Magnetic Microspheres: Synthesis of a Novel Parenteral Drug Carrier

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Abstract □ The synthesis and characterization of a novel parenteral drug carrier capable of area specific localization by magnetic means are described. The carrier consists of human serum albumin microspheres, average of 1 μm in diameter, in which a magnetizable material (magnetite) and a prototype drug (doxorubicin) are entrapped. Stabilization of the microsphere matrix by formaldehyde, 2,3-butanedione, and heat conferred equal stability to the matrix but differentially affected the *in vitro* drug release rate. Released doxorubicin was chemically identical to the starting material.

Keyphrases □ Microspheres, magnetic—synthesized as drug carriers, effect of stabilization and heat on drug release *in vitro* □ Carriers, drug—magnetic microspheres synthesized, effect of stabilization and heat on drug release *in vitro* □ Delivery, drug—magnetic microspheres synthesized as drug carriers, effect of stabilization and heat on drug release *in vitro*

Several types of carriers intended to modify the systemic distribution of soluble chemotherapeutic agents recently were proposed as possible drug vehicles. Kramer (1) entrapped daunorubicin hydrochloride and mercaptopurine in human serum albumin microspheres and suggested their use *in vivo*. The *in vivo* distribution of dactinomycin and a variety of other agents was modified by entrapping them into liposomes (2, 3).

The *in vivo* distribution of a carrier may be influenced mechanically by varying its size, as with albumin microspheres (4, 5), or chemically by altering its surface charge, as with liposomes (6, 7). In addition, the attachment of antibodies to the carrier surface was suggested as a mechanism for targeting to specific cell surface antigens (8, 9). However, intravascular administration of any such carriers, modified or not, results in their undesired accumulation by the reticuloendothelial system (10, 11). A mechanism for selectively restricting the carrier and, hence, the drug to a desired body site with minimal interference of the reticuloendothelial system is highly desirable.

This report describes the preparation and characterization of a novel delivery system for water-soluble drugs. It consists of albumin microspheres (average of 1 μm in diameter) containing ultrafine magnetic particles, magnetite (ferrosoferric oxide), and a prototype drug, doxorubicin hydrochloride. Magnetic microspheres injected intravascularly can be localized to a desired target site *in vivo* by an externally applied magnetic field of appropriate strength (12). Moreover, the intra-arterial injection of carrier-delivered doxorubicin resulted in the same target site concentrations as a 100-fold higher dose of free doxorubicin administered intravenously.

This site-specific delivery of chemotherapeutic agents allows for maximum concentration of an agent at a desired body site, thus permitting the use of much smaller doses than are normally required with generalized systemic administration. Such smaller doses would also result in decreased toxicity of the administered agent.

EXPERIMENTAL

Preparation of Microspheres—A modified emulsion polymerization method, similar to that developed by Scheffel *et al.* (13), was used to prepare the microspheres. Stabilization of the albumin matrix of the microspheres was accomplished by either heat denaturation at various temperatures or cross linking with carbonyl compounds in an ether phase reaction.

Heat-Stabilized Microspheres—An aqueous solution was prepared containing, per milliliter, 250 mg of human serum albumin[1] (I), 0.02 ml of ^{125}I-bovine serum albumin[2] (II), 32 mg of bulk purified doxorubicin hydrochloride[3] (III), and 72 mg of magnetite[4] (IV) particles 10–20 nm in diameter. A 0.5-ml aliquot of this suspension was added to 30 ml of cottonseed oil[5] and the resultant emulsion was homogenized by sonication[6] (100 w) for 1 min at 4°. The homogenate was then added dropwise into 100 ml of stirred (1600 rpm) cottonseed oil preheated to a desired temperature (110–165°).

The duration of heating (10 min) was held constant regardless of the temperature selected. After 10 min, the oil was allowed to cool to 25° with constant stirring. The microspheres were washed free of oil by adding 60 ml of anhydrous ether[7], centrifuging[8] for 15 min at 2000×*g*, and decanting the supernate. After the fourth wash, the pellet of microspheres was allowed to air dry in the dark for 24 hr at 4°. The resultant microspheres were lyophilized[9] to remove any remaining water and stored at 4° until they were used.

