Biodegradation of Synthetic Polymers. II. A Limited Microbial Conversion of ^{14}C in Polyethylene to $^{14}CO_2$ by some Soil Fungi*

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Synopsis

Polyethylene film synthetized with a randomly distributed ^{14}C marker, was exposed to biodegradative impact by cultivated soil, a mixed culture of three wood rot fungi, and *Fusarium redolens*, isolated from soil experiments. The net values of $^{14}CO_2$ evolution obtained by scintillation measurements amounted for about 0.5% in two years when calculated as a percentage of the total amount of radioactivity in the test sample. Both the soil and the different mold cultures reflected with very good agreement a definite liberation of $^{14}CO_2$ from the ^{14}C-labeled polyethylene film, significantly above that produced abiotically from aging samples. This is interpreted as due to an enzymatic cleavage and oxidative conversion of synthetic polymeric or oligomeric alkanes with limited chain length, accessible for biodegradation. Abiotic parallel experimental series maintained in a similar way, but either on distilled water only, or on media completed with silvernitrate in order to inhibit microbial growth, revealed slow but consequently progressing—evidently nonenzymatic—conversion of ^{14}C to $^{14}CO_2$. This is referred to as a borderline case of as unarrestable tardy mineralization process in the absence of light, however, autocatalytic and oxidative, an aging procedure in restricted sense.

INTRODUCTION

Many microorganisms can utilize paraffins as a carbon source[1]; the idea that some of the polyethylenes could also be catabolized by such microbes has been put forward by many research workers. However, the degradation of paraffins usually decreases when passing from liquid to solid paraffins,[2,3] and also the normal paraffins are preferred to the branched isomers by several micro-organisms.[4]

Polyethylene and paraffins were first compared in biodegradation experiments by Jen-hou and Schwartz[5] who counted the number of bacteria grown on the carbon of these alkanes as a measure of polyethylene utilization. Potts et al.[6] also studied the biodegradability of hydrocarbon samples and polyethylene after thermal degradation with four strains of fungi and estimated the mycelial growth. Other fungi, e.g., *Aspergillus oryzae*, was reported to grow on the surface of polyethylene material and led to an increased degree of crystallinity.[7]

The growth on polyethylene is usually interpreted as being limited to microbial action on the surface of an inert support without impact on the polymers.[8–10] Küster et al. claim, however, that a true biodegradation of plastics cannot be excluded.[11] At variance with most reports, Wallhäuser[12] and Griffin[13] believe that polyethylene is successively degradable in compost and they relate the decrease in tensile strength to biological degradation.

* Part I of this series: Reference 15 of this paper.

Journal of Applied Polymer Science, Vol. 22, 3419–3433 (1978)

0021-8995/78/0022-3419$01.00

Nykvist started work on ^{14}C-labeled polymers in 1973. He examined low-density polyethylene mixed with soil and measured the $^{14}CO_2$ that evolved from the system.[14] When we continued his work we found that the amount of $^{14}CO_2$ evolved decreased rapidly with time.[15] This can indicate a leveling off of the "degradation" of the polymer, or alternatively, an early exhaustion of accessible material which can be attacked by the soil organisms. It was, however, not clear whether such "degradation" could be prolonged slowly but progressively as far as a final mineralization, without a preceding abiotic physical or chemical cleavage of the macromolecules.

This report describes studies on a ^{14}C-labeled high-density linear polyethylene (HDPE). Biodegradation has been studied in the presence of cultivated garden soil, several strains of white rot fungi and the soil mold *Fusarium redolens* by specific radiorespirometric technique.

EXPERIMENTAL

Materials

A ^{14}C-labeled high-density (linear) polyethylene (HDPE) was prepared in cooperation with Unifos Kemi AB, Sweden. A ^{14}C-labeled monomer from New England Nuclear was blended with the usual monomer and the resulting ethylene gas mixture was then polymerized. The polymer contained a low concentration of ^{14}C, viz., one ^{14}C per 1.2×10^6 atoms of ^{12}C randomly distributed. From the virgin polyethylene powder, a ^{14}C-labeled film of 0.02 mm thickness was made by heating and moulding. The polymer in this way was a highly linear high-density research product with one or two CH_3 groups per 1000 carbon atoms, explicitly without addition of antioxidants. The density of the film was 0.958 g/cm^3 and the molecular weight $\overline{M}_w$ about 300,000. In nearly every run aliquots of the 2 g film were added to 250 ml of the nutrient medium after cutting the samples in small pieces of about 5×5 m.m. size.

Culture medium

The polyethylene was tested in a culture medium having the following composition[16]: ammonium tartrate, 5.0 g; potassium dihydrophosphate, 1.0 g; magnesium sulphate (calculated as anhydrous), 0.5 g; ferric chloride, 1% solution, 0.5 ml; zinc sulfate, 1% solution, 4.5 ml; as well as 1.0 ml solution of a conventional mixture of B-vitamins (viz., thiamine, biotin, inositol, c.f. ref. 16); and distilled water adjusted to pH 5.5, completing the final volume to 1000 ml.

Repeated control of the viability of the inoculated organisms, as well as search for possible contaminants in the cultures were performed through spreading and isolation on conventional malt agar plates. All media were sterilized at 120°C under pressure for 20 min.

