ESTIMATION OF FUNGI IN THE PRESENCE OF BACTERIA

by

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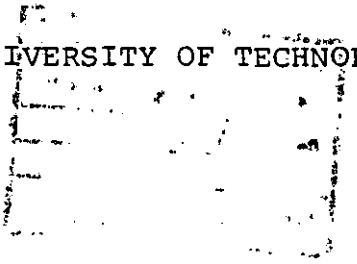

A C K N O W L E D G E M E N T S

Our ... our ... G. Shama

My special thanks go to my supervisor, Dr. R. K. Dart, for his brilliant suggestions, and guidance throughout this work. and understanding project

I am grateful to all those members of Academic staff and research colleagues in the Medicinal Chemistry who offered helpful comments.

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SUMMARY

A quantitative assay of mixed cultures of bacteria and the fungus Aspergillus flavus was carried out. The assay was based on the estimation of glucosamine formed by the hydrolysis of chitin.

The bacteria used were typical Gram-positive and Gram-negative bacteria commonly found in spoiled food. They included, Escherichia coli, Bacillus cereus, Staphylococcus aureus and Pseudomonas aeruginosa.

The experiments carried out involved mixing cultures of bacteria and fungi immediately prior to estimation or alternatively fungal and bacterial cultures were mixed, grown for 40 hours and then estimated for glucosamine.

Results obtained show that when cultures are mixed immediately prior to estimation, there is no significant effect on glucosamine levels. When Gram-negative bacteria are grown with A.flavus prior to estimation there is no significant effect on glucosamine levels compared with pure cultures of A.flavus. However, when Gram-positive bacteria are mixed with fungi grown for 40 hours and glucosamine is estimated, there is a significant reduction in the level of glucosamine formed. It was shown that this is due to bacterial growth competing with the fungi and causing a considerable reduction in the amount of fungal mycelium formed.

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I N T R O D U C T I O N

The fungi consist of a large, heterogeneous group of heterotrophic organisms living as saprophytes or parasites, or associating with other organisms as symbionts.

Fungi play an important role in the life of man, due to their beneficial and harmful activities. They are widely used for the synthesis of complex compounds, one familiar example being the production of penicillin by strains of Penicillium chrysogenum.
They have also been used in industrial processes such as brewing, baking, and the synthesis of various chemicals and pharmaceutical products. It is however the production of antibiotics by fungi which attracts most attention in this field today (Ainsworth et al., 1965).

Fungi also play an important role as pathogens of animals and plants and in addition they cause damage and deterioration in a number of materials, such as foodstuffs and textiles.

Fungi synthesize mycotoxins in the living plant, on decaying plants and also in stored products, and they have been recognized as the spoilage agents of many different foods. The association of microbial toxins in food or feed products with human and animal disease has generally been based on symptoms occurring after ingestion.

They produce many secondary metabolites, some of which are acutely toxic and some of which have other toxic manifestations, i.e. mutagenicity, teratogenicity or carcinogenicity.

The biologically active fungal metabolites that are of significant occurrence in food and feedstuffs are ochratoxin A, citrinin, patulin, penicillic acid, trichothecenes, zearalenone and the aflatoxins. (Stoloff, 1976). The greatest attention has been given to the aflatoxins, but other mycotoxins potentially capable of causing damage on ingestion have been considered.

The ochratoxins are toxic metabolites elaborated by several species of Aspergillus ochraceus as well as by Penicillium viridicatum. These toxins are commonly found in soils, and on food commodities such as nuts, dried fish, grains, beans, peas, coffee, spices, alfalfa and meats (Scott et al., 1970; Scott et al., 1972; and Saito et al., 1974).

Ochratoxin A, the main toxic component of the ochratoxins has been reported as teratogenic to mice and rats (More et al., 1974; and Hayes et al.,1974). In addition, studies have shown other toxic effects of the ochratoxins such as kidney and liver damage, collapsed lungs, ocular lesions and pulmonary tumors in pigs, rats, mice, guinea pigs and miniature swine (Krogh et al., 1968; and Swicker et al., 1973).

Citrinin is a toxic metabolite originally isolated from Penicillium citrinum. This fungus, found in all of the world's major rice-producing countries, has been frequently isolated from polished yellow rice. The occurrence of citrinin as a contaminant of feedstuffs has been associated with Penicillium viridicatum and species of Aspergillus such as Aspergillus flavus and Aspergillus terreus (Subrahmanyam, & Rao, 1974). The most

serious change caused by citrinin in experimental animals is kidney damage (Ambrose et al., 1946; Saito & Tatsumo, 1971). Studies have been carried out to evaluate the potential hazard to humans resulting from exposure to these toxins.

Patulin is a toxic and carcinogenic lactone metabolite elaborated by species of the genera Aspergillus and Penicillum.

Patulin has been found on a wide range of agricultural commodities including grain, malt, flour, sausages, bread and fruit. Apples naturally rotted by Penicillum expansum often contain patulin. The primary interest in patulin derives from the fact that it is a proven carcinogen of laboratory animals (Ayres et al., 1980).

Penicillic acid has been isolated from at least ten species of Penicillum and Aspergillus. Penicillic acid has been found in mouldy tobacco from commercial storage, in mouldy corn and beans. It is prevalent in the environment and is produced by several fungi capable of decaying food, penicillic acid has been considered as a potentially dangerous mycotoxin implicated in carcinogenesis (Thorpe et al., 1974).

Trichothecenes are a group of chemically related and biologically active secondary metabolites produced by Fusarium species. One common producer, Fusarium tricinctum has been isolated from field corn, sweet corn, sorghum, peas and rice. These toxins are considered as natural contaminants of corn and have been implicated in economically important mycotoxicoses such as mouldy corn toxicosis of cattle and poultry (Hsu et al., 1972) and stachybotryotoxicosis, a disease mainly of horses (Eppley & Bailey, 1973).

Trichothecenes have varying levels of toxicity, the LD_{50} levels in rats varying from 800 mg/kg for crotocin down to 0.5 mg/kg for verrucarin A (Bamburg & Strong, 1971).

Zearalenone is a secondary metabolite produced by Fusarium species such as Fusarium tricinctum and Fusarium moniliforme but particularly by Fusarium roseum. Zearalenone was first isolated from maize infected with the fungus Gibberella zeae, the perithecial stage of Fusarium graminearum (Stob et al., 1962). It is usually produced on maize and barley in storage. Zearalenone is notable because of its oestrogenic and anabolic activity in animals and in common with other mycotoxins is active at very low concentrations. An oestrogenic effect in pigs can be induced with concentrations in the range 5-10 ppm in feed (Sherwood & Peberdy, 1974).

Although many of these toxins have been shown to be toxic at various levels, it would appear that the majority of the mycotoxin survey work has been confined to the aflatoxins.

Aflatoxins are a group of secondary fungal metabolites which were discovered as contaminants of certain animal feeds. The generic name "Aflatoxins" was applied to the group of toxic compounds produced by some strains of Aspergillus flavus.

After their discovery in the early 1960's, research on fungal toxins and their role in human and animal diseases intensified greatly and also become more systematic.

The term aflatoxin normally refers to the group of

bisfuranocoumarin metabolites. The major members are designated as B_1, B_2, G_1 and G_2 (see fig 1). A number of other closely related compounds are also produced by fungi of the Aspergillus flavus group in minor amounts. The aflatoxins fluoresce strongly in ultraviolet light; B_1 and B_2 produce a blue fluorescence whereas G_1 and G_2 produce a green fluorescence.

The aflatoxins are the causative agents of an important disease in humans (liver cancer), which can be induced by long term and relatively low level ingestion of the toxins (Rodricks, 1976).

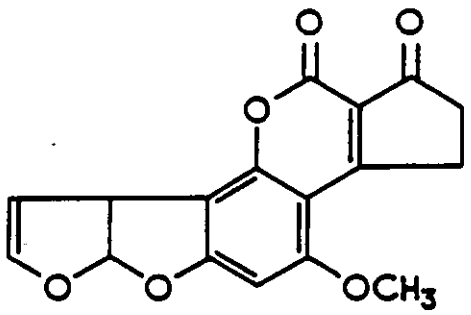
Their occurrence in agricultural commodities and the demonstration of various biological effects resulting from the ingestion of contaminated diets by animals, have emphasized the potential public health hazard which might arise from contamination of food supplies.

Aflatoxin B_1 is the most common of the group in naturally contaminated foodstuffs and is also the most potent hepatocarcinogen. It has an LD_{50} in the range of 0.5 to 1 mg per kilogram body weight for such animals as the duck, rat, dog, rabbit, hamster, guinea pig (Jennie & Marth, 1968). In some animal species e.g. dairy cattle, aflatoxins B_1 and B_2 are partially metabolized to give aflatoxins M_1 and M_2, respectively. These derivatives are almost as toxic as the parent compounds. Aflatoxin M_1 is sometimes a contaminant of cow's milk and hence may enter the human food chain.

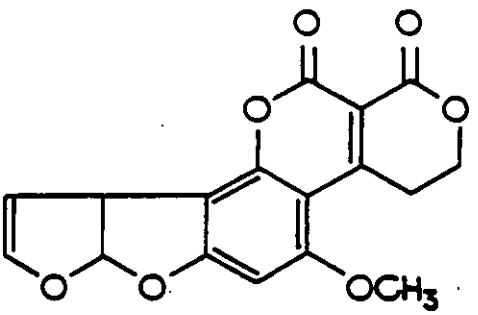
The effects of these toxins have been widely reported in the literature due to two factors. These are,widespread contamination of human food supplies especially in the tropics and the intense carcinogenity

FIGURE 1

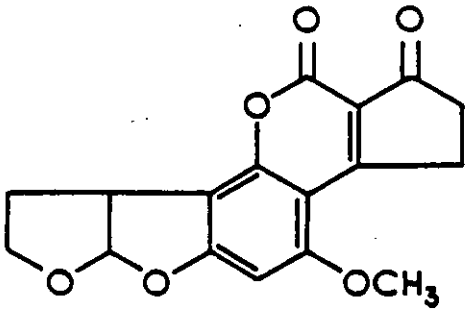
STRUCTURES OF THE AFLATOXINS



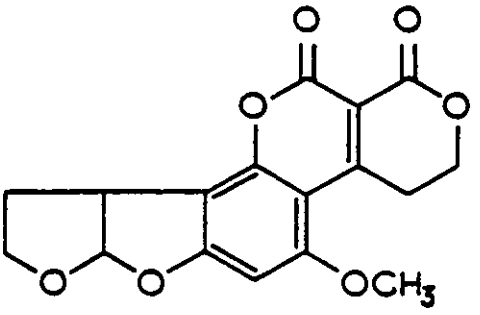
B_1



G_1



B_2



G_2

of aflatoxin B_1 in experimental animals.

An early study that associated aflatoxins in food with the incidence of human liver cancer was carried out in Uganda by Alpert et al.,(1971). Four hundred and eighty food samples were collected from various areas during 1966-1967, and almost 4% contained more than 1 ppm total aflatoxins which is regarded as heavy contamination. More importantly, there was a geographical relationship between the contamination and the distribution of liver cancer in Uganda.

Keen & Martin (1971) measured the extent of aflatoxin contamination of foodstuffs from various areas in Swaziland and at the same time estimated the geographical distribution of liver cancer based on the cancer registry data for 1964 to 1968. In areas where contamination of peanuts was high, the estimated incidence of liver cancer was also high. Further support relating dietary aflatoxins to human liver cancer are the results from an extensive and careful epidemiological study carried out in Kenya from 1967 to 1970 by Peers & Linsell (1973).

The conclusion that can be drawn from these studies in Africa and Southeast Asia is that there is strong epidemiological evidence incriminating aflatoxin B_1 as having an active part in hepatocarcinogenesis in man.

The effect of aflatoxins on animals is governed by several factors. These include dosage administered, the type of animal, the length of time that the animal is exposed to the toxin, and the age of the animal.

The carcinogenic properties of aflatoxins has been observed by feeding toxic peanut meal to rats. After

feeding a diet containing 20% of toxic peanut meal for six months, nine out of eleven rats developed multiple liver tumors, and two of these displayed lung metastases (Lancaster et al., 1961).

Marth (1967) associated aflatoxin with mouldy peanut meal which caused the death of a large number of poultry. The source of toxic material was found to be peanut meal used in the diet and imported from Brazil. The fungus Aspergillus flavus was recovered from peanut meal and found to produce the toxin.

In addition, Purchase et al., (1968) found that rats and cattle were able to modify and excrete some of the toxin in milk, (aflatoxin M_1 and M_2). The amount of aflatoxin M_1 excreted is directly related to the level of aflatoxin B_1 in the feed.

Members of the genus Aspergillus are also known to produce other toxic compounds such as kojic acid, sterigmatocystins, aspergillic acid, oxalic acid, β-nitropropanoic acid, terreic acid, fumigatin and fumagillin (Cole, 1976).

It may thus be seen that fungi are an important group of food spoilage agents. The most important genus in this respect is Aspergillus due to the prevalence of mycotoxin producing strains and probably the most important species is Asp.flavus due to its ability to produce aflatoxins in a wide variety of foodstuffs (Raymond, 1966).

The genus Aspergillus consists of a number of species, the most important of which in terms of spoilage are: Asp. flavus, Asp. glaucus, and Asp. candidus, which have been designated as "Storage Fungi" (Christensen, 1972)

due to their presence in a wide range of habitats in badly stored grain.

Toxin production is only one effect produced by the storage fungi. Others are a decrease in the germination percentage of malting barley and seeds to be used for planting, a discoloration and/or damage in seeds. Various biochemical changes which may make grains unfit or unattractive for food frequently occur. There is also the production of off flavours and heating which is usually accompanied by a drastic reduction in quality or complete spoilage (Christensen et al., 1969).

One of the most important effects however is the production of mycotoxins in seeds which are used for food and feeds. The kinds of seeds studied have included maize, rice, wheat, barley, oats, sorghum, common beans, soya beans and seed of various kinds of vegetables collected on a worldwide basis.

