Antioxidant Activity of Sodium Nitrite in Meat

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ABSTRACT

Polar lipids were extracted from raw, nitrite-treated beef and pork by a dry column procedure. The polar-lipid fraction had substantial activity in inhibiting the oxidation of linoleic acid as determined by a β-carotene bleaching method and by conjugated diene formation. The antioxidant activity of the polar-lipid fraction was stable over several months when stored in hexane at −20 C. Residual sodium nitrite, carbon-nitroso and nitrogen-nitroso compounds or products of the addition of nitrogen oxides to olefins do not seem to account for the antioxidant activity observed.

INTRODUCTION

In the preparation of cured meat, sodium nitrite is added to the cure because of its ability to impart color, flavor, bactericidal activity and stability against oxidation to the product. Research effort during the past 15 years has led to some understanding of the anticlostridial action of sodium nitrite (1), of the chemistry of nitrite in meat (2), of the function of nitrite as a flavor enhancer (3) and of the formation of N-nitrosamines (4,5). On the other hand, little has been revealed about the mechanism that allows nitrite to protect cured-meat lipids against oxidative degradation.

The role of sodium nitrite in modifying cured-meat flavor (6,7), and particularly in preventing the "warmed-over" flavor generally associated with lipid oxidation in cooked meat (8,9), has been examined. Although how nitrite might act to prevent lipid oxidation in meat is still unclear, Pearson (8) has speculated that nitrite might act on the lipid components of meat cell membranes, or perhaps deactivate the natural prooxidants present in muscle.

Sodium nitrite reacts rapidly with various meat components. Within 24-48 hr after addition, only 40-50% of the amount added originally is analytically demonstrable as free nitrite (2). Using $^{15}NO_2^-$, Cassens et al. have demonstrated (2,10,11) that only a small part (1-5%) of the sodium nitrite added becomes associated with lipids. The nature of this association remains unknown. Goutefongea et al. (12) studied the reaction of sodium nitrite in brine with whole adipose tissue, both ground and unground, and found that ca. 2-3% of the nitrite was bound by lipid and was extractable with the lipid by the Folch (13) procedure. They suggested that the nitrite binding was related to the degree of unsaturation of the lipid. The implication is that nitrite or a derivative has reacted with one or more double bonds. The reaction of various nitrogen oxides with olefins has been reported to result in the formation of nitroso-nitrite derivatives (nitrosites) as well as nitroso-nitro compounds (pseudonitrosites) (14-16). Lipid pseudonitrosites have been implicated as transnitrosating agents (17). The purpose of the current work was to develop information on the manner in which sodium nitrite retards the oxidation of meat lipids.

EXPERIMENTAL PROCEDURES

Materials

Linoleic acid (>99%) was obtained from Nu-Chek Prep, Inc., Elysian, MN. Cytochrome c (type III from horse heart) and *trans*-β-carotene (type I) were purchased from Sigma Chemical Company, St. Louis, MO.

Meat Preparation

Raw bottom-round beef, or lean, center-cut pork chops, obtained directly from a slaughter house, were trimmed of adipose tissue, and ground once in a Hobart meat grinder through a $^3/_{16}$ in. plate. To prepare "treated meat," 20 mL of a solution of 780 mg sodium nitrite in 100 mL distilled water was added to 1,000 g of meat to give an initial concentration of 156 ppm as $NaNO_2$, which is equivalent to 100 ppm as NO_2. To prepare "untreated meat," an equal amount of distilled water was used in place of the sodium-nitrite solution. Treated and untreated meat samples were mixed manually and then reground to assure thorough mixing. Portions (15 g) of each sample were wrapped in air-permeable plastic film (PVC) and stored at ca. 4 C.

Analytical Procedures

Lipid extraction. Total lipids were extracted from meat samples by the dry column procedure published by Maxwell et al. (18). Neutral and polar lipids were extracted sequentially by the method of Marmer and Maxwell (19).

TBA values. The 2-thiobarbituric acid method for the determination of malonaldehyde, according to Tarladgis et al. (20) and modified by Zipser and Watts (21), was used on ground meat samples.

Peroxide values. These were determined on extracted lipids by AOCS Official Method Cd 8-53 (22).

Sodium nitrite. Sodium nitrite determination in meat samples was carried out by a modified AOAC procedure (23).