Experimental design

The types of experiments were carried out applying different biotic factors to initiate degradation; viz., (a) two series each comprising five independent runs with cultivated garden soil (10 g wet weight per 250 ml). The two runs were started with an interval of a few months; (b) three independent runs with a heavy

inoculum of a mixed culture of three axenic strains of Swedish white rot fungi, viz., the Basidiomycetes: *Fomes annosus* (Polyporaceae), *Peniophora gigantea* (Telephotaceae), as well as a hymenomycete, viz., *Odontia bicolor,* Fries (Hydnaceae), and finally, (c) 11 independent runs with different amounts of polyethylene material (0.5, 2, and 5 g) and *Fusarium redolens* Wollenw., a Hymenomycete isolated from long-term degradation experiments with cultivated soil samples. The first two of these strains are known to be antagonists (c.f. ref. 16), and were seldom recovered at the end of the long time experiments. The mold was identified at the Centraal-bureau voor Schimmelcultures, BAARN, Netherlands (for further details cf. ref. 17). Control experiments were conducted with (d) distilled water alone in four independent runs and; with (e) an uninoculated medium treated with 0.05% silver nitrate as a growth repressor against invading aerial microorganisms (three runs). All 31 runs of the experiments were arranged randomly in the cultivation chamber in order to avoid systematic errors accountable for unevenness of the physical environment.

Equipment

The equipment is pictured in Figure 1. It consisted of five glass jars of which the one in the middle served as a cultivation unit. The first and the last contained aqueous potassium hydroxide (2*M*) to remove CO_2 from the incoming air (No. 1) and to absorb evolved respiratory CO_2 (No. 5). Vessel Nos. 2 and 4 were empty safety traps. No other precautions were taken to filter the incoming air. The whole experimental assembly was placed in a dark cultivation room at a constant temperature of 25°C. Air was bubbled through the flasks at a rate of ca. 10 ml/min.

Measurement

The KOH solution in jar No. 1 was exchanged bimonthly and that in jar No. 5 each month in connection with the radioactivity measurements. The utilization of polyethylene was followed by measuring the carbon dioxide absorbed in jar No. 5. The solution of potassium hydroxide was titrated with hydrochloric

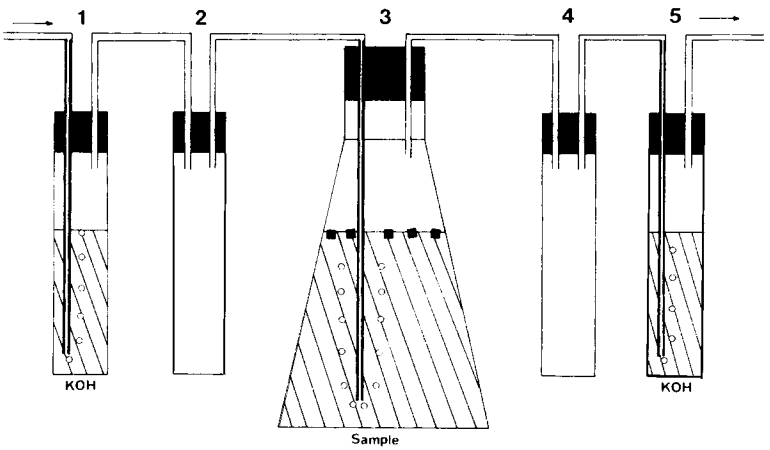

Fig. 1. Equipment for the cultivation.

acid to pH 8.35 in an automatic pH meter, and the amount of carbon dioxide formed was calculated.* After dilution to a constant ionic concentration for K^+, 1 ml aliquots were added to 10 ml scintillation solutions (Aquasol, from New England Nuclear). The amount of the ^{14}C isotope was analyzed in a liquid scintillation counter, Packard Tri Carb, Model 3375. The values were obtained as counts per minute.

These values were finally transformed to values describable as "percent degradation of polyethylene (HDPE)" after that they have been reduced by substraction of the background scintillation. As a measure of the background scintillation, similar samples without ^{14}C added were used. Ten new background samples were made up each time and the mean value was calculated.

The specific radioactivity of the labeled HDPE research material was established in a separate analysis by igniting (in an elementary analysis apparatur "Reihlen-Einbrenner-Automaten," 1941) 30 mg of the ^{14}C-labeled polymer in oxygen. The CO_2 evolved was absorbed in 20 ml aqueous potassium hydroxide (2*M*). It was confirmed by titration as above that all the polyethylene was combusted to CO_2. The content of the isotope was measured and it was found that 1 g of the labeled polymer corresponded to 5.9×10^6 counts per min.

RESULTS

Basically, high-density (linear) polyethylene (HDPE) has a chemical composition similar to that of other acyclic alkanes of the paraffin series, except that the polymeric chains in the HDPE structure have an average length corresponding 20,000 carbon atoms (or molecular weight of 300,000); the whole product reflects a rather wide distribution of molecular weights. It can, however, undoubtedly also include shorter oligomers† of molecular weight less than about 1000, but generally the major components are folded chains of molecular weight around 10,000. In view of its molecular structure with a few linear or branching methyl groups, one can speculate that some kind of microbial enzymatic attack on polyethylene is not per se unthinkable. Several authors have postulated such an attack, with a slow continuous biodegradation of polyethylene as a progressive pattern, and have sought experimental evidence to confirm these assumptions. One of the main difficulties of this type of experimentation lies in the inadequacy of techniques for assaying a limited degree of cleavage of the polymeric material during an experimental time span of several years, and still having convincing evidence that the results are uniform and reproducible. With ^{14}C-labeled ethylene built in randomly into a typical commercial type of polyethylene, we could, however, follow the evolvement of ^{14}C converted to $^{14}CO_2$ as a measure of evidently enzymatic type of oxidative splitting of some fraction of the alkane.