The invasion of seeds and grain by storage fungi is greatly influenced by the following factors: moisture content of the grain, temperature, presence of grain-infesting insects and mites, the storage time and the condition of the grain.

The temperature required for sporulation of fungi in stored products was found to be 13-41.5 ^{o}C at a relative humidity of 85-99% (Diener & Davis, 1967).

It is for this reason that periodic sampling from any lot of stored grain is recommended, each sample being tested for moisture content, damage, number and kinds of fungi.

It is evident, so far, that most of the damage

caused during storage is due to fungi. South American agricultural exports are highly susceptible to fungal attack due to a number of factors:

a) The prevailing tropical climate satisfies all the conditions for optimum fungal growth unless elaborate and expensive precautions are taken.

b) The techniques of harvesting and drying the produce cause considerable contamination by dormant fungal spores which are not detected by the grading techniques available.

With the increasingly precarious position of the world economy, it is clearly essential to make the best possible use of any proteins, such as groundnut meal, that become available for animal feeds, but it is equally essential to exclude aflatoxin contamination as far as possible.

From the previous reviews, it is obvious that all those factors engendered a tremendous impetus to study fungi, with special attention to Aspergillus flavus.

This fungus has the ability to produce aflatoxins associated with food commodities, and clearly the presence of a carcinogen in any food is considered potentially hazardous to human and animal health.

Furthermore, considerable attention is currently being given to the development of sensitive analytical control methods for detecting and estimating spoilage fungi.

Many approaches have been applied in the determination of spoilage fungi. These are, the production of pectin

degrading enzymes by the fungi, when grown in a medium incorporating pectin, the measurement of aflatoxin in different stored products and the measurement of chitin.

A number of authors worked in this field by measuring the pectinase activity as a rapid means for detecting the presence of spoilage fungi (Sreekantiah & Johar, 1963; Sreekantiah et al., 1973; Abdel-Fattah & Mabrouk, 1976 and Offem, 1980).

More work has been done by Aizenberg(1978) who studied the hydrolysis of pectin by 132 strains of different Fusarium species. The results showed that the content and qualitative composition of the pectin hydrolysis products varied with the different strains and depended on the culture age. It was also found that pectin hydrolysis by the Fusarium culture filtrate in the reaction mixture is accompanied by the formation of a number of end products of hydrolysis in addition to D-galacturonic acid.

Kratka & Vesely (1979) analysed the activity of pectinase, amylase and saccharase in Phythium spp. and studied the effect of pH and time of incubation (24 h - 15 days) on the cultivation of fungi. The result showed the same pattern in all fungal species examined.

Recently Offem (1980) studied methods for the detection and estimation of low levels of spoilage fungi. One of them was based on the estimation of pectinesterase produced by the fungus when grown in a medium supplemented with pectin. The enzyme hydrolyses the ester linkages of the pectin to produce methanol and polygalacturonic acid. The methanol was measured using gas liquid chromatography.

The results showed a straight line correlation between inoculum size (no. spores) and methanol production. Finally the method showed promise for the rapid detection and estimation of low numbers of dormant spores and can be used for quality control in industry. In addition it does not require a very high level of technical expertise.

Another approach was the measurement of aflatoxins. A variety of extraction procedures have been developed for use with various products or mouldy cultures on natural substrates, particularly in connection with chemical assays of agricultural commodities for aflatoxin contamination. The determinations have been carried out in maize, wheat, peanut, pistacho and dairy products and showed that the contamination occurred most frequently with members of the genera Aspergillus and Penicillium (Mojtahedi et al, 1979; Naoi, et al., 1979; Moreno, 1979; Takatori and Sued, 1979).

Chang et al., (1979) made a comparative study of aflatoxin by two methods, using peanut meal and peanut butter. Reverse phase liquid chromatography and T.L.C. were used to quantitate the aflatoxin. The results show the predominant aflatoxins were B_1, B_2, G_1 and G_2,

Lenovich et al., (1979) used high pressure liquid chromatography to quantitate aflatoxin which was produced in non-autoclaved and autoclaved Ivory Coast cocoa beans inoculated with Aspergillus parasiticus under optimum laboratory growth conditions. The results showed a low level of natural aflatoxin contamination in cocoa.

Lee et al., (1981) analysed a compound formed by ammoniation of the toxic and carcinogenic aflatoxin B_1. This compound was a non-fluorescent phenol and results

showed a marked reduction in biological activity with destruction of the lactone ring of aflatoxin B_1. These studies have shown that there are several sensitive methods for the detection and estimation of aflatoxins. However, aflatoxin production does not offer a means of rapid detection and estimation of fungal contamination because the aflatoxin production does not commence until cultures are approximately one week old. Also the level of aflatoxin production does not parallel fungi growth as they also metabolize it under certain conditions (Schroeder & Hein, 1967). A further problem is that due to its extreme toxicity, by the time it is detectable, the produce is useless.

The third approach is based on the measurement of chitin by its hydrolysis products (glucosamine & chitosan).

The study of the chemical constitution of fungal walls has showed that typically the walls are comprised of polysaccharides (75-80 per cent on a dry weight basis), protein, lipid and melanin. A variety of polysaccharides occur - the homopolymers β-1,3-glucan,cellulose,α-1, 3-glucan, chitin, chitosan, and a variety of heteropolymers (Rosenberger, 1976).

Evidence has accumulated linking morphological development with chemical changes in the cell wall, revealing an interesting relationship between the nature of the two major polymers present and the different taxonomic groups.

The chitin, which occurs in microfibrillar form is the skeletal component of the cell walls of most fungi.

(Bartnicki-Garcia, 1968). In higher plants, cellulose plays the role of chitin where it forms the microfibrillar component which provides the skeletal framework for the cell wall (Muhlethaler, 1967).

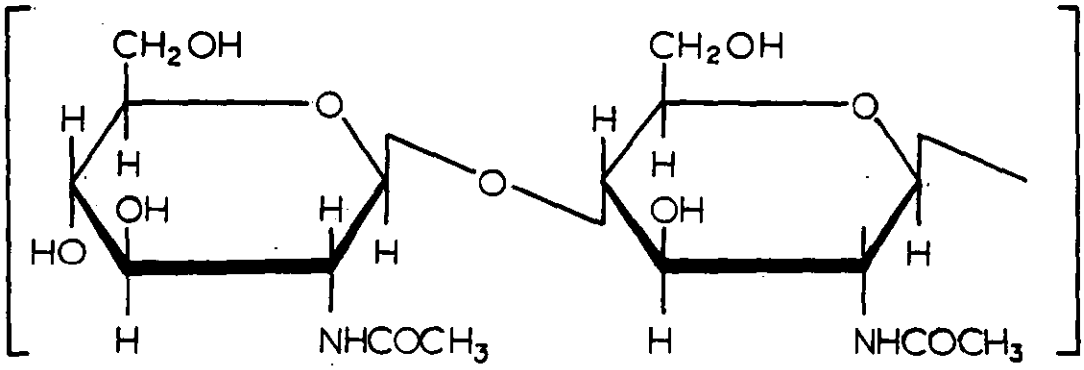
The chitin molecule consists of β-1,4-linked 2 acetamido 2-deoxy-D-glucose units; i.e. It is a long chain polymer of N-acetylglucosamine (Glc NAc) (see fig. 2). As with other polysaccharides, chitin is synthesised by glycosyl transfer from a nucleotide sugar, in this case uridine diphospho-N-acetyl-glucosamine (UDP-Glc NAc).

A knowledge of the structure and synthesis of the chitin is important for an understanding of the biogenesis of fungal walls. The first evidence for chitin biosynthesis in vitro was obtained by Glaser & Brown (1957), who demonstrated that uridine diphosphate N-acetyl-D-glucosamine (UDP-Glc NAc) was the precursor for the synthesis of a polymer recognized as "chitin" by its insolubility and its digestibility with chitinase. A number of subsequent researchers have repeatedly confirmed the ability of UDP-Glc NAc to serve as a precursor for chitin synthesis in cell-free extracts of other fungi (e.g., Porter & Jaworski, 1966; Camargo et al., 1967; McMurrough, Flores-Carreon & Bartnicki-Garcia, 1971; Peberdy & Moore, 1975; Lopez-Romero & Ruiz-Herrera, 1976).

Uridine-diphosphate N-acetyl-D Glucosamine is thought to be derived from the glycolytic intermediate fructuose-6 phosphate by the pathway shown in fig. 3. The enzyme that catalyzed the reaction in the final stage of chitin formation is chitin synthase (UDP-2-acetamido-2 deoxy-D-Glucose), this enzyme was first demonstrated using cell-free extracts of

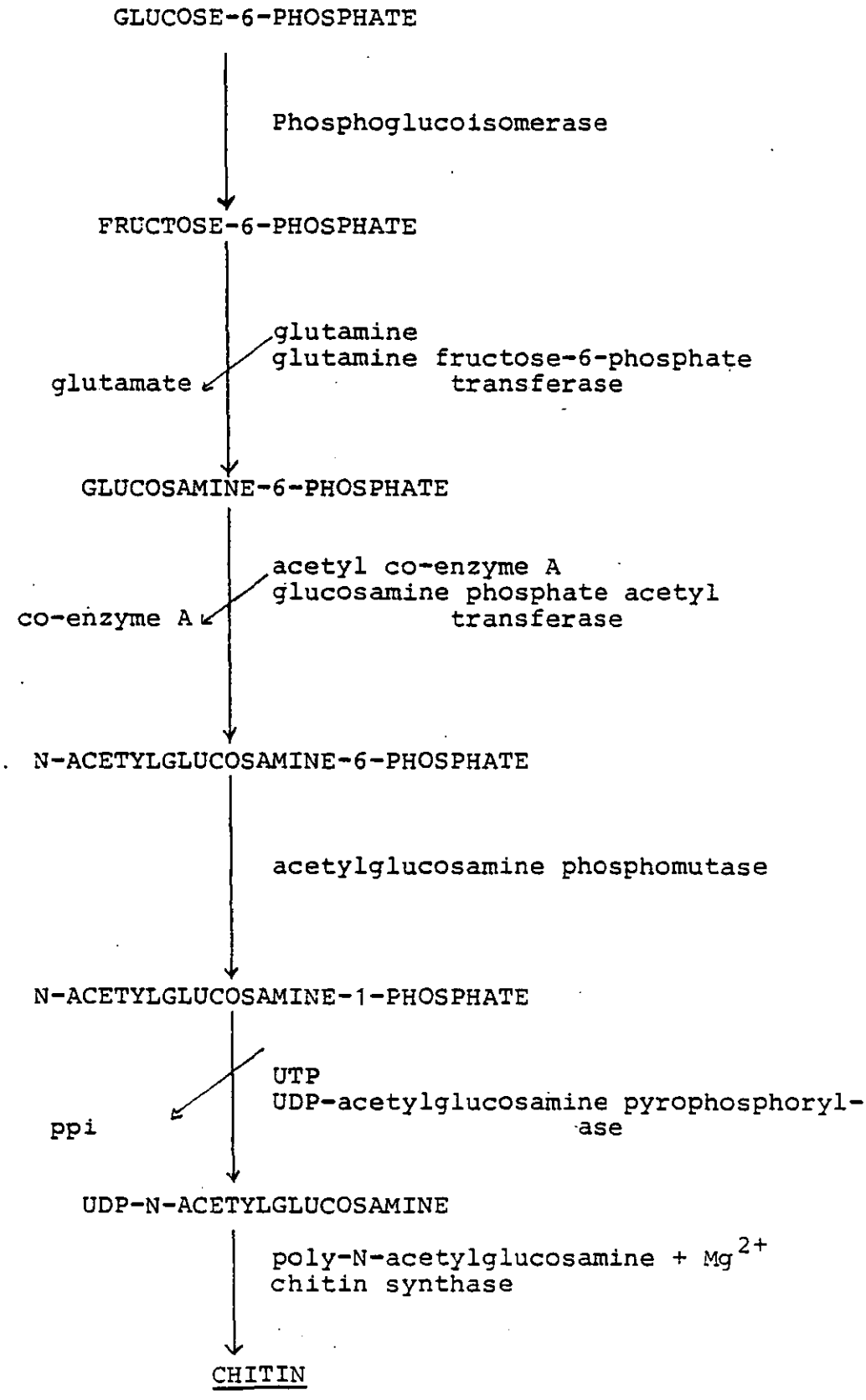
FIGURE 2

CHITIN



F I G U R E 3

Pathway of Chitin Synthesis



N. crassa (Glaser & Brown, 1957) and has been reported in many fungi such as Aspergillus flavus, Aspergillus nidulans, Mortierella vinacea, Cunninghamella elegans (Peberdy & Moore, 1975; Moore & Peberdy, 1975; Moore & Peberdy, 1976; Ryder & Peberdy, 1977).

These studies showed that the chitin synthase activity in the Mucorales is located in the cell wall fractions, during exponential growth. In the other organisms studied the enzyme activity was found mainly in the microsomal and mitocondrial fractions and was also present in an active form in mycelial material and freshly prepared protoplasts from spores and mycelium. Chitin also occurs in the exoskeletons of arthropods, and the same mechanism of synthesis has been demonstrated in both insects and crustaceans (Jaworski, Wang & Marco, 1963; Carey, 1965).

A number of workers have attempted to detect the lipid intermediate of chitin synthesis, as analogous to the biosynthesis of cellulose and bacterial cell wall polysaccharides. However, extensive work in this area has not produced any evidence for the involvement of lipid (Endo & Misato, 1969; Gooday, 1977; Ryder, 1977; Roger et al., 1980).

The occurrence of glucosamine in forms distinct from chitin has been demonstrated in plant tissues, where it is mainly used in the biosynthesis of glycoproteins and glycolipid, and in bacterial cell walls in peptidoglycan.

Chitin is structurally analogous to the glycan backbone of peptidoglycan (the 1-4-β alternate N-acetylglucosamine and N-acetylmuramic acid) which is the characteristic component of prokaryotic walls but the biosynthesis of the peptidoglycan is quite distinct and the process is totally

unrelated to the synthesis of fungal chitin.

