage from the culture. The suspension was diluted 1/10 into duplicate flasks of SDM. After ten minutes incubation at 32°C acetone was added to one flask and an acetone solution of aflatoxin B_1 to the other to give final concentrations of 1% acetone and 10 µg/ml aflatoxin B_1. Samples were removed at intervals and plated unchloroformed for infectious centres on TYA.

On examination the plates from both treated and untreated samples showed a mixture of large and small plaques. The total numbers of plaques were approximately the same in both series but the proportion of large plaques increased more rapidly in the plates from the culture containing aflatoxin B_1 than in those from the control. It was not possible to make accurate counts of the larger plaques but estimates of their numbers are given in Table 4.5.

Table 4.5 Estimates of the numbers of large and small plaques following induction

Time (minutes)	Total plaques	Large plaques	
		Control	B_1 treated
0	560	80	80
10	550	60-100	80
30	530	60-100	180
50	540	60-100	520
70	800	150	650

If it is assumed that all viable cells of B. megaterium strain 899a if plated, will eventually produce bacteriophage by spontaneous induction, then it is reasonable to suppose that the earlier lysis occurs after plating, the larger the resulting plaque will be, since more cycles of infection will have been able to occur. In that case the largest plaques represent cells which lysed very soon after plating and the smallest plaques represent cells which lysed not long before the plate was removed from the incubator. That the culture containing aflatoxin gave rise to many more large plaques than did the control culture could be explained as being due to induction by the aflatoxin.

It is not possible to count the various sized plaques accurately because of the continuous gradation in size from large to small. For the technique to be of any use it was necessary to suppress those cells which lyse spontaneously after plating and permit to lyse only those cells which were induced before plating.

An attempt was made to achieve this result by killing the lysogenic cells with streptomycin immediately after the cells induced by aflatoxin had burst. If this was to succeed the indicator organism had to be resistant to streptomycin or no plaques would be formed. A streptomycin resistant mutant of the phage-sensitive strain KM was isolated (see Materials and Methods). It was also established that the lysogenic strain 899a was streptomycin-sensitive. Strain 899a was grown, washed and resuspended as before and diluted 1/10 into SDM with and without 10 µg/ml aflatoxin B_1. Samples were taken at fifteen minute intervals and appropriate dilutions mixed with streptomycin-resistant strain KM cells (KM/Sr) in 3.5 ml soft agar. The soft agar was poured over plates containing tryptone yeast extract agar supplemented with magnesium and copper and 20 µg/ml streptomycin. A duplicate series was made in which the streptomycin was omitted. A chloroformed sample was also plated in order to assay the free phage. The results are given in Table 4.6.

Table 4.6 Effect of streptomycin on the plaque count of induced cultures

Time (minutes)		0	15	30	45	60
No aflatoxin	Strept present	24	16	8	30	67
	Strept absent	>1000	>1000	>1000	>1000	>1000
10 µg/ml aflatoxin	Strept present	22	12	21	64	340
	Strept absent	>1000	>1000	>1000	>1000	>1000
Chloroformed sample		0				

It was hoped that the time taken for the streptomycin to diffuse into the soft agar layer would allow only those cells already committed to lysis when they were plated to complete the cycle. All others would be killed before lysis could occur. There was certainly a marked reduction in the numbers of plaques on the plates containing streptomycin. There was also an increase in the number of plaques on the plates from the aflatoxin-containing culture after 45 minutes. The reduction in total numbers was so great however as to suggest that a proportion of the induced cells were killed before they could lyse. The method therefore was modified to allow more time for the cycle to be completed. Instead of incorporating streptomycin in the lower layer of the double layer plates, a third layer of plain agar containing 20 µg/ml streptomycin was poured over the upper soft agar layer. Before the streptomycin layer was applied the double layer plate was incubated for fifty minutes. Strain 899a was incubated in SDM containing 0, 2, 4, 6 and 8 µg/ml aflatoxin B_1 for 50 minutes. Samples were diluted and plated and the plates incubated for a further 50 minutes. The plates were then overlaid with a layer of plain agar containing 20 µg/ml streptomycin and returned to the incubator overnight. Results are given in Table 4.7.

Table 4.7 Effect of adding streptomycin after 50 minutes incubation

aflatoxin conc. µg/ml	0	2	4	6	8
Plaque count	8	26	102	92	87

There was no further increase in plaque count above 4 µg/ml aflatoxin but at and above that level there was a tenfold increase over the count in the control plate.

Effect of sampling and incubation times on the plate count

Further investigations were made to determine the optimum times for which the plates should be incubated before overlayering with streptomycin-containing agar and the time at which the reaction mixture should be transferred to the plates.

In the first experiment samples were removed from reaction mixtures containing 0 and 10 µg/ml aflatoxin B_1 after 50 minutes. Plates were overlayered with streptomycin agar after incubation for 70, 90, 110 and 130 minutes. Results are shown in Table 4.8.

Table 4.8 Effect of incubation time on plate count

		Incubation time (minutes)			
	aflatoxin conc. (µg/ml)	70	90	110	130
Plaque counts	0	129	165	220	210
	10	560	560	560	560

The aflatoxin-treated culture was unaffected by the duration of the subsequent incubation but the control culture showed a slowly increasing count up to 110 minutes.

In the next experiment samples from medium containing 0 and 10 µg/ml aflatoxin were removed at 40, 50, 60 and 70 minutes. Subsequent incubation was allowed to bring the total time from the start of the experiment to 150 minutes. Results are given in Table 4.9.

Table 4.9 Effect of sampling time on plate count

Sampling time (minutes)	40	50	60	70
Incubation time (minutes)	110	100	90	80
No aflatoxin	190	175	600	1200
Plaque counts				
10 µg/ml aflatoxin B_1	950	1650	1800	2000

Sampling at 50 minutes gave the largest difference between control and aflatoxin-treated culture. From the results of these two experiments a combination of 50 minutes treatment in aflatoxin followed by 90 minutes incubation before overlayering was taken as optimal.

The induction bioassay for aflatoxin

The final form taken by the assay is set out below.

Preparation of cultures:

1) Grow Bacillus megaterium strain 899a in 10 ml semi-defined medium to an OD of 100 Klett units. Centrifuge the culture at 3000 g for fifteen minutes at 4-10°C. Resuspend in 10 ml diluent peptone and centrifuge again. Resuspend in 10 ml diluent peptone.

At the same time prepare an actively growing culture of Bacillus megaterium strain MM/Sr in semi-defined medium. The optical density of the culture should be approximately 150 Klett units but the exact optical density is not too important provided the culture is sufficiently dense and is actively growing when it is used.

2) Aflatoxin treatment:

Prepare the samples for examination by dissolving dried toxin in acetone and adding the solution to 2 ml semi-defined medium containing only 0.1% glucose contained in a 25 ml Erlenmeyer flask. The final concentration of acetone should not exceed 3.0% and the final concentration of toxin should be less than 12 µg/ml aflatoxin B_1. Prepare standard media containing 0, 5 and 10 µg/ml aflatoxin B_1. All the media should have acetone added to them to bring the level up to that of the medium containing the greatest amount of acetone.

