AN ABSTRACT OF THE THESIS OF

SHUN YIH LIN for the degree of Doctor of Philosophy in Pharmacy presented on August 2, 1990. Title: Calcium Alginate Gels In Oral Dosage Form Design.

Redacted for Privacy

Abstract approved: _____

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In vivo research following ingesting of commercially available Lactobacillus tablets, which contain about $2X10^6$ cfu/tablet of Lactobacillus acidophilus and Lactobacillus bulgaricus cells in a dose of four tablets daily, showed serum lipoprotein concentrations did not change significantly. In order to increase the number of viable Lactobacillus bacteria after challenging in low pH solution (gastric fluid), enteric coating polymer was applied over dried calcium alginate beads containing Lactobacillus. Survival of Lactobacillus bacteria was generally higher from freeze dried calcium alginate beads compared to vacuum dried products. However, after pretreatment with simulated gastric fluid (pH = 1.5) for 2 hours, only the coated products from vacuum drying showed promising results. Lactobacillus bacteria were fully protected against gastric pH after formulating the bacteria inside mini-tablets which were coated with Eudragit L30D, an enteric coating polymer.

Alginic acids are naturally occurring substances found only in the brown seaweeds. Alginic acid salts formed with most di-, and polyvalent metals are insoluble in water. The most common application of alginate

precipitation in drug product formulation is based on insolubilization of alginate by addition of calcium salt. By altering the composition of calcium alginate, drug loading, enteric coating thickness, and sustained release coating thickness, the lag time for drug dissolution can be controlled. This formulation research provides oral dosage form design for targeted delivery of drug to any desired site in the gastrointestinal tract. Examples of site specific targeted delivery are given for Lactobacillus bacteria, ibuprofen, sulfasalazine, and 5-aminosalicylic acid.

CALCIUM ALGINATE GELS

IN

ORAL DOSAGE FORM DESIGN

BY

SHUN YIH LIN

A THESIS

SUBMITTED TO

OREGON STATE UNIVERSITY

IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR THE

DEGREE OF

DOCTOR OF PHILOSOPHY

COMPLETED AUGUST 2, 1990

COMMENCEMENT JUNE, 1991

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Redacted for Privacy

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Date thesis is presented August 2, 1990.

ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my parents for their love, encouragement and support. I would like to thank Dr. James W. Ayres and Dr. William E. Sandine for their assistance and advice throughout my graduate studies. I would like to thank my fellow graduate students for their help and friendship. Last, my greatest thanks goes to Yaling Lee. Her understanding and love make this meaningful.

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INTRODUCTION

Many techniques for drug encapsulation are becoming well developed and are now generally available to researchers. While the study of new approaches and materials will undoubtedly continue, the specialized use of alginate delivery systems for drugs should represent an important area in oral dosage form design. Chapter I of this thesis deals with use of traditionally formulated Lactobacillus bacteria to decrease cholesterol concentrations both *in vitro* and *in vivo*. Chapter II and Chapter III deal with utilization of calcium alginate gels in developing novel oral dosage forms which can be used for lactic acid bacteria or colonic drug delivery.

CHAPTER I

LACTOBACILLUS EFFECTS ON CHOLESTEROL: *IN VITRO* AND *IN VIVO* RESULTS

ABSTRACT

A double-blind investigation was conducted on the influence of a commercially available tablet containing Lactobacillus acidophilus and Lactobacillus bulgaricus (Lactinex™, Becton Dickinson Microbiology Systems, Cockeysville, MD) on human serum lipoprotein concentrations. Tablets containing about $2X10^6$ viable bacteria of Lactobacillus mixtures or placebo tablets were ingested by 354 nonfasting informed subjects in a dose of one tablet each, taken four times a day. There was a 3-week washout period between two 6-week treatment periods. The number of viable lactobacillus in unused returned tablets was the same at the end of the study as in the beginning. Analysis of paired data using Wilcoxon signed ranks test showed no major effects on lipoprotein concentrations for either the placebo-treated group or Lactobacillus-treated group. There were no statistically significant differences for low density lipoprotein concentrations between the lactobacilli-treated group and the placebo-treated group. The high-density lipoprotein concentrations increased 1.8 to 3.0 mg/dl in both groups for both study periods. For total cholesterol the placebo-treated group experienced a statistically significant increase in the first period according to the Wilcoxon signed-ranks test (from 208.0 mg/dl to 215.0 mg/dl, $P < 0.001$), but not according to a two samples Student T test. Total cholesterol did not change statistically significantly for the Lactobacillus-treated group in either period. Cardiac risk factor (RATIO of total cholesterol to high-density cholesterol) did not vary during the study. Lipoprotein values increased immediately following vigorous exercise compared with

following 15 minutes of resting without either placebo or treatment. Sample controls for assay and reassay gave virtually identical values (coefficient of variation 1.6%), confirming that assay results were quite reliable. Thus, ingestion of commercially available Lactobacillus tablets which contain about $2X10^6$ cfu/tablet of L. acidophilus and L. bulgaricus cells in a dose of four tablets daily did not affect serum lipoprotein concentrations.

INTRODUCTION

Americans generally have relatively high cholesterol levels that appear to enhance the risk of coronary heart disease (CHD). Thus, the goal of decreasing plasma cholesterol levels is receiving more emphasis. It has been established that lowering elevated blood cholesterol (specifically low-density lipoprotein cholesterol) will reduce the risk of heart attack due to CHD. Although dietary change is the first line of therapy in most patients with hypercholesterolemia, drug treatment must sometimes be considered. Currently, the American Heart Association (AHA) recommends that aggressive effects be made to reduce cholesterol in a full 25% of the American population (1).

In 1974, Mann and Spoerry observed that feeding Masai warriors large amounts of fermented whole milk lowered serum cholesterol (2). Mann hypothesized that dairy products contain a cholesterol-lowering "milk-factor". He proposed hydroxymethylglutarate (HMG) as a possible "milk factor", but it has not been demonstrated to be present in milk (3). Since then, others have reported that consumption of large amounts of dairy products (fermented or unfermented) lowered serum cholesterol in United States subjects (4, 5, 6). Several compounds found in milk, including orotic acid, uric acid, and bacterially produced substances have been hypothesized to be hypocholesteremic agents (7, 8, 9). Not all studies have shown that dairy products lower serum cholesterol (10, 11). Explanations for inconsistent findings are that amounts of hypocholesteremic compounds naturally present may be different in different milks, or a factor may be produced by only certain bacteria in

fermented milk.

Wostmann et al. (12) found an accelerating effect of normal intestinal microflora on systemic cholesterol catabolism and elimination in rats. This conclusion was based on lower systemic cholesterol in rats in a normal environment compared with systemic cholesterol in rats in a sterile, microbe-free environment. Mott et al. (13) mono-contaminated germ-free pigs with Lactobacillus acidophilus before moving them to a nonsterile environment and found that normal intestinal bacteria could lower serum cholesterol concentration in cholesterol-fed piglets. Harrison et al. (14) demonstrated that bottle-fed infants experienced decreased serum cholesterol with added bicarbonate or L. acidophilus in their milk formula. In 1977, Gilliland et al. (15) observed deconjugation of bile acids (both glycocholate and taurocholate) by L. acidophilus in vitro and suggested that an increased excretion of bile acid might lead to a faster rate of catabolism of cholesterol to bile acids. In 1985, Gilliland et al. (16) found that certain strains of L. acidophilus could assimilate cholesterol in the presence of oxgall (<0.5%) during anaerobic growth in MRS broth. He demonstrated different bile tolerance and cholesterol assimilation by different strains of L. acidophilus, and compared the ability of two different strains of this bacterium to decrease serum cholesterol in 5-week-old pigs. Massey et al. (17) stated that yogurt significantly reduced fasting serum cholesterol by 10 to 12% in human adult males initially, but serum cholesterol returned towards control values with continued yogurt consumption (about 2 weeks later).

The purposes of this investigation were: 1) to study in vitro

uptake of cholesterol by two bacteria utilized in a commercially available tablet; and 2) to study the *in vivo* effect of the two bacteria (*L. acidophilus* and *L. bulgaricus*) on serum cholesterol in humans when administered as a tablet which contained an equal proportion of the bacteria (approximately 10^6 or 10^7 total viable cells per tablet). The *in vivo* study involved an initial small group followed by a double blind large group trial using commercially available tablets.

MATERIALS AND METHODS

Lactobacilli and Cholesterol Assimilation from Phosphate Buffer Solution

Source and Maintenance of Cultures.

A commercially available product (Lactinex™ brand tables) that contained L. acidophilus (ATCC 4962) and L. bulgaricus (ATCC 33409) was blended using 5 tablets in 100 ml autoclaved 0.1% peptone water. These organisms are present in a ratio of approximately 1:1 and are described in the ATCC Catalogue of Bacteria, Phages, rDNA Vectors (16th edition, 1985) available from The American Type Culture Collection (Rockville, MD). A 1-ml aliquot was transferred to a culture tube containing 20 ml MRS broth (Difco, Detroit, MI). After 24 hr incubation at 37° C under anaerobic conditions using a GasPak (Becton Dickinson Microbiology Systems, Cockeysville, MD) jar, 1 ml of this subculture was transferred to another culture tube for another 24 hr of anaerobic incubation. Then 1 ml was transferred to a culture tube containing 20 ml MRS with 0.15% oxgall (Difco, Detroit, MI). One ml of subculture from the 0.15% oxgall MRS broth was inoculated into 20 ml MRS broth and incubated for 24 hr. This subculture was treated as a stock subculture for testing cholesterol assimilation by the commercially available bacteria.

Assimilation of Cholesterol.

Bacterial stock cultures were grown in three flasks containing 9 ml MRS broth for 24 hr in a 37° C walk-in incubator with stirring (Lab-line Multi-Magnestir, Lab-line Instruments Inc., IL). These subcultures were centrifuged at 12,000 rpm, 0 to 4° C for 15 min, then the pellets were resuspended and recentrifuged twice using 20 ml phosphate buffer

solution, pH=7 [413 ml monopotassium phosphate (9.073 g/l) mixed with 587 ml disodium phosphate anhydrous (11.87 g/l)] to wash the cells. The final pellet was resuspended in 20 ml buffer solution, and 1 ml of this suspension was inoculated into flasks which contained 18 ml buffer solution with or without 2 ml PPLO serum fraction (pleuropneumonia-like organism serum fraction, control 751044, Difco, Detroit, MI), and different percentages of oxgall (0, 0.1, or 0.2%). The PPLO serum fraction was used as a source of cholesterol. The concentration of bacteria after inoculation into solution was about $2X10^8$ cfu/ml. These flasks were incubated with stirring in a walk-in incubator at 37° C for another 20 hr. No effort was made to produce anaerobic conditions. Cells were removed from the broth by centrifuging for 20 min at 12,000 rpm and 1-4° C. The supernatant was collected in a test tube and analyzed for cholesterol concentration and for pH change. Deionized water (20 ml) was used to resuspend the cell pellet which was recentrifuged for 10 min at 12,000 rpm and 1 to 4° C. Then the pellet was resuspended in 4 ml deionized water. The cell suspension was filled into a Manual-Fill Mini-Cell (FA-003) and pressed at 1,000 psi using a French-Pressure Cell Press (SLM Instruments Inc., Urbana, IL). Pressed suspension was collected in a test tube and centrifuged. The supernatant was collected to analyze for cholesterol, which was released from inside the cells by the French Pressure Cell treatment.

Standard Curve for Cholesterol Calibrator.

An enzymatic procedure available from Sigma Chemical Co., St. Louis, MO was used for testing total cholesterol and high density cholesterol. The analysis kit contained both cholesterol reagent (Lot.

37F-6189) and cholesterol calibrator (50 mg/dl, Lot. 37F-6114 and 200 mg/dl, Lot. 116F-6165). The macro-method for total cholesterol (> 1-ml reaction volume) was followed according to the Sigma manual. Cholesterol concentration in the sample was determined from absorbance at 500 nm (A_{500}) as compared with a standard curve to determine concentrations of cholesterol using the commercially available kit. Different known concentrations were made from Sigma's cholesterol calibrator, 400 and 200 mg/dl, by diluting them to produce 2000, 1500, 1000, 750, 500, 250, 125, 100, or 75 mcg/ml. These different concentrations of cholesterol were measured at A_{500} using a Beckman DU40 spectrophotometer (Beckman Instruments, Palo Alto, CA) and the cholesterol assay kit.

Sample Analysis.

Cholesterol analysis was modified as follows: 1 ml sample was added to 9 ml deionized water and mixed for about 30 s; then 1 ml was mixed with 4 ml deionized water. One ml of the final diluted solution was added to a cuvette followed by one ml cholesterol reagent. The final concentration of sample in the cuvette was the same concentration as Sigma suggests for testing total cholesterol in the sample. This method avoids measuring 10 mcg/mcl (a small volume) directly into the cuvette.

Ability of Bacteria in Study Tablets to Lower Cholesterol

Experimental design.