As mentioned earlier, chitin is an important component of the walls of most fungi with the exception of a few members of the phycomycetes and can therefore be used as a measure of fungal growth (Bartnicki-Garcia, 1968). The chitin is usually determined as its hydrolysis product (glucosamine).

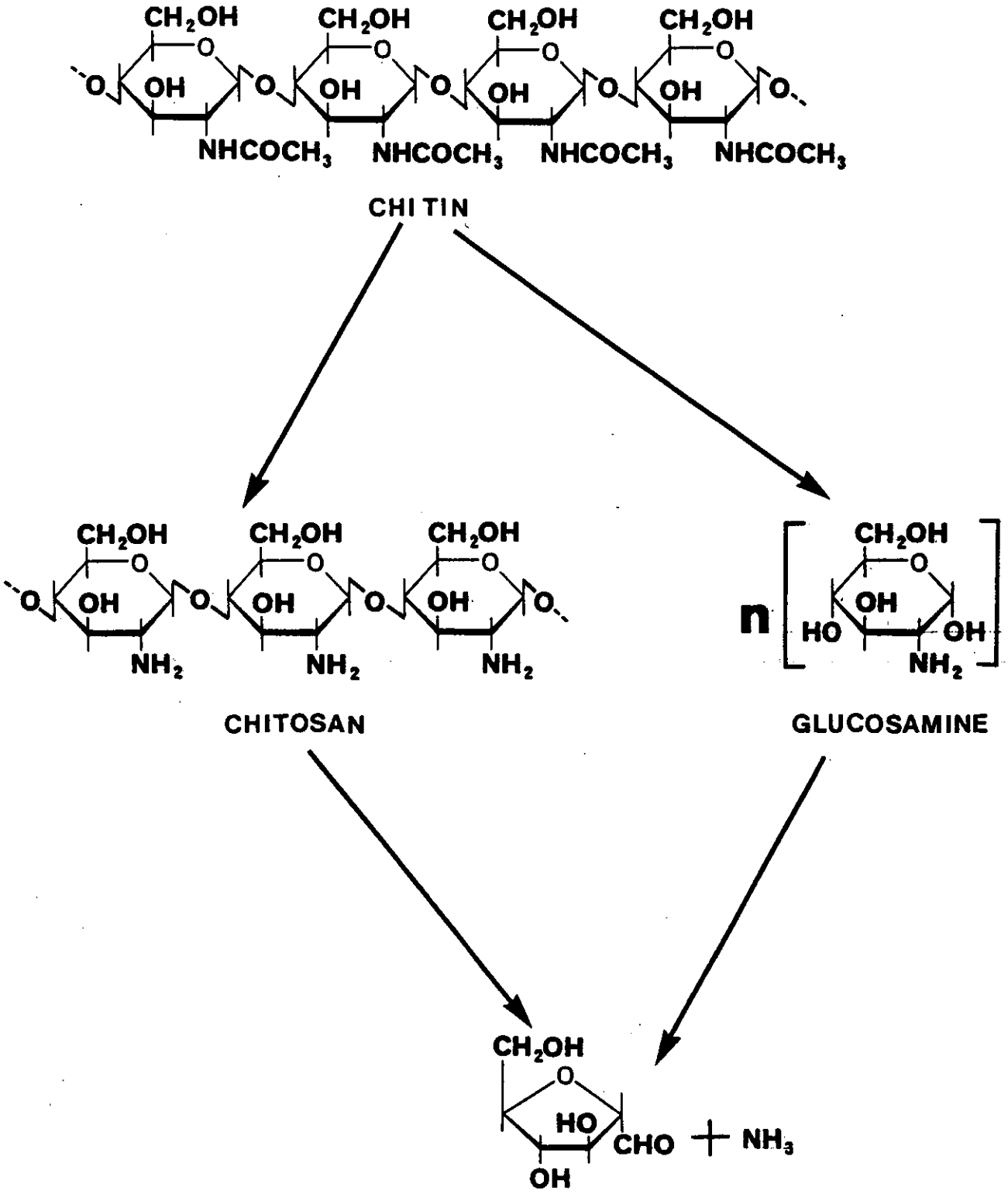
The estimation of glucosamine following hydrolysis of fungal chitin (see fig. 4), provides a method for the quantitative analysis of fungi in food and food products, but it has a disadvantage in that it cannot distinguish between viable and non-viable fungi.

The use of x-ray powder diagrams to detect chitin has become a popular technique (Aronson & Preston, 1960). However considerable progress has now been made with appropriate analytical techniques.

Arima & Uozumi, (1967) analysed chitin as glucosamine by a modification of Elson & Morgan's (1933) method in contaminated rice, and koji and found a relationship between the chitin content and mycelial dry weight which gave an indication of the level of fungal contamination.

A number of authors (Ride et al., 1971, 1972; Swift, 1973; and Jarvis, 1977), estimated the mycelial biomass of fungi decomposing plant materials by the use of a chitin assay technique. The method used was based on the alkaline deacetylation of chitin to chitosan, the glucosamine residues of which are susceptible to deamination with nitrous acid. This yields an aldehyde, which was determined with 3-methyl-2 benzothiazolone hydrazone. The results showed that the level of aldehyde expressed as glucosamine was related to fungal dry weight.

FIGURE 4



CHITIN HYDROLYSIS

Toppan et al., (1976) have reported a method for the determination of the mass of chitin containing fungal pathogens in plants. It is based upon the release of glucosamine by hydrochloric acid hydrolysis.

Holan et al., (1980) analysed chitin in biological materials. The method used was based on the measurement by gas liquid chromatography of acetic acid, liberated by acid or alkaline hydrolysis of chitin.

Other authors, Lung-Chi & Stahmann (1975); Hubbard, et al., (1979); Zacharius, (1976); Donald & Mirocha, (1977); Sharma, et al., (1977) have worked with measurement of fungal contamination by means of chitin determination.

When all these techniques are examined, it is found that they have been applied mainly to fungal contamination and have shown that the chitin assay provides the basis for an objective method which might find wide applicability in food analysis. The precision of the fungal glucosamine assay as an index of fungal contamination is reasonably high for a single operator working on some types of sample materials. The measurement of chitin offers promise in the evaluation of deterioration in stored grain and rapidly determining the quality of grain.

The contamination of food with bacteria is a well studied area, but there is little information on food spoilage due to mixed cultures of bacteria and fungi and there is virtually no information on the estimation of fungi in the presence of bacteria.

In terms of the spoilage of food, some of the most important bacterial species are Esherichia coli; Bacillus cereus; Staphylococcus aureus and Clostridium botulinum.

Therefore the purpose of this study was to carry out a quantitative estimation of glucosamine derived from chitin on mixed cultures of bacterial species and the fungus Aspergillus flavus. The bacteria selected were those likely to be found in food spoilage situations. Determination of the influence of these species on the fungal assay was also carried out.

M A T E R I A L S A N D M E T H O D S

MATERIALS

ORGANISMS

The following organisms were used in this study:

Aspergillus flavus	CMI 15959
Escherichia coli	NCTC 9001
Bacillus cereus	ATCC 11755
Staphylococcus aureus	NCIB 8625
Pseudomonas aeruginosa	NCIB 8295.

A.flavus was obtained from the Commonwealth Mycological Institute, Kew, Surrey. E. coli was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London. B.cereus was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. S.aureus and P.aeruginosa were obtained from National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

MEDIA

Fungi

Maintenance Media

A.flavus was grown on slopes at 30°C then stored at 4°C. The medium consisted of:

Maltose (Fisons Laboratory reagent grade)	38g
Neutralized Soya peptone (Oxoid Ltd.)	8g
Yeast Extract (Oxoid Ltd.)	2.5g
Malt Extract (Oxoid Ltd.)	2g
Agar Technical No. 3 (Oxoid Ltd.)	20g

These were dissolved and made up to 1 litre with distilled water. The pH was adjusted to 5.4 before sterilizing by autoclaving for 15 minutes. Subcultures were made every 2 weeks.

Growth Medium

A.flavus was grown on Czapek-Dox liquid medium made up as follows,

Sodium Nitrate	2.0g
Potassium Chloride	0.5g
Magnesium Glycerophosphate	0.5g
Ferrous Sulphate	0.01g
Potassium Sulphate	0.35g
Sucrose	30.0g

These were dissolved and made up to 1 litre with distilled water. The pH was adjusted to 5.8. 100 ml of of medium was dispensed into 500 ml Erlenmeyer flasks and sterilized by autoclaving at 115°C for 15 minutes.

Sporulation Medium

Cultures were grown in the following medium:

Tri-potassium Orthophosphate (Hydrated)	2.0g
Potassium Nitrate	2.0g
Calcium Chloride	0.25g
Yeast Extract	5.0g
Glucose	10.0g
Bacto Casamino Acids (Difco Laboratories, Detroit, Michigan)	5.0g
Metals Solution	0.1 ml
Distilled Water	1 litre

The metals solution comprised (mg/10 ml)

Magnesium Sulphate	0.5g
Zinc Sulphate	0.02g
Ferrous Sulphate	0.02g
Manganese Sulphate	0.01g
Cupric Sulphate	0.05g

This medium was adjusted to pH 5.4. 250 ml of medium were dispensed in 1 litre Roux bottles and autoclaved at 115°C for 10 minutes.

Bacteria

Maintenance Media

The bacteria were grown on Nutrient Agar slopes at 37°C then stored at 4°C. The medium consist of:

Lab-Lemco (Oxoid Ltd.)	10.0g
Neutralized Bacteriological peptone (Oxoid Ltd.)	10.0g
Sodium Chloride	5.0g
Agar Technical No.3 (Oxoid Ltd.)	20.0g

These were dissolved and made up to 1 litre with distilled water. The pH was adjusted to 7.4 and sterilized by autoclaving for 15 minutes. Subcultures were made weekly.

Growth Medium

Bacterial cultures were grown in Nutrient Broth made up as follows,

Lab-Lemco (Oxoid Ltd.)	10.0g
Neutralized Bacteriological peptone (Oxoid Ltd.)	10.0g
Sodium Chloride	5.0g
Distilled Water	to 1 litre

The pH was adjusted to 7.4. 100 ml of medium was dispensed into 500 ml Erlenmeyer flasks and sterilized by autoclaving at 115°C for 15 minutes.

Bacterial Sporulation Medium

Spores were obtained by a modification of the method in

British Pharmacopoeia (1980) using supplemented Nutrient Agar. The medium consist of:

Lab-Lemco (Oxoid Ltd.)	10.0g
Neutralized Bacteriological peptone (Oxoid Ltd.)	10.0g
Sodium Chloride	5.0g
Magnesium Sulphate	0.1g
Glucose	10.0g
Agar Technical No. 3 (Oxoid Ltd.)	20.0g

These were dissolved and made up to 1 litre with distilled water. The pH was adjusted to 7.4 before autoclaving for 15 minutes.

Buffer Solutions

The 1% phosphate buffer, containing the following constituents to pH 7.0

Disodium Hydrogen Orthophosphate.$12H_2O$	3.58g
Sodium Dihydrogen Orthophosphate.$2H_2O$	1.56g
Distilled Water	to 1 litre.

CHEMICALS

Unless otherwise specified, all chemicals were laboratory reagent grade.

Glucosamine hydrochloride was obtained from Sigma Chemical Co., U.S.A. and recrystallized before use.

p-Nitrobenzaldehyde was obtained from Fisons Ltd., Loughborough, Leics., England.

Pyridine and ethanol were obtained from Fisons Ltd., Loughborough, Leics., England, and redistilled before use.

Tetraethylammonium hydroxide was obtained from BDH Chemicals Ltd., Poole, Dorset.

EXPERIMENTAL METHODS

Fungi

Preparation and harvesting of fungal spores

The sporulation medium was inoculated with pure cultures of the fungus maintained as slopes in universal bottles. The Roux bottles were incubated under static conditions at 30°C for 3 weeks. At the end of this period, a thick mycelial mat covered with spores had grown over the surface of the medium in all cases. The spores were prepared and harvested by a modification of the procedure described by Dart (1975). The contents of the Roux bottle were tipped into a blender operated at low speed for 2 minutes to dislodge the spores for the mycelial mass. The mixture was then filtered twice through sterile loose cotton wool plugs into a sterile filtration flask. The filtrate was centrifuged for 15 minutes at 3500 x g, the supernatant decanted, and the spores washed with sterile distilled water. The centrifugation, decanting and washing processes were repeated until microscopic examination showed the spores were free of mycelial contamination. The spores were resuspended in sterile distilled water and stored in stock suspensions of approximately 400 ml volume at 0°C until required. To maintain a high percentage of viable spores in the suspensions used throughout the work, fresh stock suspensions were prepared every 2 months as described. This suspension was used as inoculum.

All spores suspensions were regularly checked for purity by plating out on fungal maintenance medium. The

plates were incubated at 30^{o}C for 48 hours. Checks were carried out using Nutrient Agar to determine whether there was bacterial contamination. Plates were incubated at 37^{o}C for 48 hours. The plates were done in 10 replicates.

FUNGAL GROWTH

The fungal growth medium was inoculated with a spore suspension to give a final concentration in the range 10^2 to 10^6 spores per ml. Inoculated flasks were incubated at $30^{\circ}C$ as shake cultures in a Gallenkamp orbital incubator rotating at 150 r.p.m. for 40 hours.

After incubation, the mycelium was harvested by filtration using a Whatman glass microfibre filter and washed three times with sterile distilled water. The mycelium was dried to a constant weight.

Before use, the spores underwent heat-shock activation by holding each suspension in a water bath at $50^{\circ}C$ for 25 minutes (Sussman 1969). The suspensions were then agitated vigorously for 15 minutes to prevent clustering of spores and ensure uniform distribution.

Total Counts

The actual number of spores per mililitre of a suspension was determined by means of a counting chamber (Improved Neubauer Chamber). The master suspension was serially diluted until reaching an appropriate dilution for counting and the process was repeated for every freshly prepared spore suspension. The total count was recorded for each spore suspension used.