Dilute the washed suspension of strain 899a one hundredth and add 0.2 ml to each of the test flasks. Swirl to mix and incubate the flasks in a shaking water bath at 32°C.

3) Sampling and plating:

After 50 minutes remove 0.5 ml from each flask into 4.5 ml diluent. Mix by shaking, and transfer 0.1 ml in duplicate to bijou bottles containing 3.5 ml soft yeast extract tryptone agar. Add three drops (approximately 0.1 ml) of the MM/Sr culture to each bijou bottle, mix by inversion three or four times and distribute the contents over the surface of a plate containing 10 ml yeast extract tryptone agar supplemented with magnesium and copper. Allow the plates to set and incubate at 30°C for 90 minutes. Then overlayer the plates with 5 ml plain agar containing 20 µg/ml streptomycin added as a sterile solution just before pouring. Incubate plates overnight.

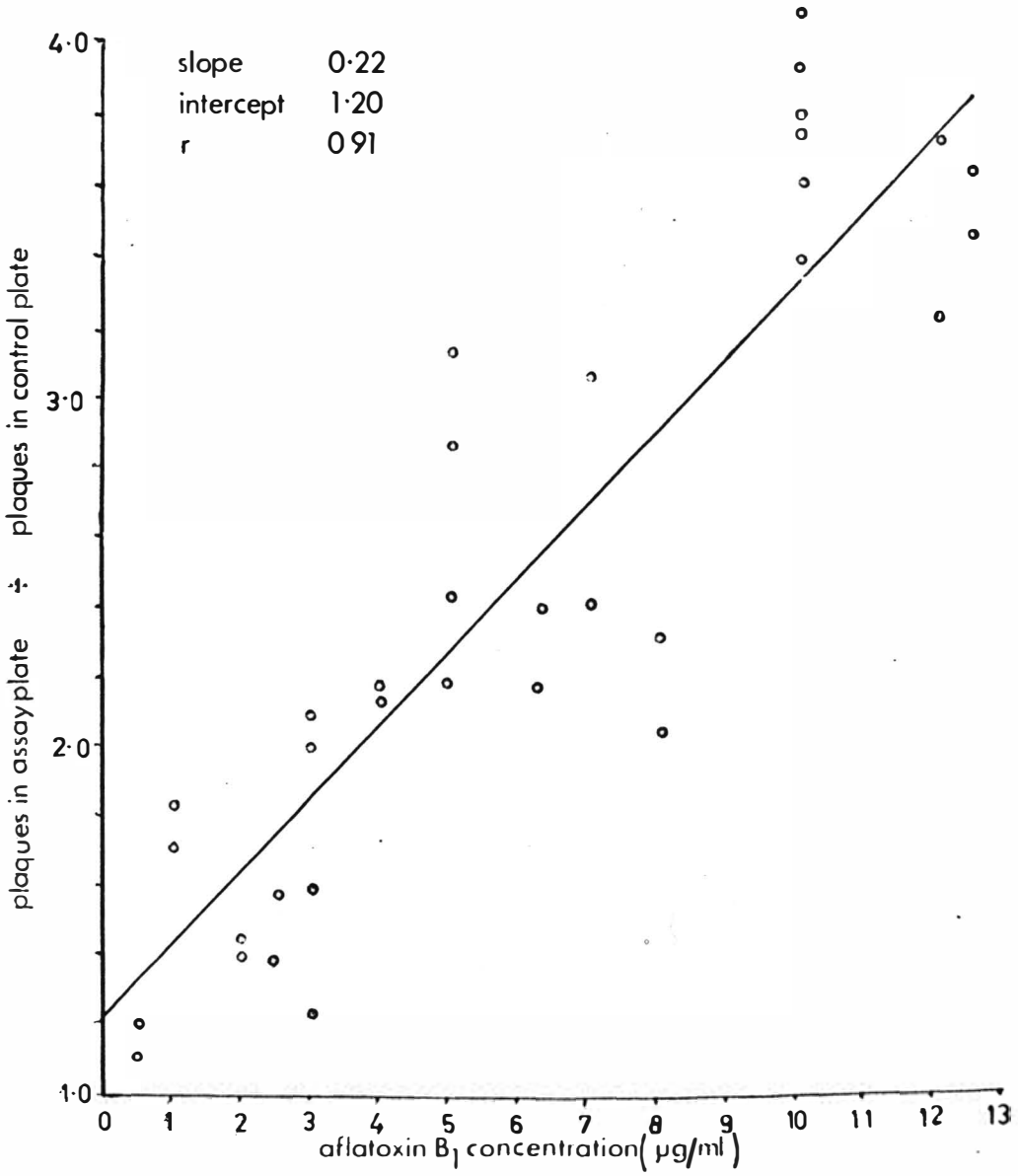
Express the results as the ratio of the count in the treated plate to the count in the control plate containing no aflatoxin.

A graph giving the results of 34 determinations from seven experiments is given in Figure 4.6. Values for 16 and 20 µg/ml aflatoxin B_1 show a lower proportionate increase than those for 12 µg/ml. It is assumed that at these levels the toxin is inhibiting the growth of the bacterial culture.

The method does not have the precision of the inhibition assay but is nevertheless sufficiently precise to be regarded as a practical assay method. The application of the method to other toxins and its specificity are dealt with in the following section.

Figure 4·6

COLLECTED INDUCTION ASSAY RESULTS FROM SEVEN EXPERIMENTS



SECTION V

APPLICATION OF THE ASSAY TECHNIQUES

INTRODUCTION.

The inhibition assay (Section II) and the induction assay (Section IV) were both developed using pure crystalline aflatoxin B_1 as the active compound under test.

The final section deals with the use of these assays to examine a number of mycotoxins structurally related to aflatoxin. The assays were also applied to the examination of infected foodstuffs. The pure mycotoxins chosen for study were aflatoxins B_2, G_1 and G_2, sterigmatocystin, dothistromin, a dehydro bisdeoxy analogue of dothistromin, citrinin, radicinin, ochratoxin A and, for the induction assay only, mitomycin C. Sterigmatocystin is a probable precursor of aflatoxin. Dothistromin and its analogue are produced by the pine blight fungus, Dothistroma pini. Citrinin is a product of Penicillium citrinum, radicinin of Alternaria radicina and ochratoxin of Aspergillus ochraceus. Mitomycin C is produced by a streptomycete and was included for its well-established radiomimetic properties. The structures of these compounds are shown in Figure 5.1. Of these compounds only ochratoxin appears similar to aflatoxin B_1 when TLC spots are examined under ultra-violet light. However when the chloroform:acetone solvent system is used to develop TLC plates, ochratoxin remains at the origin while the aflatoxins migrate. In this respect the TLC plate inhibition assay is more specific than disk inhibition assay techniques.

The results of the assays are given in Tables 5.1 and 5.2. Unless otherwise stated the results are for a single determination only. Table 5.3 shows the mean relative activities of the toxins by the two methods.