This study was conducted to determine if either Lactobacillus or placebo tablets would remove cholesterol from media in vitro. Four screw cap test tubes were used, each containing 5 ml double strength MRS

broth (Difco). They were incubated for 23 hr at 37° C under anaerobic conditions using the GasPak anaerobic system (BBL). After incubation, tubes were autoclaved for 15 min, and then 5 ml PPLO serum fraction (Pleuropneumonia-like organism serum fraction, Difco) was added into each tube using a disposable plastic syringe. Two tubes were used at 0 hr to measure the cholesterol concentration in the medium. Samples were collected at 23 hr and 45 hr after incubation under anaerobic conditions to measure the cholesterol concentration and plate viable bacteria on MRS agar by series dilution in 0.1% peptone water. These were "blanks" or "controls" as no bacteria or tablets were present. Procedure and materials were the same when lactobacilli were used for each sample. Five tablets from each group were blended for 30 s with 99 ml autoclaved 0.1% peptone water, and 1 ml of this suspension was added to each test tube. The amount of cholesterol present at time zero was assumed to be the same as for the blanks, and the concentration was calculated by correcting for the volume difference.

Preliminary In Vivo Trial

Product Information.

A commerical product was studied that contained L. acidophilus and L. bulgaricus and available without prescription (Lactinex™) for both adults and children to treat diarrhea. No claim is made regarding the product's effect on serum lipoprotein levels. Each tablet contained a total of about 10^7 cfu of L. acidophilus and L. bulgaricus.

Experimental Design.

Approval for the study was granted by the Committee for Protection

of Human Subjects of Oregon State University. Thirty-eight volunteers from the Oregon State University faculty and staff fitness class signed an approved informed consent form and were involved in this open study (see Table I.1). The study was designated "open" because there was no attempt to control exercise, weight, or diet. The only instruction was in regard to consumption of study tablets. Fifteen subjects were in a control group which followed their normal daily diet without any tablet treatment. The treatment group contained 23 subjects who followed their normal daily diet plus one Lactobacillus tablet before each meal. Tablets were taken three times a day, before meals with fruit juice or water, and could be chewed before swallowing.

Blood samples were collected by licensed nurses and analyzed by personnel at Good Samaritan Hospital (Corvallis, OR) using the Boehringer Mannheim Diagnostics/Hitachi system H-704 (Boehringer Mannheim Diagnostics Division, Indianapolis, IN). Subjects were required to fast at least 12 hours before blood collection. Subjects were required to return unused tablets and report any noticeable side effects at the end of each study period.

Table I.1 Preliminary in vivo trial treatments time schedule.

DATE	CONTROL GROUP (n= 15)	TREATMENT GROUP (n=23)
December 6, 1986	first blood sample	first blood sample
December 16, 1986	seminar[a]	seminar[a]
January 13, 1987	normal diet	normal diet +
(a 7-week period)		Lactobacillus tablet
March 2,1987	second blood sample	second blood sample
March 29, 1987[b]	normal diet	normal diet +
(a 9-week period)		Lactobacillus tablet
June 5, 1987	third blood sample	third blood sample

[a]The seminar was given by a physician from the local hospital to explain to subjects the computer printout of cholesterol data and the relationship of cholesterol to cardiac heart diseases.

[b]There was no Lactobacillus tablet treatment between March 2 and March 29, 1987.

Double-Blind Test of Lactobacillus Tablet Effects on Serum Cholesterol

Product Information.

Tablets were supplied in coded sets containing the Lactobacillus mixture or placebo tablets. Each code number was for 200 tablets divided into 4 plastic bottles of 50 tablets each, which had only the code number shown outside. Code number "xxx" was assigned to only one person, with 1-xxx given the first period of study (beginning February 16, 1988) and 2-xxx administered the second period of study (beginning April 19, 1988). Thus, subjects received two different treatments in these two periods in randomized sequence. Those who received Lactobacillus tablets in the first period received placebo tablets in the second period, and vice versa. A letter which contained the code information was retained in a sealed envelope and not opened until the end of this study (June 2, 1988).

Experimental Design.

Approval for the study was granted by the Committee for Human Subjects of Oregon State University and each subject signed an approved informed consent form. Dosage instructions were to take one tablet three times a day with meals plus one tablet at bedtime. This is lower than the dosage recommendation as presently marketed. Subjects with lactose intolerance or sensitivity to milk products including yogurt were excluded. Subjects were assigned a code number as they entered the study. There were 460 people at the beginning of the study. Most subjects were working on the campus of Oregon State University or were a spouse of an employee. Some subjects withdrew from the study because of preceived side effects, or were out of town, or generated incomplete

cholesterol data (major reason) by missing a sampling time. The final number of subjects was 334, including only 72 subjects from the faculty and staff fitness class.

The first period started February 16 and ended March 30, a 6-week study period. Unused tablets were collected and counted to calculate percentage of compliance. A 3-week wash-out period without any treatment was allowed to reduce residual treatment effects (if any), and to reset the baseline for a second period of study (cross-over). The second portion of the study started April 19 and ended June 2. Again, unused tablets were collected and counted. Subjects were required to report side effects at the end of each period. Some blood samples were divided into two portions, and analyzed on the day collected and a day later to test stability and storage effects on measured cholesterol concentrations.

The effect of resting vs. exercise on measured lipoprotein concentrations was also determined. Fourteen subjects who participate in a 45 minute aerobic exercise class on a routine basis participated. Subjects sat quietly for 15 minutes prior to collection of blood for lipoprotein assay and then exercised vigorously for 45 minutes, and then blood was again collected for lipoprotein assay. All samples were assayed at the same time in random fashion.

Statistical Analysis.

Data including total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL), triglycerides (TRI) and the ratio of TC/HDL (cardiac risk factor) were analyzed using the two-tailed Wilcoxon signed ranks test.

RESULTS AND DISCUSSION

The Lactobacillus product utilized contained two species of bacteria, L. acidophilus (ATCC 4962) and L. bulgaricus (ATCC 33409). Subculture of these two strains was inhibited by oxgall since the optical density (at A_{650}) of the fermentation medium after 20 hours decreased from 1.4 to 1.0 to 0.8 to 0.3 as oxgall increased from 0 to 0.1% to 0.15 % to 0.2%, respectively. Optical density is directly related to number of viable cells produced (see Table I.2, OD footnote). The MRS medium tested positive for cholesterol, and as expected, there was no change after 20 hr incubation at 37°C with no bacteria. It may be that steroids in MRS medium react with the cholesterol test reagent. After adding PPLO serum fraction to MRS medium (with no bacteria) with different percentages of oxgall, the assayed cholesterol concentrations did not change with time (treatments 3, 7, 11, 15, and 19, Table I.2). However, Lactobacillus bacteria decreased cholesterol from the supernatant and were most effective when there were higher viable bacterial numbers in the MRS medium than with lower bacterial numbers (Treatments 2, 4 and 8 versus 6, 10 and 16, Table I.2). Because oxgall is a normal bile salt that inhibits growth, especially L. bulgaricus, it can be expected that the ability of these bacteria to reduce cholesterol will decrease with increasing bile concentrations.

Because MRS tests positive for cholesterol, isotonic phosphate buffer solution was used to evaluate lactobacillus uptake of cholesterol to avoid the solution "background effect". It was expected that phosphate buffer solution would prevent a pH decrease caused by lactic

acid produced by lactobacilli. It is recognized that when pH in the medium drops below 5.0, the bacteria are damaged and may lose some of their metabolizing activity. With 0% oxgall, the phosphate buffer solution contained an average of 145 mcg/ml cholesterol (Table I.3). In buffer plus Lactobacillus bacteria, the cholesterol was reduced to about 13 mcg/ml or less following 20 hr incubation (Table I.3, Treatments 1 and 2). When oxgall concentration reached above 0.1% there was almost no bacterial effect to decrease cholesterol. These data again suggest the bacteria lost activity in higher concentrations of bile salts in phosphate buffer solution under the study conditions (Table I.3).

A small portion of cholesterol reduction in the supernatant was due to precipitation of cholesterol after PPLO was added to phosphate buffer solution. When PPLO was added to a flask containing autoclaved isotonic buffer solution, the solution became turbid after 30 min. Visible particles developed at the bottom of the flask, but no organisms were found when plated on MRS agar. Table I.4 shows that the cholesterol concentration (with no oxgall present) decreased an average of 40 mcg/ml after centrifuging, which is due to cholesterol precipitation. Oxgall inhibited precipitation as shown by Treatments 7 and 8 in Table I.3.

After cell walls were broken using a French-Pressure Cell Press, steroid inside the cells was released and mixed with cell fragments and precipitated particles of cholesterol (if any developed). When the suspension was centrifuged, these cell walls and any solid cholesterol particles present remained in the pellets, which were separated from the dissolved cholesterol in the supernatant. Thus, the data for "CELL" cholesterol in Table I.3 are the result of cholesterol released from

inside the cells and are not due to any precipitated cholesterol, which would be removed with cell debris. There was no detectable cholesterol in the cells from .2% oxgall medium indicating that bacterial assimilation of cholesterol was inhibited (Table I.3).

Viable bacteria were isolated from both Lactobacillus and placebo tablets. The colony type isolated from tablet code 1-001 was different from the isolate from tablet code 2-001. Colonies from tablet code 1-001 were mixed type colonies with both rough and smooth, round edges. They were short rods in chains for rough type colonies, under microscopic examination. Smooth type colonies from tablet code 1-001 appeared as two or three cocci in chains under the microscope. For tablet 2-001, only smooth type colonies appeared on the agar plate. These were very similar to the smooth type colonies in tablet 1-001. Both were gram-positive and grew in MRS broth.

Plate counts were $1X10^6$ cfu/tablet for 1-001 (Lactobacillus tablets) and $<10^4$ cfu/tablet for 2-001 (placebo tablets). Incubation for 23 hr at 37 °C was not enough time for microorganisms from tablets code 1-001 or 2-001 to propagate to above 10^5 cfu/ml (Table I.5). When the bacterial number in the broth did not increase, the cholesterol concentration did not change significantly. After 45 hr incubation, the bacterial number increased at least 1000-fold compared with time 23 hr, and the cholesterol concentration decreased about 26% for tablet 1-001. It appears that double strength MRS broth diluted 1:1 with PPLO may delay growth of bacteria from tablet 1-001 as there were only $10^{4.9}$ cfu/ml at 23 hr for 1-001. Dead cells from tablet 1-001 or 2-001 did not change the total amount of cholesterol in the broth (Table I.5).

Thus, the bacteria assimilated cholesterol only when they were alive and at numbers above 10^8 cfu/ml.

Table I.2 Effect of Lactobacillus subcultures on cholesterol in MRS broth found after incubation 20 hours at 37°C.

TREATMENT	OD	Cholesterol Concentration SUPERNATANT[a]	SUPERNATANT[b]	% R[c]	CELL[d]
1. MRS	-	77	77	0	-
2. MRS+LAC	1.4	84	65	22.6	26
3. MRS+CHO	-	120	120	0	-
4. MRS+CHO+LAC	1.6	130	80	38.5	39
5. MRS+0.1%OX	-	84	84	0	-
6. MRS+0.1%OX+LAC	1.0	84	71	15.5	26
7. MRS+CHO+0.1%OX	-	140	140	0	-
8. MRS+CHO+0.1%OX+LAC	1.4	140	75	46.4	65
9. MRS+0.15%OX	-	77	77	0	-
10. MRS+0.15%OX+LAC	0.8	84	71	15.5	20
11. MRS+CHO+0.15%OX	-	120	120	0	-
12. MRS+CHO+0.15%OX+LAC	1.3	130	110	15.4	26
13. MRS+0.2%OX	-	84	84	0	-
14. MRS+0.2%OX+LAC	0.3	84	84	0	6
15. MRS+CHO+0.2%OX	-	135	135	0	-
16. MRS+CHO+0.2%OX+LAC	0.8	140	130	7.1	13
17. MRS+0.3%OX	-	84	84	0	-
18. MRS+0.3%OX+LAC	0.1	84	84	0	0
19. MRS+CHO+0.3%OX	-	130	130	0	-
20. MRS+CHO+0.3%OX+LAC	0.4	135	130	3.7	5

Table I.2 (continued)

MRS: MRS broth 20 ml

OX: Bacto oxgall (a bile salt)

LAC: Lactobacilli bacteria (inoculum one ml 10^8 cfu/ml)

CHO: 2 ml PPLO serum fraction (cholesterol concentration 1460 mcg/ml)

a: Total cholesterol concentration (mcg/ml) at 0 hr

b: Total cholesterol concentration (mcg/ml) after 20 hr incubation

c: Percentage of reduction by comparing the loss of cholesterol concentration in supernatant (time 0 hr to 20 hr).

d: Total cholesterol in the cells after 20 hr incubation as released by French Press treatment.

OD: Optical density (at 650 nm). Note that OD is related to number of viable cells as shown by the Table below. OD was determined after 20 hr incubation.

OD	CFU/ml (approximate)
0.05	$10^{6.2}$
0.25	$10^{7.2}$
0.50	$10^{8.0}$
1.00	$10^{8.8}$
1.45	$10^{9.2}$

Table I.3 Effect of Lactobacillus bacteria ($2X10^8$ cfu/ml) in phosphate buffer solution on concentration of cholesterol found after incubation at 37° C for 20 hours.