Viable Counts

The viable counts of the spores suspensions were determined using fungal maintenance medium on the surface plate method. The spore suspensions were serially diluted. Plates were inoculated with 1 ml of the appropriate spore dilution and incubated at 30^{o}C for 48 hours. Counts were carried out in sextuplate for each spore suspension. Fungal colonies were counted on an Astell colony illuminator with the aid of an electronic colony counter, and the number of spores per mililitre were calculated.

Bacteria

Preparation and harvesting of Bacterial spores

Spores of B. Cereus were obtained from cultures grown in supplemented Nutrient Agar at 30°C for 10 days.

After incubation time the spores were harvested by centrifugation and washed five times with sterile distilled water. The supernatant fractions were decanted and the spores were resuspended in sterile distilled water and stored in stock suspension at 4°C. To maintain a high percentage of viable spores in the suspensions used, fresh stock suspensions were prepared every 2 weeks as described. This suspension was used as inoculum. Before use, the spores were heat-shocked in distilled water at 80°C for 10 minutes (Weingerger et al; 1980).

Bacterial Growth

Bacterial growth medium was inoculated from the master culture. Inoculated flasks were incubated at 37°C overnight.

After incubation, the cells were harvested by centrifugation and washed three times with sterile distilled water, followed by two volumes of phosphate buffer. The supernatant fractions were decanted, and the cells were resuspended in phosphate buffer and stored in stock suspension at 4°C. The stock suspensions were prepared every 3 days.

Total Counts

The number of cells per millilitre of bacterial suspension was determined by means of a counting chamber (Improved Neubauer Chamber) as described previously for fungal spores.

CHITIN DETERMINATION

Chitin analysis was carried out as follows by a method based on determination of the glucosamine liberated by acid hydrolysis of the chitin.

The total glucosamine was estimated according to the method described by Nakamura et al., (1969). This is based on the reaction of unacetylated hexosamine with p-nitro benzaldehyde in pyridine to yield schiff bases (p-nitro-benzylidene hexosamines) which give a red colour by addition of tetraethylammonium hydroxide in ethanol. The red colour formed by the condensation products of the hexosamine can be estimated spectrophotometrically at 504 nm.

The glucosamine in test samples could be determined by comparison with a calibration curve in the range 10 - 120 µg/ml of glucosamine.

Chitin Analysis as an Index of Fungal Contamination

Growth Curves

500 ml Erlenmeyer flasks containing 100 ml of fungal growth medium were inoculated with fungal spore suspension to give a final concentration in the range 10^2 to 10^6 spores per ml. and incubated with shaking at 30°C for 40 hours. At the end of this period the mycelium was harvested as described earlier.

The dry mycelium obtained from each concentration of spore suspension were weighed and placed in pyrex screw cap tissue culture tubes and 4 ml of 6N hydrochloric acid was added. Nitrogen gas was bubbled through the contents from a pasteur pipette to remove oxygen. The tubes are stoppered tightly and hydrolyzed for 10 hours at 100°C.

After hydrolysis, the flask and sample were cooled to room temperature before being opened. The hydrolysates were filtered to remove the residue. The filtrates were evaporated under reduced pressure and the residue redissolved in distilled water.

A 0.5 ml volume of 1% (w/v) p-nitrobenzaldehyde solution in pyridine (freshly prepared) was added to the sample, and the mixture was shaken, and left for 20 minutes at 27 - 28°C. It was then cooled in ice for 5 minutes and diluted with 10 ml of ice-cold 0.3% (v/v) tetraethyl-ammonium hydroxide in ethanol, the absorbance was measured at 504 nm against the reagent blank after the tubes had been allowed to stand for 10 - 25 minutes at room temperature (Nakamura et al., 1969).

Preliminary work was necessary to determine the optimum conditions for hydrolysis of the sample. Maximum glucosamine yield was found after 10 hours incubation of the sample in 4 ml of 6N hydrochloric acid.

Determination of Fungal Chitin in Presence of Bacterial Contamination

500 ml Erlenmeyer flasks containing 100 ml of fungal growth medium were inoculated with the fungal spore suspension to give an appropriate concentration for each experiment. Bacterial cell suspension was added to obtain the required concentration.

The estimation of chitin was carried out on 8 mixed cultures of fungal spores and bacterial cells as follows:

1. A mixture containing a fixed number of fungal spores and a variable number of E.coli cells.

The fungal spore suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 spores per ml. Bacterial cell suspension was then added to obtain a bacterial concentration in the medium ranging from 10^4 to 10^8 cells per ml.

2. A mixture containing a fixed number of E.coli cells and a variable number of fungal spores.

E.coli cell suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^7 cells per ml. Fungal spore suspension was then added to give a final concentration in the range from 10^2 to 10^6 spores per ml.

3. A mixture containing a fixed number of fungal spores and a variable number of B.cereus cells.

The fungal spore suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 spores per ml. Bacterial cell suspension was added to obtain a bacterial concentration in the medium ranging from 10^3 to 10^7 cells per ml.

4. A mixture containing a fixed number of B.cereus cells and a variable number of fungal spores.

B.cereus suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 cells per ml. Fungal spore suspension was then added to give a final concentration in the range from 10^2 to 10^6 spores per ml.

5. A mixture containing a fixed number of fungal spores and a variable number of B.cereus spores.

The fungal spore suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 spores per ml. Bacterial spore suspension was then added to obtain a final spore concentration in the medium ranging from 10^2 to 10^6 spores per ml.

6. A mixture containing a fixed number of B.cereus spores and a variable number of fungal spores.

B.cereus spores were inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 spores per ml. Fungal spore suspension was then

added to give a final concentration in the range from 10^2 to 10^6 spores per ml.

7. A mixture containing a fixed number of fungal spores and a variable number of S.aureus cells.

The fungal spore suspension was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 spores per ml. Bacterial cell suspension was then added to obtain a bacterial concentration in the medium ranging from 10^2 to 10^6 cells per ml.

8. A mixture containing a fixed number of S.aureus and a variable number of fungal spores.

S.aureus was inoculated into 500 ml flasks containing 100 ml of medium to give a final concentration of 10^5 cells per ml. Fungal spore suspension was then added to give a final concentration in the range from 10^2 to 10^6 spores per ml.

All inoculated flasks were incubated with shaking at 30^oC for 40 hours. After this period the mycelium and cells were harvested by filtration. The mycelium was washed ten times with sterile distilled water to remove the bacterial cells and dried to a constant weight. Dried mycelium was weighed and the chitin analysis was carried out as previously described.

Measurement of Growth

500 ml Erlenmeyer flasks containing 100 ml of fungal growth medium were inoculated with fungal spore suspension to obtain a final concentration ranging from 10^2 to 10^6 spores per ml. These were then incubated with shaking at $30^{\circ}C$ for 40 hours. After incubation, the dry weights of the mycelial materials were determined by filtering the culture medium through filter paper and washing six times with sterile distilled water. The mycelial materials were dried at $90^{\circ}C$ to a constant weight. Five replicates of each inoculum were taken.

Similar procedures were repeated using mixed cultures of A.flavus and B.cereus. The Erlenmeyer flasks containing 100 ml of fungal growth medium were inoculated with fungal spore suspension to obtain a final concentration ranging from 10^2 to 10^6 spores per ml. Bacterial suspension was then added tc give a final cell concentration of 10^5 cells per ml.

After incubation with shaking at $30^{\circ}C$ for 40 hours, the dry weights of the mycelial material were determined as described earlier.

R E S U L T S

The number of spores of Aspergillus flavus were estimated by measuring the glucosamine formed from the hydrolysis of chitin.

Calibration curves of glucosamine (fig. 5) and glucosamine against spore numbers (fig. 6) were obtained.

Attempts were made to determine fungal numbers in the presence of bacteria.

In one set of experiments fungal cultures were grown in the presence of bacteria and in a second set the fungi were mixed with bacteria immediately prior to the estimation of glucosamine.

The first set of experiments were divided into two groups. Those in which the number of fungi varied and bacteria were kept constant and the second in which the number of bacteria varied and the fungi were kept constant.

The first bacterium used was a typical Gram-negative bacteria E.coli. Studies were carried out in mixed cultures of fungi and bacteria cultured simultaneously (fig. 7, table 1). The results suggest that the presence of E.coli at varying concentrations does not influence the level of glucosamine production by A.flavus.

When E.coli cells were mixed with the fungi prior to glucosamine estimation the results obtained were similar to those shown in fig. 7.

No glucosamine could be detected when high concentrations of E.coli were tested alone.

The second bacterial species used was a typical Gram-positive organism B.cereus. Estimation of glucosamine content was carried out on vegetative cells of B.cereus

in the concentration range of 10^3 to 10^7 cells per ml. The results in fig. 8 show a gradual increase in glucosamine at high cell concentrations, but none can be detected at concentrations below 10^4 cells per ml.

Further work was carried out on the detection of fungal numbers in the presence of vegetative cells of B.cereus (fig. 9, table 2). A decrease in glucosamine level was observed when varying concentrations of fungal spores were cultured simultaneously with a fixed number of bacteria (fig. 9) and also when varying concentrations of bacteria were grown with a fixed number of fungal spores (table 2).

When cells of B.cereus were added to cultures of A.flavus immediately prior to glucosamine determination, the results obtained in fig. 10 were found, indicating that there was no significant change under the experimental conditions used.

Glucosamine content was also estimated in B.cereus spores (fig. 11). Results were similar to those found in vegetatives cells (fig. 8).

The results for the estimation of glucosamine in mixed cultures of fungal spores ranging from 10^2 to 10^6 spores per ml. and a fixed concentration of bacterial spores are shown in fig. 12.

Results of the estimation of glucosamine in mixed cultures of fungal spores (fixed concentration) and bacterial spores ranging from 10^2 to 10^6 spores per ml. are shown in table 3. A decrease in the level of glucosamine (fig. 12, table 3) was observed with respect to the level of glucosamine estimated in pure fungal cultures (fig.6).

The estimation of glucosamine was repeated using S.aureus (table 4).

The level of glucosamine was also estimated in mixed cultures of fungal spores ranging from 10^2 to 10^6 spores per ml. and S.aureus cells (fixed concentration). These results are shown in fig. 13.

Results for the estimation of glucosamine in mixed cultures of fungal spores (fixed concentration) and bacterial cells ranging from 10^2 to 10^6 cell per ml. are shown in table 5. A decrease in glucosamine level was observed (fig. 13, table 5).

The results presented in fig. 14 show that the addition of S.aureus to A.flavus immediately prior to glucosamine estimation has no significant effect compared with glucosamine levels in pure fungal cultures (fig. 6).

Attempts were made to carry out similar work on P.aeruginosa but the pigments produced interfered with the glucosamine assay and this line of research was not extended further.

The fungal spore suspensions used throughout the work were tested for viability and the results can be seen in table 6.

Further experiments were concerned with the ability of different species of bacteria to grow in Czapek Dox liquid medium and in conditions normally used for fungal growth (table 7). E.coli did not grow on this medium when incubated at $30^{\circ}C$ for 40 hours, whereas B.cereus and S.aureus show some growth under these conditions.

The results shown in fig. 9 when B.cereus and A.flavus were grown simultaneously show that there is a decrease in

glucosamine produced. It was thought that this might be due to a decreased production of fungal mycelium because of bacterial competition. An experiment was therefore carried out in which the dry weight of fungal mycelium was obtained from pure cultures of A.flavus and mixed cultures of A.flavus and B.cereus. The results shown in table 8 indicate that when a mixed culture is grown there is a considerable reduction in the amount of fungal mycelium produced.

FIGURE 5

CALIBRATION CURVE FOR GLUCOSAMINE DETERMINATIONS

The standard solution used was Glucosamine-Hydrochloride which had been purified by recrystallization from aqueous ethyl alcohol.

Each point is the mean of 6 determinations, highest standard deviations = 0.005%

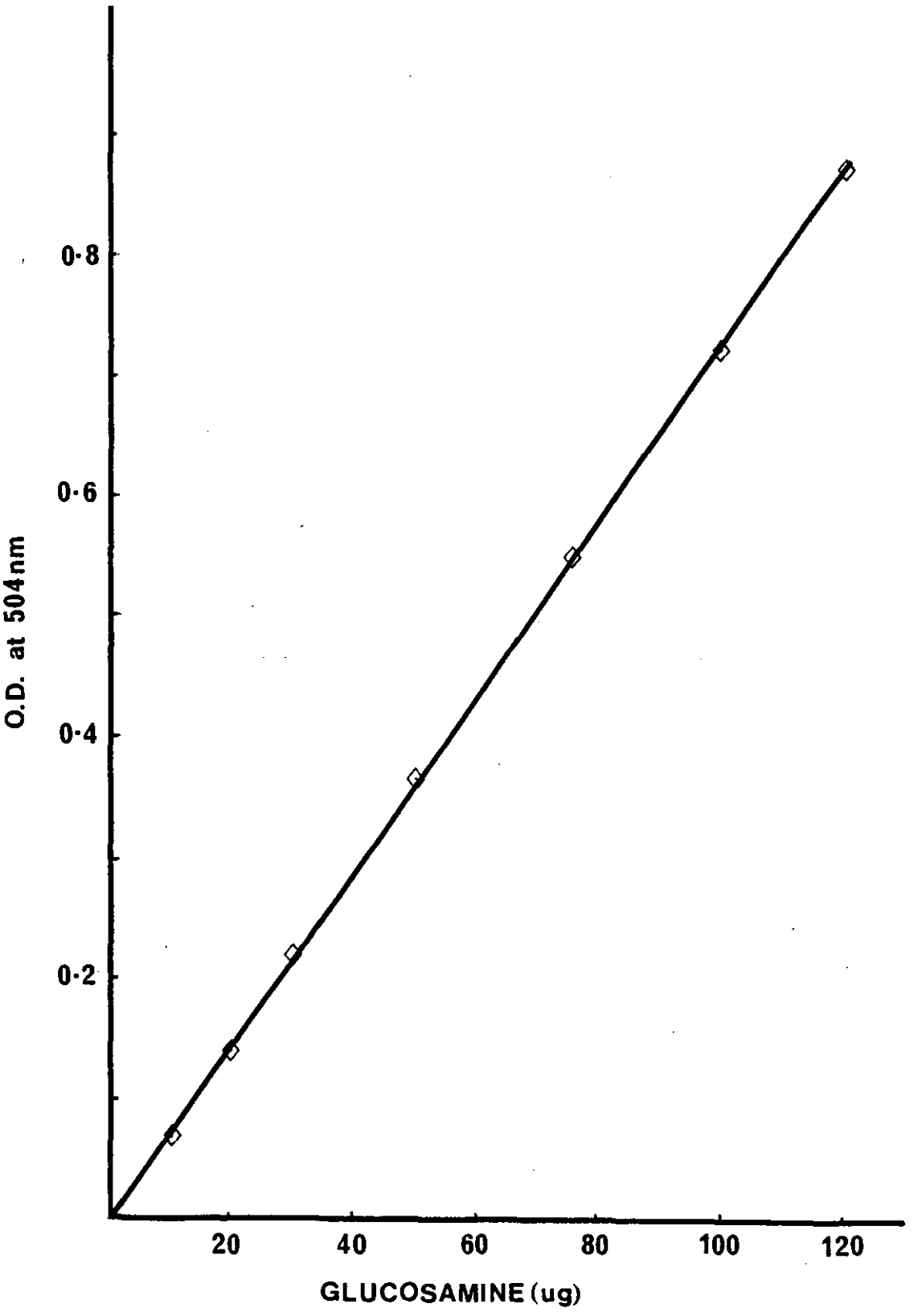

0·8
0·6
0·4
0·2
O.D. at 504nm
20
40
60
80
100
120
GLUCOSAMINE (ug)

FIGURE 6

DETERMINATION OF SPORE NUMBERS OF A.flavus FROM THE CONCENTRATION OF GLUCOSAMINE

A calibration curve was plotted between $\log_{10}$ spore number per ml (y) and glucosamine concentration (µg) after 40 hours of incubation (X).