Table 5.1 Inhibition assay of mycotoxins

Toxin	Conc./spot (µg)	Zone size (mm^2)	Relative activity*
Aflatoxin B_1	0.2	13.5	
(from standard curve)	0.4	26.0	
	0.6	39.0	
	0.8	51.0	

continued -

Table 5.1 (continued)

Toxin	Conc./spot (µg)	Zone size (mm^2)	Relative activity*
Aflatoxin G_1	1	4.0	0.03
	2	4.8	0.02
	10	31.4	0.05
	20	42.3	0.03
Aflatoxin B_2	5	0	
	10	13.7	0.019
	20	16.0	0.011
	40	22.1	0.008
Aflatoxin G_2	5	0	
	10	4	
	20	7.0	0.004
	40	9.0	0.003
Dothistromin	0.1	2.6	0.12
	0.2	5.1	0.26
	0.4	12.3	0.42
	0.5	15.2	0.43
	0.8	18.9	0.34
	1.0	21.6	0.32
	1.5	24.5	0.24
	2.0	19.8	0.14
Dothistromin analogue	0.5	0	
	1.0	8.1	0.1
	1.5	1.4	0.005
	2.0	5.5	0.029
Sterigmatocystin	1	2.3	0.007
	2	4.0	0.017
	4	7.8	0.024
	5	2.4	0.001
	8	6.0	0.008
	10	5.1	0.005
	20	5.3	0.003

continued -

Table 5.1 (continued)

Toxin	Conc./spot (µg)	Zone size (mm^2)	Relative activity*
Ochratoxin	2	0	
	4	3.1	0.005
	5	2.6	0.002
	8	6.8	0.01
	10	7.8	0.01
	15	9.0	0.008
	20	10.9	0.007
Radicinin+	2	?	
	4	39.1	0.15
	8	56.3	0.11
	16	64.0	0.06
Citrinin	0.1 - 40	0	

+ Zones were faint due to the very rapid diffusion of the compound.

* Relative activity = $\frac{\text{µg aflatoxin } B_1 \text{ giving the same zone size}}{\text{µg toxin}}$

Table 5.2 Induction assay of various compounds

Toxin	Conc. (µg/ml)	Ratio of Plaque increase+	Relative activity*
Aflatoxin B_1 (mean values)	2	1.6	
	4	2.1	
	6	2.5	
	8	2.9	
	10	3.9	
Aflatoxin G_1	50	1.4	0.013
	80	1.6	0.021
	100	2.2	0.049
	160	inhibitory	

Aflatoxin B_2 and G_2 insufficient material could be dissolved to demonstrate induction.

continued -

Table 5.2 (continued)

Toxin	Con. (µg/ml)	Ratio of Plaque+ increase	Relative activity*
Dothistromin	0.5	1.0	
	1	1.0	
	2, 5, 10	inhibitory	
Dothistromin analogue	0.5	1.0	
	1	1.0	
	2	1.0	
	4	1.1	
	5	1.6	0.3
	8	1.5	0.18
	10, 20	inhibitory	
Sterigmatocystin	20	2.9	0.40
	40	1.4	0.023
	50	2.8	0.15
	100 (mean of 2 results)	1.8	0.03
	200	1.5	0.007
Ochratoxin A	2	1.4	0.46
	4	2.1	0.99
	5	1.9	0.65
	8	2.5	0.76
	10	3.1	0.90
Mitomycin C	1	3.3	9.5
	2	4.8	8.4

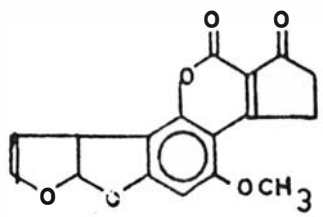
Radicinin, Citrinin — No induction at non-inhibitory concentrations

\+ = Plaques on test plate / Plaques on control plate

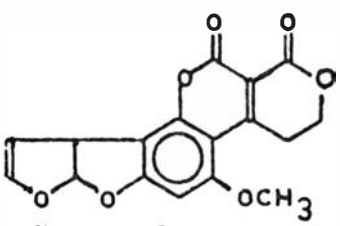
* = Conc. aflatoxin giving ratio / Conc. test toxin giving ratio

Figure 5·1

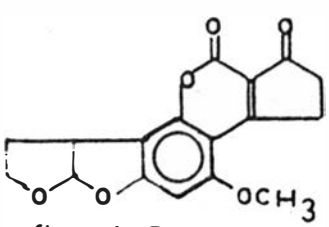
STRUCTURES OF COMPOUNDS USED IN BIOASSAYS



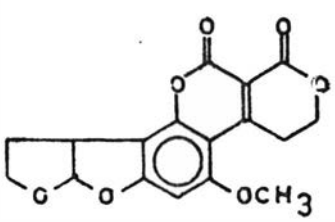
aflatoxin B_1



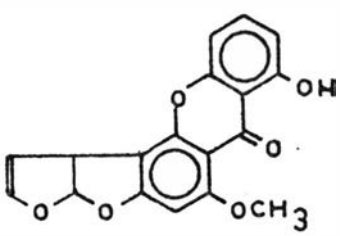
aflatoxin G_1



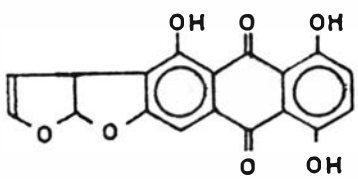
aflatoxin B_2



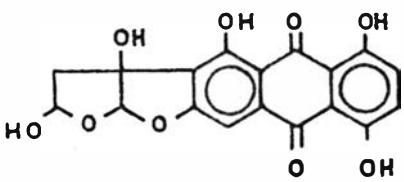
aflatoxin G_2



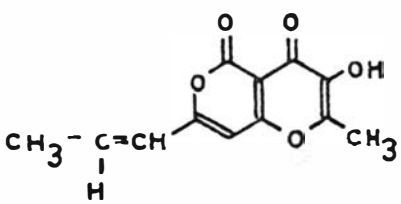
sterimatocystin



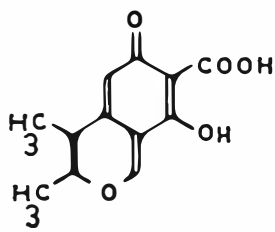
dothistromin analogue



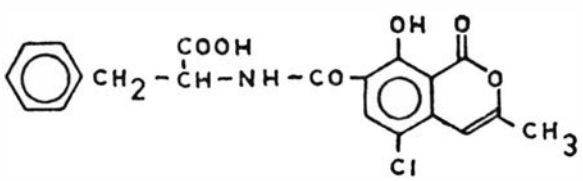
dothistromin



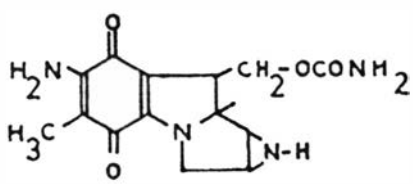
radicinin



citrinin



ochratoxin A



mitomycin C

Table 5.3 Relative activities of toxins by inhibition and induction assay

Toxin	Mean relative activity* Inhibition	Induction
Aflatoxin B_1	1	1
Aflatoxin G_1	0.033	0.028
Aflatoxin B_2	0.012	- +
Aflatoxin G_2	0.004	- +
Sterigmatocystin	0.009	Present (variable)
Dothistromin	0.3	0
Dothistromin analogue	Variable <0.1	0.24
Ochratoxin	0.007	0.75
Radicinin	0.11	0
Mitomycin	-	9.0
Citrinin	0	0

\+ = could not be determined

* = mean of the values for relative potency in Tables 5.1 and 5.2.

