INTERNATIONAL BIODETERIORATION BULLETIN

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INTERNATIONAL BIODETERIORATION BULLETIN

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INTERNATIONAL BIODETERIORATION BULLETIN

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The Bulletin acts as a vehicle for the publication of works on all aspects of biodeterioration, i.e. the deterioration of materials of economic importance by micro-organisms, insects, rodents, etc.

Contributions may be in English, French, German or Spanish and should be submitted in triplicate on international A4 size paper (21.0 cm × 29.7 cm or 8.27 in. × 11.69 in.) typewritten on one side of the paper only. A summary of 25-100 words should accompany each contribution. Illustrations should be clearly drawn in Indian ink or should be photographed. The reduction desired should be clearly indicated and illustrations when reduced are not to exceed 17 cm × 26 cm. Where figures are to be inserted in the text the approximate position for each one should be clearly marked in the typescript. The bibliographic references are to be indicated in the text as, e.g.

Reese and Levison (1952).

and in the bibliography:


Authors are requested to abbreviate journal titles according to the conventions of the World List of Scientific Periodicals.

Proofs will not be sent to authors before final publication. 30 reprints will be sent free of charge with each article. Additional reprints are obtainable: scale of charges available on application to the Editors.

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BIODETERIORATION SOCIETY NEWSLETTER

Honorary Regional Meetings

Secretaries

Any member interested in arranging Society activities outside Europe should contact one of the Honorary Regional Meetings Secretaries. They are:

Canada: Dr. R. S. Smith (Western Forest Products Lab., 6620, Northwest Marine Drive, Vancouver 8, B.C., Canada)

India: Prof. S. M. Nair, (Dept. of Museum Studies, Birla Institute of Technology & Science, Pilani, Rajasthan, India)

Japan: Prof. T. Haraguchi (Dept. of Forest Products Technology, Tokyo University of Agriculture & Technology, Fuchu, Tokyo 183, Japan)

U.S.A.: Prof. R. W. Traxler (Chairman, Dept. of Plant Pathology—Entomology, University of Rhode Island, Kingston, R.I. 02881, U.S.A.)

Forthcoming meetings

1st March 1974

"Biodegradation of town refuse and farm wastes". University of Aston in Birmingham.

5th/6th July, 1974

A.G.M. and Scientific Meeting. University of Bath.

One scientific session will be devoted to a Symposium on "Extracellular enzymes in biodegradation".

The other scientific session will be for presentation of short (15 mins.) original papers on any aspect of biodegradation.

Offers of all papers should be made to the Hon. Programme Secretary by 1st March, 1974.

29th November, 1974

Provisional title:-

"Biodegradation at limits of temperature: thermophiles and psychrophiles".

Location: London.

Further details may be obtained from the Honorary Programme Secretary:

Dr. R. H. Tilbury (Tate & Lyle Ltd., Group Research & Development, P.O. Box 68, Reading, Berks. RG6 2BX)

3rd International Biodegradation Symposium

This is to be held at the University of Rhode Island, Kingston, R.I., U.S.A. on 17th-23rd August 1975.

Further details may be obtained from the Hon. Secretary, (Dr. D. Allsopp, Biodeterioration Information Centre, University of Aston, Birmingham B4 7PF) or the U.S.A. Hon Regional Meetings Secretary, (Prof. R. W. Traxler, Dept. of Plant Pathology-Entomology, University of Rhode Island, Kingston, R.I. 02881 U.S.A.)

Second International Symposium on the Genetics of Industrial Microorganisms

This Symposium will take place on 25th-31st August, 1974 at the University of Sheffield, Ranmoor House Conference Centre, England.

The theme will be the relationship of recent advances in genetics and molecular biology to problems in the field of industrial microbiology. There will be about 40 invited papers and a larger number of unsolicited short communications. The First Circular and Preliminary Application Form are now available from The Assistant Secretary (GI M 74), Society of Chemical Industry, 14 Belgrave Square, London, England, SW1X 8PS.

The Proceedings of the Symposium will be published by Academic Press.

Conference on Stored-Product Entomology

The First International Working Conference on Stored-Product Entomology is to be held in Savannah, Georgia, U.S.A. on 7th-11th October 1974. The conference will cover all phases of Stored-Product Entomology and will include symposia, panel-discussions and submitted papers. Facilities for informal meetings will be available. The tentative programme includes symposia and panel-discussions on:

1. Tropical Stored-Product Entomology

2. Biology, Ecology and Integrated Control

3. Pesticides, Toxicty and Insect Resistance

4. Radiation and other Physical Means of Insect Control

5. Pesticide Residues, Tolerances and Registration.

The proceedings will be published in full after the Conference.

Anyone desiring further information should write to: The Organizers, Working Conference on Stored Product Entomology, c/o Stored-Product Insects Res & Dev Lab, ARS-USDA, P.O. Box 5125, Savannah, Georgia 31403, U.S.A.

6th Cornell University Conference on Agricultural Waste Management

The major focus of this Conference will be on technical and managerial aspects for the handling, stabilisation, disposal and utilisation of liquid, solid and gaseous discharges from agricultural production. The following areas will be emphasised: land disposal; food processing; economics; agricultural-ecological systems analysis; non-point pollution source identification and control; animal production; crop production; effluent guidelines.


All correspondence should be addressed to: Agricultural Waste Management Program, 1974 Conference Committee, 207 Riley Robb, Cornell University, Ithaca, NY. 14850, U.S.A.

IAMS Congress

The 1st International Congress of the International Association of Microbiological Societies is to be held in Tokyo on 1st-7th September 1974. This will be the first interdisciplinary meeting to be held since the reorganisation of IAMS, replacing meetings known as the International Congress for Microbiology and is a sequel to the 10th meeting held in Mexico City in 1970.
Information in the Congress may be obtained from: Prof. D. Ushiba, General Secretary, The Organising Committee, First Inter­sectional Congress of IAMS, Science Council of Japan, 22-34, Roppongi 7-chome, Minato-ku, Tokyo, 106 Japan.

Seminar on disposal of packaging materials

Pira (the research association for the paper and board, printing and packaging industries) is to hold a one day seminar on the disposal of packaging materials. The seminar will be held on 23rd April 1974 at the Pira headquarters at Leatherhead and is open to non-members. It is intended for package producers and users, local government and specialist firms concerned with waste disposal. The purpose of the seminar is to provide current awareness of the problem of disposability of various forms of packaging waste and to discuss the use of alternative materials, methods of waste disposal and treatment and the reutilisation of waste.

Further information from: Information and Training Department, Pira, Randalls Road, Leatherhead, Surrey, England. Telephone- Leatherhead 76161. Telex 929810.

President of BPCA

Mr. Angus Fraser McIntosh has been elected for a third term of office as President of the British Pest Control Association for 1973-74.

Mr. McIntosh, a director of Rentokil Limited, was President for 1957-59 and 1970-72 and his father was President in 1946-48.

The Association, which represents manufacturers and contractors in the fields of industrial and domestic pest control and environmental hygiene, has its headquarters at Alembic House, 93 Albert Embankment, London SE1 7TU.

The B.P.C.A. has also been instrumental in forming a European Federation of Pest Control Associations which will come into being later this year, with representatives of Britain, Holland, France and Germany.

Mr. McIntosh hopes that many more companies will join from associated fields of activity as the Association’s work and Codes of Practice become more widely recognised by Government and by Consumers.

The B.P.C.A. will be holding a major international conference in April 1975.

Shading out aquatic weeds

A system for controlling aquatic weeds by shading them from the light is being marketed in the USA. A liquid formulation, called Aquashade, is applied at the rate of 5 l in 6 million l of water, and it is claimed that a control is obtained without hazard to humans, livestock, fish or other wildlife. The product is produced by Aquashade Inc., PO Box 117, Dobbs Ferry, N.Y. 10522, U.S.A. (Wild Ling 1973, 15:35).

Coolant preservative

Bayer’s Preventol D2, a liquid preservative and disinfectant solu­ble in oil and water, is environmentally acceptable.

Recycled coolants, for turning, cutting and drilling at workbenches or in automatic metalworking machines, inevitably become infected with microbes. Infection sources include contaminated systems and machinery waste matter, sometimes even the water for dilution. The microbes cause decomposition in the coolant resulting in the formation of slime and sludge, blocked filters, corrosion, loss of lubrication properties and smells. Pathogenic microbes can also cause dangerous skin infections to anyone in contact with the coolant.

Preventol D2 gives reliable lasting protection against all such risks.

Bayer also markets Preventol AS, a slime control agent.

Preventol AS and D2 are easily biodegraded.


Biology of Plant Litter Decomposition

edited by C. H. Dickinson and G. J. F. Pugh


This publication is unusual in that it treats ecological problems in a truly biological manner, analysing separately the influences of litter type, organisms, and the environment, on litter decomposition, as well as identifying those areas which demand further investigation.

This volume will be useful to biologists, zoologists, botanists, and microbiologists—both research workers and advanced undergraduates.

For a descriptive leaflet on the above title, please write to the publishers.

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Scanning Electron Microscopy: A new technique in the study of the microbiology of works of art.

**SCANNING ELECTRON MICROSCOPY:**
A NEW TECHNIQUE IN THE STUDY OF THE
MICROBIOLOGY OF WORKS OF ART

Maria Bassi and Clelia Giacobini

**Summary.** The scanning electron microscope is now used parallel to the traditional methods of study. Examples show the positive results obtained with it in the study of paintings, archaeological ruins, materials for restoration, etc. Particular emphasis is given to the microbiological research of frescoes. Five specific biological attacks are described and the problems of each are analysed.

La microscope electronique à balayage: une nouvelle méthode d'étude de la microbiologie des œuvres d'art. Bien que les méthodes traditionnelles soient toujours utilisées dans la microbiologie des œuvres d'art, aujourd'hui on se sert aussi du microscope électronique à balayage. Cet article traite les premières études faites avec ce microscope sur les œuvres d'art et décrit en détail la microbiologie des fresques, où il y a cinq phénomènes biologiques différents.