Each point is the mean of three cultures, three determinations per culture.

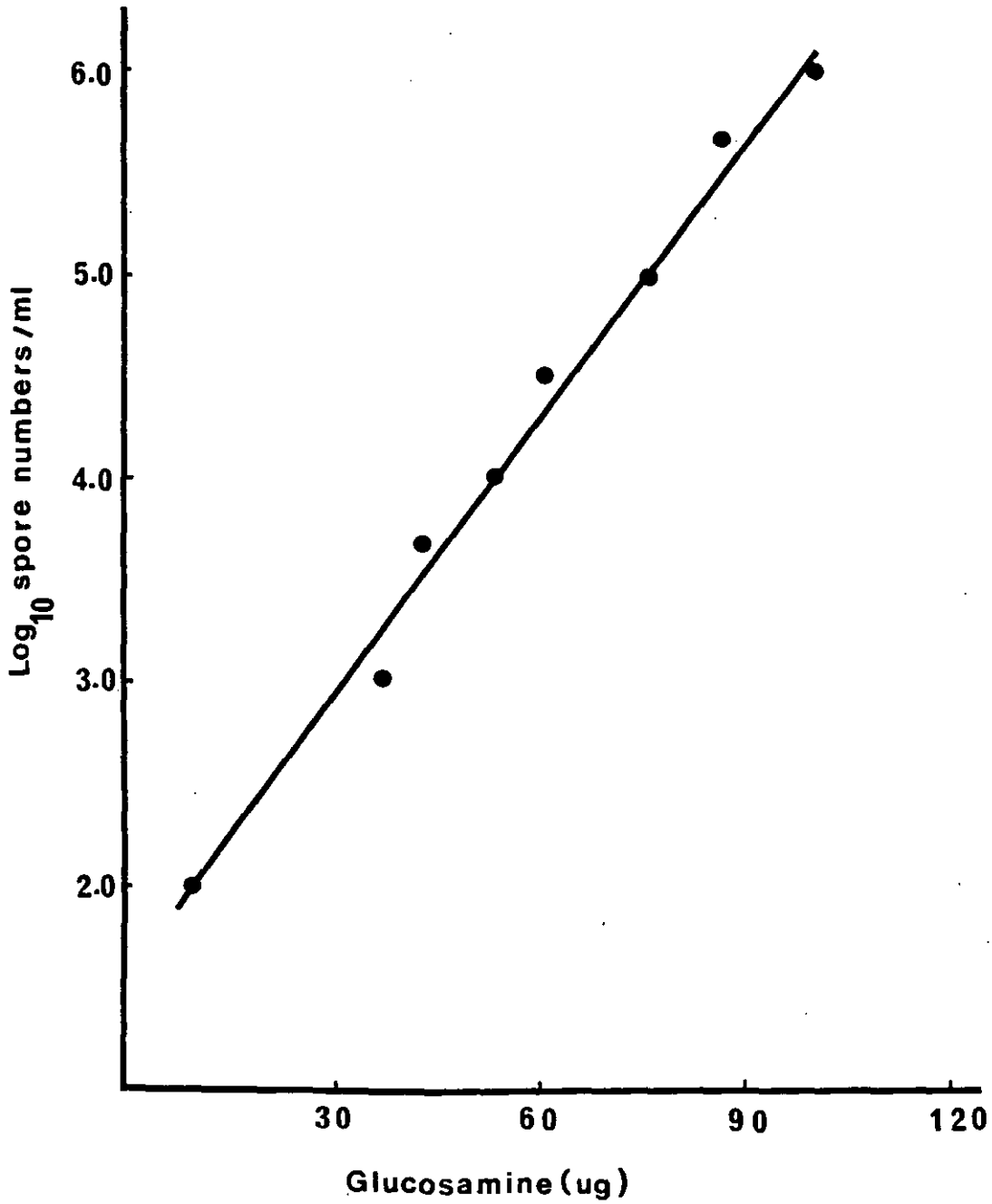

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers / ml
30
60
90
120
Glucosamine (ug)

FIGURE 7

GLUCOSAMINE ESTIMATION IN MIXED CULTURES OF *A.flavus* AND *E.coli*

A standard curve was plotted between $\log_{10}$ spore number per ml (y) and glucosamine concentration (µg) (X). Spore suspensions of *A.flavus* were cultured simultaneously with cells of *E.coli* at 30°C for 40 hours.

A.flavus

A.flavus and *E.coli*

Each point is the mean of 5 determinations.

The two sets of points shown in this graph were initially plotted separately to give two straight lines. These were very close together and statistical analysis by the method of least squares (Lee et al., 1982), indicates that they are estimates of the same straight line.

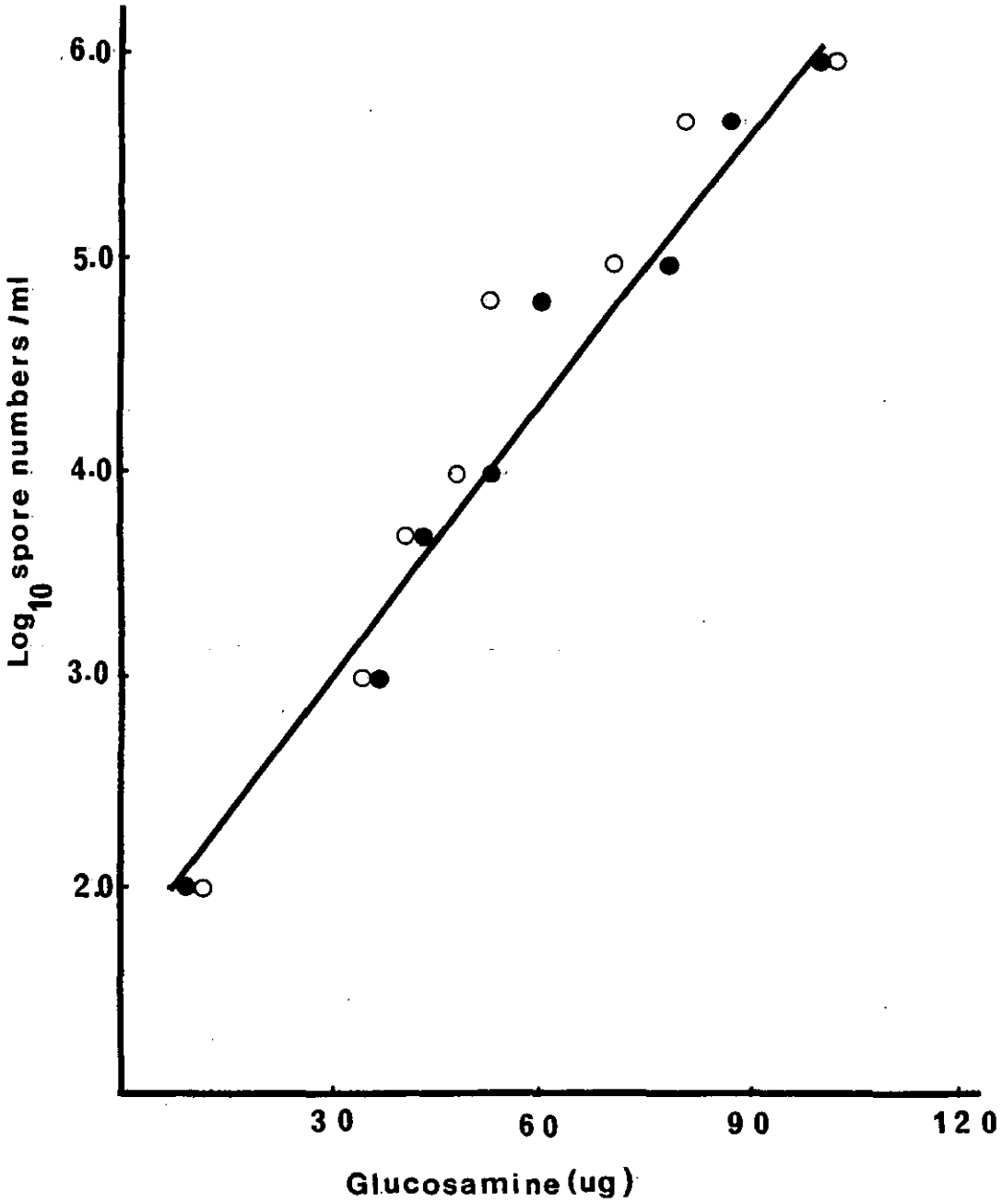

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers /ml
30
60
90
120
Glucosamine (ug)

TABLE 1

GLUCOSAMINE PRODUCTION BY A.flavus IN THE PRESENCE OF VARYING AMOUNTS OF E.coli

Varying concentrations of E.coli cells were cultured simultaneously with 10^5 spores per ml. of A.flavus in Czapek Dox medium and incubated with shaking at 30°C for 40 hours. Glucosamine production was then determined for each concentration.

Each value is the mean of three cultures, three determinations per culture.

TABLE 1

ORGANISM		Glucosamine (µg)	STD.DEV
E.coli (9001)	A.flavus (15959)		
Cell numbers/ ml	Spore numbers/ ml		
10^4	10^5	71.66	± 2.88
10^5	10^5	69.00	± 1.73
10^6	10^5	71.66	± 2.88
10^7	10^5	69.33	± 1.15
10^8	10^5	67.00	± 0.00

FIGURE 8

ESTIMATION OF GLUCOSAMINE IN VEGETATIVE CELLS OF B.cereus

The cell suspension of B.cereus were diluted serially and glucosamine determinations were made for each concentration as described in the methods section.

Each point is the mean of 5 determinations.

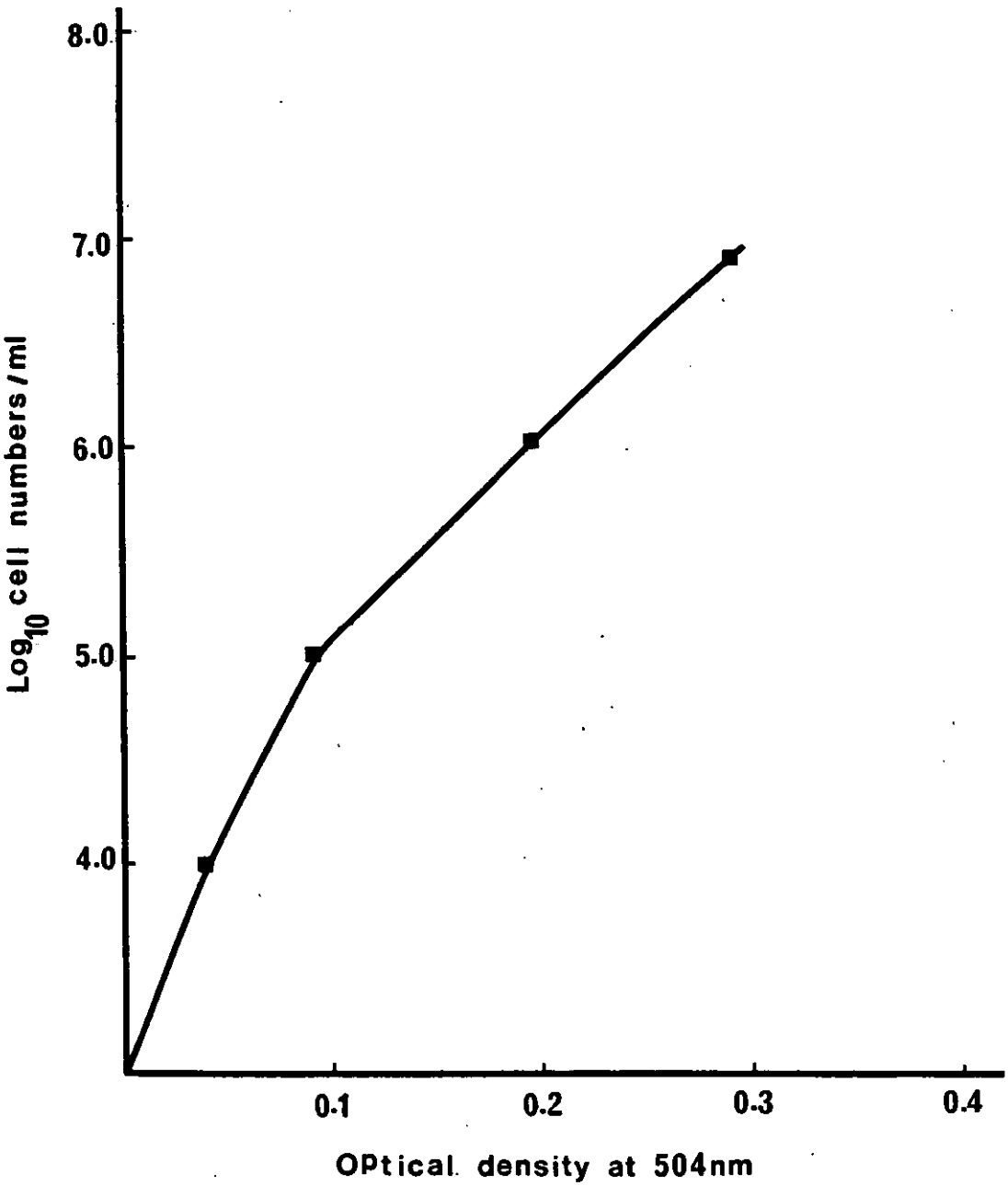

8.0
7.0
6.0
5.0
4.0
Log10 cell numbers/ml
0.1
0.2
0.3
0.4
OPtical density at 504nm

FIGURE 9

GLUCOSAMINE ESTIMATION IN MIXED CULTURES OF *A.flavus* AND *B.cereus* CELLS

Varying concentrations of *A.flavus* spores were cultured simultaneously with fixed concentration of *B.cereus* at 30°C for 40 hours.