The assay systems were not particularly suitable for some of the compounds tested. The inhibition assay did not give good results for sterigmatocystin or the dothistromin analogue which are extremely insoluble in water nor for radicinin which is very soluble in water. In the first case diffusion was too slow to allow of the formation of regular zones and in the second case diffusion was so rapid that the toxin had become diluted in the agar before growth of the bacterial lawn and very large faint zones resulted.

The relative activity figures do not necessarily give a true estimate of the relative inhibitory properties of the compounds since the zone size will reflect the speed of diffusion of a compound as well as its ability to inhibit Bacillus magaterium.

In the same way it was not possible to obtain satisfactory results for some of the compounds by the induction assay. This was due to their

poor solubility in water. Aflatoxins B_2 and G_2 will not dissolve in water in sufficiently high concentrations to show induction. Sterigmatocystin showed some slight induction but was so insoluble that it was uncertain how much of the material added to the system was actually in solution.

Despite these drawbacks the methods made possible some interesting comparisons. The relative activities of the aflatoxins, judged by the inhibition assay are in the same order as has been found for their toxicity in ducklings (Carnaghan et al. 1963). The relative activities of the aflatoxins did not differ so widely in ducklings as in the microbiological systems being 0.46 for G_1, 0.2 for B_2 and 0.1 for G_2 ($B_1 = 1$). Ochratoxin is less toxic than aflatoxin B_1 to ducklings being 0.73 or 0.12 according to Van der Werwe et al.(1965), or Purchase et al. (1967), respectively. Sterigmatocystin was claimed to be "less toxic and carcinogenic than aflatoxin" (Holtzapfel et al. 1966). No toxicity studies have been made on dothistromin or its analogue.

The ability of the compound to inhibit growth of B. megaterium is not a good indication of its ability to cause induction. Dothistromin and radicinin were both inhibitory but neither were able to cause induction. It is interesting also to note that only those compounds said to be carcinogenic were able to induce. The toxicological properties of the dothistromin analogue are not known but its similarity in structure to aflatoxin and sterigmatocystin make it possible that it would be carcinogenic. Mitomycin C is the product of a Streptomycete and was included because of its known radiomimetic properties. It has been suggested for use as an antineoplastic agent.

Although the number of toxins studied is limited the following conclusions can be drawn.

The assays are not completely specific for the aflatoxins. The inhibition assay can be made more specific when it is combined with TLC and fluorescence. The induction assay, while not specific for aflatoxin, may be strongly co-related with carcinogenicity. From a practical point of view this enhances its value as a bioassay rather than detracts from it.

Assay of unknowns and toxins from inoculated substances

Three unknown solutions A, B and C were assayed by the fluorodensitometric, inhibition and induction assay methods. The assay results and the actual contents are given in Table 5.4.

Table 5.4 Assay of unknown solutions

Unknown sample	A	B	C
Actual contents	100 µg/ml B_1	50 µg/ml B_1	125 µg/ml B_1
Fluorodensitometry	125 µg/ml B_1	55 µg/ml B_1	100 µg/ml B_1
Inhibition	100 µg/ml B_1	60 µg/ml B_1	125 µg/ml B_1
Induction	124 µg/ml B_1	41 µg/ml B_1	110 µg/ml B_1

In this particular series the inhibition assay was more accurate than the other two methods.

Samples of cheese, milk powder, and butter were inoculated with a toxigenic strain of Aspergillus parasiticus. An uninoculated control set of samples was also prepared. The samples were incubated in an humidified chamber for a month at which time chloroform was introduced into the chamber to kill the fungus. The Aspergillus inoculum had grown well on all except the butter sample. Samples were extracted by the method of Pons et al. (1963) and assayed by the three methods. The induction assay was unsuccessful due to the presence of some inhibitory material in the extract which affected the induction. Attempts to improve the purity of the extract by precipitation with hexane were only partially successful. Results are given in Table 5.5.

Table 5.5 Assay of dairy products for aflatoxin B_1

	Inoculated			Uninoculated
	Butter	Cheese	Powder	All samples
Fluorodensitometry	N.D.	11 µg/g	10 µg/g	N.D.
Inhibition	N.D.	9 µg/g	9 µg/g	N.D.
Induction	N.D.	N.D.	N.D.	N.D.

N.D. = Not detected.

Both the fluorodensitometric and inhibition assays gave similar results. Neither detected aflatoxin or inhibition in the control samples or in the butter, where the fungus had not grown well.

The induction assay results were disappointing in that the assay failed to demonstrate the inducing activity of the aflatoxin. The extract required for good induction assay results must apparently be very much purer than that required for the inhibition assays. Even silica gel column treatment did not give a sufficiently pure extract for reliable results to be obtained.

The extracts from all the samples gave clear solutions in acetone but precipitated when added to the medium. This did not happen with crystalline aflatoxins. The cheese extract was treated with hexane to precipitate the aflatoxins. The precipitate was washed in hexane, dried and redissolved in acetone. In all, the extract was precipitated and redissolved three times. Even after this treatment there was some precipitation when the extract was added to culture medium. However it was possible to demonstrate induction although the result indicated only 6.5 µg/ml compared with the 9 or 11 µg/ml indicated by the other methods.

Combined perhaps with other procedures, hexane precipitation may produce an extract which can be used with the induction assay. The problem may be peculiar to dairy products as even extracts from the uninoculated controls inhibited the induction assay.

The fluorodensitometric and inhibition assays gave results which supported each other and the induction result obtained did at least confirm the presence of an inducing compound qualitatively. With further work to improve the methods of extraction the combined techniques should be capable of the sensitive and specific analysis of foodstuffs for aflatoxins.

DISCUSSION

DISCUSSION

The aim of this work was to devise a bioassay for aflatoxin which was to be an improvement on those presently available. Two complementary assays have been described and will be discussed here separately.

The inhibition bioassay

The inhibition assay proposed by Clements (1968) involved impregnating a paper disk with test solution, allowing the solvent to evaporate and placing the disk on the surface of agar seeded with *B. megaterium*. The zones of inhibition were measured after overnight incubation.

The first step towards improving the sensitivity of the inhibition assay was the direct application of the solution of toxin to an agar plate rather than by means of a paper disk. The use of a disk restricts the lower limit of the size of the zone of inhibition to one slightly greater than that of the disk itself. Anything smaller will not be detected. Because the toxin is distributed throughout the disk the concentration/unit area may be quite low unless the disk itself is very small. By applying toxin solutions directly to the agar the area of application is kept as small as possible leading to an increase in sensitivity. This was possible because of the relatively low solubility of aflatoxins in water. Water-soluble toxins would diffuse rapidly from the point of application before growth of the bacterial lawn proceeded very far and would give rise to very diffuse zones. At low concentrations diffusion would dilute the toxins to such an extent that they would not be inhibitory.

