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TECHNICAL REPORT SUMMARY

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(Biodegradation)
EE & PC-Div.
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(Degradation)

CURRENT OBJECTIVE:

To evaluate the susceptibility of FM 3422 to biological decomposition.

REPORT ABSTRACT: (200-250 words) This abstract information is distributed by the Technical Communications Center to alert 3M'ers to Company R&D. It is Company confidential material.

Biodegradation studies are described which allow the evaluation of the susceptibility of FM 3422 to aerobic microbial degradation. Biodegradation test procedures used are modifications of common biodegradation test methods. They include semicontinuous activated sludge and shake flask die-away studies with sequential adaptive transfers. Microbial innocula were obtained from several environmental sources. Some sources were selected for their likelihood of containing microbial populations enriched in the capability of degrading FM 3422 or similar fluorochemicals. Analytical procedures used included GLC, TLC, ^{14}C-scintillation counting and analyses for released fluoride. Interpretation of biodegradation results from these studies was hindered by the low solubility of FM 3422, its volatility, and its affinity for suspended organic material. This binding made it difficult to quantitatively extract FM 3422 from the biological solids of the test cultures. Nontoxic detergent stabilized emulsions were used to circumvent problems caused by low water solubility.

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SUMMARY AND CONCLUSIONS

FM 3422 was found to be completely resistant to biodegradation under the conditions described in this report. These conditions are considered to be optimum for biological degradation. FM 3422 was also found to have a strong affinity for organic solids.

In all experiments described herein, FM 3422 was exposed as a surfactant stabilized emulsion, to heterogeneous microbial cultures, under aerobic conditions, and near neutral pH (7.1-7.5). Concentrations of FM 3422 ranged from 100-500 mg/l. Microorganisms were obtained or developed from soil or waste treatment sludges. Some microbial sources were selected because of their previous or continuous exposure to fluorochemicals. It was felt that these sources were likely to have developed naturally enriched cultures capable of degrading fluorochemicals. FM 3422 was exposed to both high (∿2000 mg/l) and low (∿100 mg/l) cell densities. Weekly transfers of the low cell density cultures allowed acclimation to FM 3422 to proceed for six months.

This study cannot rule out the possibility that conditions could be found that would allow biodegradation of this compound, nor can it exclude the possibility that a microorganism could eventually be enriched that could metabolically alter this chemical under the conditions described in this report. Nevertheless, the results of this quite extensive study strongly suggest that FM 3422 is likely to persist in the environment for extended periods unaltered by metabolic attack. Its observed affinity for organics suggests that FM 3422 released to the environment is likely to sorb to organics such as those present in soil or in the sediments of aquatic ecosystems. Table 1 summarizes the experiments conducted in the study of FM 3422 biodegradation.

TABLE 1

SUMMARY OF EXPERIMENTS PERFORMED IN FM 3422 BIODEGRADATION STUDY

Experiment	Findings
1) 10-day semicontinuous activated sludge biodegradation study of surfactant stabilized FM 3422.	No FM 3422 biodegradation products were detected in ethyl acetate extracts by TLC or GLC.
2) 6-month shake flask die-away study with weekly transfers.	Again no degradation products were detected in ethyl acetate extracts. However, >50% and sometimes >75% of the initially ethyl acetate extractable FM 3422 was missing from extracts from 7-day-old cultures.
3) Materials balance experiment.	An attempt to measure the fractions of ^{14}C-FM 3422 present in the water, ethyl acetate, and solids phases of 7-day-old cultures failed. This apparently was due to FM 3422 volatilization during solids drying. When mixed with grown cells, FM 3422 became nonextractable too rapidly to be explained by a metabolic phenomenon.
4) Fluoride release	Fluoride concentration did not increase during die-away studies with cultures acclimated to FM 3422. This suggests no modifications occur to the perfluoro portion of FM 3422.
5) Attempts to recover nonethyl acetate extractable FM 3422 from cell cultures:	
A) Hydrolysis	Neither acid or base hydrolysis freed non-extractable FM 3422.
B) Binding to Containers	Irreversible binding to culture container did not explain nonextractable FM 3422.
C) Volatilization from Cells	Nonextractable FM 3422 was 1/3 recovered by volatilizing at 100°C. from culture solids and condensing.
D) Hot ethyl acetate extracts	Ethyl acetate extracts at 60°C. did not recover additional FM 3422.
E) Reflux with Dioxane	Refluxing culture solids with 1,4-dioxane recovered all nonextractable FM 3422 from the solids of 7-day-old cultures. Complete recovery of FM 3422 shows that no biodegradation occurred.

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INTRODUCTION

Our early work evaluating the susceptibility of FM 3422 to microbial degradation has been described in a previous report (1). This work included two separate Warburg respirometric experiments which, convincingly, showed increased oxygen utilization by unacclimated biological sludge immediately following addition of unstablized emulsions of unpurified FM 3422. In these two experiments, with FM 3422 as the sole exogenous carbon source, oxygen uptake above the endogenous level accounted for 3% of the oxygen required to completely oxidize the hydrocarbon portion of the molecule. The reason for this remains unexplained. One possibility is that biodegradable fragments of FM 3422 may have been formed as a result of the emulsion formation by sonication.

Attempts to isolate FM 3422 degradation products from the Warburg flasks following the observed oxygen uptake were unsuccessful, as were those from a subsequent semicontinuous activated sludge (SCAS) biodegradation study. In the SCAS study solutions of FM 3422 in ethanol were added directly to the biological reactors. Added in this manner, the FM 3422 rapidly congealed and separated from the water phase accumulating on the reactor walls and with the biological solids. This separation from the water phase may have made FM 3422 unavailable to react with degradative enzymes.