A.flavus

A.flavus and *B.cereus*

Each point is the mean of 5 determinations.

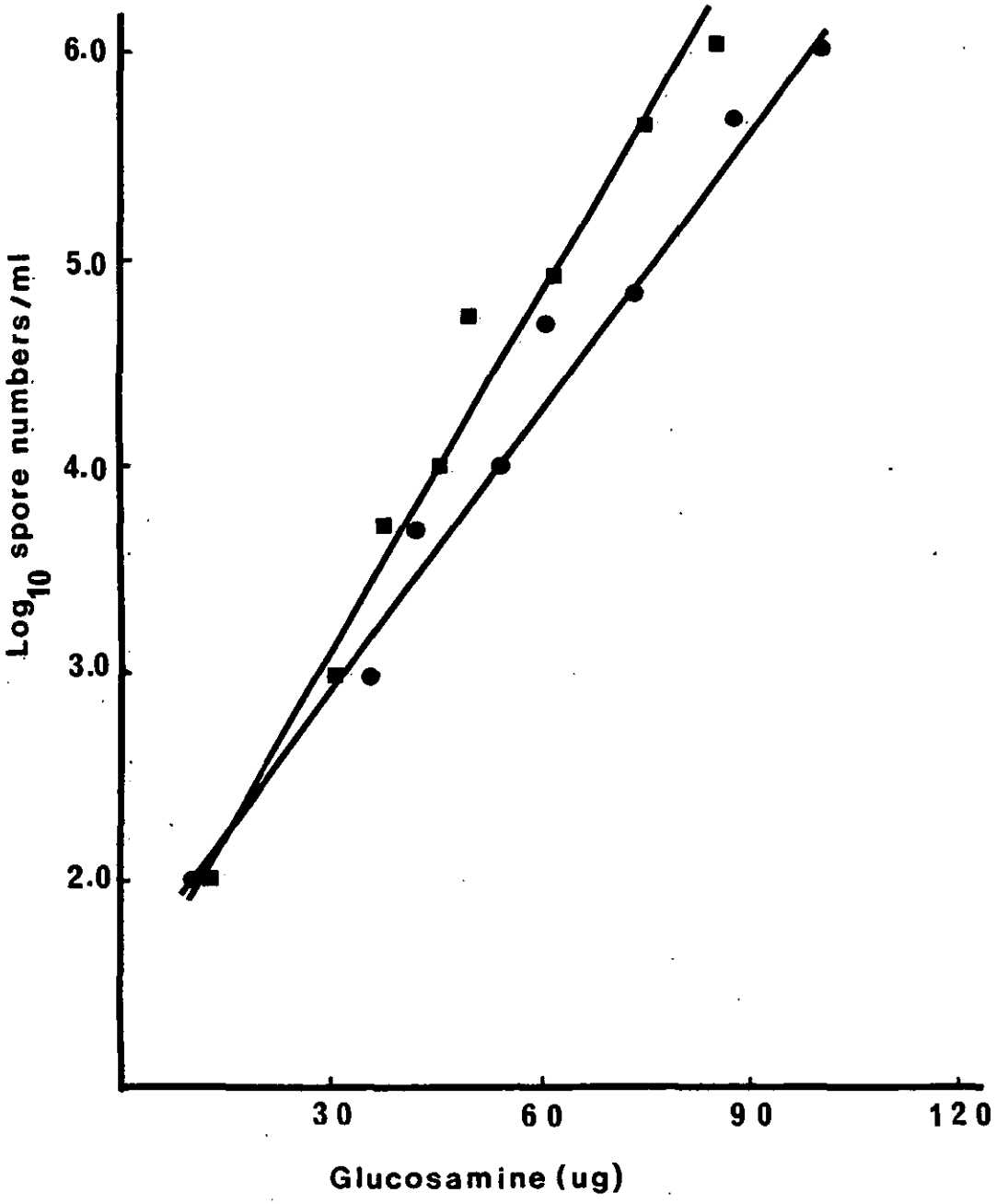

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers/ml
30
60
90
120
Glucosamine (ug)

TABLE 2

GLUCOSAMINE PRODUCTION BY A.flavus IN THE PRESENCE OF VARYING AMOUNTS OF B.cereus CELLS

Varying concentrations of B.cereus cells were cultured simultaneously with 10^5 spores per ml. of A.flavus in Czapek Dox medium and incubated with shaking at $30^{\circ}C$ for 40 hours. Glucosamine production was then determined for each concentration.

Each value is the mean of three cultures, three determinations per culture.

TABLE 2

ORGANISM		Glucosamine (µg.)	STD. DEV
B.cereus (11755)	A.flavus (15959)		
Cell numbers/ ml	Spore numbers/ ml		
10^3	10^5	71.66	±2.88
10^4	10^5	70.00	±0.00
10^5	10^5	61.66	±0.57
10^6	10^5	57.00	±1.73
10^7	10^5	51.66	±2.30

FIGURE 10

COMPOSITE GRAPH FOR A.flavus AND B.cereus CELLS

A standard curve was plotted between $\log_{10}$ spore number per ml (y) and glucosamine concentration (µg) (X). Fungal cultures were mixed with cells of B.cereus immediately prior to glucosamine determination.

A. flavus

A.flavus and B.cereus

Each point is the mean of 5 determinations.

The two sets of points shown in this graph were initially plotted separately to give two straight lines. These were very close together and statistical analysis by the method of least squares (Lee et al., 1982), indicates that they are estimates of the same straight line.

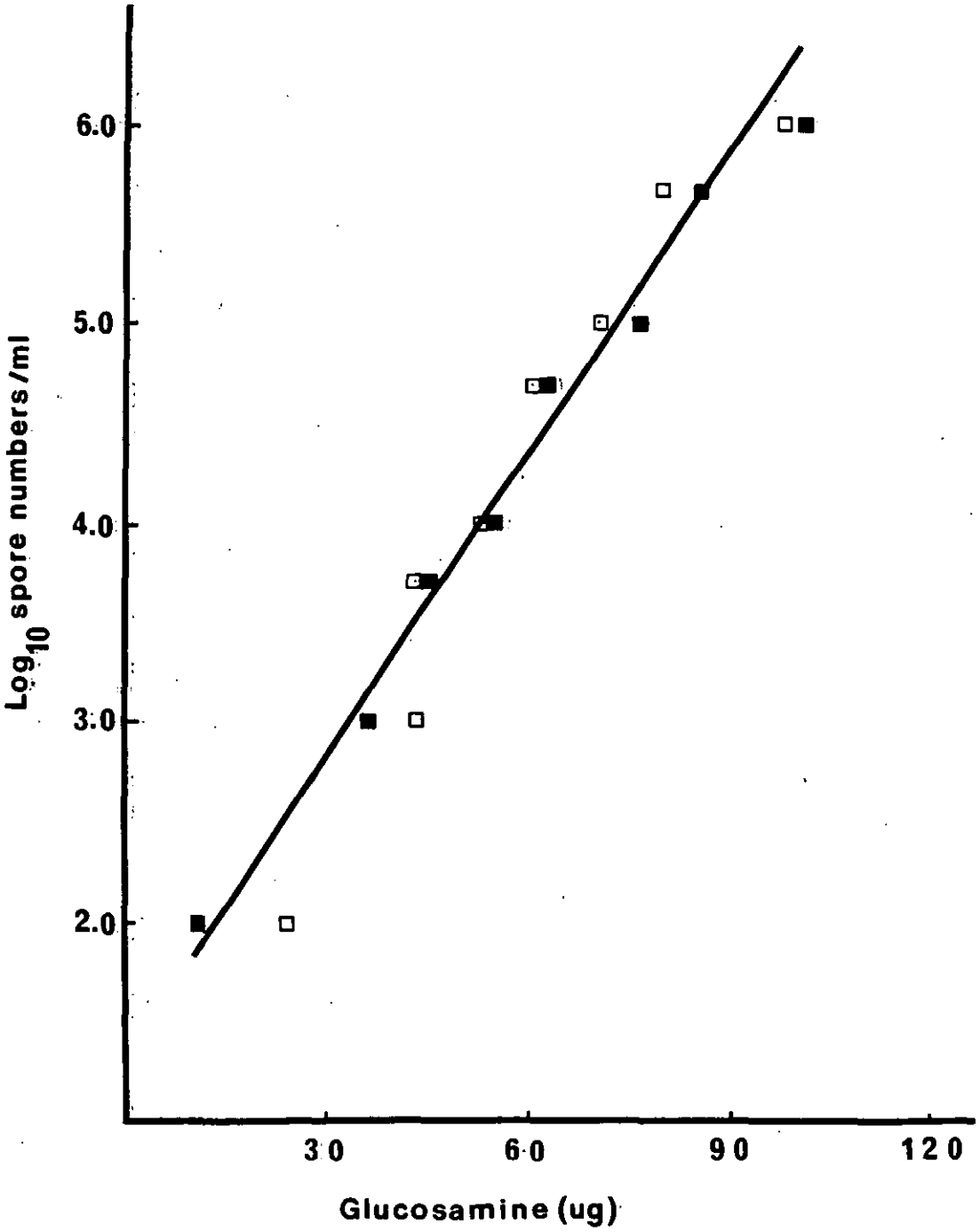

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers/ml
30
60
90
120
Glucosamine (ug)

FIGURE 11

ESTIMATION OF GLUCOSAMINE IN SPORES OF B.cereus

The spore suspensions of B.cereus were diluted serially and glucosamine determinations were made for each concentration as described in the methods section.

Each point is the mean of 5 determinations.

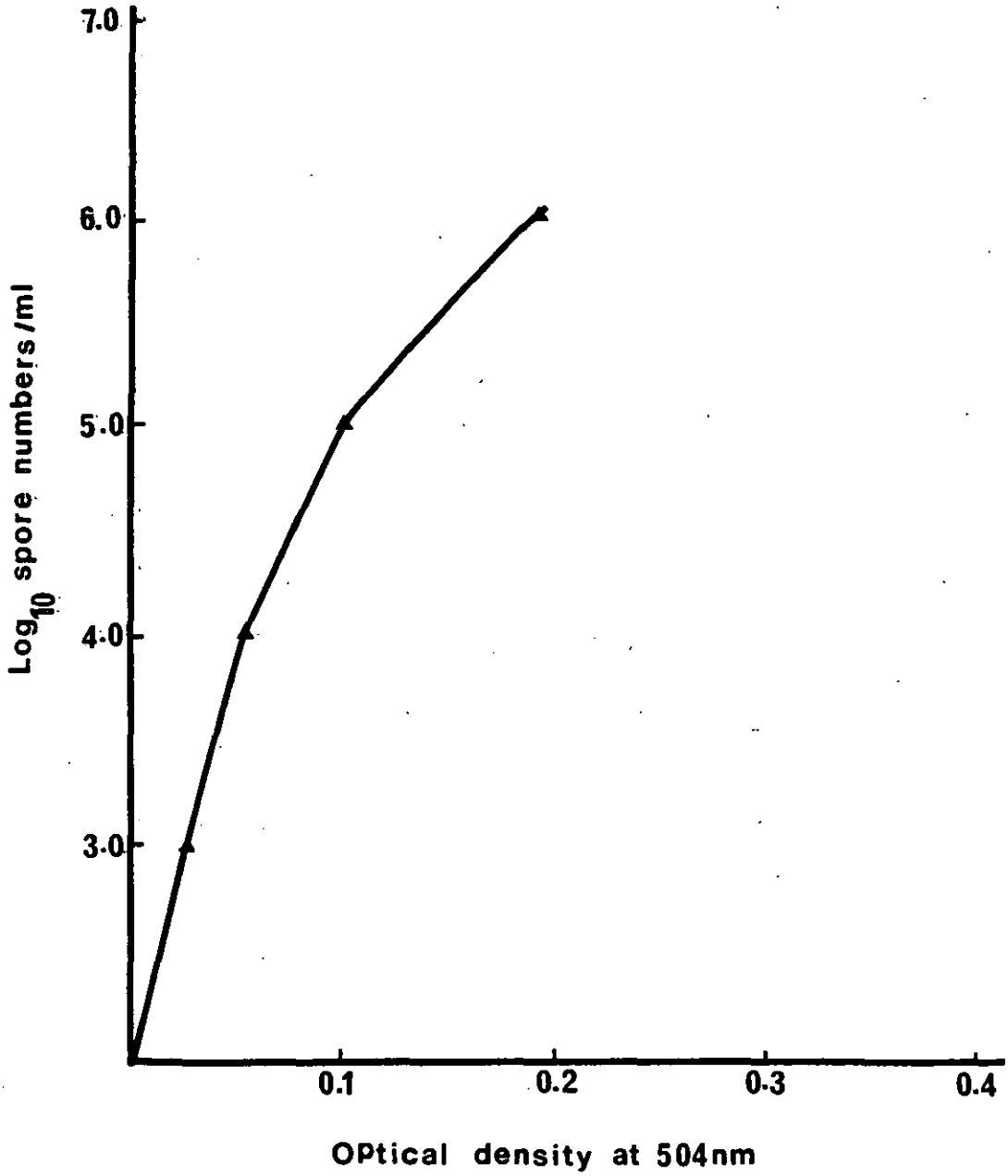

7.0
6.0
5.0
4.0
3.0
Log10 spore numbers/ml
0.1
0.2
0.3
0.4
OPtical density at 504nm

FIGURE 12

GLUCOSAMINE ESTIMATION IN MIXED CULTURES OF *A.flavus* AND *B.cereus* SPORES

Varying concentrations of *A.flavus* spores were cultured simultaneously with fixed concentration of *B.cereus* spores at 30°C for 40 hours.