Antioxidant activity—carotene bleaching method. Inhibition of linoleic acid oxidation by test substances, as indicated by spectrophotometric observation of β-carotene bleaching, was measured by the method of Ben-Aziz et al. (24) with cytochrome c as catalyst. A Bausch and Lomb Spectronic 21 (single-beam) or a Perkin-Elmer 559A (double-beam) spectrophotometer was used (at 460 nm, path length 1 cm). Potential antioxidants were dissolved in 0.1 mL of ethanol or isopropanol. Alternatively, as required by substrate solubility, potential antioxidants were dissolved in 0.1 mL of a mixture prepared as follows: ethanol (1 mL), 10% v/v Tween 80 in 95% ethanol (0.3 mL), and 0.5% w/v EDTA in distilled water (5 mL) were combined, the mixture was adjusted to pH 9 with 0.1 N NaOH, and the volume of the mixture was brought to 10 mL with distilled water. A corresponding amount of ethanol, isopropanol or the solvent mixture was present in the control not containing antioxidant. The final reaction mixture in the cuvette contained 1.5 mL linoleic acid-β-carotene solution, 0.3 mL cytochrome c in distilled water, 0.1 mL ethanol and 0.1 mL antioxidant solution or blank. Relative antioxidant activity, i.e., the ability to inhibit the bleaching of carotene, is measured and compared with that of controls containing no antioxidant.

Antioxidant activity—conjugated diene method. The spectrophotometric assay described by Ben-Aziz et al. (25) was used with cytochrome c at 4 μg/mL at pH 5. The linoleic acid concentration in the reaction mixture was 5×10^{-4} M. The final reaction mixture contained 2.3 mL linoleic-acid solution, 0.1 mL sample and 0.1 mL enzyme solution.

Addition of nitrogen oxides to olefins. General procedure: A solution of olefin (0.05-0.20 mol) was added to a reac-

tion chamber (500 mL 3-neck flask) in an appropriate solvent (150 mL). The solution was stirred and cooled to 3-5 C. Nitrogen oxide gases were generated in a second flask by the slow addition, with cooling, of sodium nitrite (35 g) in water (80 mL) to sulfuric acid (54 mL, concd.). The gases evolved were swept into the reaction flask and below the surface of the olefin solution by means of a stream of nitrogen gas or air. After gas generation was completed, the reaction mixture was allowed to warm slowly, was stored at room temperature for 1 hr, and was then washed twice with water and dried. Solvent was removed under reduced pressure. The crude product mixture was either tested for antioxidant activity, or was fractionated by chromatographic techniques and the fractions tested.

Specific procedures. Table I lists the specific olefin substrates, solvents, and carrier gases used.

HPLC-TEA. Samples (50 μL) of polar lipids were analyzed on an HPLC interfaced with a thermal energy analyzer (TEA) detector. HPLC was performed on a 25 cm 5 μ silica column. The mobile phase was programmed linearly from methylene chloride/methanol (1:1) to 100% methanol in 20 min at a flow rate of 1 mL/min. HPLC eluates were pyrolyzed in a TEA furnace at 750 C.

RESULTS AND DISCUSSION

Ground raw beef was treated with 156 ppm sodium nitrite and stored at 4 C for a period of 3 weeks. Samples were removed from storage periodically and aliquots were analyzed for TBA value and residual free nitrite. From another portion of each meat sample, polar lipids were extracted and these were analyzed for peroxide values. Table II gives a comparison of results from nitrite-treated samples with untreated controls. The data clearly demonstrate that nitrite treatment inhibits lipid oxidation. The results shown here are illustrative of several experiments in which similar data were obtained. Shortly after treatment, the free nitrite content decreased to 50% or less of its original value and then slowly decreased further over a period of several weeks. TBA numbers of the treated meat remained very low (usually less than 1 ppm) whereas those of the controls rose rapidly and then levelled off. The peroxide values of polar lipids extracted from nitrite-treated beef showed an induction period and then rose slowly; those from untreated beef rose more markedly. Both tended to fluctuate. These fluctuations have also been observed by others (26). The experiments could not be extended beyond the 3-week period, because the untreated, but not the treated, samples underwent microbial spoilage.

MacDonald et al. (6) and Hadden et al. (7) also obtained reduced TBA values on nitrite-treated pork. In our experiments, we noted high peroxide values were associated with polar, rather than neutral lipids. In fact, oxidation of neutral lipids remained quite low. This is not entirely unexpected, as most of the polyunsaturated fatty acids are found in the polar-lipid portion.