TREATMENT	pH[a]	SUPERNATANT[b]	SUPERNATANT[c]	CELL[c]
1. BUF+0%OX+CHO+LAC	7.23	142	13	77
2. BUF+0%OX+CHO+LAC	7.22	148	0	90
3. BUF+0%OX+LAC	7.26	0	0	0
4. BUF+0.1%OX+CHO+LAC	7.18	148	52	52
5. BUF+0.1%OX+CHO+LAC	7.26	142	103	39
6. BUF+0.1%OX+LAC	7.21	0	0	0
7. BUF+0.2%OX+CHO+LAC	7.24	135	135	0
8. BUF+0.2%OX+CHO+LAC	7.23	148	148	0
9. BUF+0.2%OX+LAC	7.27	0	0	0

BUF: 18 ml Phosphate buffer solution

OX: Bacto oxgall

CHO: PPLO serum fraction

LAC: Lactobacilli bacteria

a: pH value after 20 hr incubation.

b: Cholesterol concentration at 0 hr (mcg/ml).

c: Cholesterol concentration after 20 hr incubation (mcg/ml).

BUFFER SOLUTION: 413 ml monopotassium phosphate (9.073 g/l) mixed with 587 ml disodium phosphate anhydrous (11.87 g/l)

Table I.4 Effect of centrifugation on the concentration of PPLO cholesterol found in the supernatant at 0 hr and after holding for 20 hr at 37° C.

FLASK No.	Supernatant (mcg/ml) 0 hr	Supernatant (mcg/ml) 20 hr
1	201	156
2	188	136
3	175	162
4	214	143
5	208	162
6	208	162

The average concentration for 0 hr was 201 (mcg/ml)

The average concentration for 20 hr was 156 (mcg/ml)

Table I.5 The hypocholesterolemic effect of viable and dead bacteria from tablets in double strength MRS broth incubated at 37°C.

	0 hr			23 hr			45 hr		
TREATMENT	TC[1]	%[2]	CFU[3]	TC	%	CFU	TC	%	CFU
MRS + PPLO									
BLANK	426	0	-	412	-3	<4	412	-3	<5
1 001[4] D[5]	387*	0	-	412	+6	<4	412	+6	<5
1 001 V[6]	387*	0	-	368	-5	4.9	287	-26	8.7
2 001 D	387*	0	-	-		-	390	+1	<5
2 001 V	387*	0	-	390	+1	<4	390	+1	<5

1: Cholesterol concentration (mcg/ml) in MRS broth determined at wavelength 500 nm using Beckman DU-40 and a Sigma cholesterol test reagent. Numbers shown in this column are the average of two readings.

2: The percentage change of cholesterol concentration compared to time 0 hr.

3: Colony forming units/ml. Numbers in this column are the logarithm.

4: Tablet coded number.

5: Dead cells.

6: Viable cells.

*: The number was calculated from 426*10/11 = 387, which corrects for sample dilution.

Preliminary In Vivo Trial

For the control group of 15 subjects, the mean concentration of TC, LDL, HDL, and TC:HDL for December, March, and June were not statistically significantly different over time (Table I.6). Thus, serum cholesterol concentrations were constant in the control group for this preliminary in vivo trial. The TRI and VLDL did not change significantly between December and March, but decreased statistically significant during March to June (Table I.6). In the 23 subjects of the Lactobacillus tablet-treated group, the mean concentration of TC and LDL reduced statistically significantly in March and June compared with December. Concentrations of HDL, TRI, and VLDL did not change significantly (Table I.6).

These results are different from those reported with feeding of yogurt where the observed decrease in cholesterol is transient, lasting only 2 or 3 wk (17). It is especially interesting to note that TC:HDL ratio (known as the "cardiac risk factor") was unchanged in the control group but decreased with time in the Lactobacillus treatment group, becoming quite significant when December and June data are compared (Table I.6). It seems unlikely that diet or exercise changes lowered cholesterol in the Lactobacillus treatment group but not in the control group. However, that possibility must be considered. In addition, the control group had an average initial TC of only 192 mg/dl compared to 221 mg/dl for the Lactobacillus-tested group (Table I.6). Lower values of TC are more difficult to reduce than higher values. Also, the subjects in this preliminary study were aware of the treatment given, which could bias their behavior and the results.

Table I.6 Effects of lactobacilli on serum lipoprotein concentrations in preliminary trial.

TIME	TC	LDL	HDL	VLDL	TRI	TC/HDL
				(mg/dl)		
CONTROL (n=15)						
December	191.8	123.9	46.1	21.8	109.3	4.45
March	189.9	122.0	44.6	23.3	116.9	4.56
June	191.5	125.9	45.7	19.8[2]	99.1[2]	4.43
TREATMENT (n=23)						
December	220.6	148.6	50.4	21.5	107.9	4.76
March	203.0[1]	130.2[1]	49.0	23.9	119.9	4.48
June	208.9[2,3]	136.1[2,3]	50.9	21.9	109.6	4.38[3]

TC: Total cholesterol

LDL: Low-density cholesterol

HDL: High-density cholesterol

VLDL: Very-low-density cholesterol

TRI: Triglyceride

1: The average of March data was statistically significantly different compared to December data ($p < 0.05$).

2: The average of June data was statistically significantly different compared to March data ($p < 0.05$).

3: The average of June data was statistically significantly different compared to December data ($p < 0.05$).

The Double Blind In Vivo Test

Total Population.

A large number (334) of subjects completed the entire study and provided cholesterol data from February 1988 through June 1988. There were 157 people in the Lactobacillus tablets-treated group and 177 people in the placebo treatment group (first phase). The average starting serum total cholesterol (TC) was 208.0 mg/dl for the placebo group (Group 2) and 206.3 mg/dl for the Lactobacillus tablets (Group 1)-treated group (Table I.7, Figure I.1, February data). After 6-wk of treatment, the average TC in the Lactobacillus tablets-treated group increased to 210.0 mg/dl vs. 215.0 mg/dl for the placebo group. The HDL increased on average from 52.2 mg/dl to 54.0 mg/dl during the first study period for the Lactobacillus tablets-treated group and from 50.0 mg/dl to 53.0 mg/dl in the placebo group. There was also a small average increase of about 1 mg/dl for LDL in the first period (from 131.2 mg/dl to 132.3 mg/dl) for people receiving Lactobacillus and 2 mg/dl increase in the placebo group.

The Wilcoxon signed ranks test for paired samples was used to test differences between, before and after treatment for these three variables (TC, HDL, LDL). This test evaluates the number of subjects whose value of interest (TC, HDL, etc.) goes up compared to the number which go down. If there is no treatment effect, it is expected that there will be about the same number of increases as decreases over time. No statistically significant differences in TC or LDL occurred for the Lactobacillus treatment. The TC did statistically significantly increase according to the Wilcoxon test (208 to 215 mg/dl) in the

placebo group. That is, TC increased for 114 subjects but only decreased for 60 subjects. The average increase was 18.4 units and the average decrease was 14.8 units. The HDL was significantly increased in the first period of study for both treatments, although the actual change was quite small (Table I.7, Figure I.1). However, using a paired Student's T test, two tailed, the mean TC (=208) before placebo and after placebo (=215) was not statistically significantly different with this test; HDL in the Lactobacillus group also did not change statistically significantly (52.2 to 54 mg/dl), but HDL in the placebo group did change statistically significantly (from 50 to 53, p=0.02). These differences in statistical significance as a result of the test used suggest that a conservative (and reasonable) conclusion is that significant differences did not occur. None of the above is considered clinically significant in terms of influencing coronary heart disease.

After a 3-week wash-out period, blood samples were collected and used as a baseline of the second study period. Total cholesterol averaged 214.9 mg/dl for the Lactobacillus tablets-treated group and 211.3 mg/dl for the placebo group (Table I.8, Figure I.1, April data). At the end of the second study period, average TC increased to 219.3 mg/dl following Lactobacillus treatment and 213.4 mg/dl with placebo treatment (June data). The increases in TC were not statistically significant. The HDL increase was statistically significant with the Wilcoxin test for both groups but was actually quite small, about 3.5 mg/dl. The LDL decreased 2 mg/dl in the placebo group and was unchanged with the Lactobacillus treatment. Once again, there were no significant changes of LDL in these two groups. The ratio of TC to HDL, known as

the "cardiac risk factor", did not change significantly during the study for either treatment group (Tables I.7, I.8, and Figure I.2).

Tablets remaining at the end of each treatment were counted and bacterial number remained about 10^6 cfu/tablet for Lactobacillus tablets and less than 10^4 cfu/tablet for placebo tablets.

High Cholesterol Level Population.

Subjects whose TC concentration was equal to or greater than 240 mg/dl were assigned in this subgroup. Fifty-nine subjects qualified, 29 in the placebo group and 30 in the Lactobacillus treatment group in the first treatment period. A statistically significant change in the TC and LDL levels did not occur through the study for either group. A statistically significant increase in the HDL occurred with the Wilcoxin test in the second period of study for the Lactobacillus tablets treated group (Tables I.7 and I.8), but the mean concentration did not change significantly (t-test, $p=0.3$).

Medium Cholesterol Level Population.

Subjects whose TC was 239 to 200 mg/dl were assigned to this subgroup. There were 126 people in this subpopulation, 72 in the placebo group and 54 in the Lactobacillus treatment group in the first study period. The TC, HDL, and LDL responses were the same in the subpopulation as for the total population (Tables I.7 and I.8).

Low Cholesterol Level Population.

People whose TC was lower than 200 mg/dl were assigned in this category. There were 148 subjects who satisfied this criterion, 72 in the Lactobacillus treatment group and 76 in the placebo group in the first study. The patterns of the three main variables during the study

were the same as in the total population (Tables I.7 and I.8).

Regular Exercise Population.

There were 72 people in this study who were also in the Oregon State University faculty and staff fitness class. Subjects in this group were expected to have regular exercise every week and to have the same average physical activity compared to the subjects in the preliminary in vivo trial (38 subjects involved). The cholesterol concentration varied in the same pattern in this group as in the total study group. No significant difference existed in total cholesterol, LDL or TC:HDL (Table I.9). Subjects in this group were in the same environment as in the preliminary in vivo trial, except in the latter case the subjects did not know what kind of treatments they received during each study period. The results were different compared to the preliminary trial results in that with the larger population study there were no significant effects of Lactobacillus tablets on lipoproteins.

In a separate exercise group (14 subjects), blood lipoprotein concentrations were determined following 15 minutes of rest (sitting) prior to exercise and again immediately following 45 minutes of exercise. Figure I.3 shows that the total cholesterol increased in all subjects following exercise, and the average increase was 17.2 mg/dl. Other average lipoprotein increases were 5.1 mg/dl for HDL, 8.0 mg/dl for LDL, 4.2 mg/dl for VLDL and 21.2 mg/dl for TRI. Average weight loss due to perspiration during the 45 minutes was 1.1 pounds. The slopes of the lines in Figure I.3 are nearly parallel, suggesting a uniform effect of exercise on lipoproteins. The uniform response considered with randomized assay suggests the assay is quite reliable (also, see

Validity of Assay section) and the exercise effect is real. These results are consistent with suggestions that patients should sit quietly for 15 minutes prior to donating blood to be used for lipoprotein evaluation.

Table I.7 Effects of lactobacilli on serum lipoprotein concentrations in a cross-over, double-blind study (first 6-week period).

		SUBLECT NO.	TC[1]	HDL[2]	TRI[3]	LDL[4]	RATIO[5]
GROUP-2 (placebo treated, F= February, M= March)							
TOTAL[6]	0-wk(F)	177	208.0	50.0	119.7	134.1	4.39
	6-wk(M)		215.0	53.0	125.0	136.7	4.26
HIGH[7]	0-wk(F)	29	270.1	55.6	152.9	183.9	5.15
	6-wk(M)		264.0	55.0	156.0	177.9	5.01
MEDIUM[8]	0-wk(F)	72	220.5	50.0	126.7	104.6	4.71
	6-wk(M)		229.0	54.0	133.0	149.1	4.58
LOW[9]	0-wk(F)	76	172.6	47.8	100.4	104.6	3.84
	6-wk(M)		182.0	52.6	106.0	109.3	3.68
GROUP-1 (Lactobacillus tablets treated)							
TOTAL[6]	0-wk(F)	157	206.3	52.2	114.9	131.2	4.22
	6-wk(M)		210.0	54.0	120.0	132.3	4.10
HIGH[7]	0-wk(F)	30	259.0	55.7	139.7	175.4	4.99
	6-wk(M)		254.0	57.0	145.0	168.2	4.76
MEDIUM[8]	0-wk(F)	54	219.2	49.2	136.8	142.7	4.79
	6-wk(M)		224.0	51.0	135.0	146.6	4.69
LOW[9]	0-wk(F)	72	174.3	53.0	87.2	104.0	3.47
	6-wk(M)		181.0	55.0	97.0	106.3	3.45

Table I.7 (continued)

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1. Average serum total cholesterol concentration, mg/dl.
2. Average serum high-density lipoprotein concentration, mg/dl.
3. Average serum triglyceride concentration, mg/dl.
4. Average serum low-density lipoprotein concentration, mg/dl.
5. Cardiac risk factor, a ratio of TC to HDL.
6. Total= all subjects in this group.
7. High= subjects with their TC $\geq$ 240 mg/dl at the beginning of study.
8. Medium= subjects with their TC 239 to 200 mg/dl when beginning the study.
9. Low= subjects with their TC < 200 mg/dl when beginning the study.