In the present report, further attempts to characterize the susceptibility of FM 3422 to biodegradation are described.

METHODS AND MATERIALS

Most methods are described in the experimental section. The following standard methods and materials were used repeatedly throughout the experiment.

Extraction Procedure

10.0 ml samples were extracted in polypropylene centrifuge tubes by adding 10.0 ml of ethyl acetate. The samples were then capped with polypropylene caps and inverted 50 times. Phase separation was ensured by centrifuging for 10 minutes at about 12,000 x gravity. A correction has to be made for the decreased volume of the solvent phase and the increased volume of the water phase following extraction. After shaking the volume of the aqueous phase increases from 10.0 to 11.0 ml and the volume of the ethyl acetate solvent phase decreases from 10.0 to 9.0 ml. Therefore, a determination of the number of ppm of FM 3422 originally present in the water phase which is extracted into ethyl acetate is made by multiplying the concentration found in the ethyl acetate phase by 0.90.

Media

The basal salts media used in the semicontinuous activated sludge experiment consisted of:

1.0 g/l NH_4Cl

2.0 g/l K_2HPO_4

0.25 g/l $MgSO_4 \cdot 7H_2O$

Adjusted to pH 7.2 with strong acid

The standard media used in die-away and subsequent studies contained:

133 ppm of FM 3422 (emulsified)

2 g/l K_2HPO_4

2.4 g/l YM Broth

830 mg/l Alconox

∿6 g/l Ethyl acetate

pH ∿7.2

It was prepared by emulsifying 0.2 g of FM 3422 dissolved in water-saturated ethyl acetate in 100 ml of 12.5 g/l Alconox solution that had been prepared using ethyl acetate-saturated water. Emulsification was accomplished by mixing for 30 seconds with a model SDT Tissumizer. The resulting emulsion was then diluted in a K_2HPO_4 solution which in turn was diluted in a weak YM broth media.

The surfactant containing media were demonstrated to be nontoxic to microorganisms by inoculating these media and comparing the dissolved oxygen concentration depletion rates from these cultures with those of nonsurfactant-containing cultures. These comparisons were made initially, after one hour, and after one day of exposure to the surfactant-containing media.

Chemical Analyses

TLC and GLC analyses are described in the Progress Report by A. Mendel (2).

Chemicals

The following chemicals were used in this study:

1) Sublimated FM 3422 (see report of Art Mendel - (2))

2) Radioactive FM 3422 (see report of Art Mendel -(2))

3) Ethyl acetate - Mallenckrodt, A.R.

4) YM Broth - DIFCO - Bacto Dehydrated
   Bacto Yeast Extract - 14.3%
   DIFCO Malt Extract - 14.3%
   Bacto-Peptone - 23.8%
   Bacto-Dextrose - 47.6%

5) Ether - Anhydrous, J. T. Baker, "Baker Analyzed."

6) Siponate DS-10 - A purified alkylaryl - sulfonate supplied by Alcolac, Incorporated.

7) Alconox - A mixture of alkylarylsulfonates, lauryl alcohol sulfates, phosphates, carbonates and synergistic agents - supplied by Alconox, Incorporated.

8) 1,4-Dioxane - Reagent grade - repackaged, supplier unknown.

9) Aquasol - Xylene-based scintillation solution - supplied by New England Nuclear.

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EXPERIMENTAL

Semicontinuous Activated Sludge Study

In an earlier study, no degradation products of FM 3422 were detected by TLC or GLC in a semicontinuous activated sludge (SCAS) study in which FM 3422 was simply added to the media as an ethanol solution, and FM 3422 rapidly congealed and separated from the water phase (1). It was postulated that biodegradation may not have been observed because enzymes could not "attack" the coagulated FM 3422 molecules. Since emulsions of water-insoluble molecules have been shown to be more susceptible to enzyme attack (3), a second SCAS degradation study of FM 3422 was conducted using emulsified FM 3422.

Prior to the initiation of this study, several surfactants were tested for their ability to stabilize FM 3422 emulsions. The two most satisfactory were Alconox and Siponate DS-10. These were both used in the SCAS study.

The emulsions for this study were made by dissolving 3 g of FM 3422 in 3 ml of ethyl acetate. Portions of this solution were then added to 5 g/l solutions of Alconox or Siponate DS-10, and emulsified by mixing for 30 seconds with a tissumizer. The concentrations of chemicals in the emulsions thus formed were 5 g/l Alconox or DS-10, 2.7 g/l ethyl acetate, and 3 g/l FM 3422. Emulsions with only ethyl acetate and one of the two surfactants were used as controls.

Six semicontinuous activated sludge units were set up by adding 2 liters of fresh activated sludge to each unit. This sludge was collected from an aeration basin at the municipal waste treatment plant in Pig's Eye, Minnesota. The sludge was settled and the bottom 500 ml, containing all of the settled cells, was retained. One liter of medium was then added to the settled sludge in each reactor, and identical medium readded at the beginning of each test cycle. These media were made by diluting portions of the above-described emulsions in the basal salts medium described in the methods and materials section.

Table 2 shows the concentrations of ethyl acetate, Alconox, Siponate DS-10, and FM 3422 present in 1.5 ℓ contents of each reactor at the beginning of the first test cycle.

TABLE 2

CONCENTRATION OF ORGANIC COMPOUNDS IN SEMICONTINUOUS ACTIVATED SLUDGE REACTORS AT THE BEGINNING OF THE FIRST TEST CYCLE

	Initial Concentration (ppm)			
SCAS Reactor	Ethyl Acetate	Alconox	DS-10	FM 3422
1	560	830	0	0
2	560	0	830	0
3	460	830	0	510
4	460	0	830	510
5	90	170	0	100
6	90	0	170	100

The semicontinuous activated sludge procedure was described in a previous report (1), and is briefly outlined in Figure 1.