Heme proteins and nonheme iron are known to be active catalysts in the oxidation of lipids (27-29). MacDonald et al. (30) have shown that nitrite inhibits the prooxidation effect of Fe^{++} and Fe^{++}-EDTA as well as those of aqueous beef and pork extracts, and they suggested that nitrite may function as a metal chelater in tying up the trace metals present in meat. In addition to inhibiting the catalytic effects of prooxidants, sodium nitrite might also give rise to an antioxidant factor that, in order to be most effective, may be in close association with the lipid itself. To test this hypothesis, we extracted lipids from nitrite-treated and untreated meat and compared the antioxidant activity of the polar lipids. Lipids were extracted by the dry column procedure of Marmer and Maxwell (19), which is faster and more convenient than the Folch procedure (13) or the Bligh and Dyer method (31), and which permits the separate isolation of neutral and polar lipids. The extracted polar lipids were dissolved and stored in hexane, in which most associated nonlipid materials are insoluble.

TABLE I

Reaction of Olefins with Nitrogen Oxides

Olefin	Solvent	Carrier gas
Methyl oleate	$CHCl_3$	N_2
Methyl oleate	Petroleum ether	N_2
Oleic acid	Petroleum ether	N_2
Methyl oleate	Hexane	N_2
Cyclohexene	Petroleum ether: ether (1:1)	N_2
Cyclohexene	Petroleum ether: ether (1:1)	Air
Beef polar lipids	Petroleum ether	Air
Sorbic acid	$CHCl_3$	Air
Methyl linoleate	Petroleum ether: ether (1:1)	Air
Methyl linoleate	Petroleum ether: ether (1:1)	N_2

TABLE II

Lipid Stability During Meat Storage

Storage time	$NaNO_2$ ppm	Whole meat TBA values (ppm)		Polar lipids Peroxide values	
		Treated	Untreated	Treated	Untreated
48 hr	80	0.50	7.80	<15	<15
1 week	56	0.60	12.9	<15	28.8
2 week	47	0.57	13.9	<15	247
3 week	31	0.77	13.8	62.9	125

Numerous methods are available for measuring lipid oxidation (32). The method of Ben-Aziz et al. (24) was chosen because of its rapidity, convenience and adaptability for use with small samples. In this procedure linoleic acid is allowed to oxidize in the presence of cytochrome c and β-carotene, and progress of the oxidation is followed spectrophotometrically by observation of the bleaching of β-carotene at 460 nm. The reaction is essentially complete 4 min after the reagents are mixed. In this investigation, relative antioxidant activity, i.e., the ability to inhibit bleaching of β-carotene, was measured and compared with that of controls containing no antioxidant.

By this procedure, the polar-lipid fractions from raw nitrite-treated beef had substantial antioxidant activity compared with those extracted from raw untreated beef. Figure 1 compares the effect on carotene bleaching over a 4-min span by polar-lipid fractions from treated and untreated beef and by the commercial antioxidant BHT (butylated hydroxytoluene) at 1×10^{-4} molar with a control containing no antioxidant. The plots shown for the control and for treated and untreated polar lipids are averages of values obtained from 10-12 samples. These values were taken over a 3-week period while the meat was stored at 4 C. On each of the sampling days, 5 g portions of the treated and untreated meat were removed from storage, extracted and the antioxidant activity of the polar lipids isolated was determined, along with that of a control. The curve for BHT represents an average of 4 values obtained on 4 different days. Statistical analysis of the daily

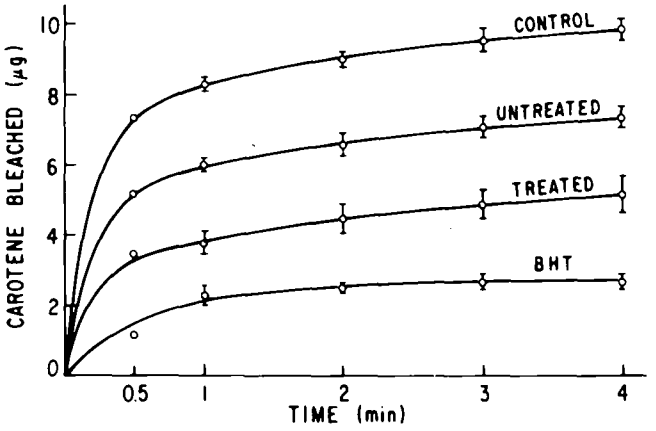

FIG. 1. Antioxidant effect on linoleic acid of polar-lipid fractions from untreated and nitrite-treated raw beef sampled over 3 weeks and compared to control and BHT. Vertical bars indicate standard error.