**Introduction**

It is a well known fact to-day that even works of art are subject to microbiological deterioration. American scientists have shown that microorganisms are among the principal agents which cause the degradation of paints and varnishes. Those familiar wall stains which were once thought to be dust marks or humidity stains are in fact caused by active microorganisms (Coffey and Goll, 1948; Drescher, 1958; Gettens et al., 1941; Klens and Lang, 1956; Ross, 1958; Rothwell, 1958; Torao, 1951; Yoshikadru, 1959). Also the degradation of canvas and panel paintings, as well as of other materials such as metal, marble, stones, plastics, etc. can be due to microbial growth. (Baldini, 1967, Barcellona Vero, 1967; Gargani, 1968; Giacobini, 1957; Giacobini, 1967a; Tonolo, 1954).

The study of microorganisms as cause of the deterioration of works of art began in Italy a few years ago at the Istituto Centrale del Restauro in Rome. Even in the early stages of these researches it was discovered that the biological degradation of works of art differed very much in nature from any other form of physico-chemical degradation. There are various species of microorganisms, ranging from bacteria to lichens, which can all cause deterioration if the substrates of the work of art and the environmental conditions are suitable.

We present here the results of our latest researches. A new investigation method has been introduced with the use of the scanning electron microscope. This microscope can be of decisive aid to us, for it has a depth of focus far greater than the light microscope and will probably become of prominent importance in identifying the structural, morphological and diagnostic characters of biological deterioration.

The Scanning Electron Microscope in the Microbiological Research of Works of Art

One of the greatest difficulties encountered in the study of biological deterioration of works of art has been the identification of microorganisms, which first of all requires their isolation in culture. This takes time and can even give negative results when a particular microorganism is not grown in the medium it requires. In other words, should the development in culture be unsuccessful it does not necessarily mean that there are no microorganisms present on the test sample. To-day, however, with the scanning electron microscope (SEM) it is possible to observe directly a fragment of the damaged surface and to state almost immediately whether microorganisms are present or not. In the case of fungi, it may be possible to recognize their genus or even their species by observing the morphological characteristics of their spores. A number of studies have already been made on the

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1This paper was presented at the XXVI National Congress of A.T.I. Sept. 22-25, 1971.
2Centro Gino Bozza, Politecnico di Milano, Pza L. da Vinci 32, Milano, Italy.
3Istituto Centrale del Restauro, Dept. of Microbiology, Pza S. Francesco di Paola 9, Roma, Italy.

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Scanning electron microscopy: A new technique in the study of the microbiology of works of art.
M. Bassi and C. Giacobini.

morphological features of spores belonging to different fungal species. For example, Raper and Fennel (1965) demonstrated the importance of the genus *Aspergillus* as a deteriorating agent in man's economy, and it is known that a great number of species belonging to this genus have a quite typical spore conformation. The details of the spore surface, such as furrows, ridges, etc., are too small to be ascertained by light microscopy but are easily detectable by scanning electron microscopy. Many of the fungal species can now be identified on the basis of SEM examination of the spores (Locci, 1972; Locci and Quaroni, 1970 and 1971). Besides, a great advantage of the scanning electron microscope is that it can be used to check the identity of the microorganisms isolated and grown in culture with that of the spoiling agent originally present on the sample.

Characteristics of the scanning electron microscope are its good resolving power (about 200 A) and great depth of focus. It gives therefore pictures that are characterized by the sharp appearance of every detail on the surface of the examined objects.

The scanning electron microscope utilizes an extremely narrow electron beam, which is directed at the specimen and scanned to and fro across its surface. The secondary electrons which emerge in this way from the specimen are collected by a detector and the changes in their amount are transformed into electric signals, which are amplified and rendered visible as variations in brightness in a Braun tube. The picture which is thus obtained on a fluorescent screen gives a three-dimensional view of the sample surface.

Metals, which have a good electrical conductivity and under the electron bombardment emit a great number of secondary electrons, can be observed in the scanning electron microscope without any previous treatment. On the contrary other materials, such as stone, wood, canvases or biological samples, must be previously coated with a thin metallic layer to ensure a good electrical conductivity and a sufficient emission of secondary electrons. The coating is achieved by placing the samples in an evaporator chamber and by evaporating in it a small amount of metal or carbon under vacuum.

The samples examined in this study have been coated with either platinum-gold or carbon in a Balzers evaporating unit type BAE 121, and viewed in a Jeol scanning electron microscope type JSM U3.

The biodeterioration of archaeological ruins, panel and canvas paintings, and restoration materials, as studied with the scanning electron microscope

The study of the microbiology of monuments and archaeological ruins encounters the same problems as are found in the microbial deterioration of stone (Barcellona Vero and Giacobini, 1969; Barcellona Vero and Monte Sila, 1971; Barcellona Vero and Silveri, 1971). In the patina where the attack occurs it is not always possible to detect immediately the presence of a biological component. Analyses under the light microscope are equally difficult to perform and are not always satisfactory, since the mineral structures often mask the presence of dehydrated biological structures.

In panel paintings the fungal colonization can be found either inside the wooden support or in the ground layer directly underneath the paint film. The microbial attacks in the ground layer are an important factor in the difficult and controversial problem of lifting colour layers from paintings. To detect the presence of microorganisms, different laboratory procedures have been attempted: colour lamellae have been analyzed under the light microscope and other lamellae developed in culture, but the results obtained in both cases were not satisfactory and were by no means clear and precise enough. On the contrary, when the lamellae were examined in the scanning electron microscope it was possible to identify the real cause of these microbial attacks.

Research into the biodegradability of restoration materials is another important application of the scanning electron microscope, which can be used in the study of the biological deterioration of substrates in the "stacco and strappo" methods used for restoring the supports of mural paintings.

The deterioration of canvas paintings has two distinct aspects: it can either be generic or have the outward appearance of a microbial attack. If the attack occurs on the face or on the back of the painting it is immediately perceptible. If, as it is most often the case, the attack occurs in the interior or in the glue between the fibres of the canvas painting, it cannot be detected until the painting is about to be restored. The identification of this attack is never easy. If it is a generic attack it is extremely difficult, if not impossible, to diagnose its cause, because of the dehydrated state of the canvas paintings and the thickness of the fibres. If mycelium is present but does not grow in culture, the problem of its unsuccessful growth remains unsolved.

Various samples of biodeteriorated paintings, different in type, nature and phenomenology, were examined in laboratory with the scanning electron microscope and excellent results were obtained. Details were revealed very clearly and a generic attack appeared to be quite different from a biological one. (Figs. 1-3).
Scanning electron microscopy: A new technique in the study of the microbiology of works of art.
M. Bassi and C. Giacobini.

Figures 1-3: Microbiology of canvas paintings.

**Figure 1.** The biodeterioration on the wooden supports at the back of the paintings.

**Figure 2.** The biodeterioration of the canvas fibres seen under the light microscope (100×).

**Figure 3.** The biodeterioration of the canvas fibres seen under the scanning electron microscope (3000×).
Current microbiological problems in frescoes

Since 1957, the microbiological problems of frescoes have been a subject of study and research in the Istituto Centrale del Restauro of Rome. In 1961, a number of microbial types which could cause deterioration were listed in a small review on the deterioration of frescoes (Tondo and Giacobini, 1961).

In 1965, further investigations into these problems were carried out and it was proposed to continue this research from a statistical point of view by collecting scientific and technical data (Giacobini and Lacerna, 1965).

In 1967, the first classification of biological attack was compiled; based on the period in which the attack appeared, the gravity of the attack, its type species, its location and how it affected the painting (Giacobini, 1967b).

In 1968, it was stated that wherever a growth took root on a fresco, the latter became a specific centre of attack having the aspect and microbiological characteristics of a deterioration phenomenon.

Finally, in 1970, emphasis was laid on the importance of studying the original colonisation and the biological population and it was proposed to use new analytical techniques which are far more precise than the methods used up to then.

At the present stage of research the biological phenomena have been clearly defined. By biological phenomena is meant a form of deterioration caused by a particular microflora which appears on frescoes found in given ecological environments.

In this paper we illustrate five current problems of microbial deterioration in mural paintings and present the first SEM micrographs of deteriorated frescoes. The problems are the following ones:

1) the extension of the attacks; the insufficient growth of the microorganisms when isolated in culture;

2) the mode in which an attack occurs; its colouring;

3) the close relationship between the biodeterioration problem and certain restoration activities;

4) the particular ecological environments where deterioration occurs (tombs, crypts);

5) the cause that induces the attack to appear, i.e. exposure to light (as with excavated frescoes brought into light for restoration).

1st biodeterioration problem

The frescoes are covered with small dark brown stains and look as though they had been splashed with dust or dirt. Microorganisms present: a fungus whose isolation in culture is still under study.

This biodeterioration problem, which was first reported to the Istituto Centrale del Restauro in 1963 and again in 1967, 1968 and 1971, has always coincided with restoration activities and the deterioration appeared in an identical form in each scene under restoration. The frescoes on the vaults and side walls were covered with small dark floccose stains which looked rather like lumps of earth or thick layers of dust. The colours white, yellow, red, light blue, green, mauve and gold, and the distempered parts were stained. The attack looked superficial, as though it could be removed with a scalpel. However, when the stains were removed, small holes were found underneath them. A fungus had caused the attack. So far no-one had been able to give an explanation as to the cause of this form of biodeterioration, but old photographs have been found showing that this problem has existed for a long time.

Microscopic analyses have confirmed the biological nature of the macroscopic degradation. With a light microscope large quantities of mycelium could be seen on the edges of coloured lamellae. The same mycelium could be observed more clearly and in greater detail in the scanning electron microscope.

Research into this fungal infection has brought up many problems, all of which are being studied. In the restoration of paintings the removal of fungal colonies by mechanical means and the use of synthetic resins may be an aid to restorers. (Figs. 4-6).

Figures 4-6: Microbiology of mural paintings, illustrating the 1st biodeterioration problem.