The liberation of radioactivity measured as $^{14}CO_2$ evolution from the high-density polyethylene film when the biotic factor in the nutrient solution was cultivated soil (type a) is shown in Figures 2 and 3. The difference between the two figures is that they relate to runs started on two different occasions with a few months interval. The amount of $^{14}CO_2$ liberated after one year from the ten samples corresponds to (0.26 ± 0.04) %, and after two years (0.36 ± 0.03) % by weight of the polymeric material added to the culture.

* One drop of trimethylamine was added to avoid loss of carbon dioxide.

† An oligomer contains several monomer units; arbitrarily its upper molecular weight has been set at about 1000 units.[18]

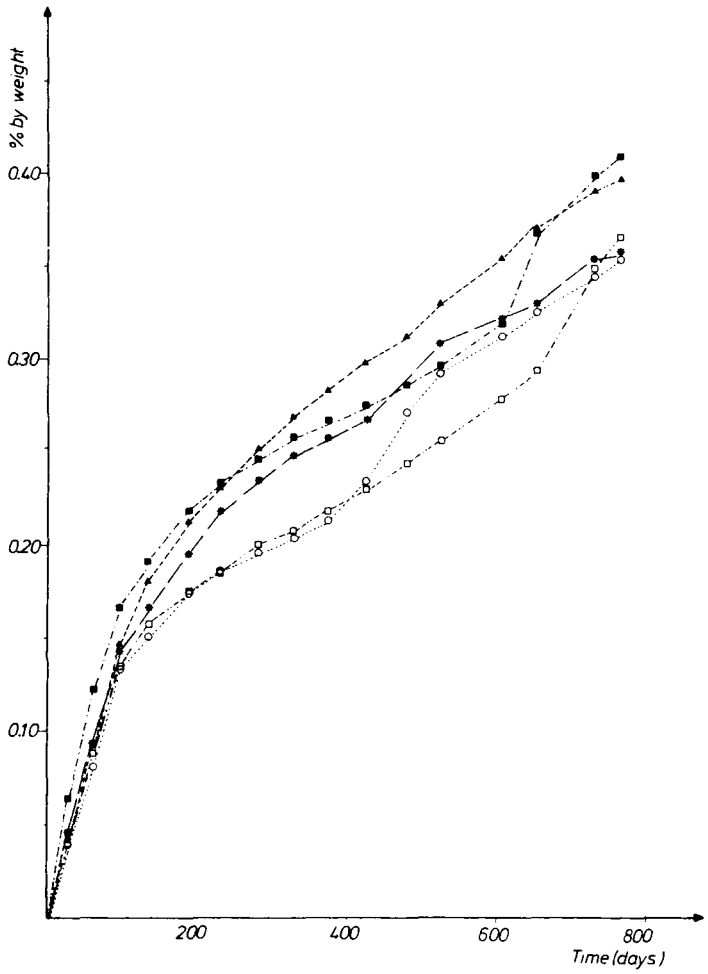

Fig. 2. Biotic liberation of $^{14}CO_2$ from randomly ^{14}C marked high-density (linear) polyethylene (HDPE) film without addition of any antioxidant. Long-term experiment in strongly aerated cultivation medium inoculated with cultivated soil. The active agents are mostly molds of *Fusarium*, *Aspergillus*, *Verticillium*, and *Acremonium* type.

When a mixed culture of three strains of wood rot fungi (type b) was used as a biodegradative agent, the amount of $^{14}CO_2$ liberated from labeled polyethylene films (Fig. 4) was similar to that found in the previous experiments (Figs. 2 and 3). The catabolic values of (0.27 ± 0.03) % after one year and (0.36 ± 0.04) % after two years were in good agreement with the biotic action of soil.

Figure 5 shows a similar set of results for cultures inoculated with *Fusarium redolens* (type c). With this biotic factor, the catabolic values were (0.27 ± 0.02) % after one year and (0.39 ± 0.02) % after two years. A comparison of Figures 2, 3, and 5 shows fairly good agreement between the values for the individual jars within each series. However, one of the five individual runs in both Figures 3 and 5 deviates from the remainder and shows a lower level of $^{14}CO_2$ evolvement.

The reason for this deviation was thought to be one of the usual variations of biological experimentation, viz., weaker inoculum, decreased vitality of the