●——● *A. flavus*

▲——▲ *A.flavus* and *B.cereus* spores.

Each point is the mean of 5 determinations.

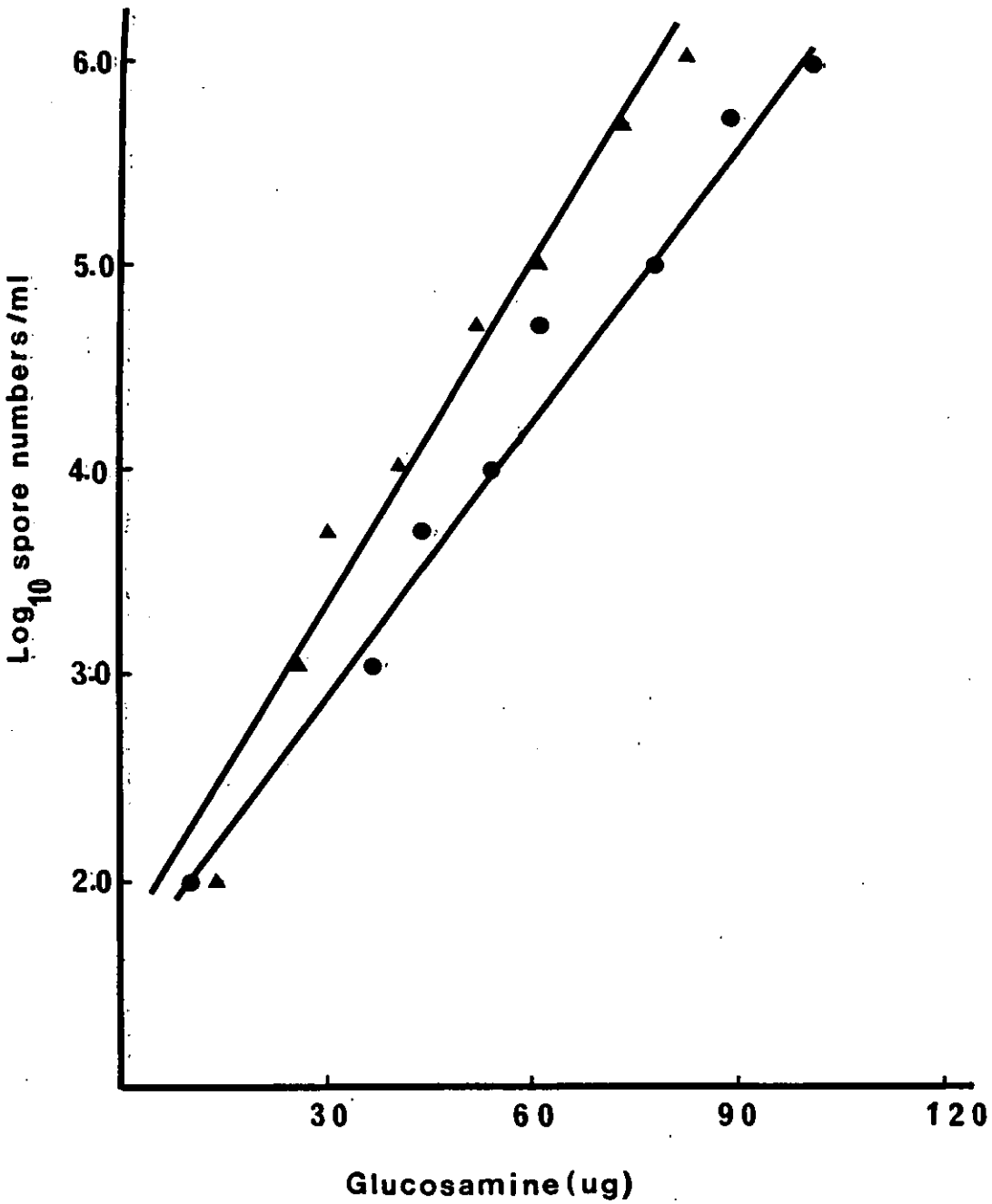

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers/ml
30
60
90
120
Glucosamine(ug)

TABLE 3

GLUCOSAMINE PRODUCTION BY A.flavus IN THE PRESENCE OF VARYING AMOUNTS OF B.cereus SPORES

Varying concentrations of B.cereus spores were cultured simultaneously with 10^5 spores per ml. of A.flavus in Czapek Dox medium and incubated with shaking at $30^{\circ}C$ for 40 hours. Glucosamine production was then determined for each concentration.

Each value is the mean of three cultures, three determinations per culture.

TABLE 3

ORGANISM		Glucosamine (µg)	STD. DEV
B.cereus (11755)	A.flavus (15959)		
Spore numbers/ ml	Spore numbers/ ml		
10^2	10^5	62.50	±4.04
10^3	10^5	62.00	±0.00
10^4	10^5	53.66	±4.04
10^5	10^5	57.66	±2.88
10^6	10^5	47.00	±3.46

TABLE 4

ESTIMATION OF GLUCOSAMINE IN CELLS OF S.aureus

The cell suspension of S.aureus were diluted serially and glucosamine determination was made for each concentration as described in the methods section.

Each value is the mean of 5 determinations.

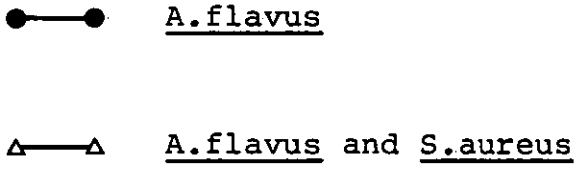
TABLE 4

ORGANISM *S.aureus* (8625)	Glucosamine (µg)	STD. DEV
Cell numbers/ml		
10^6	14.66	± 0.57
10^5	10.00	± 0.00
10^4	ND	-
10^3	ND	-
10^2	ND	-

FIGURE 13

GLUCOSAMINE ESTIMATION IN MIXED CULTURES OF *A.flavus* AND *S.aureus*

Varying concentrations of *A.flavus* spores were cultured simultaneously with fixed concentration of *S.aureus* at 30°C for 40 hours.



Each point is the mean of 5 determinations.

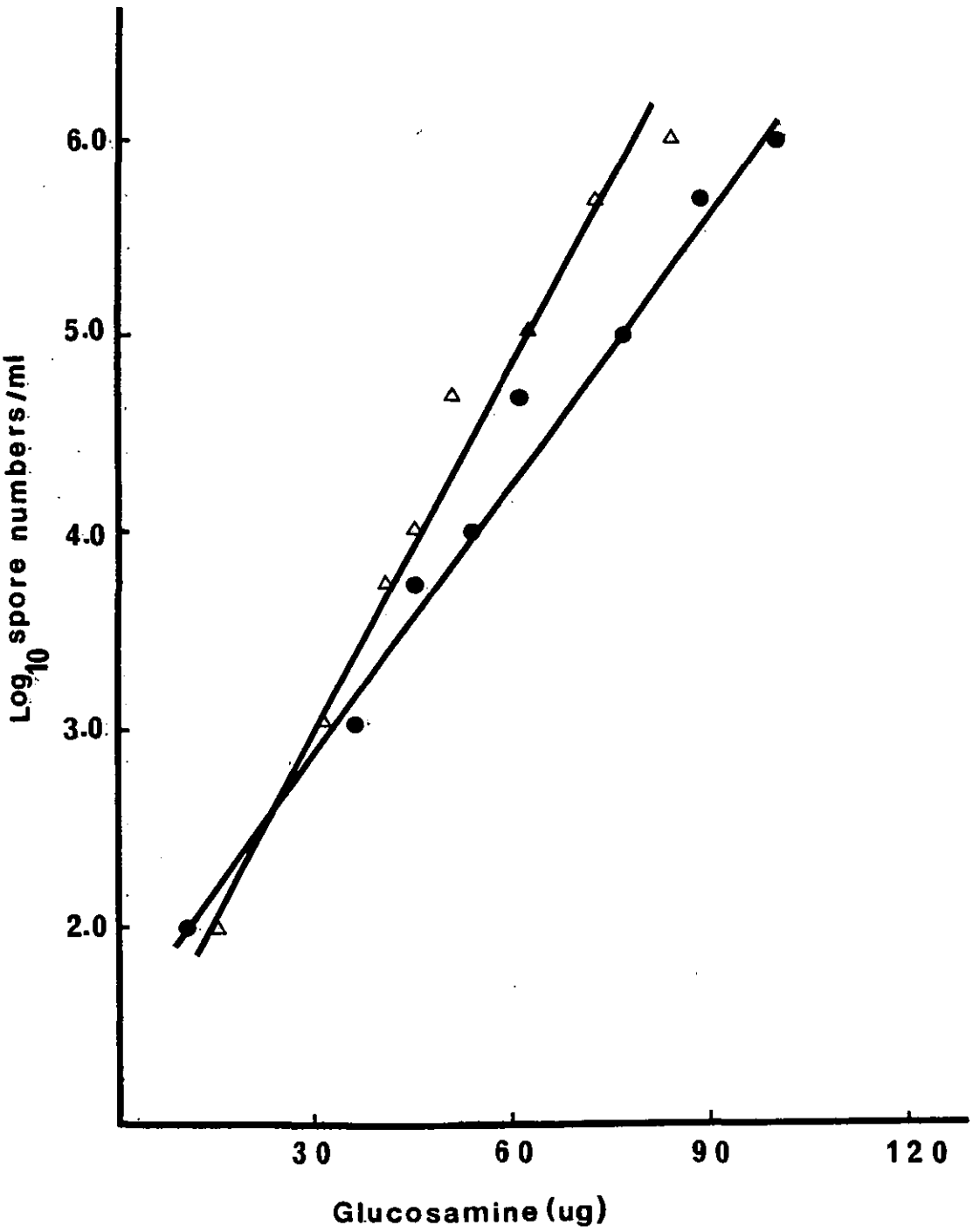

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers/ml
30
60
90
120
Glucosamine (ug)

TABLE 5

GLUCOSAMINE PRODUCTION BY A.flavus IN THE PRESENCE OF VARYING AMOUNTS OF S.aureus

Varying concentration of S.aureus cells were cultured simultaneously with 10^5 spores per ml. of A.flavus in Czapek Dox medium and incubated with shaking at $30^{\circ}C$ for 40 hours. Glucosamine production was then determined for each concentration.

Each value is the mean of three cultures, three determinations per culture.

TABLE 5

ORGANISM		Glucosamine (µg)	STD. DEV
S.aureus (3625)	A.flavus (15959)		
Cell numbers/ ml	Spore numbers/ ml		
10^2	10^5	73.00	±0.00
10^3	10^5	70.00	±0.00
10^4	10^5	69.00	±1.73
10^5	10^5	61.33	±0.57
10^6	10^5	59.66	±0.57

FIGURE 14

COMPOSITE GRAPH FOR A.flavus AND S.aureus

A standard curve was plotted between $\log_{10}$ spore number per ml (y) and glucosamine concentration (µg) (X). Fungal cultures were mixed with cells of S.aureus immediately prior to glucosamine determination.

▲——▲ A.flavus

△——△ A.flavus and S.aureus

Each point is the mean of 5 determinations.

The two sets of points shown in this graph were initially plotted separately to give two straight lines. These were very close together and statistical analysis by the method of least squares (Lee et al., 1982), indicates that they are estimates of the same straight line.