Figure 4. Apparent form of biodeterioration.
Scanning electron microscopy: A new technique in the study of the microbiology of works of art.
M. Bassi and C. Giacobini.

In the second case, when the microbial attack comes in the form of a pink powder, the frescoes look as though they had been covered with a coat of chalky but compact powder about 1 mm thick. Sometimes the powder is evenly spread out, at other times it forms circular stains and at times it is scarcely visible to the naked eye.

The frescoes in the House of the Aurighi, which were restored at different times up to 1966 and where this microbial attack was first discovered in 1970, had all the characteristics of this form of biodeterioration. The attack was more intense and more extensive on the wall below the eaves (these were clogged) and on another wall just underneath a window. Here the powder was compact and its colouring was a deep pink with violet spots. These violet spots never alter much (they sometimes become lighter) not even in the months of March and May when the pink turns to white and back again to pink at the beginning of June. Hygrometric measures are taken regularly in order to follow and study this macroscopic problem.

Since 1959, when research into this problem began, there has been so far but one positive result: the only microbial form as seen under the light microscope are large or small capsulated coccolid forms, which are constantly present.

Different attempts have been made to isolate these microorganisms but so far only negative results have been obtained.

Examination in the scanning electron microscope was attempted as well, and it was possible to detect cellular forms either on the surface of the sample or still immersed in the powder. It was also possible to isolate the cells and examine them in a transmission electron microscope. (Figs. 7-9).

Research into this problem will continue.

3rd biodeterioration problem

A different agent, the fungus Cladosporium sp., attacks frescoes which have been detached from their supports and afterwards put back again.

This type fungal attack has been said to be the most serious form of deterioration of fresco paintings in modern times (Giacobini and Lacerna, 1965). As a matter of fact, it was first brought to the attention of the Istituto Centrale del Restauro in 1961 and since then we have often been consulted on this microbiological problem.

The attack appears within the first year of restoration and the growth of the fungus fluctuates with the seasons. At the peak of its growth it appears as a brown mycelium, or as a colony in different shades of dark brown. In this stage the fungus has already penetrated as far as the plaster and may also appear in the form of faded brown stains with or without a ring around them.
Scanning electron microscopy: A new technique in the study of the microbiology of works of art. M. Bassi and C. Giacobini.

Figures 7-9: Microbiology of mural paintings, illustrating the 2nd biodeterioration problem.

Figure 7. Apparent form of biodeterioration.

Figure 8. The attack seen under the light microscope (1500 x).
Scanning electron microscopy: A new technique in the study of the microbiology of works of art.
M. Bassi and C. Giacobini.

Upon a close examination it will be noticed that the deterioration problems in the tombs frescoes and in the crypt frescoes are slightly different, although both cases are biologically identical. In tombs the frescoes are coated with calcium carbonate or are covered with various kinds of efflorescence and other atypical destructive agents. But, as a rule, there are no macroscopic attacks in the colours of the paintings, either on the surface or underneath the efflorescences. It is not unusual to find similar symptoms in the early stages of biodeterioration of frescoes in excavations (see 5th biodeterioration problem). In crypts, the biodeterioration comes in the form of a white powdery coating which scales easily, has the strong smell of wet soil and is just white mycelium, formed by *Streptomyces* that alters the aesthetical appearance and the chromatic value of paintings.

Light microscope analyses of samples showed the presence of a regular mycelium of *Streptomyces* (1000×) which later developed normally in culture, sometimes together with certain species of fungi and bacteria.

In the scanning electron microscope the characteristic structures of *Streptomyces* could be observed quite clearly. Besides, it was possible to study in detail the relationship between such microorganisms and the saline crystals which always coexist in this form of biodeterioration. (Figs. 13-14). We intend to continue our research in collaboration with biochemists and physicists to find a means of eliminating this infection by the use of a fungicidal substance.

5th biodeterioration problem

Frescoes discovered in excavations are found to be particularly vulnerable to certain microorganisms such as streptomycetes, fungi, bacteria and algae.

In this fifth form of biodeterioration two different cases must be considered. If the excavations are covered (crypts, porticoes, rooms underground or half covered over), the frescoes are coated with an extremely fine, shiny dust which causes the colours to be brushed off easily. If the whole place is immediately cleaned up and the paintings restored, the attack stops, the dust disappears and does not return.

In the open excavations the situation is far more complex. Restoration operations in the House of Jerodulds and in the Garden Houses in the Ostia excavations began in December 1969 and since then they have been protected from frost, wind and rain with large plastic sheets which let in both light and air. The following Spring (1970) this phenomenon appeared for the first time on the walls of the corridor, galleries and tablinium, and one year later in the small room to the right. But the ceiling which had once fallen in and had been immediately restored never showed any sign of attack.

At the beginning of the attack a very fine, shiny powder appears, mixed with organic salts. Later it changes into a rather thick, white and yellow layer which intermixes with the different salts and microscopic algae present. In the meanwhile these algae have developed more rapidly than the heterotrophic microflora.

Microscopic examinations (450×, 1000×) of the dust on the biodeteriorated painting and of that developed in culture gave an assessment of the microbial component of the attack which reached an estimation of 10^7 and more. Analyses performed with the scanning electron microscope detected the pre-
Scanning electron microscopy: A new technique in the study of the microbiology of works of art.
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By direct microscopic analyses it was possible to observe the mycelium in its different developmental stages and the conidia which had already penetrated the painting. The microorganisms in the samples were studied in the laboratory. The principal fungus isolated was *Cladosporium* sp., whose assessment reacted $10^7 \times 10^8$ per gram of sample. Observation of the same samples in the scanning electron microscope confirmed this result. (Figs. 10-12).

All frescoes that have been restored at one time or another are periodically examined so that, if necessary fungicide solutions can be applied to check biodeterioration.

When the supervisors of Ostia Antica were informed of the real cause of the attacks (remains of glue which provide the microorganisms with organic substances), they started replacing the organic solutions with synthetic resins, and since 1968 all the frescoes which have been detached with “Paraloid” have shown no further signs of biodeterioration.

4th biodeterioration problem

In this case the frescoes are covered with a white patina, very much like a chemical efflorescence. Microorganisms present: *Streptomyces*.

---

**Figure 9.** The attack seen under the scanning electron microscope (10000×).

**Figures 10-12:** Microbiology of mural paintings, illustrating the 3rd biodeterioration problem.

**Figure 10.** Apparent form of biodeterioration.
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Figures 13-14: Microbiology of mural paintings, illustrating the 4th biodeterioration problem.

Figure 13. Apparent form of biodeterioration.
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Figure 14. The attack seen under the scanning electron microscope (3000×).

presence of microorganisms and salt crystals together, thus confirming the fact that attacks on frescoes in excavations, crypts and tombs are quite similar. (Figs. 15-17).

The Istituto Centrale del Restauro, in collaboration with the “Soprintendenza” in Ostia Antica, is continuing its research on how to check this form of biodeterioration on frescoes which still have to be detached.

Non-biodeteriorated Frescoes

In this paper on current microbiological problems a word must be said about those frescoes which have not been attacked by microorganisms and are in a good state of preservation.

The Istituto Centrale del Restauro is often called upon to examine these frescoes and their analyses have been quite interesting for they lead to a comparative study and a classifications of all the aerial microflora found on pictorial art. This type of microflora is in fact part of the microbiology of frescoes too.

Figures 15-17: Microbiology of mural paintings illustrating the 5th biodeterioration problem.

Figure 15. Apparent form of biodeterioration.
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Frescoes in a good state of preservation do not have any of the symptoms described in this paper, nor do they show any macroscopic form of typical or atypical deterioration due to the presence of microflora. Samples observed under the light microscope do not show any cellular structure. Samples isolated in culture are completely negative, and even when examined in the scanning electron microscope they have always been found free from any type of microorganism.

but it can give clearer and more precise results. It is an aid to other optical and culture techniques and throws a new light on the biological problems of works of art, since it can clearly show the biodegradative attacks and at the same time demonstrate the presence of the microorganisms responsible for it. Consequently, the means of controlling and protecting the frescoes from biological attacks are becoming far more precise.

As for the more typical microbiological problems, observation has shown that the biological attacks appear in their own specific form only when the microclimatic factors are suitable. These factors are the types of substrates the frescoes have, the adaptability and life span of microflora, the antagonism between microorganisms of different species.

The microbiological department has already directed its research into this problem but of course only long months of observing and controlling the attacks will bear fruit.

An ideal system must exist, but how does one know when a system is ideal? We have still a long road ahead of us.

The investigations into the phenomena discussed in this paper were conducted at the Istituto Centrale del Restauro, Roma. All the experiments with the scanning electron microscope were carried out at the Istituto di Fisica Tecnica del Politecnico di Milano, Centro “Gino Bozza” del C.N.R. Milano.

Figure 16. The attack seen under the light microscope (450 ×).

Figure 17. The attack seen under the scanning electron microscope (5000 ×).

Conclusion
The different examples mentioned in this paper are representative of the applications that the scanning electron microscope can have in microbiological research in art. The scanning electron microscope by no means lessens the importance of the light microscope but it can give clearer and more precise results. It is an aid to other optical and culture techniques and throws a new light on the biological problems of works of art, since it can clearly show the biodegradative attacks and at the same time demonstrate the presence of the microorganisms responsible for it. Consequently, the means of controlling and protecting the frescoes from biological attacks are becoming far more precise.

As for the more typical microbiological problems, observation has shown that the biological attacks appear in their own specific form only when the microclimatic factors are suitable. These factors are the types of substrates the frescoes have, the adaptability and life span of microflora, the antagonism between microorganisms of different species.

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Acknowledgements
We would like to thank Prof. Pasquale Rotondi, Director of the Istituto Centrale del Restauro, for the keen interest he took in this work, and the A.T.I. which kindly gave us permission to translate this paper.