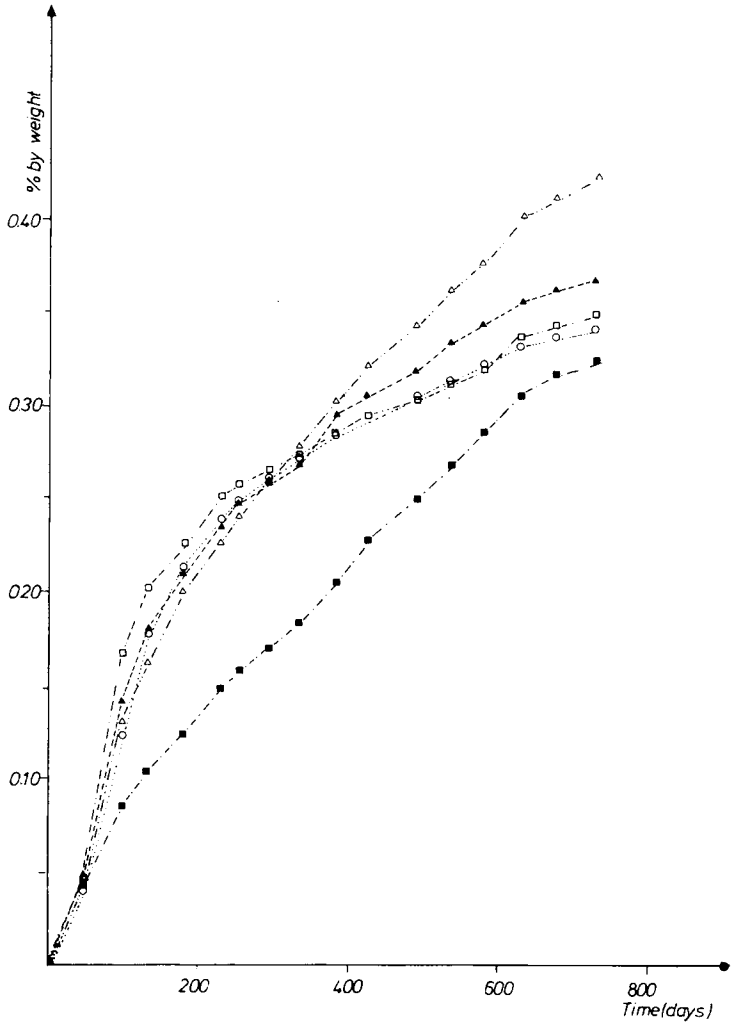

Fig. 3. Experiment repeating that recorded in Figure 2 under similar conditions. From the run represented by the lowest curve, only one fungal species could be isolated in variance with the mixed cultures of the other jars.

culture, inhibitory effect of some unidentified component in the medium, etc. To clarify this, aliquots taken from both the inoculated and the supposedly sterile control cultivation were examined microscopically and also dispersed onto the surface of agar media in Petri dishes. Attempts were made to detect other microorganisms than *Fusarium redolens*, especially in vessels inoculated with a pure culture of the latter. Naturally, in soil and in mixed fungal cultures there was little hope of achieving observable effects. The jars with *F. redolens* showed, however, after prolonged aeration such variations in macroscopic appearance that the presence of a mixed culture due to infection could be suspected. The spreading and isolation experiments showed that this was so in about half the jars after one year and in still more after two years.

The microorganisms isolated were considered to be infections in the *F. redolens* cultures as well as in the wood rot fungal cultures. They were, however, considered to be naturally occurring species in the case of the soil cultures. The

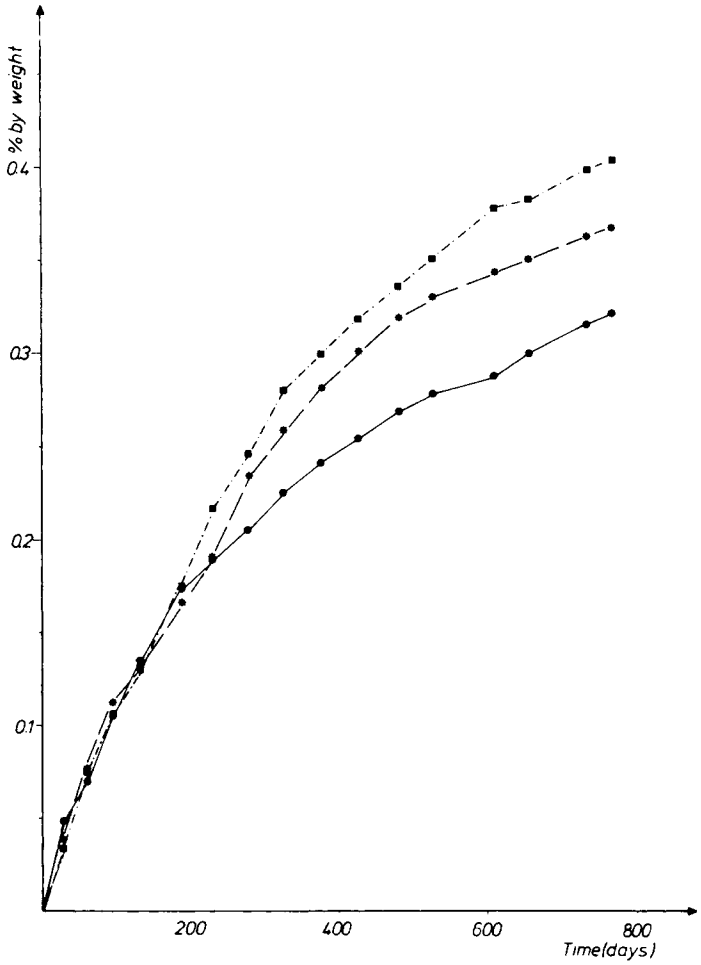

Fig. 4. HDPE biodegradation with a mixed culture of three wood rot fungi (viz., *Phomes annosus*, *Peniophora gigantea*, and *Odontia bicolor*). Cultivation conditions as for experiments presented in Figures 2 and 3.

different populations of microorganisms found in the jars with polyethylene films were quite similar, except that the flasks originally inoculated with *F. redolens* sometimes resisted invasive infection.

The recolonization of the pure *F. redolens* culture by invasive bacilli and fungi is explained by the extremely long run of the aerated cultivation where no strict precautions were taken to sterilize the air passing through the system except for the KOH gas trap of the first of five jars (cf. the Materials section). It is more curious that several fungi and some bacteria can invade the medium used and thrive in an environment where the conditions for microbial growth are poor and the carbon source is mainly of the alkane type.

At least two bacterial strains—probably bacilli—were found, as well as 6–8 different molds probably belonging to the *Aspergillus, Cephalosporium, Cladosporium, Fusarium,* and *Verticillium* families. The ratio of foreign infective cells was, however, quite low in the spreading and isolation tests when compared with the colonies accountable for the original inoculum.