Carbonyl-Stabilized Microspheres—An oil homogenate of I–IV was prepared as described. The homogenate was added dropwise into 100 ml of constantly stirred (1600 rpm) cottonseed oil at 25°. After 10 min of stirring, the microspheres were washed free of oil with ether. The microspheres were then resuspended in ether (40 mg of microspheres/100 ml of ether) containing either 0.2 *M* 2,3-butanedione[10] or 0.1 *M* formaldehyde[11] as the cross-linking agent.

The suspension was stirred rapidly for either 15 or 60 min. Excess carbonyl reagent was removed by immediately adding 100 ml of ether, pelleting the microspheres by centrifuging at 2000×*g* for 10 min, and decanting the supernate. The microspheres were washed four times in this manner and subsequently lyophilized and stored at 4°.

Analysis of Microspheres—Aliquots of the lyophilized microspheres suspended in ether were taken, and the size of the microspheres in each aliquot was determined by scanning electron microscopy.

The stability of III released from an aqueous suspension of both types of microspheres (1 mg/ml) was determined by TLC on 5 × 20-cm precoated silica gel 60[12] plates, using chloroform–methanol–acetic acid–water (60:20:14:6). The R_f values of III and its aglycone breakdown products were obtained by visualizing the native fluorescence of the drugs with a shortwave UV lamp[13]. The experimental R_f value was then compared to a reference R_f value of stock III.

The percent starting parent compound remaining intact after release from the microspheres was determined quantitatively by scraping the appropriate areas of the chromatographic plates and eluting the compounds from the silica with 5% HCl in ethanol for 12 hr at 4°. The con-

[1] Sigma Chemical.
[2] Specific activity of 1.51 mCi/mg, 1.42 mCi/ml, New England Nuclear.
[3] Adriamycin, Adria Laboratories.
[4] Ferrosoferric oxide (Fe_3O_4) in aqueous suspension, Ferrofluidics Corp.
[5] Sargent-Welch.
[6] Branson sonifier model 185.
[7] Mallinckrodt Chemicals.
[8] Sorval model RC2-B.
[9] Model 10-010, Virtis Co.
[10] Aldrich Chemical Co.
[11] Scientific Products. The 2,3-butanedione is soluble in ether; however, formaldehyde must be extracted into the ether phase. This extraction was accomplished by the vigorous shaking for 10 min of a 1:5 solution of 37% aqueous formaldehyde–ether with the addition of a saturating amount of ammonium sulfate.
[12] EM Laboratories.
[13] Model UVS-12, Ultraviolet Products.

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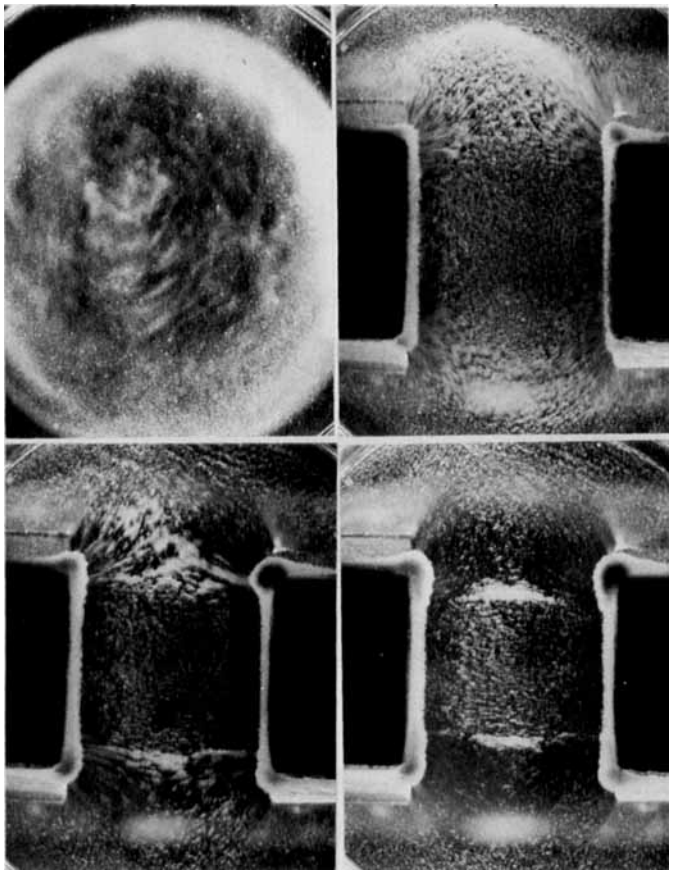