We further wish to thank Prof. Angelo Rambelli, Dr. Antonella Bartoli and Dr. Maria Luisa Velocci for their cooperation; our colleagues Lidia Barcellona Vero, Maria Tabasso and Manlio Santini who contributed to this research; Miss Lidia Rissotto, Mr. Claudio Bettini, Mr. Romolo Ferrari, Dr. Giovanni Bova, Mr. Roberto Bonecchi, Mrs. Nicoletta Barbieri Sala, Mr. Marco Papetti, Mr. Claudio Ciarniello for their excellent technical assistance.

References


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ALKANE OXIDATION AND ASSIMILATION BY BACTERIA

G. T. von dem Busche1, R. W. Traxler2 and N. Trivedi3

Summary. Data obtained with Pseudomonas aeruginosa strains 196Aa and Sol 20 shows low isocitric lyase and malate synthetase activity when grown on n-alkanes indicating the absence of the glyoxylate bypass. Gas chromatographic analysis of oxidation products indicates 1 carbon removal from n-fatty acids. C4 incorporation studies show CO2 fixation into cellular protein and nucleic acids and n-hexadecane carbon incorporation in all cellular components.


Introduction

It is now generally agreed that a major metabolic pathway by which bacteria oxidize an n-alkane involves the oxidation of the terminal methyl group to a carboxyl group (van de Linden and Thissie, 1965; McKenna and Kalio, 1965). Fredricks (1967) showed that Mycobacterium rhodochrous oxidized n-decane to decanoic acid and then via b-oxidation but that a strain of Pseudomonas aeruginosa oxidized decane via a non-specific oxidation to the decanone stage which was followed by scission of the carbon chain at the keto group. Forney and Markovetz (1970) have described a subterminal oxidation of long chain aliphatic hydrocarbons. Abbot and Casida (1968) reported the oxidation of n-hexadecane to a mixture of internal monohexadecanes by Nocardia salmionicolor. This dehydrogenation apparently did not result in the formation of unsaturated fatty acids, thus suggesting a new pathway of aliphatic hydrocarbon degradation.

In the dissimilation of the n-hexadecane molecule via b-oxidation, an organism should produce eight moles of acetyl-coenzyme A for each mole of hexadecane consumed. This means the organism faces the problem of manufacturing its essential cellular components from a 2 carbon compound. Trust and Millis (1970) showed increased amounts of isocitric lyase (E.C. 4.1.3.1) present in cells grown on hydrocarbon, suggesting that the glyoxylate bypass was the major assimilatory mechanism.


Studies by Blevins and Perry (1972) showed that cells growing on three-carbon compounds did not possess an active isocitric lyase enzyme system. They showed 14CO2 incorporation into pyruvate when propionate or n-propylamine were oxidized but insignificant amounts of 14CO2 incorporation when propane or acetone were oxidized. Leadbetter and Foster (1958) demonstrated considerable 14CO2 incorporation by Ps. methanica grown on methane in the presence of 14CO2.

One organism used in this study (Ps. aeruginosa strain 196Aa) was reported earlier by Traxler and Bernard (1969) to utilize n-alkanes under conditions of anaerobiosis. The organism also will utilize n-alkanes aerobically if high aeration is not provided and therefore may have distinctive physiological characteristics. A second organism (Ps. aeruginosa strain Sol 20) has been reported to have an active dehydrogenase (Senez and Azoulay, 1961). It was thought that these two strains might possess similar metabolic capabilities which are distinct from those reported for other strains of n-alkane oxidizing Ps. aeruginosa. Corynebacterium (TEIC) was included in certain parts of this study because its hydrocarbon metabolism is described and known to follow a conventional aerobic scheme.

Materials and Methods

The organisms used in this study were Pseudomonas aeruginosa strain 196Aa isolated from JP-4 jet fuel, Pseudomonas aeruginosa strain Sol 20 obtained from

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3Dept. of Microbiology, University of Southwestern Louisiana.

(Copy originally received February 1973; in final form June 1973).
Dr. J. C. Senez, CNRS, Marseille, France, and Corynebacterium SEIC obtained from the stock culture collection at the University of Texas, Austin. Stock cultures were carried on Trypticase Soy Agar (BBL) at 4°C.

The basal mineral salts medium described previously (Traxler and Bernard, 1969) containing 1% (w/v) n-hexadecane, 1.0% (w/v) sodium acetate or n-octane vapor was used to grow cells for these experiments. In some experiments the acetate medium was solidified by adding 1.5% washed agar (Difco) and then overlaid with liquid acetate medium. Aerobic growth was at 30°C with shaking at 120 rpm or without shaking (strain 196Aa) in Bellco side-arm flasks. Cells were recovered by centrifugation at 1475 RCF for 15 minutes at 4°C, washed twice in buffer (either Tris-HCl or 0.1M phosphate) and resuspended in buffer for use.

Fatty acids were dissolved at known concentration in 95% ethanol and aliquots of these solutions added to the test flasks so as to contain the desired concentration of fatty acid on a weight:volume basis. In the fatty acid oxidation studies the fatty acid was added to the reaction flask at a concentration of 500 μmoles. After incubation the cells were removed by centrifugation, the fatty acid layer decanted and acidified to insure the free acid form. The acidified fatty acids mixture was injected directly onto a Chromosorb 101 column contained in a Packard series 7400 gas chromatograph equipped with a flame ionization detector. Mixtures of known fatty acids were used to establish the retention time for each fatty acid in the series.

Cell-free extracts were prepared by rupture of the cells in the X-Press at -20°C. Cell debris was removed by centrifugation at 14,900 RCF for 30 minutes at 4°C. The clear supernatant was collected and estimated for protein by the Folin method of Lowery, Rosebrough, Farr and Randall (1951), using Bovine Serum Albumin as the standard.

Isocitric lyase was assayed by the method of McFadden (1969). One unit of isocitric lyase is defined as the amount of enzyme which caused the disappearance of 1 μmole of isocitrate at 30°C in 10 minutes. The malate synthetase activity was measured by the method of Dixon and Kornberg (1962). One unit of this enzyme is defined as the amount of material which catalyzes the cleavage of 1 μmole of Acetyl Co A per minute. The specific activity of each preparation is expressed as units of enzyme activity/mg protein.

All 14C determinations were performed in the Packard model 3320 Tri Carb Liquid Scintillation Spectrophotometer. Carbon dioxide was trapped in 10 ml of a Methanol-Ethanolamine (2:1) solution by slowly bubbling the gas phase from the culture flask through the solution for 5 minutes. Ten ml of flor (4.0g PPO and 0.3g POPOP, toluene to 1 litre) were added to 5 ml of the trapping solution and each sample brought to temperature before counting. Whole cells were measured for 14C content by collecting the cells on HA 0.45 millipore membranes, the cells were washed with distilled water, dried and placed in scintillation vials with 15ml of a toluene cocktail (1.0g PPO, 0.5g POPOP, toluene to 1 litre) which rendered the membrane transparent. The cell fractions were counted in 15ml of a cocktail mixture containing 8g PPO, 1g Bis-MSB, 150g Naphthalene, 20 ml Ethylene glycol, 100ml 2-ethoxyethanol brought to 1 litre with dioxane.

The method of Park and Hancock (1960) was used with slight modification to fractionate cells for component analyses. The cells (2-4mg) were suspended in cold 10% TCA for 10 minutes at 0°C, centrifuged at 3015 RCF for 10 minutes to remove low molecular weight compounds soluble in cold TCA. The cell pack was resuspended in 2ml of acidified ethanol (pH 2.5) for 10 minutes at room temperature then centrifuged at 3015 RCF for 10 minutes. The supernatant from this extraction contained the lipid. After liquid extraction the residue was treated with 2 ml of 5% TCA at 90°C for 6 minutes to extract the nucleic acids. The residue which represented the cellular proteins was solubilized in 1ml of Nuclear Chicago Solubilizer (NCB).

Results and Discussion

The study by Heringa, Huybregtse and van der Linden (1961) provided unequivocal evidence that β-oxidation of fatty acids formed from n-alkane is a dissimilatory mechanism in specific alkane utilizing bacteria. Trust and Millis (1971) have shown that with some organisms growth on an n-alkane induced isocitric lyase activity in the cells. It is generally assumed that Acetyl-CoA produced via β-oxidation of hexadecanoic acids formed from the n-hexadecane substrate induces the enzymes of the glyoxylate cycle.

In this investigation the test organisms were grown on acetate and octane vapour in mineral salts medium, the cells harvested during exponential growth, disrupted and the cell-free extracts assayed for the two critical enzymes of the glyoxylate cycle. Table 1 reports the isocitric lyase assay data and Table 2 the malate synthetase assays. It was demonstrated that isocitric lyase specific activity of acetate grown Pseudomonads was significantly higher than that of n-alkane grown Pseudomonads. In theory, if β-oxidation is operational, the carbon from n-octane or other n-alkanes will be at the acetate state for assimilation and the glyoxylate system will have to be functional. Therefore, the specific activity should be high for glyoxylate enzymes in n-alkane grown cells if indeed acetate is the primary assimilation product. This assumption is supported by the higher isocitric lyase and malate synthetase activities demonstrated on hydrocarbon grown Corynebacterium SEIC which is known to utilize alkanes by a conventional aerobic scheme.

TABLE 1. Effect of growth substrate on isocitric lyase in Pseudomonas aeruginosa 196Aa, Pseudomonas aeruginosa Sol 20 and Corynebacterium 7EIC.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Inducer</th>
<th>Specific Activity*</th>
<th>Induction Ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps. aeruginosa 196Aa***</td>
<td>Acetate</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Octane</td>
<td>0.34</td>
<td>1.9</td>
</tr>
<tr>
<td>Ps. aeruginosa Sol 20</td>
<td>Acetate</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Octane</td>
<td>0.25</td>
<td>2.4</td>
</tr>
<tr>
<td>Corynebacterium 7EIC</td>
<td>Acetate</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Octane</td>
<td>0.60</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Specific activity = units activity/mg protein  
**Ratio of the specific activity of n-alkane grown cells to acetate grown cells. All cells harvested at the same stage of exponential growth.  
***Cells were grown in non-agitated cultures to provide limited aeration.