The immiscibility of the chloroform solvent with the water in the agar medium often caused the solvent to run over the surface of the medium away from the point of application leading to irregularities in the shape and size of the zones. Although this meant that the method was of limited use as it stood, it demonstrated that the necessary sensitivity could be achieved by the direct application of toxin to small areas.

A more effective method of producing regular zones of inhibition proved to be the application of the toxin to silica gel thin layer chromatography (TLC) plates which were then overlaid with agar seeded with the test organism. However the growth of the bacterium in the agar medium overlaying the TLC plate could not easily be seen due to the opacity of the silica gel on the plate. The ability of Bacillus megaterium to reduce triphenyl tetrazolium chloride to the red triphenylformazan was used to delineate the areas where the organism had grown leaving the zones of inhibition clearly defined.

The basis of the assay having been arrived at it remained to examine its reproducibility and sensitivity. By standardising the inoculum and the thickness of the agar overlay it was possible to achieve a degree of reproducibility between assays performed at different times such that 95% of the values fell within 0.2 µg of the expected value (see Figure 2.4). The sensitivity of the assay depended on whether the TLC plate had been developed chromatographically. Development had the effect of spreading the toxin over a wider area and reducing the sensitivity of the assay. There was however a compensating gain in specificity since the Rf of the inhibitory compound could be compared with that of standard compounds. The lower limit of sensitivity using undeveloped plates was 0.1 µg aflatoxin B_1. This compares very favourably with the limit of 1 µg claimed by Clements (1968) for the disk diffusion assay.

The specificity of the inhibition assay per se is no greater than that of any other form of inhibition assay. Combined with thin layer chromatrography, however, the specificity is as good or better than the chromatographic assay itself though about ten times less sensitive. The inhibition assay can distinguish between aflatoxins B_1 and B_{2a} which have very similar Rf values in some solvent systems though the latter is practically non-toxic. It has the additional advantage that the same material can be used both for a physicochemical assay and for a biological assay. The combination of chromatography with a bioassay has been described by other workers. For example, Hoehn et al. (1970) used paper chromatography to separate cephalothin and desacetylcephalothin in body fluids. In their method the paper chromatogram was laid for fifteen

minutes on the surface of a seeded agar medium in a large plate. The paper was then removed and the plate incubated giving rise to zones of inhibition. This method is basically similar to that described for aflatoxin though rather more cumbersome due to the larger size of the paper sheets employed in the chromatography. When applied to unknown solutions and to crude extracts the TLC/inhibition assay agreed within 25% or better with the fluorodensitometric assay and with the true value where this was known.

Although the combination of thin layer chromatography and inhibition assay provides a rapid, specific and fairly sensitive assay, it is still desirable to have available an assay which is based on a property specific to the material to be examined. The induction assay was developed in an attempt to fulfil this requirement.

The induction assay

The ability to induce lysis of lysogenic bacteria is a property possessed by relatively few compounds (Lein et al. 1962). For that reason lysogenic induction seemed suited as the basis of a relatively specific bioassay. In addition the report of Legator (1966) on induction of lambda in E. coli promised a sensitivity of better than 0.1 µg/ml for the proposed assay. Legator's findings with E. coli and S. aureus could not be reproduced. Consequently the rather less sensitive induction of lysogenic B. megaterium, reported by Lillehoj and Ciegler (1970) was made the basis of the assay.

During the study of the B. megaterium phage system some interesting observations were made. These will be discussed before dealing with the induction bioassay eventually developed.

1. Metal ion requirements for lytic infection:

Magnesium ions and, to a much lesser extent, copper ions were found to be necessary for the lysis of B. megaterium strain KM by bacteriophage 899a. This requirement had not been reported by previous workers with this megaphage although Huybers (1953) had found magnesium to be necessary

for induction of this phage by ultra-violet irradiation. Metal ions are necessary for the adsorption of some bacteriophages as, for instance, calcium for coliphage T_2 and magnesium for coliphage lambda (Hayes 1968). However adsorption experiments showed that megaphage 899a adsorption was not dependent on magnesium ions.

Electron micrographs of the phage (see Figure 3.13) adsorbed to bacterial cells in the absence of magnesium show few if any empty phage heads and it could be conjectured that the presence of magnesium ions is necessary for the injection of the phage genetic material into the cell. However, attempts to demonstrate a difference in the appearance of the phage heads by electron microscopy have so far failed to produce any evidence that this is the case.

2. Effects of aflatoxin on growth, lysis and free phage number of lysogenic B. megaterium.

The following three sub-sections discuss observations which were potentially useful in obtaining dose/response relationships. The first observation (see Section IV) was of changes in the shape of the optical density curve from which it might be possible to find the proportion of cells lysed by induction. The second was of the peak of phage production where a plateau had been expected. The final sub-section discusses the development of a method to count induced cells rather than their progeny phage.

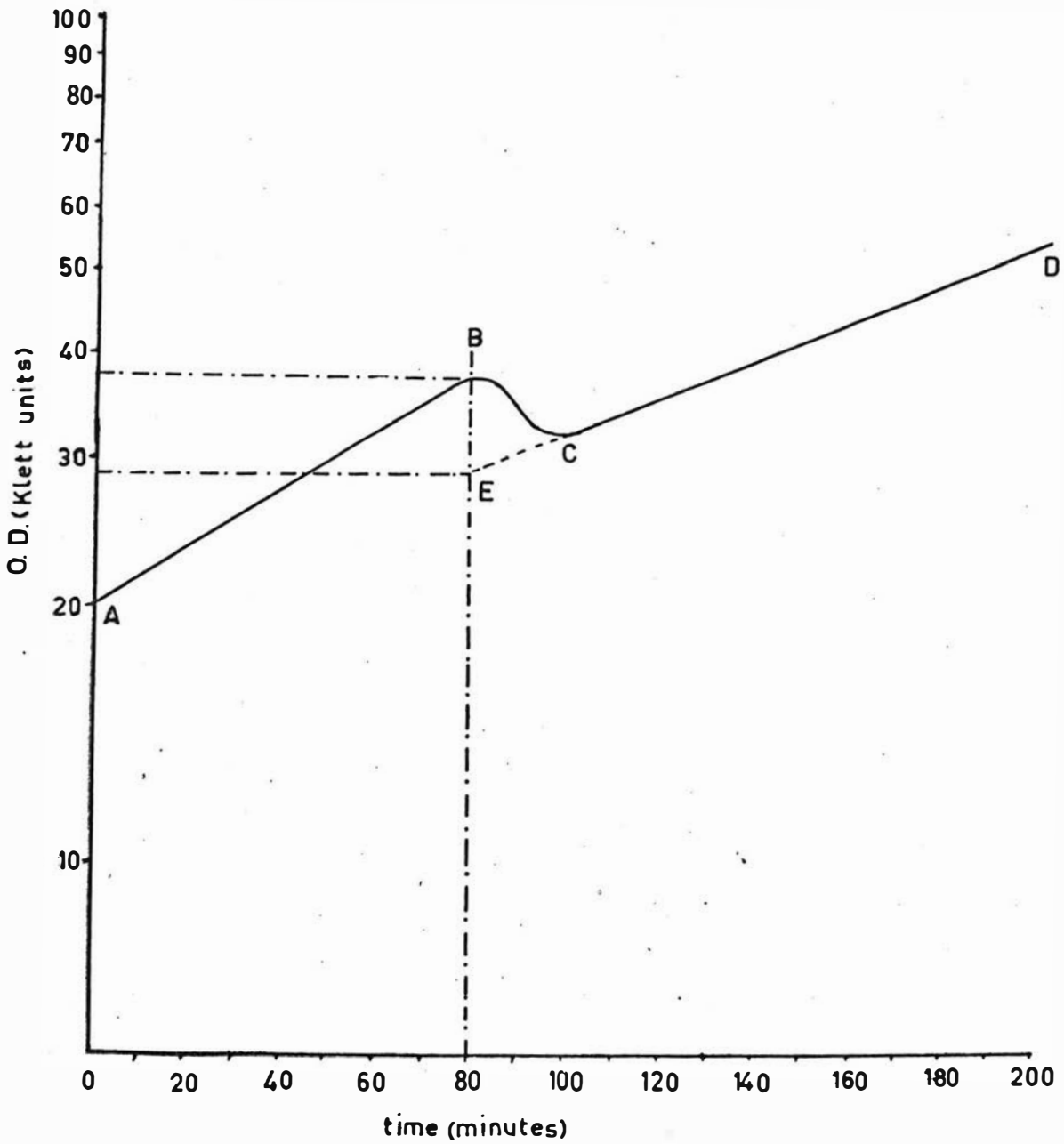
2a. The effect of aflatoxin on the growth and lysis of lysogenic B. megaterium.