This experiment was conducted for 10 days with samples taken at the beginning and end of the first and last test cycle. After the samples were taken, they were centrifuged. The solids and centrifugate were extracted separately. The centrifugate was extracted two times, first at the pH of the sample (∿pH7) and again after adjusting to pH 1 with concentrated sulfuric acid. The cells were extracted only once at pH 7. The ethyl acetate extracts were then evaporated, resuspended in methanol and separated by TLC.

Problems with foaming developed in all reactors during the first test cycle. This foaming caused the loss of some suspended solids in reactors 1, 2, 3, and 4. Little or no solids were lost from reactor 5, and most of the suspended solids were lost from reactor 6. In all subsequent test cycles, foaming and loss of solids were controlled by blowing a stream of air on the water surface of each reactor. This also increased the rate of evaporation, and reactors were brought back to their original volume with deionized water prior to draining or sampling.

There were no new spots that developed on TLC plates that would indicate that a degradation product of FM 3422 was accumulating. This was true both in the extracts of the suspended solids and in the extracts of the centrifugates.

There were indications that biodegradation of the surfactant took place during the course of the final test cycle. Spots which could be attributed to Alconox or DS-10 were present in the extracts of the initial samples. These spots were replaced by new spots from later samples and these new spots were completely gone (except in reactor 4) at 24 hours.

It appears that FM 3422 had not degraded, at least to a detectable level, under these conditions with high cell densities and a 10-day acclimation period. These are considered to be optimum conditions for biodegradation.

Shake Flask Die-Away Studies with Adaptive Transfers

Growth of a heterogeneous cell population in nutrient media containing emulsified FM 3422 with sequential adaptive transfers was initiated for two reasons. First, it was very difficult to quantitatively measure the removal of FM 3422 from SCAS studies. This was due to FM 3422's very low water solubility and its affinity for cellular material. On the other hand, the entire contents of die-away cultures could easily be extracted eliminating this problem of nonhomogeneous sampling. The second reason for starting shake flask studies is that this adaptive transfer procedure is an accepted method of enriching organisms from nature that are capable of growth on xenobiotics or rarely-occurring organic materials.

In an attempt to obtain organisms capable of growth on FM 3422, inocula were obtained from a number of sources. These sources included three soil samples from 3M's Decatur, Alabama plant, taken from areas that were likely to have been exposed to fluorochemicals, biological sludge from the Decatur plant, and sludge from the Metropolitan Waste Treatment Plant at Pig's Eye, Minnesota. Separate shake flask cultures were started from these various inocula, but they were eventually combined into a single master culture.

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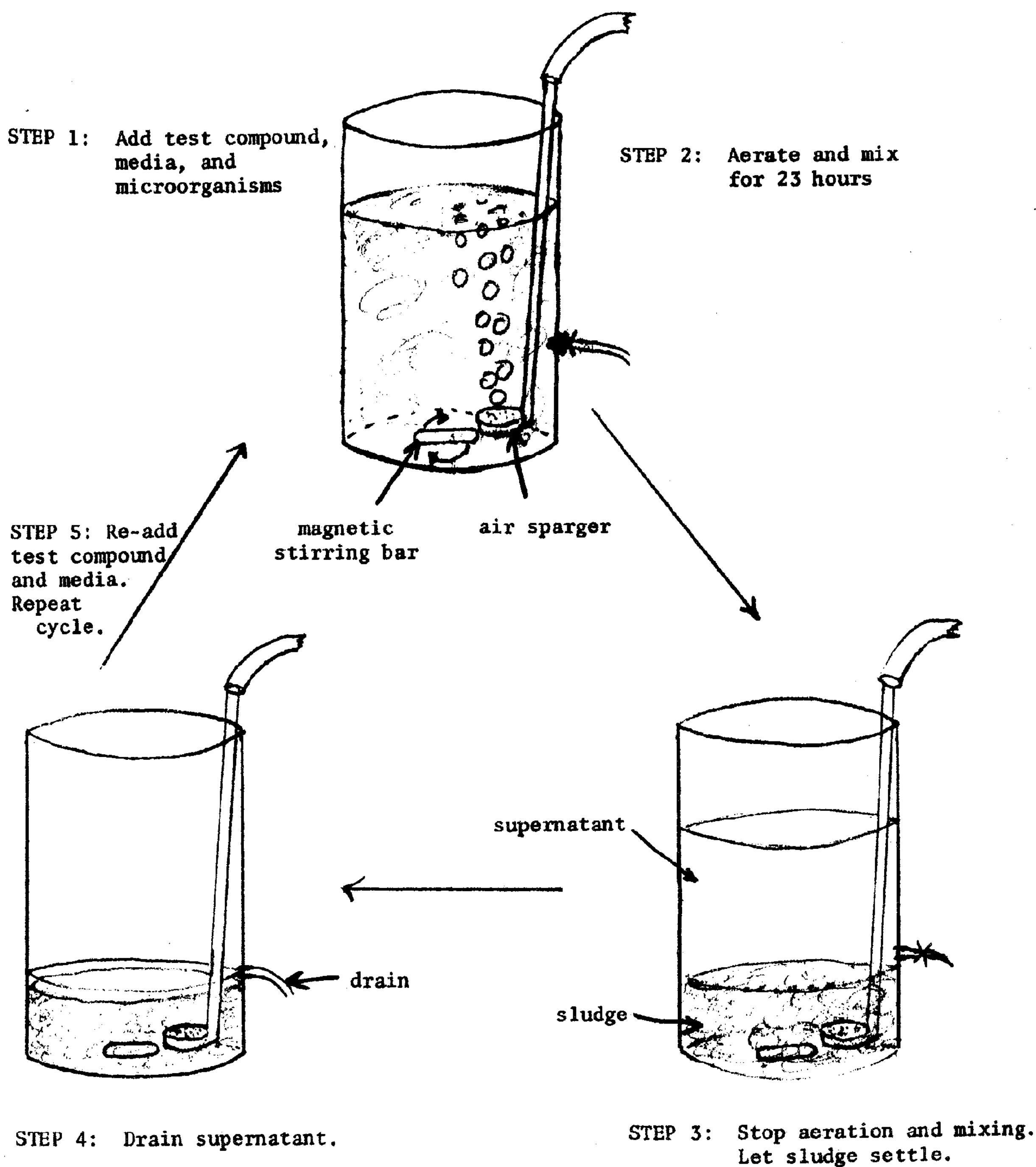

FIGURE 1. Test cycle for semicontinuous activated sludge reactor.