TABLE 2. Specific activity of malate synthetase in n-octane grown Pseudomonas aeruginosa 196Aa, Pseudomonas aeruginosa Sol 20 and Corynebacterium 7EIC.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Inducer</th>
<th>Specific Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps. aeruginosa 196Aa</td>
<td>Octane</td>
<td>0.06</td>
</tr>
<tr>
<td>Ps. aeruginosa Sol 20</td>
<td>Octane</td>
<td>0.04</td>
</tr>
<tr>
<td>Corynebacterium 7EIC</td>
<td>Octane</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Specific activity = units of activity/mg protein

Malate synthetase activity is normally present at low levels and is increased only when the glyoxylate bypass is operational. The data in Table 2 indicates baseline specific activities in hydrocarbon grown Pseudomonads but increased activity in octane grown Corynebacterium 7EIC. This data suggests an operational glyoxylate cycle in the Corynebacterium but not in either of the Ps. aeruginosa strains.

In order to further examine the fatty acid oxidation mechanism in the two strains of Ps. aeruginosa, resting cell-suspensions were reacted with fatty acids and n-alkane and the oxidation products determined by gas-liquid chromatography. Data is reported for an even carbon chain and odd carbon chain fatty acid and n-decane.

Figure 1 shows the oxidation products of caprylic acid (C8) by Ps. aeruginosa strain 196Aa. Easily identified peaks were present for the C8, C7, C5, C2 and C1 acids, with minor C3, C4 and C6 peaks. The presence of the odd carbon length compounds (C6 and C3) indicates that β-oxidation is not the function mechanism of fatty acid dissimilation. This is further supported by the data in Figure 2 which shows the products of the odd carbon length fatty acid, Pelargonic acid (C9). The most apparent peaks are for the C9, C8 and C7 acids. In both experiments the C1 and C2 compounds accumulate, indicating that they are not readily assimilated by the organism.

Figure 1. Gas Liquid Chromatography of the Oxidation Products of Caprylic Acid (C8).

Organism: Ps. aeruginosa strain 196Aa, resting cells in phosphate buffer, pH 7.0, sampled after 30 minutes respiration.

GLC Data:  
Column: Chromosorb 101, 1.8g  
Detector: Flame ionization  
Carrier Gas: 20 ml/min of Nitrogen  
Program: 180 to 285°C, final hold of 2 min  
Program Rate: 10°C/min  
Amount Injected: 5 μl

Figure 2. Gas Liquid Chromatography of the Oxidation Products of Pelargonic Acid (C9).

Organism: Ps. aeruginosa strain 196Aa, resting cells in phosphate buffer, pH 7.0, sampled after 15 minutes respiration.

GLC Data:  
Column: Chromosorb 101, 1.8g  
Detector: Flame ionization  
Carrier Gas: 20 ml/min of Nitrogen  
Program: 180 to 285°C, final hold of 2 min  
Program Rate: 10°C/min  
Amount Injected: 5 μl

Ps. aeruginosa from n-decane, myristic acid and caprylic acid. This mechanism suggests that the accumulation of C1 and C2 which is then assimilated using the glyoxylate bypass mechanism. The fatty acids produced from the oxidation of n-alkanes by these two strains of Ps. aeruginosa are shown in Figure 3. The C10, C9, C8, C7 and C5 acids are easily identified and again their presence does not indicate a beta-oxidation mechanism.

TABLE 3. Retention times for oxidation products of Ps. aeruginosa strain Sol 20 determined by GLC.

<table>
<thead>
<tr>
<th>Fatty Acid Component</th>
<th>Retention Time in Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decane</td>
</tr>
<tr>
<td>C-1</td>
<td>0.75</td>
</tr>
<tr>
<td>C-2</td>
<td>1.50</td>
</tr>
<tr>
<td>C-3</td>
<td>3.25</td>
</tr>
<tr>
<td>C-4</td>
<td>4.25</td>
</tr>
<tr>
<td>C-5</td>
<td>5.50</td>
</tr>
<tr>
<td>C-6</td>
<td>7.00</td>
</tr>
<tr>
<td>C-7</td>
<td>8.75</td>
</tr>
<tr>
<td>C-8</td>
<td>10.00</td>
</tr>
<tr>
<td>C-9</td>
<td>11.25</td>
</tr>
<tr>
<td>C-10</td>
<td>14.00</td>
</tr>
</tbody>
</table>

Table 3 reports the retention times for fatty acid components produced by Ps. aeruginosa strain Sol 20 from n-decane, myristic acid and caprylic acid. This organism also produces a homologous series of fatty acids varying in chain length by one carbon.

The above data indicates that the metabolism of n-alkanes by these two strains of Ps. aeruginosa does not proceed via a beta-oxidation mechanism to acetate which is then assimilated using the glyoxylate bypass mechanism. The accumulation of C1 and C2 components and the low quantities of C3 product demonstrated in the chromatography experiments suggests that assimilation by the Pseudomonads might involve propionate. An alpha oxidation of fatty acids (Finnerty and Kallio, 1964) to produce propionate is a possible mechanism in these organisms.

Wood and Werkman (1938) first demonstrated carbon dioxide fixation by heterotrophic bacteria. Since this time other reactions involving carbon dioxide fixation by heterotrophic bacteria and in animals have been demonstrated. The reactions generally involve carboxylation of specific acceptors and do not lead to total synthesis from carbon dioxide. Leadbetter and Foster (1958) showed considerable 14C incorporation by Ps. methanica grown on methane in the presence of 14CO2.

Cultures of all three organisms were grown in mineral salts medium with octane as the carbon substrate provided in the vapor phase to prevent the presence of excess substrate. The cells were harvested, washed and resuspended in fresh mineral salts at an O.D. of 0.1. To this suspension were added 1% (v/v) of n-hexadecane and 0.2M 14CO2 in the bicarbonate form containing 30,000-50,000 C.P.M. of 14C activity. The growth system was contained in a tightly sealed growth chamber to prevent loss of gas to the atmosphere during 48 hours incubation at 30°C. The O.D. of the suspension increased from the initial value of 0.1 to 0.5 at the end of the incubation period.

After incubation, the reaction mixture was acidified to convert all bicarbonate to carbon dioxide which was removed from the growth chamber by slow bubbling under reduced pressure through a methanol-ethanolamine solution to trap the carbon dioxide. The cells were collected on an 0.45µ HA millipore membrane, washed with distilled water to remove any adhering extracellular nutrients, dried and placed in a scintillation vial with toluene and fluor to determine the 14C activity in the cells. The culture filtrate was then resuspended for counting to determine the 14C activity in low molecular weight, water soluble intermediates secreted by the cells.

Table 4 shows that the two strains of Ps. aeruginosa were more active than the Corynebacterium strain in the fixation of carbon dioxide. In all three organisms, the majority of the fixed carbon dioxide was associated with the cells and only a small percentage of the activity was found in the culture filtrate.

TABLE 4. 14CO2 fixation during growth on n-hexadecane.

<table>
<thead>
<tr>
<th>Organism</th>
<th>DPM</th>
<th>% Total 14CO2 Fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Culture Filtrate</td>
</tr>
<tr>
<td>Ps. aeruginosa 196Aa</td>
<td>2842</td>
<td>375</td>
</tr>
<tr>
<td>Ps. aeruginosa Sol 20</td>
<td>2106</td>
<td>242</td>
</tr>
<tr>
<td>Corynebacterium 7E1C</td>
<td>1169</td>
<td>126</td>
</tr>
</tbody>
</table>

Figure 4. Gas Phase $^{14}$CO$_2$ during growth of *Ps. aeruginosa* 196Aa on n-hexadecane under conditions of limited aeration. 0.2 M NaHCO$_3$ containing 50,000 CPM C$^{14}$.

An examination of the $^{14}$CO$_2$ content of the gas-liquid phases during the course of growth on n-hexadecane demonstrates the kinetics of CO$_2$ fixation (Figure 4). During the initial 3 hrs. of the experiment, which is the lag phase of the culture under these conditions, there is no change in the $^{14}$CO$_2$ content of the gas-liquid phases. When growth is initiated at 3 hrs. there is a rapid decrease in the free CO$_2$ so that within 2 hrs the activity in these phases has dropped from an initial value of 50,000 C.P.M. to less than 5,000 C.P.M. The value for $^{14}$C as CO$_2$ in these phases fluctuates slightly for the remainder of the experiment.

This experiment shows that carbon dioxide fixation is initiated at the beginning of the exponential phase of growth, and not during the lag phase. The fluctuations noted during the remainder of the growth period are interpreted as the result of cyclic decarboxylation of intracellular intermediates and refixation of the evolved carbon dioxide.

There are various reactions in which the fixation of the $^{14}$CO$_2$ could be involved in the total assimilatory metabolism of these organisms. In order to obtain some preliminary data on the nature of the incorporation products of both the carbon dioxide and the n-alkane, $^{14}$CO$_2$ and 1-$^{14}$C-n-hexadecane were supplied to separate cultures of *Ps. aeruginosa* 196Aa under growth conditions. In the experiments with $^{14}$CO$_2$ the growth substrate was unlabelled n-hexadecane, and unlabelled CO$_2$ was used in the experiments with labelled hexadecane. The cultures were grown to maximum turbidity, harvested, washed and fractionated by the method of Park and Hancock (1960). Each fraction was measured for $^{14}$C activity.