A more close identification of these strains was found to be desirable and was

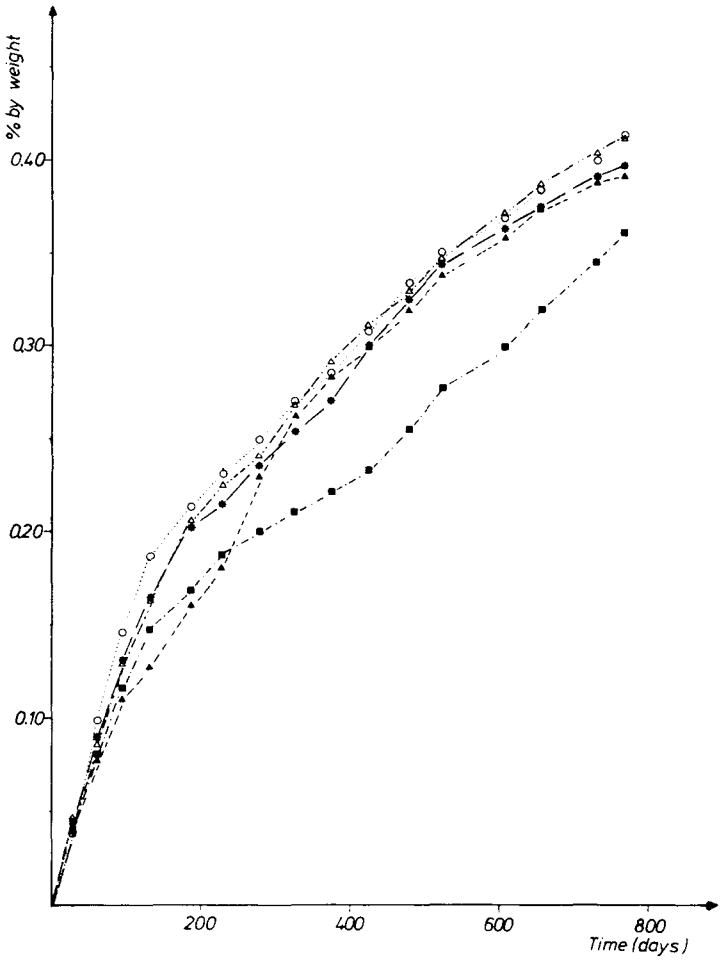

Fig. 5. Biodegradative attempt on HDPE with *Fusarium redolens* in strongly aerated cultivation medium. During the long-term run, several molds of the *Verticillium*, *Acremonium*, and *Aspergillus* type, as well as some Bacilli, invaded the originally pure *F. redolens* cultures and could be isolated from these. Despite this, the agreement between the $^{14}CO_2$ evolution curves is evident.

initiated, although the actual results obtained herewith were not much affected by the taxonomic evaluations. With few exceptions, there was surprisingly little difference between the various mixed cultures regarding the conversion rate of ^{14}C to $^{14}CO_2$ in the long-term runs. As a rule the lowest and slightly uneven curves in the mixed culture experiments (Figs. 2 and 3) represent the runs where the smallest number of species were present. On the other hand in one single case, in the experiment with *F. redolens* the lowest and somewhat deviating curve in Figure 5 corresponded to the presence of the highest number of invasive contaminants (4 mold strains).

It is in some respects more surprising that, in such a run covering so long a period, the agreement between the results was as good as seen, and that the general shape of the $^{14}CO_2$ curves obtained was so strikingly consistent.

The same cannot be said of the *F. redolens* series (type c) of three parallel cultures presented in Figure 6, where the deviations were considerable. The quantity of ^{14}C-labeled polyethylene film in this experiment was two and a half

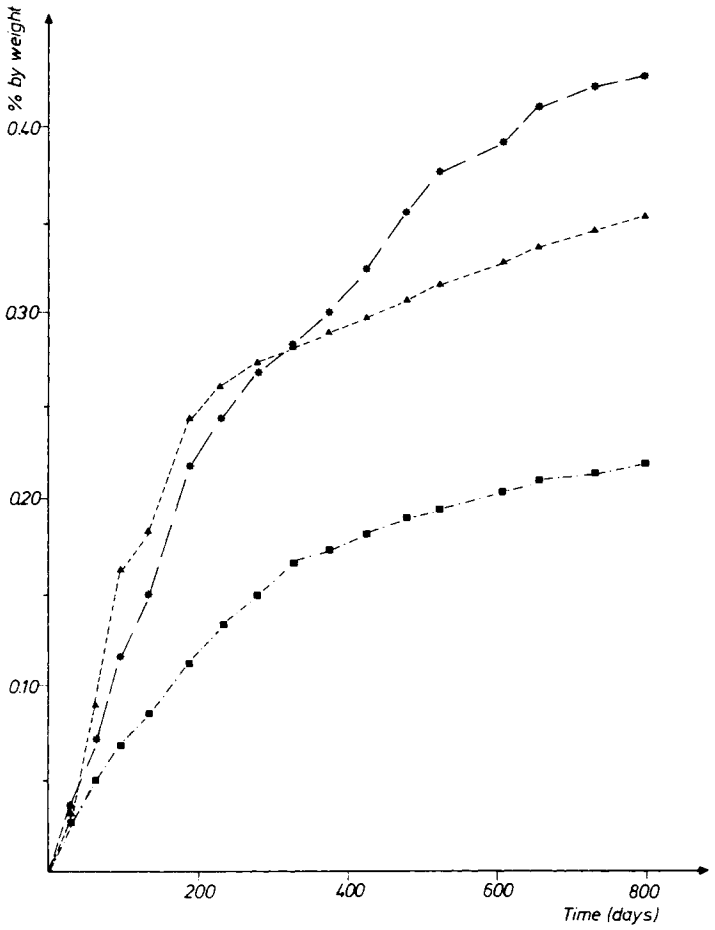

Fig. 6. Biodegradation by *F. redolens* as well as other invasive moulds of HDPE. Cultivation conditions as in Figures 1–5 except that the added amount of polyethylene film was 2.5 times as much per jar (5 g per 250 ml) as in the earlier experiments (Fig. 5).

times that used in the runs reported in Figures 2–5, and amounted to 5 g film per 250 ml nutrient medium. Consequently, both oxygen supply conditions and mixing effects through the mechanical stirring of the aeration were poor. Also the film was packed tightly on the top of the medium and gave limited access for air to the growing mold. Curiously, in this experimental series the infections (upper and lower curves only) appeared at a rather late stage and evidently had only a limited influence on the results.