Initially, the adaptive transfers were conducted in glass Erlenmeyer flasks containing 50 ml of culture medium. The total contents of the flask were acidified with sulfuric acid to pH 1, supplemented with a saturated NaCl solution, extracted two times, and the inside of the flask washed with solvent two times. Total phase separation was accomplished by centrifugation. Extracts and washings were combined prior to GLC analysis. Acidification and salt addition were later eliminated from the extraction procedure. Salt addition proved to be of no benefit and sulfuric acid addition caused the formation of a new unidentified peak in the GLC analysis. Acid addition was also of no value in improving the extractability of FM 3422. This newly-formed peak apparently was a chemical modification of FM 3422, and it was converted back to FM 3422 on thin-layer plates. Since it was present both in extracts taken initially and at the end of the growth period, it was obviously not a metabolic product.

The preparation of the media used in early shake flask cultures differed from the later improved and standard procedure described in the methods and materials section only in that water-saturated ethyl acetate, and ethyl acetate-saturated water were not used in its preparation. As a result, however, the FM 3422 was not as completely emulsified in this media. The nonemulsified FM 3422 was intentionally separated and not included in the culture media. Thus, the initial FM 3422 concentration was variable and its concentration known only to be something less than 133 ppm, which was the concentration that would have been present had all the FM 3422 been emulsified. As with the standard media, this initially-used media was demonstrated to be nontoxic to microorganisms by the fact that cell respiration occurred normally in its presence.

Initially, cultures were shaken at room temperature (20-25°C.) and transfers made every 8 days. Transfer times were later changed to 7 days for convenience. Cultures were extracted both at the beginning and end of each culture growth period.

The results were quite variable from transfer to transfer. This can be seen in Figure 2. In most cases, the amount of FM 3422 that could be extracted with ethyl acetate at the end of the incubation period was less than that which was extractable immediately following culture inoculation. Usually, 50% or more of the initially-extracted material was not recoverable from the week-old grown cultures. No degradation products were discovered by either TLC or GLC of the ethyl acetate culture extracts. At this point, it was not known whether the unrecovered FM 3422 were being completely degraded, metabolically modified to materials which could not be extracted, or whether it or its metabolites were bound to the biomass of the culture container.

A number of experiments were subsequently conducted to determine what caused a portion of the FM 3422 to become nonextractable by ethyl acetate.

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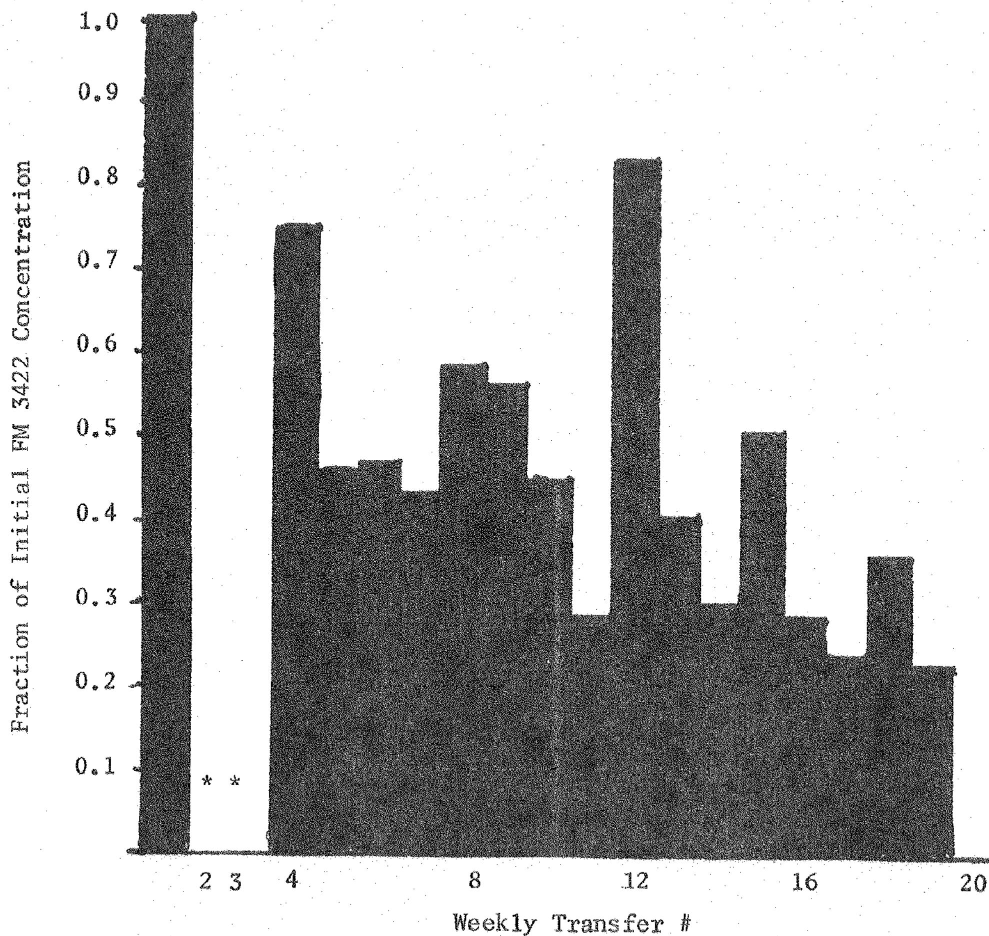

*Initial concentrations were not measured in the second and third weekly adaptive transfer periods.