Table 5 indicates that the bulk of the $^{14}$C from carbon dioxide fixation is found in the cellular protein fraction but that a high percentage is also incorporated into the nucleic acids. The amount of activity found in the lipid and low molecular weight fractions is insignificant compared to the protein and nucleic acids fractions. The 5.7% incorporation in the cold TCA fraction probably represents the pool of amino acids used in protein synthesis.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Component</th>
<th>DPM</th>
<th>% Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold TCA</td>
<td>Low mol. wt.</td>
<td>1466</td>
<td>5.7</td>
</tr>
<tr>
<td>Ether:</td>
<td>Lipids</td>
<td>426</td>
<td>1.7</td>
</tr>
<tr>
<td>Hot TCA</td>
<td>Nucleic Acids</td>
<td>9846</td>
<td>38.5</td>
</tr>
<tr>
<td>Residue</td>
<td>Protein</td>
<td>14810</td>
<td>51.9</td>
</tr>
</tbody>
</table>

*Cells were grown in non-agitated cultures to provide limited aeration.*

In contrast to the data from carbon dioxide fixation, the $^{14}$C incorporation from n-hexadecane is more evenly distributed among the various fractions (Table 6). This is to be expected as the components which are not synthesized from carbon dioxide must be derived from the hexadecane carbon.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Component</th>
<th>DPM</th>
<th>% Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold TCA</td>
<td>Low mol. wt.</td>
<td>750</td>
<td>19.0</td>
</tr>
<tr>
<td>Ether:</td>
<td>Lipids</td>
<td>1400</td>
<td>35.5</td>
</tr>
<tr>
<td>Hot TCA</td>
<td>Nucleic Acids</td>
<td>400</td>
<td>10.2</td>
</tr>
<tr>
<td>Residue</td>
<td>Protein</td>
<td>1395</td>
<td>35.1</td>
</tr>
</tbody>
</table>

*Cells were grown in non-agitated cultures to provide limited aeration.*

It is concluded that the carbon from hexadecane is funnelled into all cellular components but that the carbon dioxide which is fixed is incorporated into mainly the protein and nucleic acids components of the cell. The small amounts of $^{14}$C activity found in the Lipid and Low Molecular Weight fractions of $^{14}$CO$_2$ fed cells are not considered to indicate major assimilatory mechanisms.

**Summary and Conclusions**

The data on isocitric lyase and malate synthetase specific activity in n-alkane grown *Ps. aeruginosa* strains 196Aa and Sol 20 suggests that the glyoxylate bypass has no significant role in hydrocarbon assimilation by these organisms. This suggestion is supported by the gas chromatographic data which demonstrate

a homologous series of fatty acids formed which vary by 1 carbon in chain length. It is concluded that the assimilatory mechanisms involve compounds with a carbon chain greater than two carbons in length.

In addition, 14C studies indicate CO₂ fixation during growth on n-alkanes with the majority of this carbon appearing in the proteins and nucleic acids of the hydrocarbon grown cells. Using Cl⁴C-1-n-hexadecane it was shown that this carbon is distributed among all cellular components.

The authors do not dispute the role of β-oxidation and acetate assimilation by other hydrocarbon utilizing bacteria of even the same genus, but believe this data substantiates the existence of yet other mechanisms.

Acknowledgements

This investigation was supported entirely by the Department of Microbiology, University of Southwestern Louisiana. Parts of this work were used in partial fulfillment of the requirements for the degree of Master of Science by G. T. von dem Bussche. The authors express their thanks to Dr. J. M. Sobek for his advice and assistance with the 14C studies and cell fractionation procedures.

References


La résistance contre les termites des composes de bois-plastique.
Les composes qui se forment par moyen de la polymérisation des monomères après s'être imprégnés dans le bois, contiennent un intérêt spécial pour les pays tropicaux où leur stabilité dimensionnelle pourrait comporter de très grands avantages. La résistance contre les termes de plusieurs composes a été déterminée avec l'emploi du bois de hêtre ou de sycomore, qui contenaient le polyacrylonitrile, le polyester flexible ou rigide, le méthacrylate de méthyle ou le styreène acrylonitrile. Ces composes s'exposèrent aux colonies de Reticulitermes santonensis dans le laboratoire, et il se révéla que la dégradation qui en résultait dépendait de la dureté du produit achevé.

Introduction
There is increasing interest in wood-plastic composites as materials for use where their special properties such as dimensional stability, hardness, finish in depth and enhanced appearance are of prime importance as in flooring, bobbins, tool handles and gun stocks. They are of particular interest for tropical countries where their dimensional stability could be a major advantage; thus the termite resistance of wood-plastic composites is an important property.

These composites are made by impregnating wood with a monomer, which is then polymerised within the wood resulting in a solid plastic integral with the wood substance. The resultant product is harder than the original wood but more brittle. It is possible by varying the plastic monomer and the timber species to produce a wood-plastic composite for a specific application.

When materials are not directly toxic to termites the incidence of attack is more often in relation to the hardness of the material.

The termite resistance of plastics has been studied by a number of workers. Gay and Wetherby (1962) using laboratory tests showed that resistance is affected by the thickness of plastic films and by the hardness of products such as piping and cable sheathing. Rigid and semi-rigid polyvinyl chloride were virtually unattacked but, when plasticised for use as cable sheathing, PVC was very susceptible. Similarly high density polyethylene was more resistant to termites than low density polyethylene. It was confirmed by Becker (1963 and 1964) that the hardness of plastics was one of the most important factors in determining its susceptibility to termite damage. In laboratory tests covering 50 types of plastic exposed to five termite species he showed that generally those materials with a hardness value less than 30 when measured with a microindentation tester were resistant to attack. Materials with hardness values above 45 were susceptible with a gradient between these figures. The exceptions were those softened by heat or moisture or those where hardness was variable within the material.

These hardness values consist of an arbitrary scale with the higher values denoting the softer materials. They cannot be related directly to the hardness figures obtained by means of the Janka method used in the following test. This method of determining hardness records the load required to embed a hardened steel ball up to half its diameter in the test specimen. Results have been recorded in kilograms and the harder the material, the greater is the required load.

Wood-plastic composites were exposed to termite attack by Neel et al. (1966). Maple, birch and pine were prepared with vinyl acetate, methyl methacrylate...

or a co-polymer of methacrylic acid and methacrylate. On exposure to termites in the laboratory and in field trials vinyl acetate wood composites were attacked by termites and heavy attack occurred at the lower loadings of plastic retained by maple. Methyl methacrylate and the co-polymer were resistant to attack. Tests undertaken at the Princes Risborough Laboratory have included five plastic monomers which were used to treat two species of timber. Sycamore (Acer pseudoplatanus) and beech (Fagus sylvatica) were selected for treatment since the former will take up more impregnation solution than beech, the ratio being approximately 1.8:1. The prepared wood-plastic blocks were tested for both hardness and termite resistance.

Materials and Methods

Beech and sycamore strips of 20 × 20 mm cross-section were treated with monomers which were then polymerised as indicated in Table 1. Sufficient material was treated to provide eight strips 150 mm long for assessment of hardness for each treatment of each timber and from the original strips, 20 mm cubes were cut for exposure to termites. In addition untreated beech and sycamore strips were retained to provide control samples. After polymerisation all treated material was heated at 103°C for 48 hours to disperse any surplus monomer remaining in the wood.

TABLE 1. Preparation of Wood-Plastic Composites 20 × 20 × 150 mm

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Method of Impregnation</th>
<th>Method of Polymerisation</th>
<th>Timber</th>
<th>Loading obtained % dry weight of wood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyacrylonitrile</td>
<td>exposure to saturated</td>
<td>irradiation, cobalt 60</td>
<td>beech</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>vapour</td>
<td>source</td>
<td>sycamore</td>
<td>14.6</td>
</tr>
<tr>
<td>Flexible polyester</td>
<td>vacuum with benzyl</td>
<td>heat</td>
<td>beech</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>peroxide initiator</td>
<td></td>
<td>sycamore</td>
<td>132</td>
</tr>
<tr>
<td>Rigid polyester</td>
<td></td>
<td></td>
<td>beech</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sycamore</td>
<td>139</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>vacuum</td>
<td>irradiation, cobalt 60</td>
<td>beech</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>source</td>
<td>sycamore</td>
<td>131</td>
</tr>
<tr>
<td>Styrene</td>
<td></td>
<td></td>
<td>beech</td>
<td>68</td>
</tr>
<tr>
<td>acrylonitrile</td>
<td></td>
<td></td>
<td>sycamore</td>
<td>117</td>
</tr>
</tbody>
</table>

The termites used in the test were Reticulitermes santonensis (Feytaud) from laboratory cultures which were started from colonies collected in the field near Saintes (France).

Hardness Tests

Hardness tests were carried out according to BS 373:1957 using the Janka method, but with a hardened steel ball of 5.6 mm (0.22 inch) diameter instead of 11.3 mm (0.44 inch) diameter to reduce the risk of splitting the specimens. The depth of penetration was 2.8 mm (0.11 inch) and the speed of loading 6.40 mm (0.25 inch) per minute. The test was applied to both radial and tangential faces.

Termite Tests

For each timber and each plastic three 20 mm cubes were exposed individually to termite attack. For each sample a glass jar was partly filled with sand which was thoroughly mixed with 20% by weight of water. The sample was buried in the sand against one side of the jar so that it could be observed during the test. A small piece of wood from the termite cultures was pushed to the bottom of the sand in the centre of the jar and 200 workers, one soldier and two nymphs of Reticulitermes santonensis were added. The jars were closed by covering with aluminium foil and stored at 27°C and 80% relative humidity.

A set of samples was exposed to termite attack one month after manufacture, but despite the previous aging process (48 hours at 103°C) some monomer still remained and could be detected by smell in many of the jars. This was particularly noticeable with styrene acrylonitrile and polyacrylonitrile where all termites died within a week. A similar set of samples was stored, therefore, for 22 months before exposure to termites as described above, and it is to this second set of samples that the results of termite exposure refer. The oven-dry weights of the samples were recorded before the test and after exposure to termites for six weeks at 27°C and 80% relative humidity, the blocks were removed, weighed, oven dried and reweighed to assess moisture content and termite attack which was assessed by loss in oven-dry weight. The numbers of surviving termites were recorded.

Three jars were also set up with termites, but without wood-plastic blocks to assess survival without feeding.

Results

The hardness figures and a summary of results of termite test are given in Table 2. It will be seen that although the sycamore took up nearly twice the amount of plastic as beech, the hardness relationship of sycamore to beech remains as for untreated wood, i.e. all beech blocks were harder than similarly treated sycamore blocks. The low uptake of polyacrylonitrile was due to the method of treatment with monomer, and the hardness of these blocks was similar to untreated wood.