Table I.8 Effects of lactobacilli on serum lipoprotein concentrations in a cross-over, double-blind study (second 6-week period).

		SUBJECT NO.	TC[1]	HDL[2]	TRI[3]	LDL[4]	RATIO[5]
GROUP-2 (Lactobacillus tablet treated, A= April, J= June)							
TOTAL[6]	0-wk(A)	177	214.9	52.2	118.6	139.0	4.38
	6-wk(J)		219.3	55.9	118.0	139.8	4.12
HIGH[7]	0-wk(A)	29	265.1	55.9	147.8	179.6	4.99
	6-wk(J)		266.9	59.7	144.8	178.3	4.68
MEDIUM[8]	0-wk(A)	72	228.2	52.2	129.8	150.1	4.73
	6-wk(J)		231.6	55.9	128.9	150.0	4.43
LOW[9]	0-wk(A)	76	183.2	50.9	97.0	112.9	3.82
	6-wk(J)		189.5	54.5	97.4	115.6	3.62
GROUP-1 (placebo treated)							
TOTAL[6]	0-wk(A)	157	211.3	53.0	108.3	136.7	4.20
	6-wk(J)		213.4	56.7	111.6	134.3	3.99
HIGH[7]	0-wk(A)	30	260.4	55.8	130.4	178.5	5.00
	6-wk(J)		261.7	58.7	148.6	173.1	4.77
MEDIUM[8]	0-wk(A)	54	223.1	50.0	122.8	148.5	4.70
	6-wk(J)		224.7	53.7	120.6	146.5	4.42
LOW[9]	0-wk(A)	72	181.9	54.2	88.1	110.2	3.60
	6-wk(J)		184.7	58.1	88.8	108.8	3.33

Table I.8 (continued)

1. Average serum total cholesterol concentration, mg/dl.
2. Average serum high-density lipoprotein concentration, mg/dl.
3. Average serum triglyceride concentration, mg/dl.
4. Average serum low-density lipoprotein concentration, mg/dl.
5. Cardiac risk factor, a ratio of TC to HDL.
6. Total= all subjects in this group.
7. High= subjects with their TC $\geq$ 240 mg/dl at the beginning of study.
8. Medium= subjects with their TC 239 to 200 mg/dl when beginning the study.
9. Low= subjects with their TC < 200 mg/dl when beginning the study.

Table I.9 Serum lipoprotein concentrations in subjects with both exercise class and Lactobacillus treatment (double-blind *in vivo* test).

TIME	TRI[1]	TC[2]	HDL[3]	LDL[4]	VLDL[5]	RATIO[6]
GROUP-1[7] (n=48)						
0-wk	111.7	200.1	48.1	129.5	22.3	3.85
6-wk	112.7	204.6	51.4	130.7	20.0	3.98
9-wk	100.0	202.5	50.9	131.6	20.0	3.98
15-wk	103.7	208.5	54.6	133.2	20.7	3.82
GROUP-2[7] (n=24)						
0-wk	96.5	198.6	51.6	127.7	19.3	3.85
6-wk	102.5	205.0	54.3	130.3	20.5	3.78
9-wk	98.6	207.0	53.5	133.8	19.7	3.87
15-wk	104.4	208.1	57.2	130.0	20.9	3.64

1: Serum plasma triglyceride concentration (mg/dl).

2: Serum plasma total cholesterol concentration (mg/dl).

3: Serum plasma high-density lipoprotein concentration (mg/dl).

4: Serum plasma low-density lipoprotein concentration (mg/dl).

5: Serum plasma very-low-density lipoprotein concentration (mg/dl).

6: Cardiac risk factor (TC/HDL).

7: Group-1 were treated with placebo tablets from Feburary to March and Lactobacillus tablets from April to June. Group-2 had the opposite treatment compared to group-1.

Figure I.1 Mean lipoprotein concentrations in group 1 and group 2. Group 1 received Lactobacillus tablets from February (F) to March (M), and placebo tablets from April (A) to June (J); vice-versa for group 2. TC: total cholesterol; HDL: high-density lipoproteins; LDL: low-density lipoproteins; 1: group 1; 2: group 2.

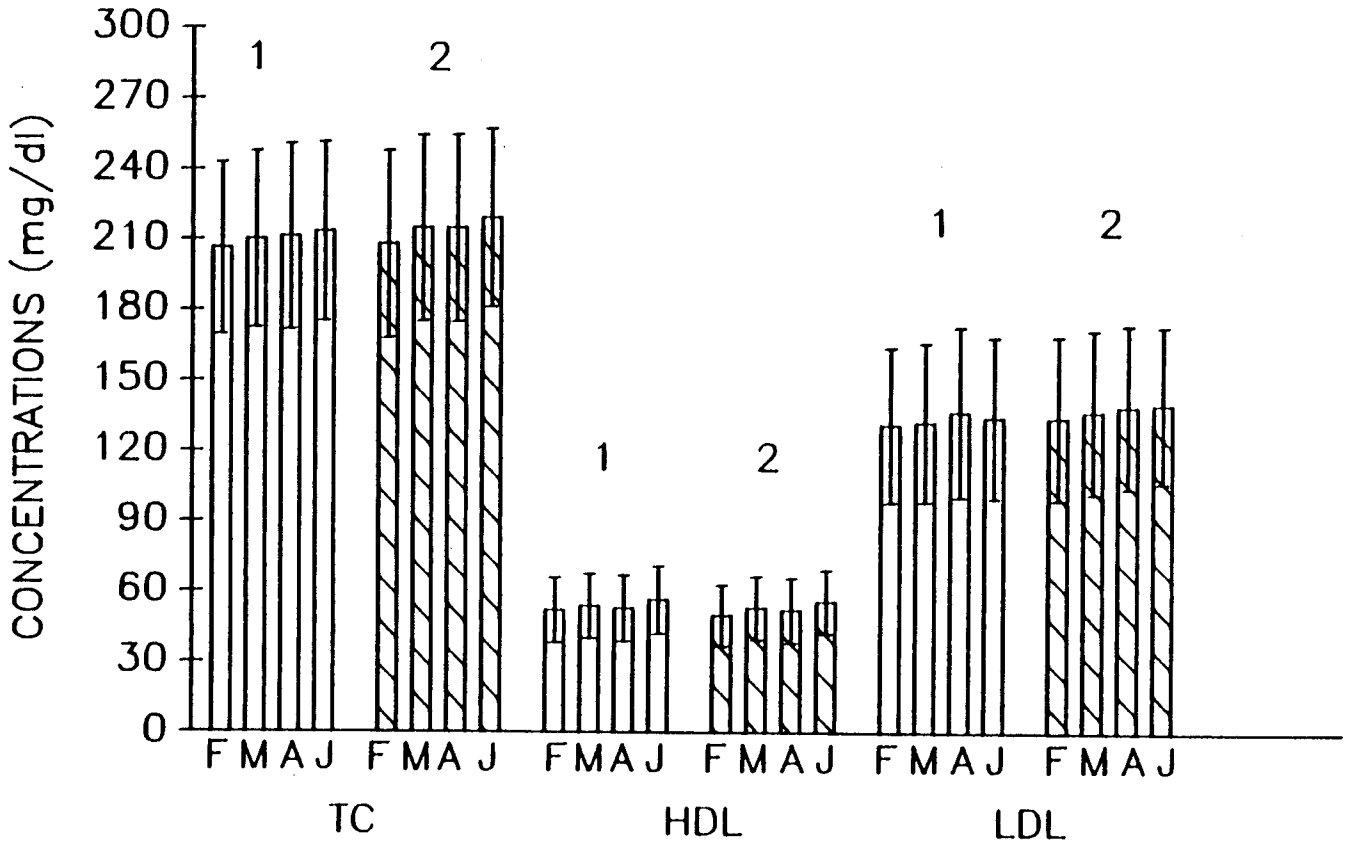
CONCENTRATIONS (mg/dl)
300
270
240
210
180
150
120
90
60
30
0
1
2
1
2
1
2
F M A J F M A J
TC
F M A J F M A J
HDL
F M A J F M A J
LDL

Figure I.1

Figure I.2 Mean lipoprotein concentrations in group 1 and group 2. Group 1 received Lactobacillus tablets from February (F) to March (M), and placebo tablets from April (A) to June (J); vice-versa for group 2. TRI: triglyceride; VLDL: very-low-density lipoproteins; RATIO: TC/HDL; 1: group 1; 2: group 2.

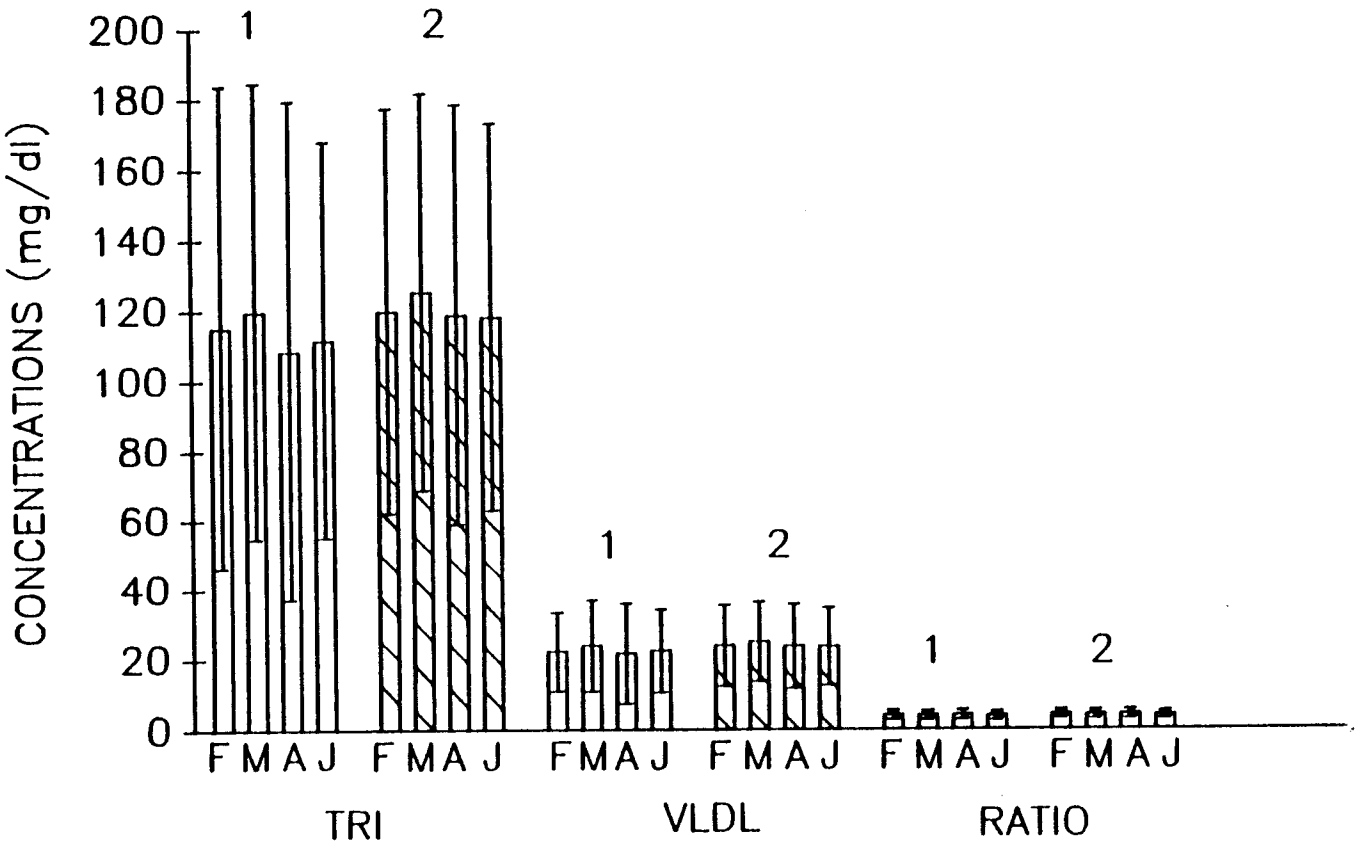
CONCENTRATIONS (mg/dl)
200
180
160
140
120
100
80
60
40
20
0
1
2
1
2
1
2
F M A J F M A J F M A J F M A J F M A J F M A J
TRI
VLDL
RATIO

Figure I.2

Figure I.3 Total cholesterol concentrations before and after exercise. ○: before exercise and following 15 minutes resting in the sitting position; ●: immediately after 45 minutes of vigorous exercise.