TABLE III

Raw Beef—Carotene Protection[a]

	Untreated	Treated	BHT
0.75 min	10.3%	32.3%	73.5%
1.0 min	9.5%	35.7%	72.6%
2 min	14.5%	37.3%	77.3%
3 min	15.7%	38.0%	77.7%
4 min	15.0%	37.8%	78.7%

[a]$\frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100.$

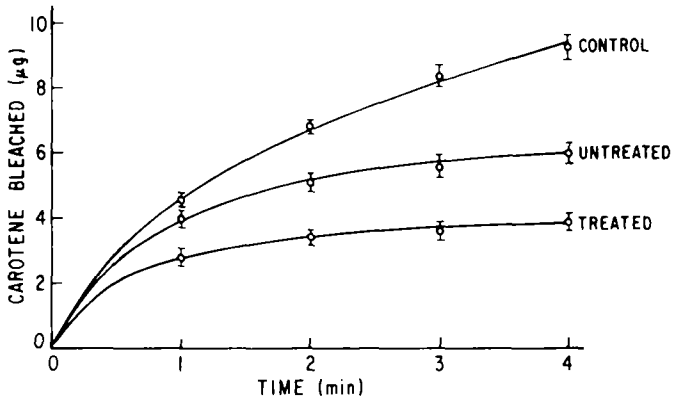

FIG. 2. Antioxidant effect on linoleic acid of polar-lipid fractions from untreated and nitrite-treated raw pork sampled over 2 weeks and compared to control. Vertical bars indicate standard error.

TABLE IV

Raw Pork—Carotene Protection[a]

	Untreated	Treated
0.5 min	6.7%	23.3%
1 min	13.0%	37.0%
2 min	25.0%	50.0%
3 min	33.3%	57.1%
4 min	35.5%	58.1%

[a]$\frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100.$

values (at 2 and 4 min) indicated that daily values of each type of sample (treated, untreated and control) did not differ significantly ($p < 0.05$), whereas the differences between sample types were significant.

Carotene protection afforded by the antioxidants can be derived from the β-carotene bleaching data by the equation

$$CP = \frac{C - S}{C} \times 100$$

where CP is the percentage of carotene protection and C and S are the micrograms of β-carotene bleached in the control and the sample, respectively. Table III gives β-carotene protection data derived from the values plotted in Figure 1. The data indicate that natural antioxidants present in untreated raw beef provide some protection against lipid oxidation and that nitrite treatment generates substantial additional antioxidant activity in the polar lipids. The polar lipids from treated meat at ca. 2.0×10^{-4} molar concentration are less effective antioxidants than BHT at 1×10^{-4} molar. This comparison, however, neglects the fact that the concentration of the antioxidant factors present in the treated polar lipids is, as yet, unknown. Preliminary comparison of the treated and the untreated polar lipids by thin layer chromatography (TLC) shows no obvious differences between the two. Hence, we suspect that the concentration of the antioxidant factors in the treated polar lipid fractions is not very great, or that the factors differ little in polarity from one of the major components of the fraction.

The data presented above (Fig. 1, Table III) give the results of one of several experiments carried out on raw beef. Similar results were obtained in the others. In all cases, the protective index of the polar lipids from treated meat, i.e., the percentage of carotene protection afforded by the treated polar lipids divided by the protection afforded by the untreated polar lipids, ranged between 1.5 and 2.5, 2 min after reagents were mixed. Analogous experiments were carried out on lean pork meat. The results from one of these experiments are shown in Figure 2 and Table IV. In raw pork meat, the polar lipids extracted from nitrite-treated meat also had substantially higher antioxidant activity than those from untreated meat.

Having established that polar lipids from nitrite-treated meat did possess significant antioxidant activity compared with polar lipids from untreated meat, we wished to confirm this activity by a second assay method. Two active polar-lipid samples were analyzed by the β-carotene and the better-known diene conjugation (25) procedure. The percentage of antioxidant activity values obtained by the 2 assays were 43% and 46% on one sample and 55.6% and 54% on another, confirming the validity of the results of the β-carotene assay.

To test the storage stability of the antioxidant activity, 2 samples of active polar lipids dissolved in hexane were stored at −20 C, and their activity was tested periodically over a month. The activity of both samples remained constant over that period. One of the samples was tested again 6 weeks later and was found to have undiminished activity.