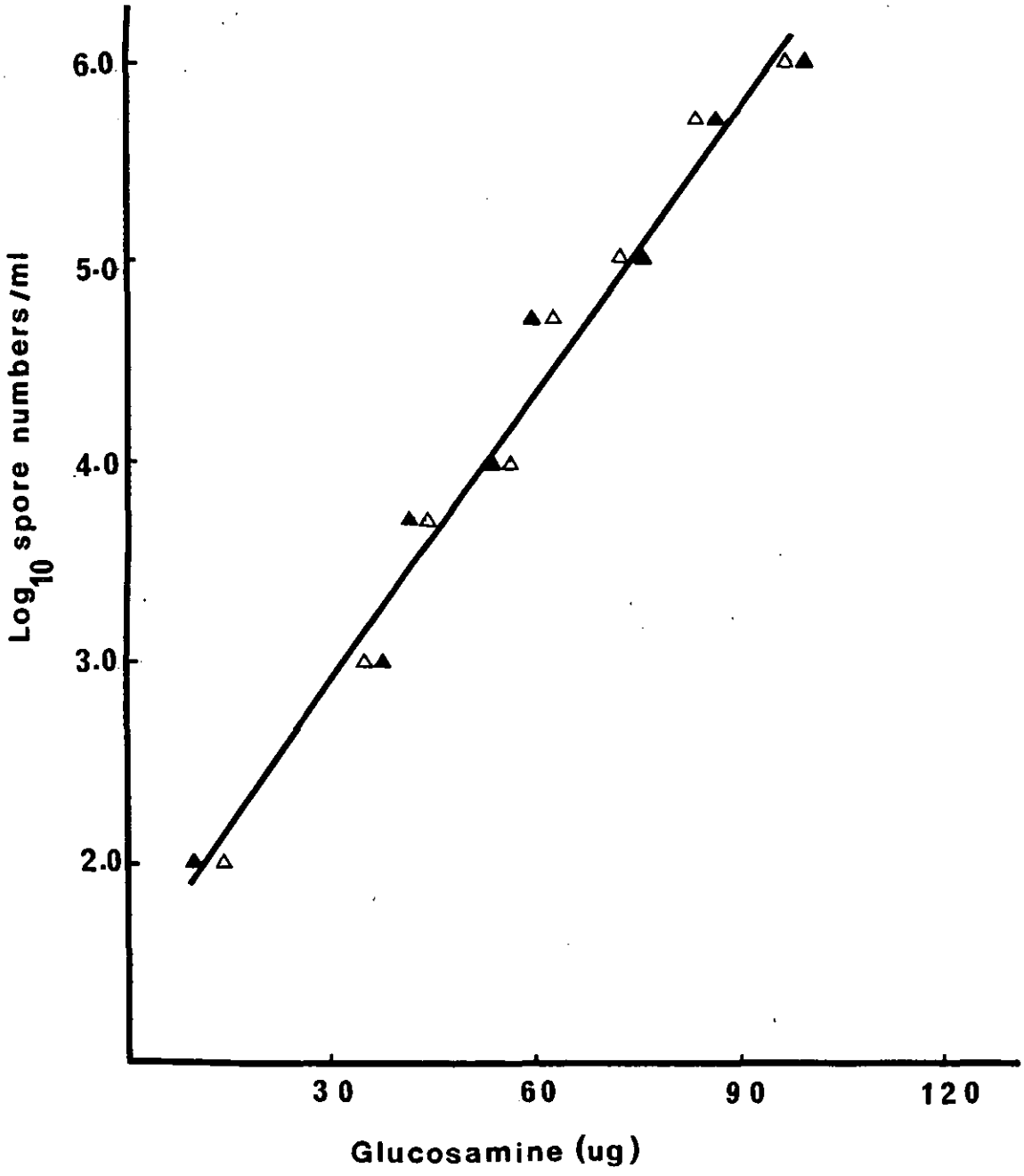

6.0
5.0
4.0
3.0
2.0
Log10 spore numbers/ml
30
60
90
120
Glucosamine (ug)

TABLE 6

RELATIONSHIP BETWEEN TOTAL AND VIABLE COUNT

OF A.flavus SPORES

TABLE 6

ORGANISM	Total Counts	Viable Counts
A.flavus (CMI 15959)	Spore numbers/ ml	Spore numbers/ml
Spore Suspension 1	$5.17\ 10^6$	$1.42\ 10^6$
Spore Suspension 2	$4.51\ 10^6$	$1.20\ 10^6$
Spore Suspension 3	$4.595\ 10^6$	$1.55\ 10^6$
Spore Suspension 4	6.10^6	$1.40\ 10^6$
Spore Suspension 5	4.010^6	$1.07\ 10^6$

TABLE 7

GROWTH OF DIFFERENT BACTERIA STRAINS IN CZAPEK-DOX LIQUID MEDIUM AT 30°C

Bacterial cells were inoculated with Czapek Dox medium at pH 5.8 and incubated at 30°C with shaking for 40 hours.

Each value is the mean of 5 replicates.

TABLE 7

ORGANISM	STOCK SUSPENSION	MASTER SUSPENSION	40h GROWTH Czapek-Dox Medium	±SD
	cell numbers/ml	cell numbers/ml	(total counts)	
E.coli	7.5×10^{8} cells ml^{-1}	7.5×10^{4} cells ml^{-1}	12×10^{4}	±2.88
B.cereus	9.2×10^{6} cells ml^{-1}	9.2×10^{5} cells ml^{-1}	18×10^{6}	±3.55
S.aureus	1.4×10^{7} cells ml^{-1}	1.4×10^{5} cells ml^{-1}	2.4×10^{6}	±0.41

TABLE 8

RELATIONSHIP BETWEEN DRY WEIGHT OF PURE CULTURE OF A.flavus AND DRY WEIGHT OF MIXED CULTURES OF A.flavus AND B.cereus

Each point is the mean of 5 determinations.

TABLE 8

ORGANISM	Mycelial Dry weight (mg)	Std. Dev	ORGANISM		Mycelial Dry weight (mg)	Std. Dev
A.flavus (15959)			A.flavus & B.cereus			
spore numbers/ ml			spore numbers/ ml	cell numbers/ ml		
10^6	9.40	±0.01	10^6	10^5	7.70	±0.25
10^5	9.00	±0.92	10^5	10^5	8.25	±0.05
10^3	6.00	±0.50	10^3	10^5	5.84	±0.01
10^2	5.50	±0.01	10^2	10^5	5.20	±0.25

D I S C U S S I O N

D I S C U S S I O N

There is a growing international interest in the problems caused by fungal spoilage especially in tropical and subtropical regions of the world. It is obvious that there is a real need for a rapid, simple method of fungal analysis in food stuffs and several methods have been developed (Rodricks, 1976; Jarvis, 1977; Offem, 1980).

There are several sensitive methods for the detection and estimation of aflatoxin. (Mojtahedi, et al., 1979; Naoi et al., 1979; Moreno, 1979; Takatori & Sued, 1979). However, aflatoxin production does not offer a means for the rapid detection of fungal contamination.

The determination of pectinase activity has been considered as a rapid method for detecting spoilage fungi (Sreekantiah & Johar, 1963; Screekantiah et al., 1973; Abdel-Fattah & Mabrouk, 1976 and Offem, 1980). This method although rapid suffers from several drawbacks especially when working with mixed cultures of fungi and bacteria, as it is known that pectic enzymes can be produced by both fungi and bacteria (Duel & Stutz, 1958; Phaff, 1947).

The method of analysis used in this work has been based on the hydrolysis of chitin.

Most glucosamine determinations have used various modifications of the colorimetric method of Elson & Morgan, (1933), which is not specific. In addition it is extremely sensitive to a number of variables and many compounds interfere with the determination of glucosamine by this method.

The method for glucosamine estimation used in this study was described by Nakamura et al., (1969). This allows an evaluation of the deterioration of food and feed products by estimating the total amount of fungus mycelium present and it is applicable to a wide range of samples. Furthermore, it is particularly valuable for laboratories which do not have facilities to carry out microbiological assay.

Although there has been a considerable amount of work on food spoilage by fungi and bacterial contamination, especially dairy produce, there is little information on food spoilage due to mixed cultures of fungi and bacteria, and virtually no information on the estimation of fungi in the presence of the bacteria.

The work reported in this thesis was a quantitative estimation of Aspergillus flavus by a glucosamine method in mixed cultures of bacteria and the fungus, Aspergillus flavus.

Calibration curves were obtained for the level of glucosamine against the number of fungal spores per ml inoculated (fig. 6). This shows that the method could reliably estimate relatively low levels of fungal contamination under the experimental conditions described.

Experiments were divided into several groups:

1. Varying concentrations of fungi were mixed with fixed concentrations of bacteria (10^5 cells per ml), immediately prior to estimation of glucosamine. The results obtained were compared with those of pure fungal cultures.
2. Varying concentrations of fungi were aseptically

mixed with a fixed concentration of bacteria and the mixed cultures were grown for 40 hours. Glucosamine was then estimated. The results obtained were compared with those of pure fungal cultures.

3. Varying concentrations of bacteria were mixed with a fixed concentration of fungi (10^5 spores per ml) and the mixed cultures were grown for 40 hours. Glucosamine was then estimated.

All these experiments were repeated five times. In each case the mean of the data was taken and the results were considered separately for each different bacteria. In pure fungal cultures the results were obtained from three cultures and three determinations per cultures, the mean of this data was also taken.

The results of the two first groups were evaluated by comparison of several regression lines (Lee et al., 1982), using the method of least squares to determine whether they differ sufficiently to be distinct or whether the differences are sufficiently small to allow the data to be pooled and treated as one common line.

The mean of the data for each individual experiment was subjected to computer analysis giving coefficients for the slopes, the intercept and F-values. In all cases the results gave points that seem to be scattered about a straight line.

Specific sets of results will now be considered.

E.coli cells tested for glucosamine, showed that at a high concentration of cells (10^8 cells per ml) no glucosamine could be detected.

Glucosamine estimation in mixed cultures of A.flavus and E.coli growing simultaneously (fig. 7) suggest that the presence of E.coli at varying concentrations does not influence the amount of glucosamine production by A.flavus. This could be confirmed with the figures in table 1, which showed that when A.flavus was cultured with different concentrations of E.coli cells, the results were similar to those found in fig. 7.

After data processing the calculated F-value indicate that there is no significant difference between glucosamine values found in pure fungal cultures and the values found in mixed cultures of A.flavus and E.coli.

As the results show the presence of E.coli does not interfere with the glucosamine level produced by A.flavus. These observations could be explained by the fact that E.coli did not grow well under the experimental conditions described, which are normally used for fungal growth, this finding was confirmed by the data in table 7, which indicates that bacterial competition by high levels E.coli is minimal under conditions allowing heavy growth of A.flavus.

The determination of glucosamine in vegetative cells of B.cereus (fig. 8) shows that glucosamine is detectable at high concentrations whereas below 10^4 cells per ml no glucosamine could be found. It should be noted, however, that the amount of glucosamine in B.cereus cells was low in relation to the amount of glucosamine found in pure fungal cultures at the same concentration.

The estimation of glucosamine in mixed cultures of A.flavus and B.cereus cells growing simultaneously (fig.9)

showed that the presence of B.cereus cells (10^5 cells per ml) did not produce any effect on glucosamine level in the range 10^2-10^3 fungal spores per ml. But when the fungal spores were increased above this concentration the presence of B.cereus cells (10^5 cells per ml) caused a decrease in glucosamine level in relation to the glucosamine detected in pure fungal spores (fig. 6). It was important at this stage to determine the effect produced by different concentrations of B.cereus cells (table 2). The results show that concentrations in the range 10^3-10^4 bacterial cells per ml had a negligible effect on glucosamine level, whereas that in the range 10^5-10^7 bacterial cells per ml, the effect on glucosamine produced was significant. The results obtained from this experiment confirm the findings in fig.9.

Estimation of glucosamine was also carried out on pure spore suspensions of B.cereus (fig. 11) and the results showed that the level of glucosamine detectable was similar to the content in vegetative cells which means that the amount of glucosamine was low in relation with pure fungal cultures.

When glucosamine was estimated in mixed cultures of B.cereus spores and A.flavus growing simultaneously (fig. 12), the results revealed that the decrease in glucosamine level was slightly different from the results obtained in mixed cultures of B.cereus and A.flavus. The effect of B.cereus spores starts to be observed at concentrations of 10^3 fungal spores per ml. The effect produced by different concentrations of B.cereus spores on glucosamine level produced by A.flavus was also studied

(table 3). It was found that when B.cereus spores were in the range 10^3-10^6 spores per ml a decrease in glucosamine level was noticed, the results in this table support the finding in fig. 12.

The results obtained from mixed cultures of B.cereus (vegetative cells and spores) with A.flavus were processed separately and compared with the data of pure fungal cultures. The F-values calculated indicate that there is a significant difference between glucosamine level found in pure fungal cultures and the glucosamine level found in mixed cultures of A.flavus and B.cereus (vegetative cells and spores), this allowed the data to be treated as separate lines (fig. 9 and fig. 12).

Bacillus is another bacterial genus likely to be found in food spoilage. The determination of glucosamine level produced by A.flavus in the presence of B.cereus showed a decrease in glucosamine level, i.e. the presence of Bacillus in vegetative or spore forms interferes with glucosamine production by fungi. These results suggest that this could be due to a decrease in production of fungal mycelium because of bacterial competition. This is supported by the figures in table 8, which showed that the dry weight of fungal mycelium from mixed cultures of fungi and B.cereus cells is considerably reduced compared with the amount of fungal mycelium obtained from pure fungal cultures.

Furthermore, when B.cereus cells were cultured under conditions favourable for fungal growth (table 7), it was found that B.cereus was able to grow well under these conditions. The decrease in mycelium production may be due to utilization of the carbon source by the

bacteria for its growth or the production of some toxic bacterial metabolite reducing fungal growth.

Studies were also carried out using S.aureus (table 4). At high bacterial concentrations the glucosamine content was small, and at concentrations of 10^4 bacterial cells per ml no glucosamine could be detected.

When the glucosamine level was estimated in mixed cultures of A.flavus and S.aureus growing simultaneously (fig. 13), it was found that it produced a similar effect as that of B.cereus on the fungi i.e. a decrease in glucosamine level was observed. The effect of S.aureus on glucosamine level produced by A.flavus was noticed at a concentration above 10^4 fungal spores per ml. On the other hand when the fungus (10^5 spores/ml) was grown with different concentrations of S.aureus cells, at concentrations of 10^2-10^3 bacterial cells per ml no effect was observed on glucosamine level. However, at concentrations of 10^4 bacterial cells per ml, a decrease on glucosamine level starts to be found (table 5).

After data processing the calculated F-values indicate that there is a significant difference between glucosamine values found in mixed cultures of A.flavus and S.aureus, which allowed the data to be treated as two different lines (fig. 13).

As mentioned earlier the first group of experiments (page 64) comprise the results when fungi were mixed with the different bacteria immediately prior to the estimation of glucosamine. These results suggest that the bacterial species does not significantly alter glucosamine levels

under these conditions.

When the data from these results was processed and compared with the data obtained from pure fungal cultures, the F-values calculated indicate that the lines were not significantly different which allowed the data to be pooled and treated as one common line (fig. 10 and 14).

When the results obtained from experiments in group two and three are considered, the evidence suggests that when A.flavus is grown with Gram-negative bacteria (E.coli), the presence of this bacterium does not interfere with glucosamine production. In contrast when the glucosamine was estimated from mixed cultures of A.flavus and Gram-positive bacteria, a decrease in glucosamine level was observed compared with that of pure fungal cultures. This was shown to be due to a decrease in fungal mycelium production in the presence of Gram-positive bacteria.

The present studies suggest that the glucosamine assay might provide a basis for the analysis of fungal contamination as long as the analyst is aware that contaminating bacteria of certain species can influence the final result.

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