FIGURE 2. Ratio of final to initial FM 3422 concentration in equal volume ethyl acetate extracts of week-old shake flask cultures from adaptive transfer experiment.

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TLC and GLC Analysis of Extracts from Shake Flask Studies

In one TLC experiment, ethyl acetate extracts of 3 cultures were found to contain a material which produced very distinct spots with a smaller R_f than FM 3422. The TLC visualizing procedure suggested that the new spots contained fluorocarbons. Two of the cultures producing these spots contained 135 ppm of FM 3422 prior to extraction.

The third culture contained less than 3 ppm of FM 3422 (introduced with the FM 3422 acclimated inocula). Despite this great difference in initial concentration, the "new" spots from the extracts of the three cultures proved to be of approximately equal intensity, but FM 3422 was detected by TLC only in the extract of the two 135 ppm cultures.

The extracts of these three cultures were combined. The material causing this new spot isolated by preparative TLC and again repurified by thin-layer chromatography. Attempts were made to characterize this material. GLC of this material in our lab only yielded a material with GLC characteristics identical to FM 3422. Measurements were not made of the amount of material injected. IR spectra of this material showed it to have a carbonyl absorption at ∿6μm and an absorption fairly characteristic of a fluorocarbon compound between 8 and 9μm. Bands similar to FM 3422, but not overlapping with FM 3422, were also present. This suggested a complex with a material like urea. Mass spectra obtained following direct insertion of the unknown into the mass spectrophotometer showed ammonia, CO_2, glycerin, and FM 3422. The relative concentration of FM 3422 to other materials could not be determined. The presence of urea was not noted. FM 3422 appeared in the mass spectra work to volatilize at a higher temperature than pure FM 3422; this may have been due to its being complexed. GC-mass spec of the unknown material yielded mainly glycerin as identified by the mass spec. (In fact, more than one peak was identified by mass spec as glycerin.) Unlike the work in our lab, FM 3422 was not detected, although the same liquid phase, Carbowax 20M, was used at a higher temperature. This product has not been further characterized. It does not appear to be a major degradation product.

Fluoride Release Determinations

Measurements of F^- concentrations, taken initially and after 7 days, have shown no change in either control cultures or cultures that showed ∿50% decreases in ethyl acetate extractable FM 3422 concentration.

In this experiment, nine cultures were prepared and inoculated with a small amount of a heterogeneous culture that had been maintained for 6-8 transfers on FM 3422-containing media. Three of these cultures were grown in the absence of FM 3422 and extracted, three were grown in the presence of emulsified FM 3422 and extracted, and three were extracted prior to cell growth and immediately after FM 3422 addition. The ethyl acetate phase was removed from all cultures. Fifty-one, sixty-one, and fifty-six percent of the initially extractable FM 3422 could not be extracted from the three cultures that had been grown in the presence of FM 3422. Less than 0.1 ppm of F^- was present in any of the nine cultures. Had all of the nonextractable FM 3422 been degraded in the culture grown with FM 3422 with complete F^- ion release to the media, ∿40 ppm would have been found. These results indicate that, as expected, the perfluorinated portion of the FM 3422 molecules is not metabolically attacked.

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Attempts to Free Nonextractable FM 3422 by Hydrolysis

A possible explanation for the observation that 50% or more of the initially-extractable FM 3422 frequently cannot be extracted into ethyl acetate after incubation with acclimated microbial cultures is that it has been conjugated or polymerized forming a nonextractable material. The objective of this experiment was to determine if acid or base hydrolysis could convert FM 3422 back to an extractable form.

In this experiment, cultures which had been grown in the presence of emulsified FM 3422 on the standard media were extracted with ethyl acetate. The concentration of FM 3422 in the ethyl acetate extract was determined. The cultures were then extracted again after hydrolysis at 80°C. and at pH 1 or pH 13 for various lengths of time. Concentrated H_3PO_4 was used for acidification and 20 molar NaOH was used to increase the pH. Following hydrolysis, all samples were neutralized and adjusted to equal volumes before extraction at room temperature.

The results of this experiment are shown in Table 3. An average of 28.4% of the FM 3422 was extractable before cellular growth. Very little of the remaining FM 3422 could be extracted even after hydrolysis. In fact, it appears that longer exposure to these hydrolysis conditions decreased the amount of FM 3422 that could be recovered in a second extraction.

TABLE 3

RECOVERY OF "NONEXTRACTABLE" FM 3422
FOLLOWING ACID OR BASE HYDROLYSIS AT 80°C

pH	Time at 80°C	% of "Nonextractable" FM 3422 Recovered
7	0	2.7
1	0	12
1	0.25 hr.	3.9
1	2.5 hr.	1.5
1	25 hr.	0.8
13	0 hr.	8.2
13	2.5 hr.	3.2
13	25 hr.	0.4

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Materials Balance Experiments

Biodegradation studies were conducted using ^{14}C-FM 3422. The primary objective of these experiments was to determine the relative portions of FM 3422 present in the aqueous, solvent, and solids phases of extracted cultures after growth in the presence of emulsified ^{14}C-FM 3422.