Figure 1—*Sequential time sequence photographs of magnetically responsive drug-bearing microspheres in aqueous suspension after horseshoe magnet is applied. Progressive alignment of microspheres (negative contrast) along magnetic field lines is evident. Elapsed time between photographs 1 and 4 is approximately 2 sec.*

centration of III and the pooled group of aglycone breakdown products was determined by spectrophotofluorometry[14].

The magnetite contained within the microspheres was determined quantitatively by hydrolyzing an aliquot of the microspheres in concentrated nitric acid and assaying the resultant hydrolysate for iron by atomic absorption spectroscopy[15]. Transmission electron microscopy of the microspheres was utilized to assess incorporation of magnetite into the albumin matrix and was described in detail elsewhere (14).

The efficiency of III incorporation was checked by extraction of the drug as follows. A 1-mg portion of the drug-containing carrier was incubated in 5 ml of 5% HCl in ethanol for 24 hr at 4°. The suspension of microspheres was then centrifuged for 10 min at 2000×*g*, and the III content was determined by spectrophotofluorometric analysis of the supernate as described. Extraction of the drug from the microspheres by this method was essentially complete since subsequent incubation of the microspheres for 24 hr at 25° in both saline and acid alcohol failed to show detectable III in the supernates.

The structural integrity of the albumin matrix of the microspheres before and after cross linking or heat denaturation was determined by the following procedure. The microspheres were suspended (0.5 mg/ml) in 0.154 *N* NaCl with 0.1% polysorbate 80[16] (V) and sonicated for 5 min in an ultrasonic water bath[17]. The sonicated suspensions were allowed to incubate at 37° for either 24 or 48 hr. After incubation, an aliquot of the suspension was centrifuged for 10 min at 2000×*g* to remove microspheres not totally disrupted. The resulting supernate was removed and treated with 24% aqueous trichloroacetic acid for 2 hr to precipitate free albumin and then centrifuged at 2000×*g* for 10 min.

The trichloroacetic acid-soluble supernate represents iodine-125 not

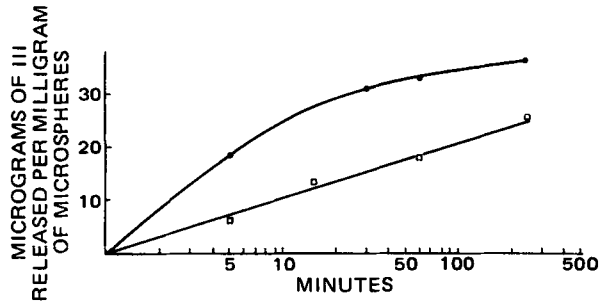

Figure 2—*Release of III from 0.1* M *formaldehyde-stabilized microspheres in saline-polysorbate 80 at 37°. Key: ●, cross linked 15 min; and □, cross linked 60 min.*

associated with albumin, whereas the trichloroacetic acid-precipitable pellet represents ^{125}I-albumin released from the albumin matrix. The deterioration of the microsphere matrix was then determined from the trichloroacetic acid-precipitable ^{125}I-albumin counts[18] and expressed as a percentage of the initial counts of ^{125}I-albumin associated with the microspheres prior to suspension and sonication.