There was no visible damage to either wood species containing styrene acrylonitrile or methyl metacrylate, nor to beech containing rigid polyester.

All remaining samples showed more than 200 mg weight loss and termite damage was evident. In all plastic-impregnated blocks with the exception of those containing polyacrylonitrile the uptake of water under the damp test conditions was greatly reduced as compared with untreated wood.

There was a direct correlation between hardness and loss in weight due to termite attack as shown in Figure 1 where $r = -0.945$. 76

### TABLE 2—Termite resistance of wood-plastic composites.
**Summary of Results (Average of 3 samples per variable)**

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Timber</th>
<th>Loading of plastic % dry weight of wood</th>
<th>Hardness† (BS 373) kg</th>
<th>Condition of blocks after 6 weeks test</th>
<th>Moisture content % dry weight of wood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Average loss in weight of test blocks</td>
<td>Average survival of termites per cent</td>
</tr>
<tr>
<td>None</td>
<td>beech</td>
<td>0</td>
<td>154</td>
<td>538</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>93</td>
<td>142</td>
<td>380</td>
<td>75</td>
</tr>
<tr>
<td>Polycrylonitrile*</td>
<td>beech</td>
<td>9.3</td>
<td>142</td>
<td>78</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>14.6</td>
<td>71</td>
<td>729</td>
<td>58</td>
</tr>
<tr>
<td>Flexible polyester</td>
<td>beech</td>
<td>75</td>
<td>396</td>
<td>483</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>132</td>
<td>274</td>
<td>285</td>
<td>30</td>
</tr>
<tr>
<td>Rigid polyester</td>
<td>beech</td>
<td>78</td>
<td>660</td>
<td>16X</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>139</td>
<td>518</td>
<td>212</td>
<td>28</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>beech</td>
<td>66</td>
<td>635</td>
<td>53X</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>131</td>
<td>538</td>
<td>69X</td>
<td>33</td>
</tr>
<tr>
<td>Styrene acrylonitrile</td>
<td>beech</td>
<td>68</td>
<td>691</td>
<td>+20X</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>sycamore</td>
<td>117</td>
<td>567</td>
<td>+14X</td>
<td>13</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

x No visible termite damage
†Janka test with ball reduced to 5.6 mm
*Monomer introduced by exposure to saturated vapour, remaining monomers introduced by vacuum impregnation

**Figure 1**

Relationships between hardness of plastic impregnated wood and amount of block eaten by Reticulitermes santonensis

The relationship between hardness and loss of weight due to termite attack is logical and has been demonstrated by other workers for plastics alone. The natural resistance of different timbers has also been shown to bear a similar relationship (Behr et al., 1972) and these authors stated that resistance to termites increased significantly at hardnesses above 454 kg (1000 lb).

The tests at Princes Risborough Laboratory using wood-plastic composites gave negligible termite damage to composites with hardness above 540 kg and resistance may be said to occur between 520 and 540 kg.

**Discussion**

Blocks with no visible damage showed some weight losses but the highest of these was 69 mg. Survival by termites with styrene acrylonitrile blocks was similar to jars where no blocks were present, thus no feeding had taken place. Termite survival was somewhat higher in other jars containing composites which showed no attack but it is possible that this might be attributable to variations in the amount of original culture wood present in the jars.

The final moisture contents of wood-plastic composites were of interest since after six weeks' burial in damp sand, with the exception of polycrylonitrile, these were much lower than untreated wood. This would indicate that in less severe conditions their susceptibility to fungal decay would be greatly reduced in comparison with untreated wood.

**Conclusions**

The incidence of termite attack of wood-plastic composites is dependent upon the hardness of the finished product. No attack occurred when hardness of the finished product was above 540 kg. The resistant composites were beech and sycamore containing styrene acrylonitrile, or methyl methacrylate and beech with rigid polyester. Termite attack was evident in sycamore containing rigid polyester and in both beech and sycamore containing flexible polyester and polycrylonitrile, but the latter material was present at very low loadings.

The presence of plastics in the wood blocks tested, with the exception of polycrylonitrile (at low loadings) greatly reduced the uptake of moisture from a damp substrate as compared with untreated wood.

References


Evaluation of biocides for use in aviation fuel.

EVALUATION OF BIOCIDES FOR USE IN AVIATION FUEL

P. B. Park

Summary. This paper outlines briefly the problems associated with microbial growth in jet aircraft fuels, and the philosophy of additive usage in preventing them. The type of testing that needs to be carried out on candidate fuel additives to satisfy the fuel specifications and engine and airframe manufacturers requirements is also discussed as are some factors affecting operational costs.

Evaluation de l'emploi de biocides dans le carburant d'aviation. Un court résumé des problèmes qui peuvent se présenter en ce qui concerne la croissance microbienne dans les carburants d'aviations à réaction, et de la philosophie qui se rapport à l'emploi des additifs pour éviter ces problèmes. Discussion sur le type d'essai qu'il faut exécuter pour les additifs potentiels afin de satisfaire les spécifications des carburants et les demandes des fabricants de moteurs et de cellules d'aviation, et aussi quelques éléments qui influent sur le coût opérationnel.

Introduction

Aircraft fuel handling systems are complex affairs and at many points microbial contamination, in some form or other, from a variety of sources is inevitable. (Elphick, 1971). Gross contamination in fuel is normally removed before delivery to the aircraft by means of gravitation and a nominal 5 μ filter. This means that although microbial matter is almost certainly delivered with the fuel, it is as discrete particles, usually fungal spores, rather than large mycelial mats. The majority of these particles pass with the fuel into the engines and are burned off without germinating and present no problem. A few probably act as foci for water droplet formation which occurs as the solubility of water in the fuel drops with the decreased temperatures at altitude - this water is greatly supplemented on descent when warm, moist air is drawn into the tanks producing water by condensation on the cold tank surfaces.

Most of this water drains to the inboard of the tank where it can be removed easily, but some small droplets (they may be less than 1 ml. in volume) adhere by surface tension to the tank surfaces (Scott, 1971). Under favourable environmental conditions it is in these undrainable droplets that spore germination and mycelial growth sometimes occurs.

In an effort to overcome the effects of microbial contamination of aircraft fuel tanks many operators have shown an interest in the use of chemical fuel additives. At the same time chemical manufacturers have shown some degree of willingness to provide chemical additives but they are sometimes not aware of the problems associated with testing for approval for their use within the aircraft industry.

The use of Additives

The overall philosophy towards fungal contamination must be one of prevention. It is not sufficient to provide a tank coating which will prevent corrosion, as the presence of fungal mats inside the tanks cannot be tolerated. The growths, living or dead, present a corrosion hazard should the lining become damaged and there is always a danger of filter clogging due to detached mycelial mats.

An obvious preventative approach to the problem is by the use of biocides or biostats and, to be effective, these must be distributed to all the water droplets dispersed throughout the tank.

Additives in fuel tank linings appear to present an attractive solution - the ideal biocide would only leach out into the water phase where it is needed and hence be economic with no wastage. The big drawback to this technique is the short life encountered in practice since the tank lining film is only of the order of 0.001 in. thick and replacement is a costly and time consuming exercise which is not practicable at the required frequency.

The current approach is to use the fuel as a carrier for biocides which are fuel soluble but preferentially soluble in water.

There are two fuel additives in current use which are applied in different ways (Robertson, 1970). The military operator uses a material which acts primarily in an anti-icing role and additions are made to all fuel uplifts. Most civil aircraft have heated fuel filters, making an icing inhibitor unnecessary, and those civil operators requiring an additive normally opt for the less expensive approach of using a material

1Aircraft Laboratories, British Aircraft Corporation Ltd., Commercial Aircraft Division, Weybridge, Surrey, England.

(Copy originally received May 1973; in final form August 1973).

which needs only to be added every 2-3 months to obtain adequate microbial control. Both additives have high water to kerosene partition co-efficients (in the order of 200-300:1) and rapidly partition into the dispersed water globules (Elphick, 1968). In the case of intermittent use, additions are usually made when the aircraft is grounded for servicing for a period of at least 24 hours. The high partition co-efficient ensures that the reversal of additive into subsequently untreated fuel is very slow and in practice longer periods between dosages than those theoretically calculated for control are achieved.

Test Procedure

Although final testing and approval for aircraft use must be the responsibility of the engine and airframe manufacturers, some preliminary testing by the additive manufacturer may well save his time and money on developing a product not acceptable to the aircraft industry.

The first requirement of a biocide or biostat is that it should be effective against the correct spectrum of organisms. In the case of subsonic jet aircraft fuels Cladosporium resinae and Pseudomonas aeruginosa are the two most predominant species found (Leathern and Kinsel, 1963; Edmonds and Cooney, 1967; Anon., 1961). Little experience has been gained to date on supersonic aircraft but it can be expected that both the above organisms will be present to some extent with the addition of other, thermophilic, organisms—possibly fungi of the Penicillium-Aspergillus-Paecilomyces type, although others obviously cannot be ruled out.

All aluminium alloys are susceptible to corrosion to a greater or less extent and therefore any additive should be non-corrosive.

Since the fuel is to be the carrier for the additive it is obvious that the additive should be sufficiently soluble in, or miscible with, jet fuel to provide an effective concentration in the water phase with a fuel: water ratio in the order of 1000:1. The presence of fuel or its normal additives may inhibit the biocide and the effective dosage has to be established using a realistic fuel/water system.

Aircraft operating temperatures vary considerably and normally can be expected to range from −40°C to +80°C or even higher in the supersonic case. The additive will need to be stable over this range.

Preliminary toxicology testing may also be carried out at this time with advantage.

Specifications exist to control the quality of aircraft fuel; these are published by Esso or Shell. Test methods are published by the Institute of Petroleum and the American Society for Testing and Materials, although it is advisable that an established petroleum laboratory should be approached to undertake testing of the proposed fuel/additive mixture to the relevant fuel specifications. The testing will cover such properties as specific gravity, flash point, sulphur content, copper and silver corrosion, gum content, freezing point, acidity, water separation index, thermal stability, etc. of the proposed fuel/additive mixture. Ideally, the presence of the additive in the fuel should not be detectable in normal specification tests.