Several experiments were performed to test the question of whether the antioxidant activity in the polar lipid is caused by an addition product of a nitrite residue to an unsaturated fatty acid. Polar lipids that have active antioxidants from nitrite-treated beef were separated and detected by HPLC interfaced with a thermal energy analyzer (TEA), a detector specific for nitric oxide (33). Chromatograms were compared with those obtained from a sample of polar lipids from untreated beef. Both samples gave identical tracings, suggesting that the active material did not contain nitroso or nitro compounds that were

thermally labile under the TEA conditions used, or that such compounds were present in amounts that were too low to be detected. If nitroso or nitro derivatives of fatty acids had been present in greater than 1% concentration in the HPLC fraction, they should have been detected by this methodology.

To test the possibility that nitrogen oxide addition to olefins might account for the observed antioxidant activity, nitrogen oxide addition products to olefins were synthesized. Methyl oleate, methyl linoleate, oleic acid, cyclohexene and polar lipids from untreated beef were all treated with nitrogen oxides generated from sodium nitrite and acid in the absence of air (generating predominantly nitric oxide) and in the presence of air (yielding predominantly dinitrogen trioxide). Table I provides a list of experiments performed. Confirming analogous work by others (14-16), reaction products were formed in all experiments by the addition of nitrogen oxides to the olefins. The antioxidant activity of all crude products (after the removal of solvent), as well as that of various chromatographically isolated fractions, was tested. No antioxidant activity could be detected in any of the synthetic products, except that slight activity was found in one product derived from methyl oleate. However, even this slight amount of activity (12%) could not be confirmed in later repeat experiments.

The antioxidant activity of sodium nitrite was compared (at 1×10^{-4} M) with those of a control and of 4 antioxidants in commercial use: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and sodium ascorbate (ASC). The results are shown in Figure 3 and indicate that sodium nitrite is inactive in this procedure, whereas activity of the others increases ASC < PG < BHT, BHA. Citric acid was also examined at the 1×10^{-4} M level and showed no activity. These results indicate that sodium nitrite remaining in the tissue is probably not responsible for the antioxidant action found, in the unlikely event that the nitrite became associated with the polar lipids.

In view of MacDonald's finding (30) that nitrite inhibits the prooxidative effect of free and bound iron, testing the question of whether the relative antioxidant effect we observed was not, in reality, the result of inhibited prooxidant activity in the treated vs the untreated samples was important. We analyzed 2 active (treated) polar-lipid samples (one from beef and one from pork) for iron content by atomic absorption spectroscopy and compared the results with those from parallel inactive (untreated) samples. The active samples contained more iron than the inactive samples. We concluded that the observed antioxidant effect is not the result of decreased prooxidative activity by iron.

A final set of experiments was designed to test the effect of unbound iron, alone and in combination with nitrite, on antioxidant activity. Ferrous sulfate was added to the β-carotene assay solution to reach final concentrations of 50 and 200 ppm. The activity of these samples was indistinguishable from that of controls. To some of the iron-containing assay solutions, sodium nitrite was added to reach final concentrations of 50 and 100 ppm. The activity of solutions containing both ferrous sulfate and sodium nitrite were also indistinguishable from those of the controls. We conclude that under the assay conditions used in this work, neither iron nor nitrite account for the antioxidant activity observed in nitrite-treated polar lipids.

The information developed in the current work demonstrates that the addition of sodium nitrite generates an antioxidant factor in raw beef and raw pork. This factor, which requires further definition, is produced consistently, is associated with the polar-lipid fraction, and does not appear to involve an addition product of nitrogen oxides to olefinic double bonds. It also seems unrelated to a possible reduction of the prooxidant activity of iron compounds by sodium nitrite. Studies to identify the antioxidant factors are continuing.

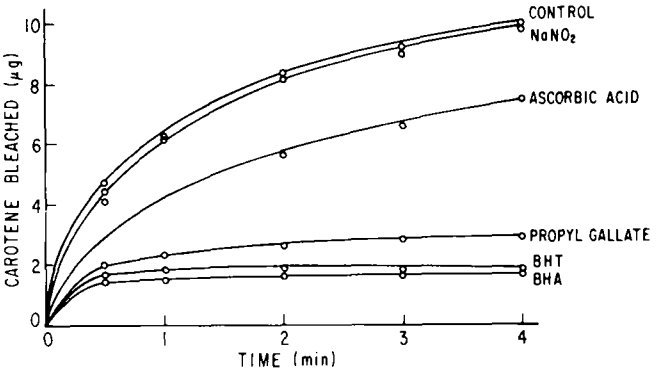

FIG. 3. Comparison of antioxidant effect on linoleic acid of sodium nitrite with that of common antioxidants at 1×10^{-4} M. BHT = butylated hydroxytoluene, BHA = butylated hydroxyanisole.