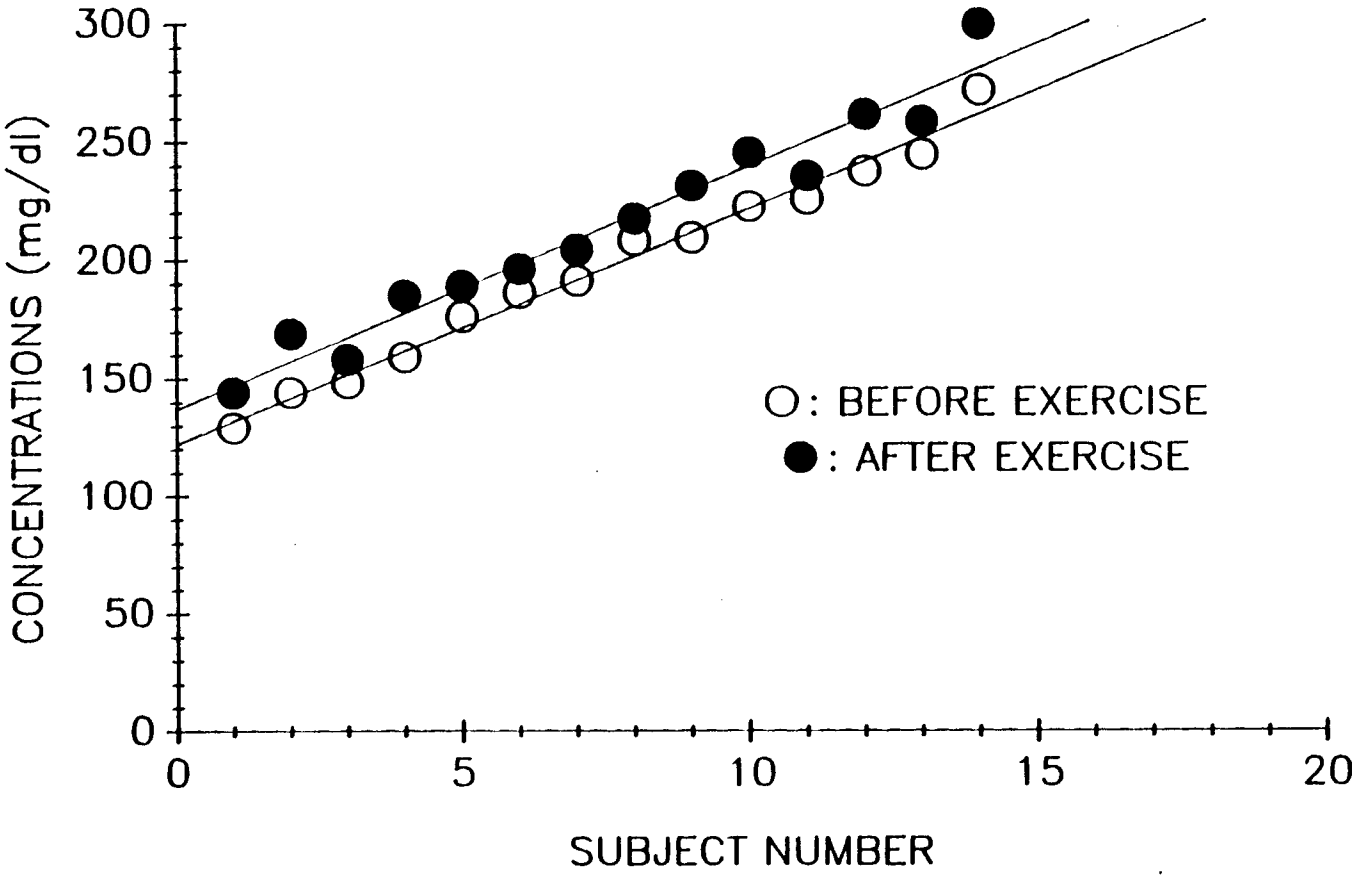
300
250
200
150
100
50
0
CONCENTRATIONS (mg/dl)
0
5
10
15
20
SUBJECT NUMBER
O: BEFORE EXERCISE
●: AFTER EXERCISE

Figure I.3

Side Effects.

There were no statistically significant differences of mean serum lipoprotein concentrations for subjects who identified side effects during the study period. About 15% of subjects who received the placebo treatment in the first study period reported side effects and this number decreased to 6% when they received Lactobacillus treatment in the second period of the study. In the other group, about 21% of subjects reported side effects when they received Lactobacillus in the first period and the number decreased to 7% when the treatment changed to placebo (Table I.10). The most common side effects reported were flatulence (gas) and diarrhea (loose stool). For both treatments, subjects reported less side effects during the second phase. Side effects reported by subjects were almost the same for both groups in both periods. Serum lipoproteins of subjects whose compliance was higher than 90% showed no statistically significant differences, as was also seen when evaluating all subjects.

Validity of Assay.

Thirty-seven blood samples were divided into two portions and analyzed both on the day of collection and the day after. The TC concentration did not vary much and no statistically significantly difference occurred between these repeat measurements (Figure I.4). According to a comprehensive chemistry survey done in 1987 supported by the College of American Pathologists (CAP), the Boehringer Mannheim Diagnostics/Hitachi systems 704 (the method used to analysis subjects blood samples) for measuring serum total cholesterol concentrations had a standard deviation less than 3%. In addition, repeated testing of CAP

cholesterol standards during each analytical day of this study gave reproductible results with a coefficient of variation of only 1.6%. Thus, assay results were quite reliable.

Table I.10 Side effects and compliance reported by subjects in both Lactobacillus and placebo tablet treated groups.

SIDE EFFECTS	FIRST 6-WEEK TREATMENT		SECOND 6-WEEK TREATMENT	
	PLACEBO (group-2)	LACTOBACILLUS (group-1)	PLACEBO (group-1)	LACTOBACILLUS (group-2)
TOTAL SUBJECTS	177	157	157	177
CONSTIPATION (firm stool)	5	6	2	1
FLATULENCE (gas)	11	15	2	3
DIARRHEA (loose stool)	10	9	6	4
STOMACH UPSET	1	3	1	2
TOTAL SIDE EFFECTS	27	33	11	10
% OF TOTAL SUBJECTS	15	21	7	6
COMPLIANCE[1]	NUMBER OF SUBJECTS			
100%	15	17	46	68
99 - 90%	99	84	50	64
89 - 80%	36	24	36	25
79 - 70%	9	11	9	8
<70%	18	21	16	12

1: Percent of tablets consumed based on the number which should be consumed determined by counting the number of tablets returned. All unconsumed tablets may not have been returned which would inflate these compliance figures.

Figure I.4 Stability and storage effects on measured total cholesterol (TC) concentration. ○ : first day measurement of individual TC (mg/dl); ● : second day measurement of individual TC (mg/dl).

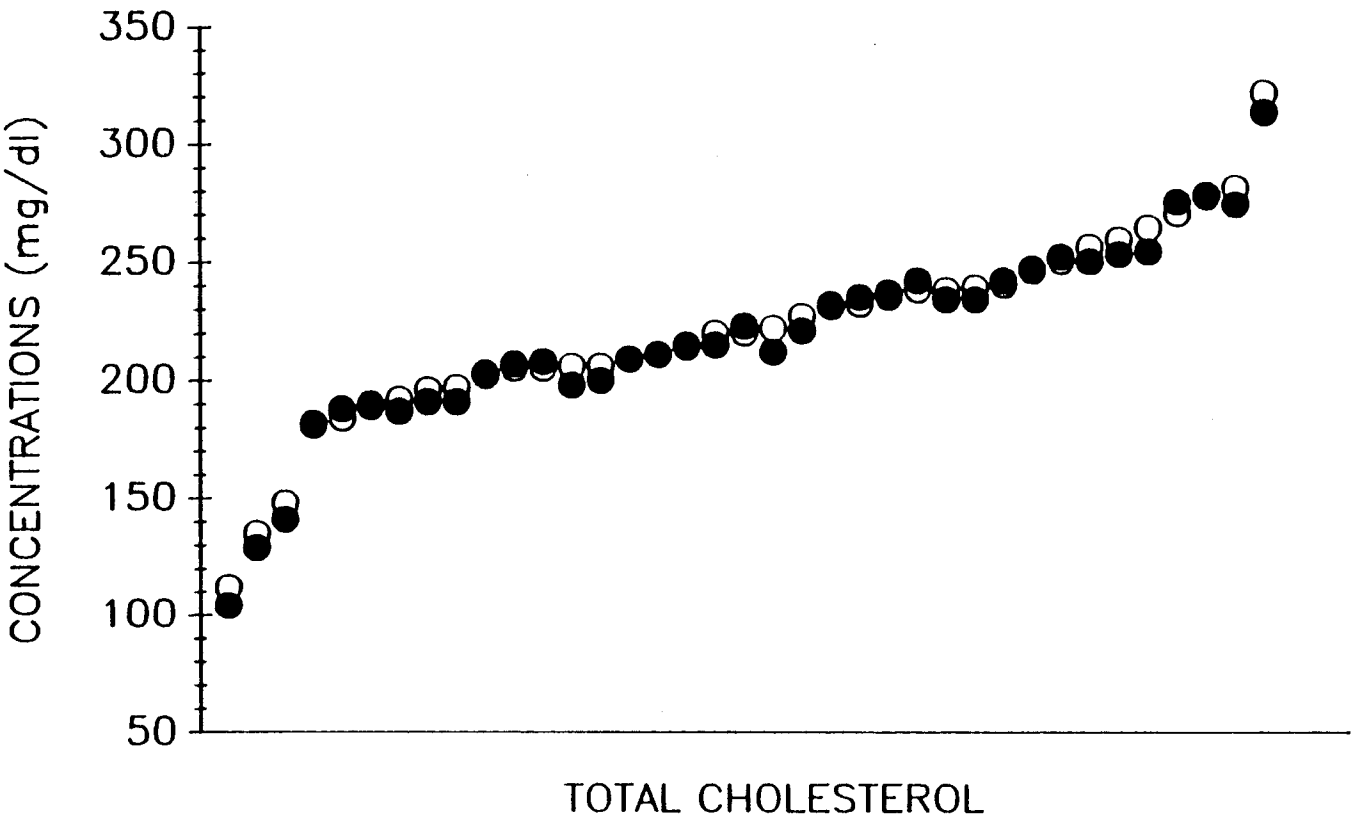
350
300
250
200
150
100
50
CONCENTRATIONS (mg/dl)
TOTAL CHOLESTEROL

Figure I.4

Generally, TC concentrations are a good reflection of LDL concentrations and both have a significant relationship to coronary heart disease. Epidemiologic studies show an inverse correlation between HDL levels and coronary heart disease in populations with relatively high levels of total plasma cholesterol. In those populations, a 1% increase in HDL correlates with (on an average) a 5% decrease in coronary heart disease risk (1). None of the large in vivo study results reported herein suggest any significant effects for either treatment. These findings are interesting in light of several reports in the literature which suggest that lactobacilli can lower blood cholesterol. Large individual variations did occur, with cholesterol levels in some people increasing as much as 50 mg/dl regardless of whether the Lactobacillus or the placebo was received. In other people cholesterol decreased as much as 50 mg/dl regardless of whether they were receiving Lactobacillus or placebo. The majority of changes were much smaller with the cholesterol changing less than 10 mg/dl in about one-half of the subjects. For example, at the end of the first placebo treatment period the average TC change was 7 mg/dl with a standard deviation of 20.4 mg/dl. Thus, although the average fluctuation in TC was small, the individual biological variation was quite large independent of treatment. The HDL changed an average of 3 mg/dl with a standard deviation of 7 mg/dl and TRI changed only about 5 mg/dl with a standard deviation of 37 mg/dl (Figures I.1 and I.2).

This is the first study where the number of participants consuming Lactobacillus was sufficiently large (334 completed the study) to prevent large individual variation from biasing the results. From the

in vitro study, when these bacteria were maintained at about 10^8 cfu/ml in the medium, they decrease cholesterol concentration substantially. However, Robins-Browne and Levine (18) reported that orally fed $2X10^8$ viable *L. bulgaricus* and *L. acidphilus* (in equal proportions) do enter the small intestine but elevated counts in jejunal fluids only persisted for 3 to 4 hr and the number of viable Lactobacillus bacteria was only 10^3 cells. Other studies (18, 19) demonstrate that bacterial strains which remain viable after passage through the human stomach may only remain in the small intestine for several hours. It is difficult to extrapolate the data reported herein involving 10^6 cfu/tablet to an expected effect with dairy products. Sweet Acidophilus milk, for example, contains at least 10^6 cfu/ml and will provide at least 3 to 8 x 10^8 bacterial cells/day if 250 to 750 ml are consumed. This is more than 100 times the dose used in the current study, but such doses are reported to not produce much change in the number of viable *L. acidophilus* in the intestine (18, 19). A major consideration in the choice of lactic acid bacteria to be used as possible hypocholesterolemic agents may be to choose a strain or dosage form that will allow establishment of large numbers of viable bacteria in the digestive tract.

REFERENCES

1. Gotto et al. 1984. Recommendations for treatment of Hyperlipidemia in Adults. Am. Heart Associ. 69:1065A.

2. Mann, G. V., and A. Spoerry. 1974. Studies of a surfactant and cholesteremia in the Maasai. Am. J. Clin. Nutr. 27:464.