The effect of aflatoxin on the optical density of cultures of B. megaterium 899a was reported in Section IV. When exponential phase cells were sub-cultured into fresh medium, there was no difference in the initial rate of growth between the aflatoxin-containing culture and the control. After an interval of 90 minutes, however, there was an inflexion in the growth curve of the aflatoxin-treated culture lasting approximately 30 minutes followed by a resumption of growth but the new rate of increase in optical density was lower than the initial rate. Some idea of the

Figure 6·1

THE USE OF OPTICAL DENSITY TO MEASURE THE RATIO OF LYSED CELLS

O.D. at B = 36
O.D. at E = 28
ratio lysed cells = $1 - \frac{28}{36} = 0.22$



probable proportion of the culture lysed by induction can be gained from the inflexion. If the slope of resumed growth (C-D) is extrapolated toward the OD axis, an optical density at the time of the inflexion (B) can be found (E) (see Figure 6.1). This may be compared with the actual optical density at the time of the inflexion. This figure would only be approximate as it is difficult to be certain of the time at which the inflexion began or of the maximum optical density attained before lysis started.

It was suggested in Section IV that the lower rate of optical density increase after the inflexion might be attributed to the continued lysis of a proportion of the culture which was otherwise continuing to grow at its original rate. This would give the appearance of a decreased rate of growth.

This possibility was investigated by computer analysis of a model system. Details are given in Appendix II. It was found that the graphs produced by the model compared well with the graphs obtained in experiments.

It would be expected that the proportion of cells lysed would depend on the concentration of aflatoxin and could be assessed by the means described here. The proportion of cells lysing did vary with different aflatoxin concentrations but the variation between experiments using the same concentration of aflatoxin was too great for this to serve as a quantitative bioassay.

2b. Changes in free phage numbers in cultures B. megaterium treated with aflatoxin

When lysogenic cultures of B. megaterium are treated with aflatoxin there is a sudden increase in the numbers of free phage occurring 80-90 minutes after treatment. This is followed by an almost equally rapid decrease in numbers. The level to which the phage numbers fall is still greater than that in untreated cultures and continues to rise slowly after a minimum has been reached.

The sudden increase in phage numbers can reasonably be interpreted as the lysis and phage release caused by the induction of the prophage by aflatoxin. The decrease immediately following the increase is less easily explained. In Section III the adsorption of megaphage 899a to

B. megaterium strain KM is described. Unfortunately no investigations of the adsorption of phage to strain 899a were made but it is assumed in the following discussion that the kinetics of adsorption of phage to strain 899a are similar to those to strain KM. It was not possible to derive an adsorption constant using cell numbers since the numbers could not accurately be assessed. A constant using optical density in Klett units as a measure of cell mass was derived instead thus:-

$$K = \frac{1}{CDt} \; 2.3 \log \frac{Po}{P}$$

where OD = optical density
t = time in minutes
Po = initial phage count
P = phage count at time t

Data from a number of adsorption experiments at optical densities ranging from Klett 26 to Klett 400 gave a mean value of $9X10^{-4}$. Using this value one finds that at an optical density of 50 Klett units, 90% of the phage would be adsorbed in 130 minutes. In the experiments where enough data is available to make an estimate, the peak of free phage occurred between 80 and 90 minutes and had been reduced to a 10th of the peak value by between 120 and 150 minutes. Thus the time in which the phage numbers declined by 90% could be as short as 30 or as long as 70 minutes. It seems therefore that adsorption is not of the same order as the decrease of phage in aflatoxin-treated cultures after the initial peak of phage production and cannot account for all of that decrease. It has already been commented that the transient and variable nature of the peak made it unsatisfactory as a measure of aflatoxin concentration. Part of the variation between experiments could be due to variations in adsorption rate in cultures of different optical density.

2c. The triple layer induction assay.

Because of the difficulties associated with getting a reproducible count of free phage released, attention was redirected to the measurement

of induced cells. The main problem was to distinguish cells induced at a particular time from later, spontaneous induction. It did not seem likely that chemically induced cells could be distinguished from spontaneously induced cells but it did prove possible to develop a system where the number of cells induced by aflatoxin treatment could be compared with the spontaneously induced cells in an untreated control. This was achieved by allowing sufficient time for the treatment to be effective and then removing the cells into an agar medium. Sufficient time was then allowed for the lytic cycle to be completed and any remaining unlysed cells were killed by treatment with streptomycin. The phage-sensitive organisms, being streptomycin-resistant, were still able to grow and be infected by the phage released from induced and lysed cells.

This system appeared to give a sufficiently good correlation between aflatoxin concentration and response to provide a quantitative bioassay. The reproducibility of the dose/response relationship is not as good as that attainable by the inhibition system but is potentially more specific. Sensitivity is also less than that of the inhibition system by a factor of twenty. Improvements in sensitivity might be obtainable using the potentiating system of Ames et al. (1973) using liver extracts to convert the toxins into their active form.

The assay of other known compounds by the induction system supported the suggestions of Legator (1966) and others that compounds known or suspected to be carcinogenic are also capable of causing induction. Aflatoxin B_1, sterigmatocystin and ochratoxin A have all been reported to be carcinogenic (Fewell 1969) and all were active in the induction bioassay. The usefulness of the assay system for toxins in dairy products is limited at present by the difficulty in obtaining sufficiently pure extracts of toxin from these products. Further investigation of methods of extraction and purification should provide the solution to this difficulty.

Comparison with other assays

Frequently the assays suggested for aflatoxins involve extraction, purification and concentration of the toxins before the assay is to be

performed. This calls for lengthy and involved procedures. The exceptions are those bioassays where the suspect materials are included in the feed of experimental animals. Even though such bioassays do not involve the preliminary purification steps, the time required before a response can be measured is several days and may be several weeks.