Drug Release—The drug release rate from the microspheres *in vitro* was determined as follows. An 8-mg portion of microspheres (1 mg/ml) suspended in V was sonicated for 2 min in the ultrasonic water bath to produce a homogeneous suspension of microspheres. The magnetically responsive microspheres were pulled to one side of the vessel using a horseshoe magnet[19]. The supernate, containing drug released from the microspheres during sonication, was removed. Microspheres were then rapidly resuspended in 8 ml of V and gently hand agitated for 10 sec.

The reaction vessels were then placed in a water bath at 37° and covered to protect III from light. At appropriate time intervals, microsphere-free supernate samples were obtained by magnetically drawing the microspheres to the side of the vessel and removing a 1-ml aliquot of the supernate. The amount of drug released over time was determined spectrophotofluorometrically as described.

RESULTS AND DISCUSSION

A relatively homogeneous population of microspheres is produced by the methods described with microspheres ranging from 0.2 to 1.5 μm and having an average diameter of 1 μm. The magnetic responsiveness of the drug carrier is shown in Fig. 1 where the sequential alignment of the microspheres along magnetic field lines and also around the poles of the magnet is evident. Details of their magnetic responsiveness under flow conditions corresponding to the human circulation were reported elsewhere (14, 15).

Doxorubicin was relatively stable to temperatures used in the preparation of heat-stabilized microspheres as determined by TLC, with only a moderate increase in aglycone breakdown products. Control starting III (nonheated) consisted of 97% pharmacologically active drug and 3% aglycones. The aglycones are pharmacologically inactive (16). Compounds released from microspheres prepared at 135–145° for 10 min consisted of 74% pharmacologically active III (identical R_f as starting III) with the remaining 26% consisting of aglycones.

Carbonyl-stabilized microspheres showed no increase in aglycone formation when compared to control III. Under the conditions employed, the carbonyl agents did not appear to react with the free amine of the released III as evidenced by TLC. The *in vitro* biological activity of III released from heat-stabilized microspheres will be described in detail elsewhere[20]; both starting and released III can identically and stoichiometrically inhibit ^{3}H-uridine uptake by rat fibrosarcoma cells.

Despite the fact that microspheres prepared at 25° retain their morphology in ether, they require some form of stabilization if they are to be transferred to an aqueous solvent. In fact, structural integrity of the microspheres as judged by sonication was directly related to stabilizing treatment. When microspheres were prepared at 25° with no stabilizing treatment, there was 16% deterioration after 24 hr of incubation at 37° and 37% deterioration after 48 hr as determined by ^{125}I-albumin disassociated from the microspheres. In contrast, ≤3% deterioration of the microspheres was detected after 24 and 48 hr when microspheres were

[14] American Instrument Co. model SPF-1255 (excitation = 470 nm, and emission = 585 nm); level of detectability was 1×10^{-6} *M* III.
[15] Iron analysis performed by Scientific Control Labs, Chicago, Ill.
[16] Tween 80 (Sigma Chemical Co.) in 0.154 *N* NaCl.
[17] Model M.E. 2.1, Mettler Electronics Corp.

[18] ^{125}I-Activity was determined in a Packard model 578 well-type γ-counter.
[19] Edmund Scientific No. 70,572.
[20] Paper in preparation.

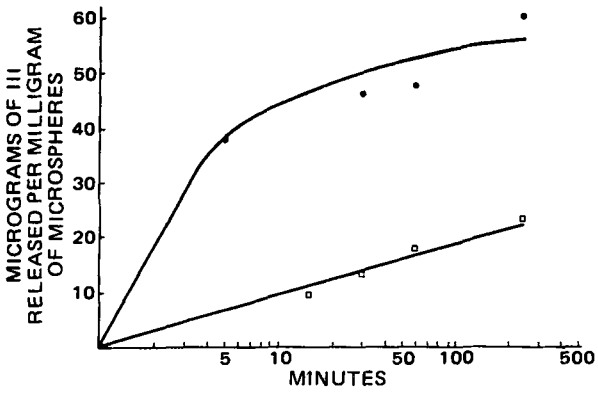

Figure 3—*Release of III from 0.2 M 2,3-butanedione-stabilized microspheres in saline–polysorbate 80 at 37°. Key: ●, cross linked 15 min; and □, cross linked 60 min.*

cross linked for 15 min or longer with either 2,3-butanedione or formaldehyde. Heat treatment above 100° had a similar effect.