3. Mann, G. V., and C. R. Nair. 1977. A factor in milk which influences cholesteremia in rats. Atherosclerosis 26:363.

4. Hepner, G., R. Fried, S. St. Jeor, L. Fusetti, and R. Morin. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19.

5. Rossouw, J. E., E. M. Burger, P. Van der Vyver, and J. J. Ferreira. 1981. The effect of skim milk, yoghurt and full cream milk on human serum lipids. Am. J. Clin. Nutr. 34:351.

6. Tompson, L. U., D. J. Jenkins, M. V. Amer, R. Reichert, A. Jenkins, and J. Kamulsky. 1982. The effect of fermented and unfermented milks on serum cholesterol. Am. J. Clin. Nutr. 36:1106.

7. Grunewald, K. K. 1982. Serum cholesterol levels in rats fed skim milk fermented by Lactobacillus Acidophilus. J. Food Sci. 7:2078.

8. Richardson, T. 1978. The hypocholesterolemic effect of milk - a review. J. Food Protect. 41:226.

9. Ward, P. C., R. D. McCarthy, and A. Kilara. 1982. Isolation of an inhibitor of hepatic cholesterolgenesis from human milk. Atherosclerosis. 41:185.

10. Hussi, E., T. A. Miettinen, A. Ollus, E. Kostiainen, C. Ehnholm, B. Haglund, J. K. Huttunen, and V. Manninen. 1981. Lack of serum cholesterol-lowering effect of skimmed milk and butter milk under controlled conditions. Atherosclerosis 39:267.

11. Pulusani, S. R., and D. R. Rao. 1983. Whole body, liver and plasma cholesterol levels in rats fed Thermophilus, Bulgaricus and Acidophilus milks. J. Food Sci. 48:280.

12. Wostmann, B. S., N. L. Wiech, and E. Kung. 1966. Catabolism and elimination of cholesterol in germfree rats. J. Lipid. Res. 7:77.

13. Mott G. E., R. W. Moore, H. E. Redmond, and R. Reiser. 1973. Lowering of serum cholesterol by intestinal bacteria in cholesterol-fed piglets. Lipids 8:428.

14. Harrison, V. C., and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr. 28:1351.

15. Gilliland, S. E., and M. L. Speck. 1977. Deconjugation of Bile acids by intestinal Lactobacilli. Appl. Environ. Microbiol. 33:15.

16. Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by Lactobacillus Acidophilus. Appl. Environ. Microbiol. 49:377.

17. Jaspers, D. A., L. W. Massey, and L. O. Luedecke. 1984. Effect of consuming yogurts prepared with three culture strains on human serum lipoproteins. J. Food Sci. 49:1178.

18. Robins-Browne, R. M., and M. M. Levine. 1981. The fate of ingested Lactobacilli in the proximal small intestine. Am. J. Clin. Nutr. 34:514.

19. Conway, P. L., S. L. Gorbach, and B. R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1.

CHAPTER II

FERMENTATION AND DOSAGE FORMULATION OF LACTOBACILLUS BACTERIA

INTRODUCTION

Lactobacillus acidophilus is one of the predominant lactobacilli present in the intestine of humans and animals (1, 2). The ingestion of lactic acid bacteria, which was initially proposed by Metchnikoff as a means to reduce intestinal putrification and prolong life (3), has been extensively investigated as a beneficial dietary adjunct for gastrointestinal disorders in humans and animals (4). More recently, Lactobacillus therapy has been advocated for the prevention and treatment of travelers' diarrhea, a condition usually associated with enterotoxigenic strains of Escherichia coli (5, 6, 7). Some workers have shown a correlation between L. acidophilus consumption and a decreased need for laxatives in constipated elderly people (8). Gilliland et al. reported a significant hypocholesterolemic effect on pigs using L. acidophilus (9). However, Lin et al. in the largest study ever conducted of Lactobacillus effects on cholesterol in people (10), demonstrated that oral lactobacilli had no significant cholesterol reduction effects. This finding occurred in spite of the authors deliberate use of a Lactobacillus mixture demonstrated to assimilate cholesterol in vitro.

Many antitumor and anticarcinogenic properties of lactobacilli also have been reported (11, 12, 13). In contrast to these reported desirable effects, many investigators have concluded that oral administration of lactobacilli is not helpful in the conditions mentioned (14, 15, 16, 17, 18). Because of the large number of reports at each end, as well as all along the spectrum of possible benefit, it

seems most likely that the studies have been variable in the number of viable lactobacilli delivered through and into the upper intestine. This variation in "bioavailability" is well known in the pharmaceutical and medical literature for drug products which are far easier to prepare than oral dosage forms of lactobacilli.

Many commercial dried L. acidophilus containing products, claiming to contain large numbers of viable L. acidophilus, are now available at retail stores for use as dietary adjuncts for humans. However, many of these products actually have very low numbers of viable L. acidophilus (19, 20). The ineffectiveness of some dried acidophilus products in counteracting GI tract maladies in humans could be related to these low numbers of viable cells in these products (15). Although drying is used as a means of preservation of bacterial cells, both drying and subsequent storage are known to be lethal to a large fraction of the cells, often resulting in > 99% kill of a given population (21). Freeze-drying of microorganisms has been practiced commercially for more than two decades. In a typical process, starter culture is frozen in liquid nitrogen at -196° C, then subjected to vacuum drying for 12 to 16 hr to yield freeze-dried starter culture with less than 1% moisture content (22).

Unfortunately, the bacterial cells are likely to lose viability both during and after freeze-drying, and after digestion if taken orally. Major factors that cause cell injury during drying are summarized by Marth (23). Among them, cold shock, change of cell wall permeability, and metabolic injury appear most important. For many microorganisms, the stomach represents a hostile environment that can

discourage bacterial growth or survival (24, 25). Several other environmental factors also affect the survival of freeze-dried bacteria; the type and concentration of the drying medium, the residual moisture of the dried product, and oxidation are considered to be the most important (26). The amount of water remaining after drying affects not only the viability of bacteria but also the rate of loss of viability during subsequent storage (27). The optimum residual moisture content varies with the composition of medium used to suspend organisms, with the storage atmosphere, and probably with the species and physiological state of the organisms (28, 29, 30). It is important to choose an appropriate suspending medium to increase the survival rate of microorganisms during and after freeze-drying. Some low-molecular-weight compounds such as sodium glutamate and aspartate are reported to protect lactic acid bacteria during freeze-drying (31).

In addition to drying, microencapsulation of bacteria has also been studied to increase shelf-life of cultures and increase durability of the cells (32). There has been considerable interest during the last decade in the immobilization of enzymes and cells. Immobilization is usually carried out by one of the following procedures: 1) covalent binding, 2) adsorption, 3) entrapment or microencapsulation, or 4) cross-liking (33). Alginic acid is a naturally occurring polysaccharide isolated from brown seaweeds and is a random copolymer of D-mannuronic acid and D-guluronic acid. One of the most useful characteristerics of alginates is the ability to form gels. A product is generally formed by first adding bacterial cells as a suspension to an aqueous solution of the gelling material. This material is then formed into droplets either

by forcing it dropwise through a nozzle or orifice or by dispersing it into a hardening liquid medium. The droplets are subsequently stabilized with entrapped organisms via polymerization or other types of crosslinking. For example, alginate droplets can be stabilized with divalent ions such as Ca^{2+}, and carrageenan droplets are crosslinked typically with K^{+}. Both of these materials interact reversibly with the cation and tend to disintegrate when it is removed. The structure of calcium alginate gels have been varyingly described as 1) porous structures, 2) honeycomb-like structures with pores ranging in diameter from 0.1 to 5 mm, 3) macroporous structures with pore diameters in the order of 0.1 mm, and as 4) solid structures (34). Calcium alginate is one of the most widely employed carriers for the immobilization of both microbial cells and plant cells (35). Such immobilized preparations have found use in three major areas: 1) for the production of biotechnologically interesting substances, 2) in analysis, and 3) in medicine (35).

Before Lactobacillus bacteria can be utilized as dietary adjuncts to produce a therapeutic effect, those bacteria must pass through stomach acidity and have a suitable transit time in the intestinal tract. Robins-Browne et al. suspended Lactobacillus bacteria in skim milk and studied the fate of ingested lactobacilli in the proximal small intestine (36). They found only 10^3 to 10^4 cfu/ml in the small intestine after ingesting $2X10^8$ cells, and the transit time was about 3 hr in fasting subjects and 6 hr in nonfasting subjects. It has been known that survival of lactic acid bacteria within the human stomach is closely related to pH, which can vary largely among individuals. The

ability of L. acidophilus NCDO 1748 to pass the human stomach was studied by Pettersson et al. (37). After consuming 500 ml of acidophilus milk which had 10^9 cfu/ml of bacteria, the number of L. acidophilus was 10^7 cfu/ml in the lower small intestine, 1.5 hr after consumption. In 1988, Kim et al. published an in vitro study of polyvinyl acetate phthalate coated L. plantarum bacteria upon exposure to simulated gastric fluids (32). After 6 hr treatment in this low pH environment (pH = 2), survival of bacteria was maintained around 100%. Uncoated L. plantarum bacteria had about 20% survival after 2 hr exposure to this low pH stress. Recently, some researchers have placed their emphasis on selection and preparation of resistant L. acidophilus strains (38, 39, 40). Kleeman and Klaenhammer reviewed the need to select strains that can survive and establish within an environment as hostile as the gastrointestinal tract (38). Conway et al. reported that the ability of various strains to survive in human gastric stomach and to adhere to human ileal cell suspensions could be enhanced by 1% skim milk (39). It seems feasible that if some minimum amount of Lactobacillus bacteria (10^7 to 10^8 cfu/g) can pass through the human stomach without being hurt, and then release quickly in the intestine, desirable therapeutic effects of the bacteria may occur.

Objectives of this study were to develop a method for preparation of a viable and stable, dry, microorganism-containing oral dosage form, which can: 1) protect bacteria against the low pH environment of the stomach, 2) have a shelf-life of about 2 years at room temperature, and 3) have about 10^8 cfu/dose. This product could be administered for the conditions identified in the introduction to this paper.

MATERIALS AND METHODS

Cultures.

Lactinex granules (Lot 1397) and Lactinex paste (Lot 4704 and 4762) were both supplied by BBL Microbiology Systems (Cockeysville, MD). These granules and paste contained equal portions of dried cultures of Lactobacillus acidophilus and Lactobacillus bulgaricus. For fermented evaporated milk cultures, overnight cultures of strains L. acidophilus (ATCC 4962) and L. bulgaricus (ATCC 33409) grown in 11% non-fat dry milk were used as starter.

Chemical Compounds.

Three antioxidants were used as received: (1) L-cysteine HCl monohydrate (LOT 95F-0614, Sigma), (2) sodium bisulfite (LOT 7444, Mallinckrodt), and (3) d-a-tocopherol (LOT 16F-0666, Sigma) which contained 1000 I.U./g vitamin E. Two different lipophilic substances were used to entrap bacteria: light mineral oil (density 0.83 g/c.c.) and Hershey's cocoa butter. Malt extract broth (Control 0113-01, Difco), glycerol, monosodium glutamate, and polyvinylpyrrolidone (mw. 40,000) were used as stabilizers. Magnesium phosphate tribasic was used as an internal pH neutralizer during cell propagation. Alginic acid sodium salt (LOT 115F-0670, Sigma) is a biodegradable polymer which was used to microencapsulate bacteria. The hardening agent was calcium chloride dihydrate (Control 4152, Mallinckrodt). Polymers used for coating were Aquateric (FMC Corp., PA), Aquacoat (FMC Corp., PA), and Eudragit L30D (Rohm Pharma, GMBH). Substances used to plasticize coating polymers were dibutyl sebacate (Lot 28F0389, Sigma), polyethlene

glycol 6000 (Lot 70F0648, Sigma), diethyl phthalate (Lot 07710JW, Aldrich), and triethyl citrate (Lot 00815LV, Aldrich).

Study-1. Effect Of Additives On Drying Lactobacillus Bacteria

Table II.1 is a list of formulations used in study-1. Three different liquid phase combinations were investigated (water only, water + liquid oil, and water + solid oil) for their influence on Lactobacillus survival in study 1. For solid oil phase products, cocoa butter was melted in a water bath with stirring and the temperature was maintained close to 40° C. After the cocoa butter melted completely, the temperature was lowered to 37° C, followed by variable addition of other ingredients as identified by superscripts in Table II.1 (see footnote to Table II.1). The water phase and remaining formulation ingredients (Table 1) were mixed and kept in a ice bath with stirring. With the temperature in water cooled to 4° C, the oil phase (cocoa butter) was poured into the water phase slowly to allow bacteria in the oil to be trapped in solid oil particles formed by cooling and suspending in the sodium alginate phase. Substances added into water or mineral oil were blended in a commercially available blender. When mixed with another phase (if any), stirring was continuous using a magnetic stirring bar.