In this experiment, the standards media was prepared both with and without ^{14}C-FM 3422 (100 ppm). This media was inoculated with a heterogeneous culture that had been maintained for approximately 20 transfers at weekly intervals on the standard FM 3422 media. Three of the new cultures containing ^{14}C-FM 3422 were extracted immediately after inoculation, and three identical cultures were extracted after one week of growth. Three cultures not containing FM 3422 were also grown for one week. At this point, ^{14}C-FM 3422 emulsion was added to these cultures, bringing their concentration to 100 ppm. The cultures were then shaken for an additional fifteen minutes and extracted. Three one-ml samples from the aqueous and ethyl acetate phases were taken from each of the nine extracted cultures, added to Aquasol(R) in scintillation vials, and counted. The solids from the grown cultures (essentially no solids were present initially) were transferred to combustion cups, dried in an oven at 103° C. for two hours and combusted using Agrichem's Packard combustion equipment. The $^{14}CO_2$ from combustion was trapped in a scintillation fluid containing an organic amine and counted. Samples were counted with an internal standard quench correction, and their radioactivity compared to known volume samples of ^{14}C-FM 3422 emulsion added directly to Aquasol.

This experiment was conducted two times. In the first attempt, the ^{14}C-FM 3422 emulsion was inadvertently not stabilized with Alconox, and it broke rapidly. Also, ^{14}C-FM 3422 emulsion was not added to the cultures that had been grown before FM 3422 addition because the week-old unstabilized emulsion had already broken.

The results of the first attempt at this experiment are shown in Tables 4 and 5.

	Culture Extracted Initially	Culture Extracted After 1 Week
Ethyl acetate	103%±11%	102%±6%
Water	0.04%±0.008%	0.02%±0.02%
Solids	--	0.3%±.4%
Total Recovered	103%±11%	102%±6.4

Table 4. Percent ± 1 Standard Deviation of initially added ^{14}C-FM 3422 recovered in first of 2 Materials Balance experiments.

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		Method of Determining FM 3422 Concentration	
		Electron Capture GLC	Scintillation Counting
Culture Extracted Initially	Extract Concentration	91 ppm±4.2 ppm	115 ppm±12 ppm
	% of Added Material Accounted For.	82%	103%
Culture Extracted After 1 Week	Extract Concentration	83 ppm±5.0 ppm	113 ppm±7 ppm
	% of Added Material Accounted For.	75%	102%

Table 5. Concentration of FM 3422 in ethyl acetate extracts ± 1 standards deviation from the first Materials Balance experiment as determined by scintillation counting and by electron capture GLC.

The results of the second run of the Materials Balance experiment are shown in Tables 6 and 7.

	Culture Extracted Initially	Culture Extracted After 1 Week	Grown Cultures Extracted After Fifteen Minutes
Ethyl acetate	89.7±3.6	39.9±9.8	33.6±3.0
Water	4.0±1.3	0.4±0.3	1.0±0.4
Solids	--	3.9±6.6	0.1±0.05
Total Recovered	93.7±4.9	44.2±16.7	34.7±3.4

Table 6. Percent ± one standard deviation of initially added ^{14}C-FM 3422 recovered in second of two Materials Balance experiments.

	Method of Determining FM 3422 Concentration	
	Electron Capture GLC	Scintillation Counting
Culture Extracted Initially	99.8 ppm±0.7 ppm	99.6 ppm±4.0 ppm
Culture Extracted After 1 Wk.	44.0 ppm±8.7 ppm	44.3 ppm±10.8 ppm
Grown Culture Extracted After 15 Minutes	31.1 ppm±2.7 ppm	37.3 ppm±3.3 ppm

Table 7. Concentration of FM 3422 in ethyl acetate extracts from the second Materials Balance experiment as determined by scintillation counting and by electron capture GLC.

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The results of these experiments were valuable despite the fact that complete recoveries of FM 3422 were not obtained. In the first attempt, it was found that when FM 3422 was added as an unstabilized emulsion, the ability to extract this material was not lost after 7 days of shaking. We have observed that unstabilized emulsions break within a few hours on sitting with the precipitation of coagulated clumps of FM 3422. This presumably happened in this experiment as well, and in this form, it remains extractable despite contact with a viable biological culture. It is possible that surfactant facilitates the uptake of FM 3422. However, it is more likely that emulsification increases the number of FM 3422 molecules contacting and, subsequently, binding with microbial solids.

As noted above, all of the ^{14}C-FM 3422 was not accounted for in the second attempt at this experiment. As is seen in Table 6, most of this material could be recovered initially, but it was not recovered after significant biological solids had accumulated. The most probable explanation for this loss of radioactive FM 3422 is that it was absorbed by the cellular material and bound so strongly that it could not be extracted by ethyl acetate. However, it apparently was not so strongly bound that it could not be volatilized from the cellular material by heating to 103° C. Loss to volatilization is the most probable explanation for the low recoveries.

This experiment also suggests that absorption is a physical phenomenon. FM 3422 was also not extractable from the pregrown cultures that were shaken with emulsified FM 3422 for only 15 minutes. This rate of removal is too rapid to be explained by a metabolic phenomenon, particularly since the organisms had not been acclimated to FM 3422. It can't be determined from this experiment whether or not the FM 3422 which is presumably bound to the cells is eventually metabolized to another chemical form. This would require recovery of the bound FM 3422 and confirmation by a method such as GLC that the recovered material is still FM 3422.

Adsorption on Culture Containers

A possible explanation for the decrease in extractable FM 3422 in grown cultures is that the material was being strongly bound to the polypropylene test culture containers. This possibility was considered remote since similar results were obtained in both glass and polypropylene containers. Nevertheless, it was investigated in the following procedures.