By varying the amount of magnetite added to the starting solution, the percent magnetite dry weight composition of the microspheres could readily be controlled. The magnetite content has important implications with respect to the magnetic responsiveness of the carrier. Though increasing the magnetite content would confer greater magnetic responsiveness to the microsphere and thus permit the use of smaller extracorporeal fields, it would decrease the available drug space in the microsphere, thus requiring larger dosages of carrier to deliver equivalent drug concentrations. Depending on the type of drug and the desired target site, the optimum magnetite content would range between 20 and 50% magnetite by dry weight of the drug–carrier complex (14, 15).

After preparation of the carrier, essentially all of the starting III was associated with the resultant microspheres as judged by extraction with acid alcohol. Regardless of the method of preparation, of the 90 μg of III associated with 1 mg of carrier, approximately 40% (36 μg) was liberated rapidly on sonication in an aqueous solvent. The rate of III release from heat-stabilized and carbonyl-stabilized microspheres is shown in Figs. 2–5. As expected, increased stabilization of the microspheres, either by longer cross-linking treatment or by increased temperature, decreased the release rate of III. The generated release curves are comparable to those presented by Scheu *et al.* (17) for coacervate-produced microspheres.

Detailed kinetic studies were not warranted in this study since a significant amount of drug was still associated with the carrier at $t = 5$ min in both heat- and carbonyl-stabilized preparations. This time period represents a realistic interval for carrier injection and *in vivo* transit time to the desired target site. Furthermore, *in vivo* extrapolation from *in vitro* data is not readily feasible because of a number of complex blood flow variables. However, data from Fig. 5, where microspheres were incubated in human serum, reveal an inhibited release of III compared to Fig. 4, in which the release medium was V. This result suggests that *in vivo* release times would correspond more closely to Fig. 5 than Fig. 4. Experimental work is in progress to evaluate the biological fate of the magnetite *in vivo* after carrier administration. Preliminary acute (7 days) and chronic (90

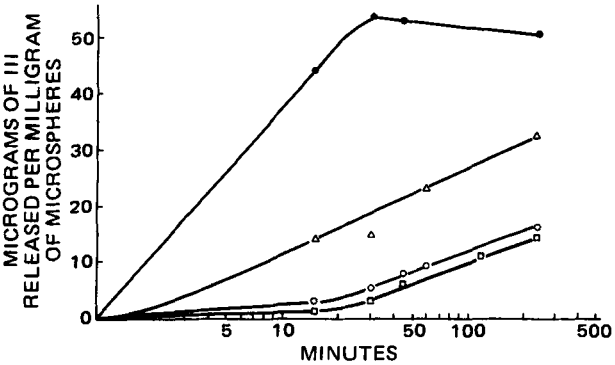

Figure 4—*Release of III in saline–polysorbate 80 at 37° from heat-stabilized (10 min) microspheres prepared at various temperatures. Key: ●, 25°; △, 115–127°; ○, 125–135°; and □, 135–145°.*

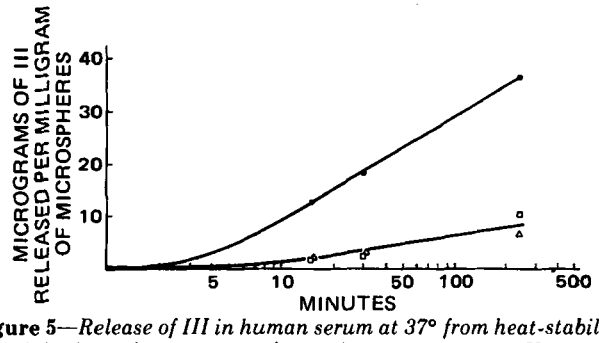

Figure 5—*Release of III in human serum at 37° from heat-stabilized (10 min) microspheres prepared at various temperatures. Key: ●, 25°; △, 115–127°; and □, 135–145°.*

days) toxicity studies in BDF_1 mice showed minimal adverse effects (12).