For formation of beads, mixtures were extruded from a 10 cc. plastic syringe through a No. 20 G hypodermic needle. The syringe was attached to a single drive Harvard infusion pump. Pump speed was altered often to form uniform droplets at the tip of the needle, which then dripped into a stirring 1 M calcium chloride solution. Calcium alginate beads

for formulae 35 to 41 were produced using a device which included a stainless steel container with multiple extruder and an air compressor. There were no beads formed for formulae which did not have sodium alginate (formulae 25-27, and 39). Temperature of the calcium chloride solution was controlled by using an ice bath when making bacteria trapped in solidified cocoa butter. Calcium alginate beads were collected and dried using air drying, freeze drying, or vacuum drying. Freeze drying and vacuum drying were done by Oregon Freeze Dry Inc. and air drying was done in a air hood at room temperature. Vacuum drying conditions for formulae 35 and 36 were 60° F for the first day followed by drying at 80° F for a second day with vacuum pressure between 120 and 130 torr. For formulae 37 and 38, vacuum drying conditions were changed to 60° C for 4-8 hr at vacuum equal to 29 inches of mercury. For formulae 39 to 41, vacuum drying conditions were 104° F overnight at vacuum equal to 29 inches of mecury. Viability of Lactobacillus bacteria was tested on MRS agar plates. Samples were dissolved overnight in previously autoclaved phosphate buffer solution (pH close to 7.4) and then blended before series dilution for plate counts. Diluted sample solutions were spread on MRS agar plates and incubated at 37° C for two days.

Table II.1 Formulations in study-1 which were used to produce lactobacilli trapped in gelled calcium alginate beads.

No.	LACTINEX	Na-Alg	MALT	GLY	MSG	PVP	$Mg_3(PO_4)_2$	CYS	Vit-E	Na-2S	OIL	BUTTER	H_2O	DRYING		
														AD	FD	VD
1	5	2											100	+		
2	5	2									25		100	+		
3	5	2	5	12.5			1.5	0.1					90	+		
4	5	2	5										100	+		
5	5	2	3	12.5			1.5						90	+		
6	5	2	3	12.5									100	+		
7	5[1]	2	5[1]	12.5			1.5	0.1[1]			41		90	+		
8	5[1]	2	5[1]	12.5						1[1]	25		90	+		
9	5[1]	2									25		100	+		
10	5[1]	2	5[1]	12.5			1.5			1[1]	41		90	+		
11	5[1]	2	3	12.5				0.1[1]			33		100	+		
12	5[1]	2	5[1]	12.5			1.5	2.3[1]			41		90	+		
13	5[1]	2	3[1]	12.5			1.5	0.1			25		90	+		
14	5[1]	2	3[1]	12.5			1.5				33		90	+		
15	5[1]	2	3[1]	12.5			1.5	0.1[1]			33		100	+		
16	5[2]	2					1.5	0.02				28	100	+		
17	5[2]	2					1.5[2]	0.02[2]				28	100	+		

Table II.1 (continued)

No.	LACTINEX	Na-Alg	MALT	GLY	MSG	PVP	$Mg_3(PO_4)_2$	CYS	Vit-E	Na-2S	OIL	BUTTER	H_2O	DRYING		
														AD	FD	VD
18	5[2]	2					1.5[2]	0.1[2]				28	100	+		
19	5[2]	2						0.02[2]				28	100	+		
20	5[2]	2	3				1.5[2]	0.1[2]				28	100	+		
21	5[2]	2	3				1.5	0.1[2]				28	100	+		
22	5[2]	2	3				1.5[2]	0.16[2]				28	100	+		
23	5[2]	2	3				1.5		2.5[2]			28	100	+		
24	5[2]	2	5	12.5			1.5		2.2[2]			28	90	+		
25	50														+	
26	50			2.5											+	
27	50			14											+	
28	5.5	2	3	12.5			1.5	0.1					100		+	
29	5.6	2	5				1.5						100		+	
30	4.8[1]	2	3	12.5			1.5				33		100		+	
31	6.5[2]	2	3				1.5	0.1				28	100		+	
32	5.5	2		0.3									100		+	
33	6	2	3										100		+	
34	5.1	2											100		+	
35	5.2	2	3	12.5			1.5						100			+
36	5.2	2	3				1.5	0.1			12		100			+

Table II.1 (continued)

No.	LACTINEX	Na-Alg	MALT	GLY	MSG	PVP	$Mg_3(PO_4)_2$	CYS	Vit-E	Na-2S	OIL	BUTTER	H_2O	DRYING		
														AD	FD	VD
37	5	2	3	12.5			1.5						100	+	+	+
38	5	2	3				1.5	0.1			12		100	+	+	+
39	50														+	+
40	15	2.5	3		3	3	1.5				8		100	+	+	+
41	13	2.2	3	10	3	1.5		0.08					100	+	+	+

Amounts shown in this table are in grams.

LACTINEX: Lactinex granules or pastes (formulae 1 to 24 used Lactinex granules Lot 1397 and formulae 25 to 41 used Lactinex pastes Lot 4704 and 4762, both provided by BBL).

Na-Alg: sodium alginate, low viscosity.

MALT: malt extract

GLY: glycerol

MSG: monosodium glutamate

PVP: polyvinylpyrrolidone (mw. 40,000)

CYS: L-cysteine HCl

Vit-E: vitamin E

Na-2S: sodium bisulfite

OIL: light mineral oil or cocoa butter

DRY: air-dry (AD), freeze-dry (FD), or vacuum-dry (VD)

1: substances were added into oil phase (light mineral oil) before mixing with water phase.

2: substances were added into oil phase (cocoa butter) before mixing with cold water phase.

+: indicates drying method employed.

Study-2. Effect Of Additives On Lactobacillus Acidophilus And Lactobacillus Bulgaricus Grown In Evaporated Milk

This section contains experimental details for investigation of growing the lactobacilli with a variety of additives, followed by also varying additives post fermentation. The underlined section headings (1-4) list the fermentation media ingredients.

1. 1400 ml Evaporated Milk + 42 g PVP K-30 (polyvinylpyrrolidone) + 21 g $Mg_3(PO_4)_2$ + 7.4 g Yeast Extract + 20 ml L. Acidophilus + 20 ml L. Bulgaricus.

The medium was incubated with stirring in a walk-in incubator at 37° C for 15 hr. After fermentation, 1200 ml of the milk culture was collected. 24 g of MSG (monosodium glutamate) and 30 g of sodium alginate were added into this fermented milk. The pH of this alginate-fermented bacterial mixture was adjusted to 7.0 with 1 N NaOH and combined as follows:

1a. No extra treatment (0% whey).

1b. 100 g whey powder added into 400 ml of the alginate bacterial mixture (20% whey). The shape of gelled calcium alginate product produced by this mixture after extrusion from the needles was a string instead of spheres.

1c. 100 g whey powder was mixed into 200 ml of the alginate bacterial mixture (33% whey). Calcium alginate beads were not produced for this formula.

1d. 40 ml mineral oil was mixed with 400 ml of the alginate bacterial mixture (10% v/w oil). This mixing produces an oil-in-water emulsion. Gelled calcium alginate beads or

strings were collected after 1 hr or 12 hr "soaking" in the 1 M calcium chloride solution. Samples were dried by either freeze drying or vacuum drying.

2. 1000 ml Evaporated Milk + 0.5 g Yeast Extract + 15 g $Mg_3(PO_4)_2$ + 20 ml L. Acidophilus + 20 ml L. Bulgaricus.

The medium was incubated for 15 hr with stirring at 37° C in a walk-in incubator. After fermentation, the pH was adjusted to 7.0 with 1 N NaOH. 15 g trimagnesium phosphate, 20 g MSG, and 20 g sodium alginate were added into this fermented milk culture and combined as follows:

2a. No extra additives.

2b. 170 g whey powder was mixed with 170 ml alginate bacterial mixture (50% whey).

2c. 15 ml mineral oil was mixed with 150 ml alginate bacterial mixture (10% v/w oil) to produce an oil-in-water emulsion.

2d. 3 g PVP K-30 was mixed with 150 ml alginate bacterial mixture (2% PVP).

2e. 4 g PVP K-30 + 200 g whey powder were mixed with 200 ml alginate bacterial mixture (2% PVP + 50% whey).

2f. 3 g PVP K-30 + 15 ml mineral oil were mixed with 150 ml alginate bacterial mixture (2% PVP + 10% v/w oil).

Alginate bacterial mixtures (except formulae 2b and 2e) were dripped into 0.5 M calcium chloride solution and kept at 4° C for 10 hr. Samples were collected and then dried using either freeze drying or vacuum drying.

3. 1400 ml Evaporated Milk + 21 g $Mg_3(PO_4)_2$ + 7 g Yeast Extract + 14 ml L. Acidophilus + 14 ml L. Bulgaricus.

The medium was fermented either at 33° C for 20 hr or 37° C for 15 hr using a computer controlled fermentor (nicknamed "Oscar" in our laboratory) which can control pH and temperature automatically. NH_4OH was used as external neutralizer to maintained pH = 5.0. The sodium alginate solution contained 44 g sodium alginate, 20 g $Mg_3(PO_4)_2$, 40 g MSG, 30 g PVP K-30, and 800 ml water. 400 ml of sodium alginate mixture was mixed with 700 ml fermented milk (from either 33° C or 37° C fermented milk culture). The alginate bacterial mixture was dripped into 0.5 M calcium chloride cooled in an external ice bath. Samples were collected after 1 hr or 12 hr soaking in calcium chloride solution and tested for viability of Lactobacillus bacteria after being freeze dried or vacuum dried.

4. 2000 ml Evaporated Milk + 10 g Yeast Extract + 30 g $Mg_3(PO_4)_2$ + 40 ml L. Acidophilus + 40 ml L. Bulgaricus.

The medium was fermented at 33° C for 33 hr or 37° C for 24 hr using the Oscar fermentor and using NH_4OH for continuous external neutralization (pH = 5.0). Treatments of 37° C or 33° C fermented milk culture were:

4a. 400 ml fermented milk culture was mixed with sodium alginate solution which contained 12 g sodium alginate, 9 g PVP, 9 g MSG, 6 g $Mg_3(PO_4)_2$, and 200 ml water. 0.5 M calcium chloride solution with or without 1.5% of PVP + 1.5% MSG was used as hardening agent. Samples were collected after 0.5 hr in calcium chloride solution and were freeze dried or vacuum dried.

4b. The formula was almost the same as in 4a except one extra additive (12 g malt extract) was added into the sodium alginate solution.

4c. Besides those additives in 4a, other ingredients were also added into sodium alginate solution: 12 g malt extract, 0.6 g cysteine and 70 ml mineral oil. The calcium chloride solution contained 2% each of PVP, MSG, and malt extract. Some calcium alginate beads were air dried in the hood for 3 hr before being freeze dried or vacuum dried.

Study-3. Effect Of Enteric Coating On Lactobacillus Bacteria Survival

1. 2100 ml Evaporated Milk + 10 g Yeast Extract + 30 g $Mg_3(PO_4)_2$ + 42 ml L. Acidophilus + 42 ml L. Bulgaricus.

The medium was fermented for 20 hr at 37° C using the computer controlled fermentor (Oscar). pH was controlled to remain at 5.0 with NH_4OH. Sodium alginate solution (contained 30 g Malt extract, 22.5 g MSG, 22.5 g PVP K-30, 15 g $Mg_3(PO_4)_2$, 150 ml mineral oil (forms an oil in water emulsion), 30 g sodium alginate, and 500 ml water) was autoclaved before being mixed with 1000 ml fermented milk culture. The pH of fermented milk culture was adjusted to 6.2 using 3 N NaOH before mixing. 0.5 M calcium chloride solution containing 1.5% of MSG + 1.5% PVP K-30 + 2% Malt extract (protectives) was used as hardening agent. This is referred to later as a "mass balance" hardening solution because the concentration of "protectives" inside and outside the beads is equal. Calcium alginate beads were collected after 1 hr in calcium chloride solution and dried by freeze drying or vacuum drying. Only the freeze dried product (moisture content = 7%) was used for enteric

coating because the vacuum dried product had about 26% moisture. A STREA-1 spray coater (Aeromatic Corp.) + modified fluid bed dryer was used for coating.

Enteric coating conditions:

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat (3% coat)	Aquateric (pH adjusted)	35% DBS + 5% tween 80	air inlet = 15 psi liquid inlet = 3 ml/min Temp = room Temp. blower = 4 nozzle size = 0.8 mm
enteric coat 3% coat 6% coat 9% coat 12% coat	Eudragit L30D	30% PEG + 3% Talc	air inlet = 10 psi liquid inlet = 3 ml/min Temp = 30° C blower = 5 nozzle = 1.2mm mm

Coated samples were tested for viability of Lactobacillus bacteria after treatment with simulated gastric fluid (pH = 1.5) for 2 hr at 37° C using a USP dissolution apparatus, basket method at 75 rpm.

2. 2100 ml Evaporated Milk + 10 g Yeast extract + 30 g $Mg_3(PO_4)_2$ + 42 ml L. acidophilus + L. bulgaricus.