A media was prepared that differed from the standard 133 mg/l FM 3422 emulsion media only in that it did not contain the nutrient YM broth necessary as a nitrogen source for cell growth. Ten ml of this media was added to each of nine tubes. Three were extracted immediately, three were capped with polypropylene caps, and three were plugged with foam stoppers. The capped and stoppered tubes were shaken for one week and extracted and analyzed by GLC using the standard procedures.

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Sample	Cap	Time in Polypropylene Tube Prior to Extraction	% Initially Added FM 3422 Recovered
I #1	Polypropylene	15 min.	79
I #2	Polypropylene	15 min.	79
I #3	Polypropylene	15 min.	78
P #1	Polypropylene	7 Days	75
P #2	Polypropylene	7 Days	78
P #3	Polypropylene	7 Days	84
F #1	Foam	7 Days	92
F #2	Foam	7 Days	93
F #3	Foam	7 Days	92

Table 8. Percent of initially added FM 3422 extracted from polypropylene tubes.

The results of this experiment are shown in Table 8.

The reasons for the low FM 3422 recoveries obtained in this experiment are not known. There also is no explanation for the greater recoveries obtained with the tubes that were shaken with foam stoppers. In the week-long shaking procedure, the contents of the tubes never came in contact with either the polypropylene cap or the foam stopper. Also, the foam stoppers had not been used before in any experiments.

Despite the incomplete recovery of FM 3422, these results do not explain the phenomenon repeatedly seen in earlier experiments. In early experiments, high recoveries of FM 3422 were obtained initially before the cell culture had developed and low recoveries of FM 3422 were obtained in ethyl acetate extracts done after the culture had grown for seven days. In this experiment with negligible microbial populations, the initial FM 3422 recoveries were not significantly different from the recoveries after 7 days. It, therefore, appears that the increasing loss of ability to extract FM 3422 from the culture with time requires the presence of biological solids.

Recovery of FM 3422 From Cells By Volatilization and Condensation

As previously noted, the most probable explanation for the loss of FM 3422 in the Materials Balance experiment is that it volatilized from the solids phase during the two hours drying period. For this reason, it was believed that it might be possible to quantitatively recover sorbed FM 3422 by volatilizing it from cells and collecting it by condensation on a "cold finger."

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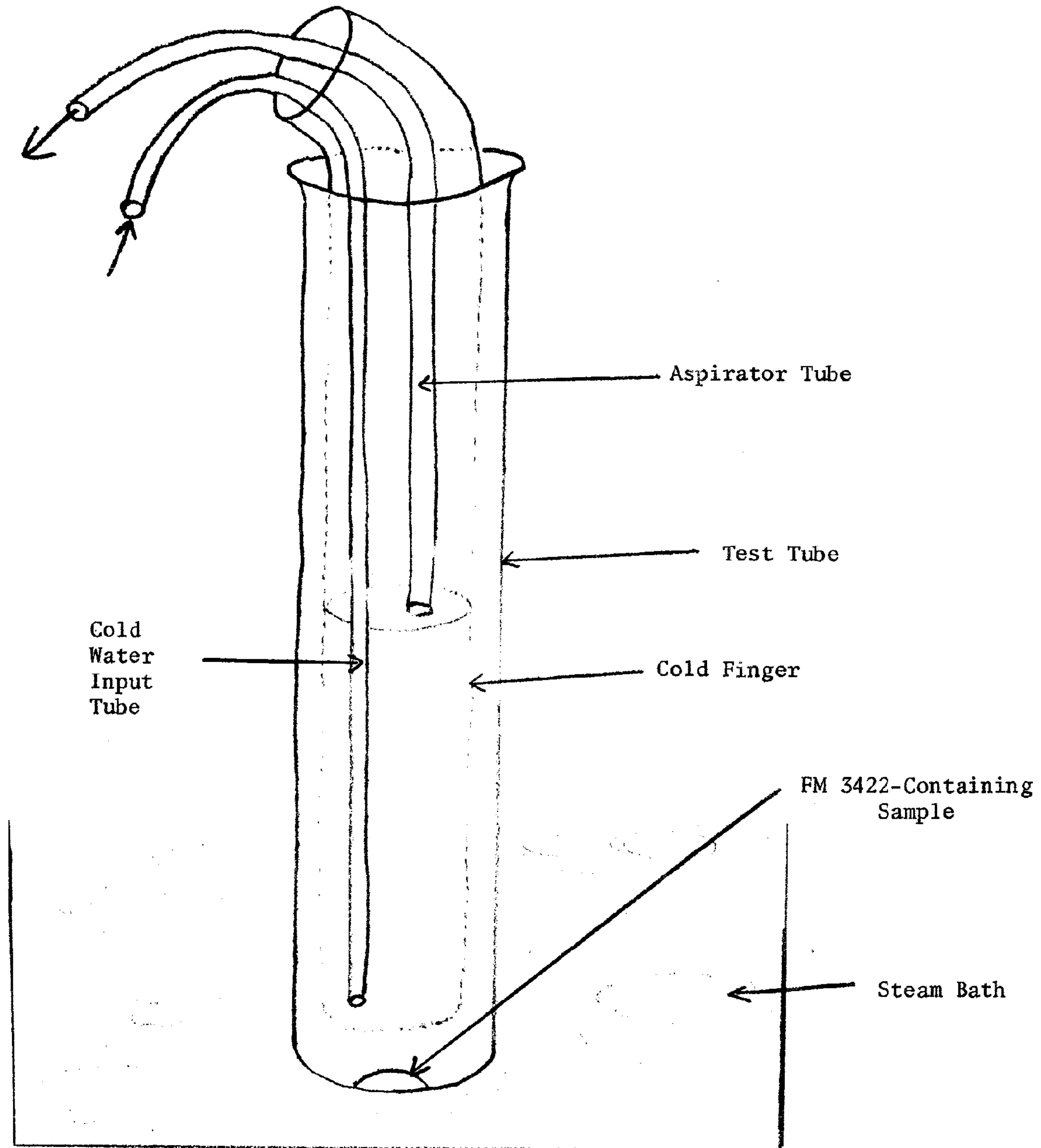

Figure 3. Apparatus used to recover FM 3422 from cells by volatilization and condensation.