The variations were also quite wide in another series (type c) where exceptionally low amounts of labeled polyethylene film (0.50 per 250 ml fluid) were used (Fig. 7). Experimental error and variations were more significant in this series but a consistent regressive increase in the $^{14}CO_2$ readings was still well recognizable.

Figures 8 and 9 show results for control systems consisting respectively of film in distilled water (type d) and film in nutrient solution plus $AgNO_3$ (type e) in order to depress all microbial growth originating from aerial contamination. The amounts of $^{14}CO_2$ evolved were in both cases low, after one year only (0.05 ± 0.02) % and after two years (0.10 ± 0.02) %. The slope of these low curves shows a very

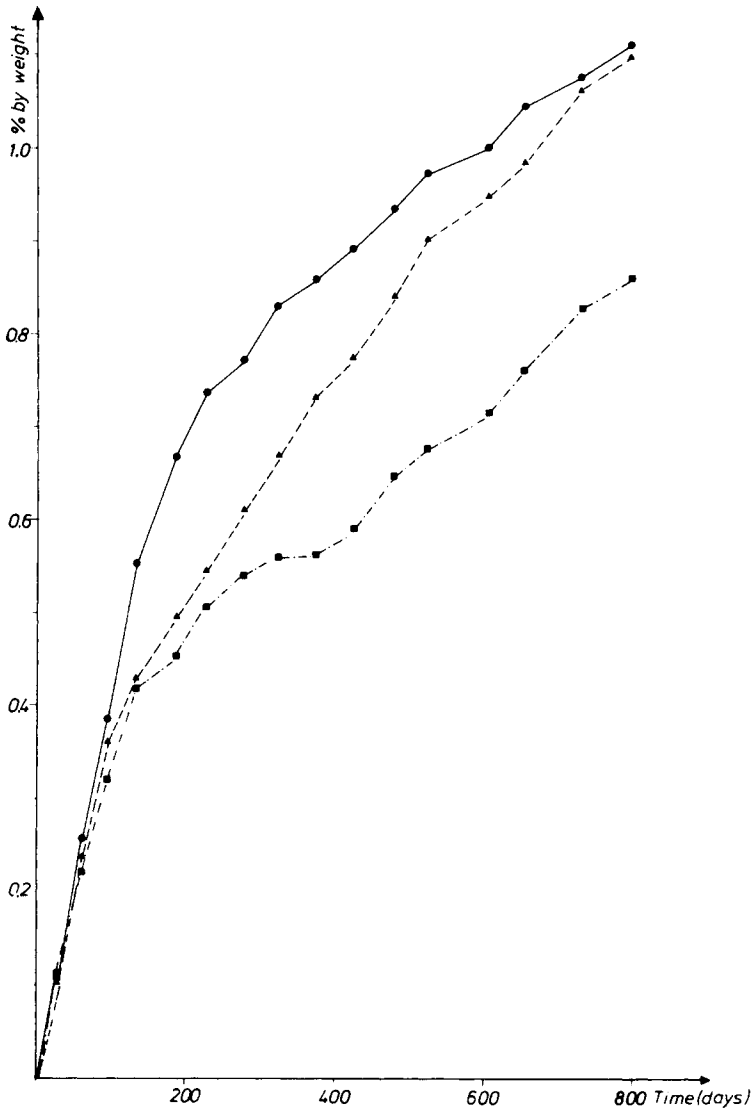

Fig. 7. Biodegradation of low amounts of HDPE in the medium (0.5 g per 250 ml) by *F. redolens*. From the runs illustrated by the two higher curves only *Fusarium* was recovered, while that of the lower curve also yielded a strain of *Aspergillus* (*versicolor*?). Other conditions as before.

slight increase in the liberation of $^{14}CO_2$ with time. One suggestion for this "aging effect" is that an actual minute liberation of $^{14}CO_2$ may occur abiotically in the experiments, even in very carefully controlled nonagressive chemical and physical surrounding, because of the aeration. The continuous flow of air over such a long period contributes to the oxidation to carbon dioxide of one-carbon fragments split off from polymeric chains. Incidentally, some of these runs also showed signs of aerial infection, despite the fact that the experimental conditions practically excluded such a possibility.

The question arises as to how much radioactivity "leaked out" from the polyethylene material. Table I gives a general answer. After the intense aeration, the radioactivity measurements showed that the ^{14}C released from the