The described low temperature conditions employing either carbonyl reagents or no deliberate denaturing step at all should permit encapsulation of many temperature-sensitive drugs that formerly could not be incorporated into solid albumin microspheres. Several theoretical explanations may be offered for the formation of stable microspheres without "overt" denaturation of the albumin. One that seems likely is that albumin molecules, aligning on the oil–water interface, undergo a change in conformation such that hydrophobic regions of the molecules are preferentially exposed externally to the oil. Thus, a hydrophobic "crust" or mantle is formed that is not reversible for a period of time, which permits microspheres to maintain their morphology for a limited time when transferred to an aqueous environment.

Using model phospholipid interfaces, Kimelberg (18) described conformational changes of proteins aligning at such interfaces. What has not been reported to date is that nonstabilized microspheres are durable enough to maintain morphologic integrity in aqueous suspension for a sufficient time to permit their use as either therapeutic vehicles or substrates for subsequent denaturation by low temperature methods. The nonaqueous carbonyl reagent method of stabilizing the albumin matrix, in addition to maintaining the spherical morphology of the carrier generated in the oil emulsion, appears to slow the diffusion of III from the carrier.

The use of microspheres or any drug–macromolecular complex as vehicles for targeting chemotherapeutic agents rests solely on the ability of the carrier to achieve either cell or area specificity. Efforts at cell-specific targeting of cancer chemotherapeutic agents are mainly focused on exploitation of biochemical differences between cells (19, 20). Unfortunately, target cell specificity has not been achieved in most cases, as evidenced by the numerous side effects apparent with generalized systemic drug administration.

Area-specific drug delivery using a carrier may be of value not only in greatly decreasing unwanted systemic distribution of drugs but also in eradicating localized disease states. Magnetic microspheres offer a new modality for drug delivery. The narrow and relatively uniform size range of these microspheres makes them ideal for small vessel passage, thus permitting uniform saturation of target capillary beds. With the ability to control the rate of drug release from the carrier and the ability to localize both carrier and drug *in vivo* to a desired site, a different modality of drug delivery may be readily explored.

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Antifungal Properties of *n*-Alkoxyacetic Acids and Their Methyl Esters

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Abstract □ Eleven *n*-alkoxyacetic acids and their methyl esters, in which the alkyl group was C_1–C_9, C_{11}, or C_{13}, were tested against *Aspergillus niger*, *Trichoderma viride*, and *Myrothecium verrucaria* in Sabouraud dextrose agar at pH 4.0 and 5.6. Toxicity to *Candida albicans*, *Trichophyton mentagrophytes*, and *Mucor mucedo* was determined in the same medium at pH 5.6 and 7.0 in the absence and presence of 10% beef serum. The fungitoxicity of the acids was influenced by chain length, pH of the medium, and absence or presence of adsorbents. The toxicity of the esters was influenced primarily by chain length and to a lesser extent by the medium pH and the presence of beef serum. The order of activity of the *n*-alkoxyacetic acids was according to the number of linear atoms in the chain: 11 > 12 > 10 > 9 > 8 > 7 > 6 > 14 > 16. *T. mentagrophytes* was the organism most strongly affected by these compounds, with the esters being slightly more active than the acids. Compared to other fatty acid analogs, the order of fungitoxicity, on a weight basis, was 2-alkynoic acids > 2-alkenoic acids > alkanoic acids > 2-bromoalkanoic acids > 2-fluoroalkanoic acids > *n*-alkoxyacetic acids.