Since the preparation steps are common to virtually all the assays, only the time taken for the assay itself need be considered when comparing the time taken to perform the assay. The most rapid technique is the fluorodensitometry of thin layer chromatography plates or similar procedures. Results may be available by this technique within a few hours. This is, however, a physicochemical method and gives no indication of biological activity except by inference.

The duckling assay

Extracts from suspect materials are administered by crop tube to ducklings weighing 50 $\pm$ 10 g. A single dose can be used and assessed by a combination of LD_{50} and liver damage. The effects of aflatoxins on the liver are at a maximum three days after administration (Butler 1964). Armbrecht and Fitzhugh (1964) claimed an increase in sensitivity by administering multiple doses over seven days. They recommended an LD_{50} estimation as a quantitative assay and an estimation of liver damage after multiple dosage as a qualitative assay. In his review, Legator (1969) regards the duckling assay as, at best, semiquantitative. In addition it is clearly time-consuming and demands a degree of interpretative skill in assessing the changes in the livers of affected animals.

Neither of the two microbiological assays described in this work is as lengthy as the duckling assay and both are more sensitive. While it is true that ducklings are more closely related to humans than bacteria, the specificity of induction, as a biological effect, seems equally as great as bile duct proliferation. The main advantage of the microbiological assays is that the measurement of the result is objective, whereas the assessment of liver damage in ducklings is necessarily subjective.

Disk diffusion assay

The TLC/inhibition assay was developed from the disk assay and has the advantages of rapidity and ease of handling with the additional advantages of increased sensitivity and specificity. The disk assay has a minimum practical sensitivity of about 2 µg aflatoxin B_1, compared with a sensitivity of 0.1 µg aflatoxin B_1 attainable by the TLC/inhibition assay.

Other animal assays

All bioassays using LD_{50} and pathological effects will suffer from the same disadvantages as the duckling assay. This includes assays using fertile eggs, though greater sensitivities and a good dose/response relationship are claimed for these methods. Nevertheless they are time and space-consuming and depend to some extent, on subjective assessment of pathological changes. A possible exception is the assay using young zebra fish (Abedi and Scott 1969). This is both rapid and sensitive but the work reported effects using purified compounds and it is not clear what the effect of crude extracts on these fish might be. In any case the maintenance of material for the assay would be troublesome.

Conclusion

The combination of thin layer chromatography, inhibition and induction proposed as in assay system in this work appears to be a practicable one for the monitoring of foods and feedstuffs. It is clear that the system must be evaluated more strictly by applying it to the assay of aflatoxins extracted from a variety of infected materials before it can be accepted. Even if it passes this evaluation it cannot be claimed as the final answer to the problem of aflatoxin bioassay. However it is potentially an advance in bioassay procedures and may lead to even better methods.

Aflatoxin has not yet been shown to be a problem in New Zealand. This is, at least in part, because of the limited investigational work which has been done. It is important to have methods available to detect and control outbreaks of the disease in this country and to reassure overseas customers that our produce is unlikely to be contaminated.

With suitable modification the system could be applicable to the assay of other mycotoxins. Thin layer chromatography is sufficiently versatile to enable most of the mycotoxins to be detected. Selection of an appropriate organism, sensitive to the toxin under examination, would enable the inhibition assay to be undertaken. The usefulness of the induction assay would depend on the properties of the toxin. Gas liquid chromatography may be suitable both to assay mycotoxins and to prepare them in a sufficiently pure form for bioassay.

It may never be certain whether aflatoxin and other mycotoxins are mutagenic, carcinogenic or teratogenic in man, but no one would willingly expose themselves to risk in the light of the known effects of these compounds in other biological systems. Assays based on biological, rather than solely chemical or physical, characteristics may reduce the chances of toxic compounds being included in the human diet unawares.

NOTES ON THE APPENDICES

The first appendix contains observations arising from the investigations of the induction bioassay but not immediately relevant to the assay itself. These are in the nature of preliminary observations and so have been presented in the form of an appendix. The mathematical analysis of the dynamics of induction kindly made by Doctor A. Tyree is also included here as Appendix II.

APPENDIX I

Introduction

Hong et al. (1971) reported observations made on Salmonella typhimurium sensitive to bacteriophage P22, in which strains of the organism lacking the ability to produce adenosine 3':5' cyclic monophosphate (cAMP) gave clear plaques when infected by the phage. However the normal turbid, centred plaques could be obtained with these strains if they were plated in medium containing cAMP. Other mutants exist which lack the cAMP receptor protein and these strains also form clear plaques with phage P22. Addition of cAMP to the medium does not enable mutants of this kind to produce normal plaques.

From this work Hong and his co-workers concluded that cAMP/receptor protein complex was necessary for the establishment of lysogeny. They claimed that this also explained why lysogeny is generally less frequent in cultures of actively growing cells and more frequent in cultures where energy is in short supply, since these growth conditions often correspond with lower and higher levels, respectively, of cellular cAMP.

The induction of lysogenic bacteria often involves interference by the inducing agent with the control mechanism which maintains lysogeny. It is possible that lysogeny may be re-established if cAMP were present in the cell.

The physiological state of the cells might also affect their response to aflatoxin. Both these possibilities were examined in the following experiments.

A. Modification by adenosine 3':5' cyclic monophosphate of megaphage induction

Bacillus megaterium strain 899a was grown in SDM to mid-exponential phase and diluted one tenth in the same medium containing various additions.

a) 10 µg/ml aflatoxin B_1

b) 10 µg/ml aflatoxin B_1 + 1mM cAMP

c) no addition

These sub-cultures were incubated in a shaking water bath at 35°C and the optical density recorded at intervals over three hours. The typical inflexion in the growth curve occurred between 85 and 110 minutes in the culture containing aflatoxin only. The culture containing both aflatoxin and cAMP exhibited only a minor inflexion. Generation times for the three sub-cultures are given in Table 7.1 below.

Table 7.1 Modification by cAMP of the effect of aflatoxin B_1 on generation times of B. megaterium.

	Generation times (minutes)	
	Before inflexion	After inflexion
Control	66	66
10 µg/ml B_1	60	90
10 µg/ml B_1 + 1 mM cAMP	65	70

The cAMP appears to have reduced the lysis of cells induced by aflatoxin. No phage counts were made so it was not possible to confirm a corresponding fall in phage numbers. The results suggest, however, that the inducing effect of aflatoxin can be reversed or inhibited by adenosine 3':5' cyclic monophosphate.

B. The effect of physiological state of culture on induction of megaphage by aflatoxin B_1

Cultures approaching or actually in stationary phase induced by glucose limitation were examined as described below.

Bacillus megaterium strain 899a was grown in a SDM containing only 0.1% glucose. Sub-cultures were made by diluting one tenth into the same medium containing 10 µg/ml aflatoxin B_1 at 20, 80, 140 and 200 minutes after the onset of stationary phase. The optical density of the sub-cultures was followed for three hours following dilution.