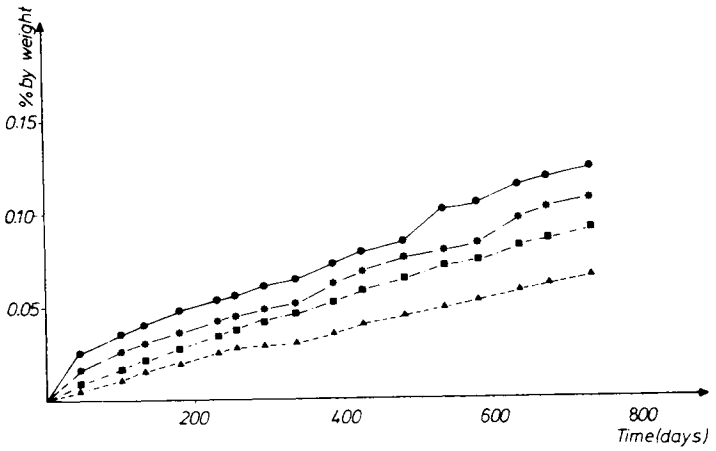

Fig. 8. Control experiments (cf. Figs. 2–5). Identical aeration conditions but with distilled water instead of cultivation medium and no inoculation. Some bacilli and an invading *Aspergillus (versicolor?)* were isolated from each jar after aeration for more than one year. Because of the lack of added nutrients only slight propagation of the contaminants could be observed. Evolvement of $^{14}CO_2$ was quite limited.

polyethylene film appeared mainly as $^{14}CO_2$. Recovery from the nutrient medium and from the mycelial mat was only about 5%–10% of that of the respiratory radioactivity. This is a good indication for a probable Pasteur effect shifted to the fermentative side in these experiments.

Although the presentation in Table I does not make this evident, it should be emphasized that the percentage recovery of ^{14}C radioactivity was strictly proportional to the amount of polyethylene material added to the different experimental jars with the same volume of nutrient medium.

Finally, a comparison of more complex nature can be made if averages from Figures 2 and 3 are plotted in one curve and presented together with averages from Figures 8 and 9. The visualization of these curves in one figure is justified since the experiments were actually conducted in the same place but with a time

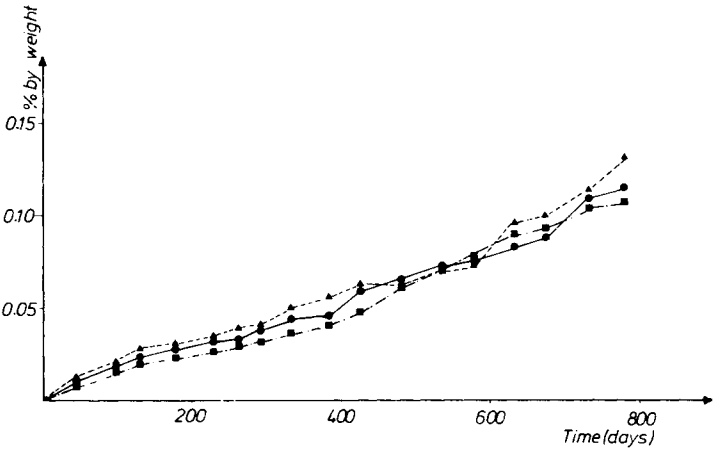

Fig. 9. Control with uninoculated nutrient medium together with 0.05% $AgNO_3$ as growth inhibitor. *Fusarium redolens* as well as an *Aspergillus* were recovered after aeration for more than one year. Conditions otherwise as in the earlier figures.

TABLE I
Recovery of ^{14}C Originally Randomly Embedded in High-Density Linear Polyethylene without Antioxidantia. Vertical Columns Represent Scintillation Counting Values Found in the Cell-Free Nutrient Medium, in the Mycelia Proper, and in the Trapped $^{14}CO_2$ Evolved Metabolically. All Readings Presented are Arithmetic Means of Three to Five Runs Accompanied by Standard Deviation

	% ^{14}C in nutrient medium	% ^{14}C in mycelium	% ^{14}C as $^{14}CO_2$	% ^{14}C total
(a) cultivated soil	0.02 ± 0.01	0.02 ± 0.01	0.36 ± 0.04	0.40
(b) wood rot fungi	0.05 ± 0.01	0.02 ± 0.01	0.36 ± 0.04	0.43
(c) *F. redolens*	0.05 ± 0.01	0.02 ± 0.01	0.39 ± 0.02	0.46
(d) distilled water	0.05 ± 0.03	—	0.10 ± 0.02	0.15
(e) $AgNO_3$	0.08 ± 0.01	—	0.12 ± 0.02	0.20

displacement (Fig. 10). In any circumstances, the rather good agreement between the values of the parallel runs in these experiments, as well as the steadily increasing high differences between the values of the uninoculated and the cultivated samples make it evident that some kind of biotic degradation of some fraction of the polymeric material must have been the ultimate cause of the liberation of ^{14}C embedded in polyethylene as well as of its conversion to $^{14}CO_2$, as measured as the end product of the degradation cycle.

DISCUSSION

Technical and economic aspects of the durability of synthetic polymers are of great concern today and will certainly remain so for quite a time. Among the inert plastics high-density polyethylene is not only the most frequently used but is generally considered to be one of the most resistant to destruction, especially through biological degradation in natural surroundings. This evidently applies to moulded high-density linear polyethylene (HDPE) in commercially marketed products. It must, however, be born in mind that such products always contain a definite amount of added antioxidants in order to prohibit spontaneous increase of carbonyl groups in the HDPE. The HDPE used in our work was a research product, not a commercial sample and contained no antioxidative additives.

The present work deviates from many previous attempts in the dimensions accessible for quantitative study since it has been possible, to measure accurately the actual conversion of HDPE carbon to CO_2 at a level of 0.4%. In this way also the degradation of minute amounts of low molecular weight alkanes present in very high molecular weight HDPE (viz., $\overline{M}_n \sim 300{,}000$ as calculated from mean values of solution viscosity) could be followed gradually in long-term experiments.