The feasibility of this approach was evaluated by the following experiment which was run in duplicate. FM 3422 (1 mg) was placed in the base of a large test tube. A second smaller test tube was placed inside the first, and was effectively made into a cold finger by continuously running water into its base and aspirating it from the top. The base of this apparatus was then placed in a steam bath (Figure 3). After 15 minutes, the cold finger was removed and the sublimated FM 3422 washed from its surface into a known volume of ethyl acetate. GLC analysis indicated that 32% and 42% of the originally added FM 3422 was sublimated and collected in two trials.

The above sublimation procedure was then repeated in duplicate using week-old 10 ml cell cultures in the standard media. The small inoculum for these cultures came from a heterogeneous FM 3422 acclimated culture. These cultures were extracted with equal volumes of ethyl acetate (10 ml), and the liquid phases removed. The undried biomass was transferred to the outer tube of the above-described apparatus and exposed to the steam bath for 45 minutes. The ethyl acetate washings from the cold finger were analyzed by GLC, but very little of the nonextracted FM 3422 was recovered. The cells were dried under nitrogen and again exposed in the steam bath for an additional two hours.

The results of this experiment are shown in Table 9. The FM 3422 that was not extractable into ethyl acetate and presumably bound to the cells, was not quantitatively recovered by sublimation and condensation. Perhaps complete recovery would have been possible had the cells been exposed to the steam bath for a longer period. Although this experiment did not rule out the possibility that some of the bound FM 3422 may have been chemically modified by metabolism, it did indicate that a large fraction of the bound FM 3422 was unchanged.

	Culture #1	Culture #2
Extracted into Ethyl Acetate	15.6%	21.0%
Volatilized from "Wet" Cells	0.5%	0.3%
Volatilized from N_2 Dried Cells	25.6%	21.8%
Total FM 3422 Recovered	41.7%	43.1%

Table 9. Percent of initially added emulsified FM 3422 recovered from cells by ethyl acetate extraction followed by volatilization and condensation of remaining FM 3422.

Hot Ethyl Acetate Extracts

In an attempt to increase the recovery of the FM 3422 sorbed to the culture biomass, extractions were done using hot ethyl acetate. This procedure involved addition of 2.5 ml of FM 3422 emulsion to week-old, 10 ml cultures that had been grown on nutrient media. FM 3422 emulsion was also added to two, 10 ml deionized water samples. The resulting 12.5 ml samples, which contained 133 ppm FM 3422, were shaken for 15 minutes on a rotary shaker to allow sorption of the FM 3422 by the biomass, and extracted with equal volumes of ethyl acetate at room temperature or at 40° C. or 60° C. Phase separation was insured by centrifugation at 12,000 rpm and the concentration of FM 3422 in the ethyl acetate phase was determined by gas chromatography.

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The results of this extraction procedure are shown in Table 10.

Sample	Aqueous Phase Composition	Extraction Temperature (^{o}C)	ppm FM 3422 in Ethyl Acetate Extract
1	Deionized Water	22	115
2	Deionized Water	22	133
3	Microbial Culture	22	52
4	" "	22	55
5	" "	40	35
6	" "	40	31
7	" "	60	51
8	" "	60	56

Table 10. Hot ethyl acetate extracts of water or biological cultures after 15 minutes of shaking with emulsified FM 3422 (133 ppm).

These results indicate that hot ethyl acetate extraction does not improve the extractability of FM 3422 from the biomass. The amount of FM 3422 extracted at 60°C. is no higher than that extracted at 22°C. The reason for the lower concentration of FM 3422 in the 40°C. extract is not known. The results also confirm the previous observation that emulsified FM 3422 is absorbed by cells within 15 minutes. This rapid loss from a nonacclimated culture suggests a physical/chemical absorption process rather than biological degradation.

Dioxane Extraction of FM 3422 from Culture Solids

In a preliminary experiment, it was found that all of the FM 3422 not extracted by ethyl acetate could be recovered by refluxing the culture solids with 1,4-dioxane. Attempts to repeat this observation were initially hindered by problems with the electron capture detector of our gas chromatograph. These problems were apparently caused by the presence of dioxane. Central Research overcame this analytical problem and the experiment was repeated. The results (Table 11) demonstrate quite conclusively that FM 3422 is not metabolically altered in seven days by shake flask cultures that have been maintained with weekly transfers on FM 3422-containing media for over six months.

Culture Extracted with Ethyl Acetate at:	% of FM 3422 Extracted By Ethyl Acetate	Procedure for Extracting Culture Solids	% FM 3422 Recovered From Solids	Total % Recovery of FM 3422
15 min.	99.3	No solids present	--	99.3
15 min.	99.3	No solids present	--	99.3
15 min.	101.3	No solids present	--	101.3
7 days	25.8	(Refluxed in Dioxane for 2 hrs.)	77.9	103.7
7 days	27.1	" "	80.1	107.2
7 days	31.1	(Dried solids with N_2 & refluxed in Dioxane for 2 hrs.)	75.0	106.1
7 days	30.4	(Refluxed in Dioxane for 1 min.)	73.5	103.9
7 days	29.6	(Extracted with Dioxane at Room Temperature)	68	97.6

Table 11. Recovery of FM 3422 from culture solids by extracting or refluxing with Dioxane.

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Eric A. Reiner

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