The results given in Figure 7.1 show that the age of the culture had no effect on the time elapsing before lysis. It did however have a marked effect on the growth of the sub-culture. This is the well-documented phenomenon of increased lag phase co-relating with duration of stationary phase. The rate of cell growth and the time for completion of phage

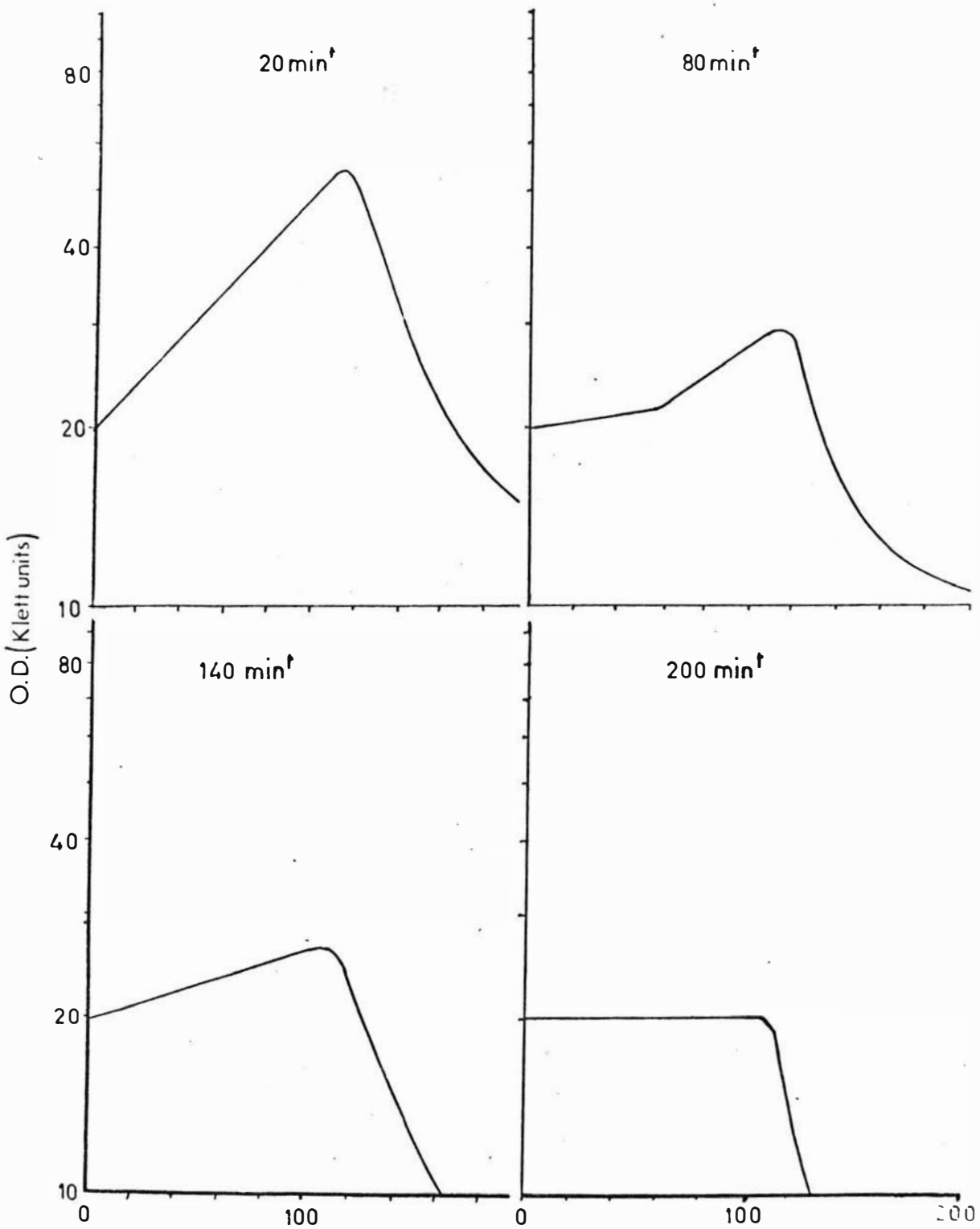
replication seem to be completely independent, the burst time being constant irrespective of the rate of growth of the culture. The time elapsing before lysis was 110 minutes in all four sub-cultures.

Some metabolic activity not resulting in growth must be occurring in the culture sub-cultured last, since lysis was able to occur and it is known that phage replication and lysis will only take place in actively metabolising cells.

The observation has interest as indicating a potential way of improving the bioassay. Cultures kept in stationary phase for about three hours and then sub-cultured show no subsequent change in optical density up to the time of induced lysis. By using this property it should therefore be possible to standardise the numbers of cells in an assay medium over the duration of the assay by standardising the numbers present at the start.

Figure 7·1

EFFECT OF AGE OF CULTURE ON LAG PHASE AND LYSIS



†time of sub-culture after start of stationary phase

APPENDIX II

MATHEMATICAL ANALYSIS OF THE DYNAMICS OF INDUCTION

The mathematical model developed in conversation with Mr Freke leads to an equation of the type known as a differential-difference equation. Ignoring statistical effects, it is assumed that the time interval between induction and lysis is a fixed positive constant t_o. Letting $p>o$ measure normal growth and $i>o$ measure induction, the model is:

$$(1) \qquad A'(t) = (p - i)\,A(t)$$

$$(2) \qquad H'(t) = iA(t) + p\,(H(t) - H(t - t_o))$$

where differentiation is with respect to time and where $A(t)$ is the number of bacteria at time t which have not yet been induced.

$H(t)$ is the number of bacteria which have been induced (including those which have undergone lysis).

The term $(H(t) - H(t - t_o))$ in equation (2) then is the number of induced bacteria which have not yet undergone lysis.

In order to solve the equations, initial conditions must be imposed; these are

$$(3) \qquad A(o) = 1$$

$$(4) \qquad H(t) = o \text{ for } t \leq o$$

Equation (1) is, of course, a standard first order differential equation. Equation (2) may then be solved using Laplace Transform Techniques (Bellman - Cooke).

The solutions are

$$(5) \qquad A(t) = e^{(p - i)t}$$

$$(6) \qquad H(t) = ie^{pt} \sum_{k = o}^{(t/t_o)} (-1)^k \frac{p^k}{k!} e^{-kpto} I_k(t - kt_o)$$

where $I_k(t) = \int_o^t (t - w)^k e^{-iw}\, dw$

and (x) is the greatest integer less than or equal to x.

Using the recurrence relations

$$(7) \qquad I_k(t) = \frac{1}{i}\left(t^k - kI_{k-1}(t)\right)$$

$$(8) \qquad I'(t) = kI_{k-1}(t) \quad \text{for } k>o$$

and

$$(9) \qquad I_o(t) = \frac{1}{i}\left(1 - e^{-it}\right)$$

it is easily verified that (6) is in fact a solution to equation (2) for all $t \geq o$.

If $\log (A(t) + H(t) - H(t-t_o))$ is plotted against time, then the characteristic graphs obtained by Mr Freke should result. Although computer similation using the CSMP language strongly support this, the rather intractible form of the solution (6) has frustrated an analytic verification.

Reference

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