Keyphrases □ *n*-Alkoxyacetic acids and methyl esters—antifungal activity evaluated *in vitro*, effect of chain length, pH, and adsorbents □ Antifungal activity—*n*-alkoxyacetic acids and methyl esters evaluated *in vitro*, effect of chain length, pH, and adsorbents □ Structure–activity relationships—*n*-alkoxyacetic acids and methyl esters, antifungal activity evaluated *in vitro*

As part of a search for fungitoxic agents, the structure–activity relationships of fatty acids were studied (1–6). In addition, compounds were evaluated for potential use against opportunistic fungi, which are frequent invaders of immunosuppressed and debilitated patients (4–10). Such fungi include species of *Candida*, *Aspergillus*, *Mucor*, and *Cryptococcus* (11).

BACKGROUND

Fatty acids possess significant antifungal activity. For systemic use, in spite of their low toxicities, they have been ineffective, possibly because they are metabolized readily by the host through the usual fatty acid pathways. The fungitoxicity of 2-fluoro fatty acids paralleled that of the nonfluorinated fatty acids (1). Since it is believed that 2-fluoro fatty acids do not undergo β-oxidation (12), they possess at least one potential advantage over the nonfluorinated fatty acids for systemic antifungal activity.

Another approach for preparing fatty acids that do not undergo β-oxidation is to replace the β-methylene group with an oxygen (13). In the structure $ROCH_2COOH$, the C–O–C angle is 111° and the corresponding C–C–C angle is 112°. The C–O bond length is 1.43 Å, and the length of the C–C bond is 1.54 Å. Replacement of the CH_2 group by O would not greatly alter the steric configuration of the analog, and it would not be β-oxidizable because of the lack of β-hydrogens.

The major factors influencing the fungitoxicity of fatty acids and derivatives are the partition coefficient and the absence or presence of adsorbents such as albumin in the growth medium. Chain length, pKa of the acid, and pH of the medium are among the factors determining the partition coefficient of the test compound (6). The deactivating effect of the adsorbent can be explained as being due to hydrogen bonding (5).

No systematic antimicrobial studies with 3-oxa acids or esters have been reported. 11-Fluoroundecyloxyacetic acid and several other 3-oxa acids inhibited *Mycobacterium tuberculosis in vitro* at 50 μg/ml (14).

The present study was concerned with the preparation of the straight-chain 3-alkoxyacetic acids and their methyl esters in which the alkyl groups included 1–9, 11, or 13 carbon atoms. Methoxy- and ethoxyacetic acids were purchased[1]. The following acids were prepared by published methods: *n*-propyloxyacetic acid, *n*-butyloxyacetic acid, *n*-pentyloxyacetic acid, *n*-hexyloxyacetic acid, *n*-heptyloxyacetic acid, *n*-octyloxyacetic acid (15), *n*-undecyloxyacetic acid, and *n*-tridecyloxyacetic acid (13).

The following methyl esters were also previously reported: methyl methoxyacetate, methyl ethoxyacetate, methyl *n*-propyloxyacetate (16), methyl *n*-butyloxyacetate (17), and methyl *n*-octyloxyacetate (18). *n*-Nonyloxyacetic acid was prepared by condensing *n*-nonyl alcohol with chloroacetic acid by means of sodium in toluene, and the previously unknown methyl esters were obtained from the acids by heating with methyl alcohol in the presence of a catalytic quantity of thionyl chloride.

The data characterizing the new compounds are in Table I. The purity of all compounds was verified by GLC.

The fungi used were *Aspergillus niger* (ATCC 1004), *Trichoderma viride* (ATCC 8678), *Myrothecium verrucaria* (ATCC 9095C), *Candida albicans* (ATCC 10231), *Trichophyton mentagrophytes* (ATCC 9129), and *Mucor mucedo* (ATCC 7941).

The compounds were screened against *A. niger*, *T. viride*, and *M. verrucaria* in Sabouraud dextrose agar[2] at pH 4.0 and 5.6 according to published methods (1). Graded levels of test compound dissolved in dimethyl sulfoxide were incorporated into the growth medium, which was then inoculated with the respective fungus. The inoculum consisted of

[1] Eastman Kodak Co., Rochester, N.Y.
[2] Difco, Detroit, Mich.

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