An assay of radioactivity stemming from the randomly distributed ^{14}C in the polymer is a direct approach to the problem of limited biodegradation. It could be argued that the disappearance of ^{14}C from the plastic film would be a better measure of degradation. This is evidently so unless we wish to restrict the question exclusively to a biological type of degradation as it was in the present case. Among the possible biodegradation processes, the highly oxidative utilization of *n*-alkanes is the most effective process and is best studied,[19] at variance with anaerobic processes prevalent for marine bacteria.

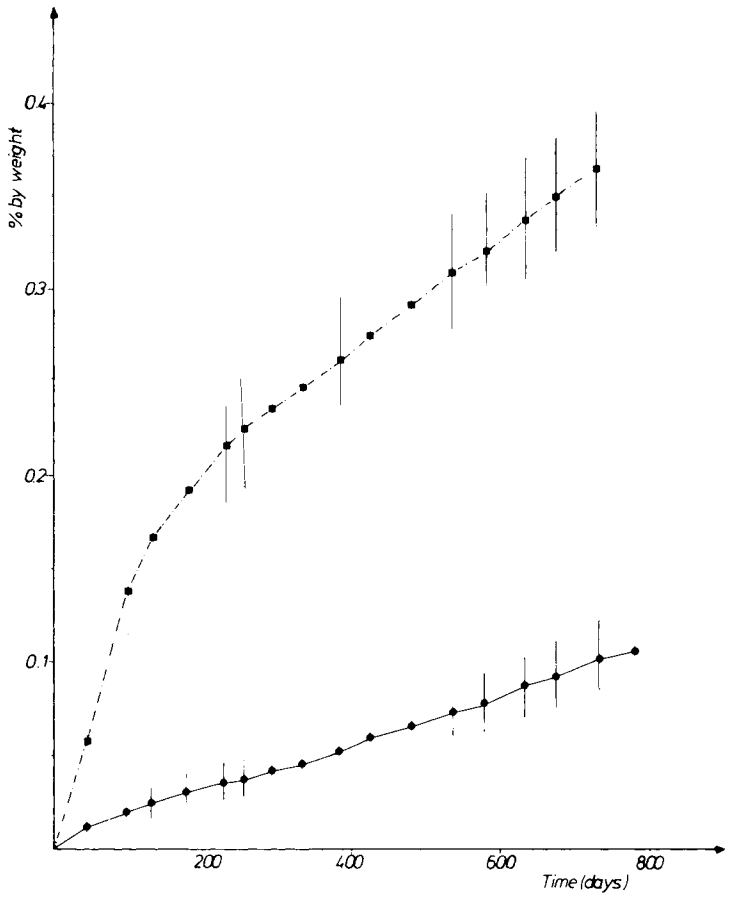

Fig. 10. Comparison of the degradation of HDPE maintained on inoculated media with that of HDPE on uninoculated distilled water and on uninoculated nutrient medium containing $AgNO_3$, respectively. The last two served as controls with very limited growth potential. The upper curve represent the mean with deviations of the curves of both Figures 2 and 3. The lower curve summarizes the results presented in Figures 8 and 9.

Some explanation must be offered for the rather restrictive radiorespirometric registration of $^{14}CO_2$ alone in the main presentations of our results on the biodegradative process. The explanation is, however, subsequently given in Table I. According to our complementary assay of ^{14}C radioactivity in the fermented culture medium and in the mycelium, the radioactivity appears preferably in $^{14}CO_2$ as gas and only to a very limited extent in volatile products in the medium or bound to the fungal material. This might well be the consequence of a Pasteur effect which the strong aeration exerted on the fungal growth by enhancing respiration of the mycelia and depressing the process of conversion of HDPE bound ^{14}C to nonrespiratory metabolic carbon components of the test organisms.

The experiments were continued over a long period of time. This was justified since the assay values gradually and proportionally increased except towards the end of the second year when a tendency for the curves to level off was observed. Long-term experiments are common when very limited and slow natural phenomena are investigated. In the case of microbial growth one can always

hope that a variation or mutation of a more progressive type of cell has a better change to occur and to be observed if the cultivation period is long. This has, however, evidently not happened in the present case since no dramatic increase in $^{14}CO_2$ liberation could be observed in any single experimental run. Nor has such a phenomenon yet been observed in a natural environment where the slow physical and chemical decay of polyethylene ensures ample opportunity for the manifestation of such a mutant. It is important in this connection that after the termination of the experiments we found fungal life present in all vessels, like *F. redolens* in agreement with the original inocula and also often some sort of invasive contaminations of the aforementioned species.

While infections were expected under the conditions used, the comparison of the $^{14}CO_2$ curves obtained was surprising for two reasons. On the one hand because of the uniformity of the runs in most of the experiments, and on the other hand because of the lack of strong regression in the pure *F. redolens* cultures. According to general experience with catabolic processes in nature (viz., cellulose and lignin mineralization) biodegradation progresses best with mixed populations of micro-organisms and is restricted or incomplete with pure cultures of single strains.

It is assumed that the source of ^{14}C metabolization in these degradational experiments must have been mainly low molecular weight polyethylene, but this conclusion must be corroborated by further study, especially on material of a more accessible form, e.g., powder, possessing a high-surface volume ratio compared with polyethylene film.

These investigations are a part of a research project on biodegradation of synthetic polymers, supported by the Swedish Board for Technical Development (STU) and the Swedish Polymer Research Foundation (SSP). Thanks are expressed to Professor Bengt Rånby, head of the Department of Polymer Technology, for proposing this study, and to Dr. Z. G. Bánhidi, Division of Microbiology, for evaluation and discussion of the results. I also acknowledge the late Professor Erik Björkman and his assistant Lise-Lotte Beyer-Ericsson, Institute of Forest Botany and Pathology, Royal College of Forestry, Sweden, for supplying the fungi.

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Received August 31, 1977