If the product is acceptable to the fuel specifications it must then be approved by the engine manufacturers who must be satisfied that no clogging of the fine jets or filters will occur and that combustion products, both partial and complete, will have no adverse effects on the engine system materials or on engine performance. From the engine manufacturers’ point of view the additive should only contain carbon, hydrogen and oxygen, but in practice certain other elements might be acceptable. Metals, the halogens, sulphur and phosphorus are particularly undesirable and should be minimised or, preferably, excluded from the additive.

Each airframe manufacturer has its own range of materials which may vary with aircraft type and often with the mark within a type. Only relatively broad guidelines can, therefore, be given as to an additive’s properties. It must be nominally non-corrosive to aluminium alloys, copper alloys, titanium and steels. It should not affect the various rubber and elastomeric seals and sealants or paint systems. It is difficult to catalogue all the materials which are used in aircraft fuel systems and, since in many cases test methods are specific to individual airframe manufacturers, each should be approached for advice on their own particular system.

Toxicity testing should be carried out by a responsible organisation and should show that the candidate additive is acceptably non-toxic in normal usage.

Cost and Operational Factors

After the additive has been fully developed and approved, the customer, i.e. the aircraft operator, must be satisfied of the cost effectiveness of the product not only in terms of direct treatment cost but also with regard to time between treatments and aircraft ‘down time’ for treatment. Depending on the method of usage the fuel supplier may be called upon to provide jet fuel pre-doped with the additive. Unless the additive and its cost is acceptable to all customers at a given location, special injection equipment or even segregated storage and delivery facilities may have to be provided. In most cases a premium will have to be charged for such arrangements and this aspect of the additive’s usage will affect its cost to the potential user (Robertson, 1970).

Final cost considerations are, of course, a matter between the chemical manufacturer and the individual customer but in most cases the operator will consult the aircraft manufacturer or fuel supplier as to the suitability of a particular product for his own specific problem.

References


THE "MINI-FUNGUS-CELLAR"
A MYCOLOGICAL TEST METHOD
FOR WOOD PROTECTION PRODUCTS
J. Hansen

Summary. A description is given of an accelerated laboratory test of the effectiveness of chemical wood preservatives against fungi, the test having a close resemblance to practical conditions. The test pieces are large enough to allow the preservatives to be applied to the wood exactly as prescribed by the manufacturer, yet small enough to allow large experimental series to be made using reasonable laboratory facilities. The treated test pieces are exposed to a strong attack of pure cultures of wood destructive fungi. The effect of the attack is evaluated by means of the weight loss of the pieces together with a visual evaluation.

Introduction
The efficacy of chemical wood preservatives against wood destroying fungi can be tested according to different standard methods such as DIN 52 176 (Germany), NF. X. 41.502 (France), ASTM D 1413-61 (USA). These methods employ fully impregnated test pieces and cannot be used to test the efficiency of a mode of application.

If a product intended for brushing or dipping or if a complete protective system consisting of, for instance, brushing first with a colourless product, thereafter with a pigmented and finally with a water repellent product is to be tested with wood destroying fungi, the prepared test pieces should satisfy the following conditions:

1. Be large enough to permit application of the product (or products) in the same way as in practice.
2. Be of such dimensions that after treatment a relatively large proportion of the wood-cells inside the test pieces will not have had any chemical applied to them. This is true for the testing of products to be applied by brushing or by dipping.

3. Have a dimension and a surface sufficiently large so that both a natural and an accelerated weathering test can be carried out.
4. Not be larger than can be tested using reasonable laboratory facilities.

When the test pieces have been treated and dried they should be exposed to a strong fungal attack in the form of direct contact with untreated fungus-infected wood under good conditions for fungal growth.

Materials
Container
1 plastic container for instance a Stockbox (No. 3-201 Rakosystem, 5620 Bremergarten (Switzerland) with cover, Internal dimensions: 560 × 368 × 215 mm, External dimensions: 600 × 400 × 220 mm.

Rockwool
A piece of Rockwool size: 560 × 368 × 40 mm.

Soil
8-9 l. garden-soil with a moisture content of 40-50 % or granular Rockwool-fibre *) moistured to 50-55 % with a nutrient solution containing 3 % malt extract and 1 % inorganic concentrate (Table 2).

*) Biological Laboratory, Sadolin & Holmblad Ltd., 70 Holmbladsgade, DK-2300 Copenhagen S, Denmark.

(Copy originally received December 1972; in final form April 1973.)
Granular Rockwool fibre is an inorganic material with a chemical content as shown in Table 1.

Even if the constituents of the Rockwool fibre are the same as in plant nutrients one cannot in practice assume that the Rockwool fibre can release any of these for nutritive purposes.

The capillary action in the Rockwool fibre will cause added water to be distributed uniformly.

**TABLE 1. Chemical analysis of “Green Grodan Wool”**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Formula</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon dioxide</td>
<td>SiO₂</td>
<td>47%</td>
</tr>
<tr>
<td>Aluminium oxide</td>
<td>Al₂O₃</td>
<td>14%</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>TiO₂</td>
<td>1%</td>
</tr>
<tr>
<td>Ferrous oxide</td>
<td>FeO</td>
<td>8%</td>
</tr>
<tr>
<td>Calcium oxide</td>
<td>CaO</td>
<td>16%</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>MgO</td>
<td>10%</td>
</tr>
<tr>
<td>Manganese oxide</td>
<td>MnO</td>
<td>3%</td>
</tr>
<tr>
<td>Sodium oxide</td>
<td>Na₂O</td>
<td>2%</td>
</tr>
<tr>
<td>Potassium oxide</td>
<td>K₂O</td>
<td>7%</td>
</tr>
</tbody>
</table>

**TABLE 2. Constituents of Inorganic Concentrate.**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Formula</th>
<th>Amount per litre (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>NH₄NO₃</td>
<td>3.0</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>K₂HPO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>Mono potassium phosphate</td>
<td>K₂HPO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄·7H₂O</td>
<td>2.0</td>
</tr>
<tr>
<td>Microsolution</td>
<td>see below</td>
<td>1.0</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>MnSO₄·H₂O</td>
<td>1.2</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>H₃BO₃</td>
<td>1.2</td>
</tr>
<tr>
<td>Cupric Sulphate</td>
<td>CuSO₄·5H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>Zinc Sulphate</td>
<td>ZnSO₄·7H₂O</td>
<td>0.4</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>Na₂MoO₄·2H₂O</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Wood**

Test pieces and inoculation pieces are required (Table 3). They should be of a timber species of low natural decay resistance and not more than one third of the cross-section should be heart-wood. Test pieces must be free from defects and taken from timber which has nor been floated or ponded, or otherwise treated to permit either excessive absorbity or resistance to fungal attack. The annual rings should be orientated at an angle of approximately 45° to the broad face.

Suitable test fungi are: *Merulius laerymans*, *Coniophora puteana* and/or *Lenzites trabea*.

*In Denmark it is sold as Green Grodan wool by Grodania A/S, Hovedgaden 570, DK-2640, Hedehusene, Denmark.

**Test Methods**

**Preparation and treatment of test pieces**

Bring the test pieces to a constant moisture equilibrium in the conditioning room. After suitable labelling weigh the test pieces to the nearest 0.01 g.

**TABLE 3. Wood.**

<table>
<thead>
<tr>
<th>Code letter in figures</th>
<th>Description</th>
<th>Number required</th>
<th>Dimensions (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Test pieces</td>
<td>5</td>
<td>28 × 6 × 2.5</td>
</tr>
<tr>
<td>B</td>
<td>Between pieces</td>
<td>12</td>
<td>28 × 2 × 2.5</td>
</tr>
<tr>
<td>C</td>
<td>End-pieces</td>
<td>4</td>
<td>28 × 8 × 1.0</td>
</tr>
<tr>
<td>D</td>
<td>Side-pieces</td>
<td>4</td>
<td>51 × 8 × 1.0</td>
</tr>
<tr>
<td>E</td>
<td>Supporting pieces</td>
<td>4</td>
<td>45 × 2.5 × 1.5</td>
</tr>
</tbody>
</table>

These are then treated as prescribed by the manufacturer. Some test pieces can then be exposed outdoors or in a weather-o-meter.

**Preparation of inoculation pieces**

2 end-pieces, 2 side-pieces and the four supporting-pieces are sterilized by autoclaving for 1 hour and then placed close to each other in a separate container with the supporting-pieces, at the bottom.

The moisture in these pieces is then increased by spraying with sterile water. Inoculation is then carried out using small pieces of the test fungus growing on malt agar. When the attack has started, especially all over the supporting-pieces, they are ready to be used in the Fungus cellar.

**Construction of the Fungus cellar**

The container is first thoroughly cleaned, for instance with a 3% solution of a quaternary ammonium compound and then the 4-5 cm layer of Rockwool is placed at the bottom of the container. Next a 3-4 cm layer of garden-soil with a moisture content of 40-50% or the Green Grodan wool as described above.

The 2 end-pieces (C), the 2 side-pieces (D) and all the between-pieces which have not been inoculated, the Rockwool and the soil are sterilised by autoclaving and the test pieces sterilised by electron radiation (Hansen, 1972).

The wood system is then built up as shown in figures 1, 2 and 3. The 2 end-pieces and 2 side-pieces that have been infected with the fungus are placed on the outside of the whole wood system. (They are marked with * in the figures).

**Figure 1. Test-box from above.**

Figure 2. Section through the length of a test piece.

Incubation
Place the whole test-system in an incubation room at 22° C and 75% R.H. for a period of 6 months.

Figure 3. Section through a supporting piece.

Determination of results
The weight losses of the test pieces are calculated allowing for initial moisture content and the weight of solids applied during treatment after drying at 105°C.

After measuring the weight loss the test pieces are cut into 3 or 4 pieces to allow visual examination of the fungal attack.
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