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Continuous Synthesis of Glycerides by Lipase in a Microporous Membrane Bioreactor

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ABSTRACT

A membrane bioreactor was developed for continuous synthesis of glycerides by lipase to overcome the drawbacks associated with the usual operation in an emulsion system. One unit (total area: 726 cm^2) of flat, plate-type dialyzer was used as the membrane bioreactor at 40 C. The glycerol solution, containing bacterial lipase and water, was supplied continuously to 1 side of a sheet of microporous polypropylene membrane (strongly hydrophobic) and the effluent was recycled, while undiluted liquid fatty acid (oleic or linoleic) was fed continuously to the opposite side of the membrane and came in contact with a glycerol-water-lipase solution to cause the reaction. The product, glycerides, was obtained at the outlet, in a pure state, with no other phase. Highest conversion (ca. 90%) was obtained when the water content of the glycerol solution was 3-4%. As the accumulation of water produced by the reaction lowered the conversion, molecular sieves in a column that the glycerol solution passed through were used for optimal water content. The reaction could be continued at least for 1 month, yielding a conversion above 70% when 1% $CaCl_2$ was added in the glycerol solution. The main component of glycerides formed was almost equimolar amounts of mono- and diglycerides.

INTRODUCTION

Glycerol and polyglycerol esters of fatty acids are industrially prepared and commercially available as food flavors, pharmaceuticals, fragrancies and emulsifiers (1,2). The industrial processes are based on direct esterification of fatty acids with glycerol and polygycerol of various chain length in the presence of inorganic catalyst at elevated temperatures (200-250 C). The chemical reaction is tedious, nonselective and consumes a large amount of energy. Furthermore, the product obtained usually needs further purification through a bleaching process (3).

In recent years, the synthesis of glycerides by lipase or cell-bound emzymes has received keen attention (4-6). The enzymatic process has certain advantages over the chemical process because the former involves mild reaction conditions and stereo and positional specificities, saves energy and has enormous catalytic activity. Because lipase is soluble in an aqueous glycerol solution, but not in higher fatty acid, enzymatic synthesis of glycerides has been carried out in an emulsion system where the reaction takes place at the interface of oil droplets and aqueous glycerol solution.

Such a conventional emulsion system for the industrial synthesis of glycerides (and for the hydrolysis of fat as well) has, however, certain drawbacks. First the emulsification of oil needs surfactant and a large power input, e.g., stirring at high speed. Second, after the reaction has finished, the separation of emulsified oily products is complicated and needs powerful centrifugation. Third, the reaction system of free lipase plus emulsified substrate is economically difficult to operate continuously. Fresh lipase must be continuously supplied to the bioreactor because the lipase adsorbed on the surface of the emulsified oil droplets is lost from the bioreactor with the droplets of products. Fourth, the immobilization of lipase involves restricted diffusion of substrate and product. One can easily imagine that particulate droplets have limited access to the enzyme, resulting in a very low reaction rate. The activity of immobilized lipase is commonly only several percent of the original activity of the free lipase. Last, control of the water content of the reaction system is difficult. The result is very important because the catalytic action of lipase is reversible. Lower water content favors the synthetic reaction, whereas, at a higher water content, hydrolysis prevails.

These limitations present obstacles to the application of lipase as a successful industrial biocatalyst in glyceride synthesis. In view of the above disadvantages of the emulsion system, an attempt has been made to carry out the continuous synthesis of glycerides from glycerol and liquid fatty acids by a lipase in a nonemulsion system. A special bioreactor, with a hydrophobic microporous membrane, has been developed for the purpose. In the present article, the configuration of the bioreactor, optimal conditions of operation and the operational stability of the lipase are described for the continuous synthesis of fatty acid glycerides.

CONFIGURATION OF THE BIOREACTOR

One unit of a plate-type dialyzer, made of plastic material (Hospal Hemodialyzer RP-6), was used as the microporous membrane bioreactor. The setup of the bioreactor is shown in Figure 1. A frame was covered with membranes (the size of 1 sheet of membrane was 11.6 cm × 31.3 cm), making a narrow compartment. The membrane is made of

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