The medium was fermented for 20 hr at 33° C in the computer controlled fermentor and pH was controlled to remain at 5.0 using NH_4OH. Sodium alginate solution (containing 22.5 g MSG, 22.5 g PVP K-30, 15 g $Mg_3(PO_4)_2$, 30 g sodium alginate, and 500 ml water) was autoclaved before mixing with 1000 ml fermented milk culture (same as 1 above except there is no mineral oil, and fermentation was at 33° C rather than 37° C). pH of this fermented milk culture was adjusted to 6.6 using 3 N NaOH before mixing with sodium alginate solution. 0.5 M calcium chloride solution (containing 1.5% MSG and 1.5% PVP K-30) was used as harding agent. Calcium alginate beads were collected after 1 hr in calcium chloride solution. Only the freeze dried product (moisture content 5%) was used for enteric coating because the vacuum dried product had 23% Moisture.

Enteric coating conditions:

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat (3% coat)	Aquateric (pH adjusted)	35% DEP + 1% tween 80	air inlet = 15 psi fluid inlet = 6 ml/min Temp = 40° C Blower = 4 nozzle size = 0.8 mm
enteric coat 10% coat 15% coat 20% coat	Aquateric	35% DEP + 1% tween 80	air inlet = 10 psi fluid inlet 3 ml/min Temp = 40° C Blower = 4

30% coat	nozzle size = 1.2 mm

Coated samples were tested for viability of Lactobacillus bacteria after 2 hr in simulated gastric fluid (pH = 1.5). A USP dissolution apparatus was used in this study (basket method, 75 rpm, 37° C).

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3. 2100 ml Evaporated Milk + 10 g Yeast extract + 30 g $Mg_3(PO_4)_2$ + 42 ml L. acidophilus + 42 ml L. bulgaricus.

The medium was fermented for 20 hr at 33° C in the fermentor and pH was controlled to remain at 5 using NH_4OH. Sodium alginate solution (10 g MSG, 10 g PVP K-30, 10 g $Mg_3(PO_4)_2$, 30 g sodium alginate, and 500 ml water) was autoclaved before mixing with 1000 ml pH adjusted fermented milk culture (pH = 6.5). 0.5 M calcium chloride solution containing 2% of MSG + PVP K-30 was used as hardening agent. Calcium alginate beads were collected after 2 hr in calcium chloride solution and washed with deionized water for one minute. Conditions for vacuum drying were 6 hr at 60° C, and then some of this vacuum dried product was also freeze dried. Both freeze dried and vacuum dried products were used for enteric coating. The moisture content was 4% for both products.

Coating conditions:

A. Vacuum dried product

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat (3% coat)	Aquacoat	15% DBS + 15% TEC	air inlet = 15 psi fluid inlet = 6 ml/min Temp = 40° C.

			Blower = 4
			nozzle size = 0.8 mm
enteric coat	Eudragit L30D	30% PEG +	air inlet = 20 psi
3% coat		6% Talc	fluid inlet = 6 ml/min
6% coat			Temp = 40° C.
9% coat			Blower = 5
12% coat			nozzle size = 0.8 mm
20% coat			

B. Freeze dried product

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat	Aquacoat	15% DBS +	air inlet = 10 psi
(6% coat)		15% TEC	fluid inlet = 6 ml/min
			Temp = 40° C.
			Blower = 3
			nozzle size = 0.8 mm
enteric coat	Eudragit L30D	30% PEG +	air inlet = 15 psi
5% coat		1% Talc	fluid inlet = 6 ml/min
10% coat			Temp = 40° C.
15% coat			Blower = 5
20% coat			nozzle size = 0.8 mm

Samples were tested in simulated gastric fluid for 1 hr or 2 hr at 37° C. (75 rpm, basket method).

4. Enteric coating of Lactinex granules (Lot: 1411)

Lactinex granules were sieved using No. 28 mesh sieve, and only those which did not pass the sieve were used. These granules were directly coated with Eudragit L30D at different percentages, from 5 to 35%. The coating solution was plasticized with 15% DBS and 15% TEC, and stirred for one hour at room temperature before application. The coating conditions were: air inlet = 10 psi, fluid inlet = 5 ml/min, temp. = 40° C, blower = 5, and nozzle size = 0.8 mm. No sealing coat was applied in this enteric coating of Lactinex granules.

Study-4. Survival Lactobacillus in Enteric Coated Mini-Tablets.

1. 1000 ml MRS medium + 3 g Lactinex granules (Lot: 1399).

The medium was incubated at 37° C. with stirring for 24 hr in a walk-in incubator. The "MRS Cell culture" was then concentrated to 100 ml using a Minitan filter (Millipore). Corn starch (20 g) was added and allowed to hydrate in this concentrated bacterial culture for 24 hr at 4° C. Lactobacillus mini-tablets were made by mixing 4 g MSG, 4 g PVP K-30, 100 g whey powder, and 110 g flour into this starch-MRS mixture, and then hand pressed on tablet triturate plates to produce tablets with diameters about 5 mm. These mini-tablets were rounded by tumbling in a closed container. Vacuum dry conditions were: Temp = 50° C., vacuum = 29 inches of Hg, time = 5 hr.

Enteric coating conditions:

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat (2% coat)	Eudragit L30D (pH adjusted)	15% DBS + 15% TEC	air inlet = 20 psi fluid inlet = 4 ml/min Temp = 40° C. Blower = 7 nozzle size = 0.8 mm (without Wurster column)
enteric coat 5% coat 10% coat 15% coat	Eudragit L30D	15% DBS + 15% TEC	air inlet = 20 psi fluid inlet = 5 ml/min Temp = 40° C Blower = 8 nozzle size = 0.8 mm (without Wurster column)

Dissolution of coated mini-tablets was conducted only in simulated gastric fluid at pH = 1.3 for 2 hr (basket method, T = 37° C and rpm = 75).

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2. 2100 ml Evaporated Milk + 10 g Yeast Extract + 30 g $Mg_3(PO_4)_2$ + 40 ml Lactinex strains (Lot 1397).

The medium was fermented for 20 hr at 37°C in a computer controlled fermentor and the pH was controlled to remain at 5 using NH_4OH. 800 ml of fermented milk culture was used and the pH was adjusted to 6.5 using 3 N NaOH, and then cooled in an ice bath and concentrated to 390 ml using a Minitan ultra-filtration system. 14 g MSG, 14 g PVP K-30, 7 g

$Mg_3(PO_4)_2$, 350 g whey powder and 35 g corn starch were added and allowed to hydrate in this concentrated bacterial culture for 24 hr at 4° C. Lactobacillus mini-tablets were made by mixing 300 g flour with this mixture, and hand pressed on a tablet triturate plate to produce tablets with diameters about 5 mm. These mini-tablets were rounded by tumbling in a closed container. Vacuum drying conditions were: Temperature = 50° C, vacuum = 29 inches of Hg, time of 5 hr.

Enteric coating conditions (without using Wurster column):

Treatment	Polymer	Plasticizer	Spray coating conditions
sealing coat	Eudragit	15% DBS +	air inlet = 25 psi
2% coat	L30D	15% TEC	fluid inlet = 4 ml/min
(pH = 5.2)			Temp = 40° C
			blower = 7
			nozzle = 0.8 mm
enteric coat	Eudragit	15% DBS +	air inlet = 20 psi
5% coat	L30D	15% TEC	fluid inlet = 5 ml/min
			Temp = 40° C
			blower = 7
			nozzle = 0.8 mm

Dissolution of coated mini-tablets was conducted in both simulated gastric fluid (1000 ml, pH = 1.3) and simulated intestinal fluid (1000 ml, pH 7.4), basket method at 37° C with rpm = 75. The moisture content in these vacuum dried mini-tablets was 1.5%, determined by measuring the difference of weight loss before and after 4 days oven-drying (with air-flow) at 55° C.

3. 2100 ml Evaporated Milk + 10 g Yeast Extract + 30 g $Mg_3(PO_4)_2$ + 40 ml Lactobacillus Mixture (Lot 1397).

600 ml of fermented milk culture was used, and concentrated to 450 ml using the Minitan ultrafiltration system. 45 g corn starch, 450 g whey powder, 9 g MSG, and 9 g PVP K-30 were mixed with this concentrated milk culture, and allowed to hydrate for 5 hr at 4° C. Lactobacillus mini-tablets were made by mixing 300 g flour with this mixture, and hand pressed on a tablet triturate plate to produce tablets with diameters about 5 mm. These mini-tablets were rounded by tumbleing in a closed container. Different vacuum drying times were applied to these mini-tablets to study the effect on bacteria survival. The conditions for vacuum drying were Temperature = 50° C, Vacuum = 29 inches of Hg, Time = 2, 3, 4, or 5 hours. Each batch of dried mini-tablets was spray coated with Eudragit L30D. The coating conditions were almost the same as in 2 (study-4), except the fluid inlet was changed to 4 ml/min when doing the 5% enteric coating.

4. 100 g Whey Permeate + 30 g Casein Hydrolysate + 10 g Trptone + 20 g Yeast Ectract + 2 ml Tween 80 + 0.045 g $MnSO_4$ + 20 g $Mg_3(PO_4)_2$ + 40 ml Lactobacillus Mixture (Lot 1397).

Medium was fermented for 21 hours at 37° C. NH_4OH was used to maintain pH around 5, and the final pH of fermented medium was adjusted to 6.3 using 3 N NaOH. 800 ml of this fermented medium was concentrated to 100 ml using the Minitan system. 50 g whey powder, 50 g microc-crystalline cellulose RC-581 (from FMC, Co.), 3 g MSG, 3 g PVP K-30, and 30 g flour were added and mixed into this concentrated medium. Mini-tablets were prepared after 20 minutes of mixing. Procedures for making

mini-tablets, vacuum drying, and enteric coating were almost the same as in 2 (study-4), except the vacuum drying time was changed to 3 hours instead of 5 hours.

RESULTS AND DISCUSSION

Study-1

Immobilization of whole cells in calcium alginate is mild and easy. It has been reported that the viability of Pseudomonas dentrificans trapped in calcium alginate beads was 80-85% in phosphate buffer solution (41). Garde et. al. found that after immobilization of Rhodopseudomonas capsulata in calcium alginate beads, there was one bacterium per cavity which increased to 8-12 bacteria after 24 hours of incubation (42). For lactic acid bacteria like L. helveticus, the number of living lactic acid bacteria cells was about $3.0X10^7$ to $8.9X10^7$ /g calcium alginate beads, which increased to $1.2X10^8$ /g beads after 20 days fermentation in milk (43). Calcium alginate beads can be dissolved using calcium chelating agents such as phosphates and citrates. The calcium alginate beads made in study-1 were soaked in phosphate buffer solution (pH 7.4 ± 0.1) overnight and blended to ensure that beads were completely dissolved. During overnight soaking the beads swell to several times the size of their dry state. Plate counts (cfu/g) in the column "WET" in Table II.2 are the number of Lactobacillus bacteria trapped inside the wet calcium alginate beads. Results show that bacterial numbers were very stable after suspending Lactinex in sodium alginate solution and then forming into calcium alginate beads. Microencapsulated Lactobacillus bacteria coated with sodium alginate reportedly showed a 40-fold increase in stability compared to uncoated particles after 15 days of storage at 22° C (32).

There were three different basic types of Lactobacillus bacterial

preparations in study-1: bacteria were suspended in either water, liquid mineral oil, or a solidified oil phase (cocoa butter). In this early study, viable cell counts were measured but mass balance of cells was not determined. Some cells are lost in product preparation and the percentage recovery was not calculated. Effect of additives on viability of bacteria during air drying was determined by comparison of actual plate count numbers (cfu/g) compared to a control.

Product formulations for study-1 are in Table II.1 and results are shown in Table II.2. When Lactinex cells were suspended in the water phase, addition of mineral oil to form an emulsion did not improve survival during air drying (compare formula 2 with formula 1 in Table II.2). Malt extract addition into the water phase stabilized the Lactinex bacteria to 3 hours of air drying (compare formulae 3-6 to formula 1). Glycerol (10% v/v) in the alginate solution did not provide additional benefit compared to malt extract without glycerol after 3 hours of air drying (compare formulae 4 and 6). This is interesting since glycerol is considered by many researchers to be an absolute requirement for good survival during freezing and freeze-drying. A mixture of neutralizer ($Mg_3(PO_4)_2$), cryoprotectant (glycerol) and malt extract with or without antioxidant (cysteine) did help protect Lactobacillus bacteria against air drying (compare formulae 3 and 5 to formula 1); but most of these additives were not necessary as formula 4 (malt extract) was equally good compared to formulae 3, 5, and 6.

For formulae 7-15, bacteria were suspended in oil before being mixed with the water alginate phase. It was hypothesized that mineral oil might form a barrier between the Lactinex granules and water, and

thus protect bacteria during drying. When using only mineral oil, the decrease of cfu/g was still too rapid (formula 9). The decline in viable cfu/g could only be changed by adding more substances into either the oil phase or water phase (formulae 7-8 and 10-15). When cysteine was added into the oil phase and trimagnesium phosphate in the water phase (formulae 7 and 15), the protection was decreased compared to having cysteine in the water phase (formula 13) or without trimagnesium phosphate (formula 11). Using other antioxidants like sodium bisulfite or vitamin E instead of cysteine gave about equivalent results during air drying (compare formulae 10 and 12 to formula 13).

When cocoa butter has been melted slowly below 40° C, it solidifies when the temperature is lowered to room temperature (25° C), and then will not melt again until the temperature reaches 37° C. This characteristic of cocoa butter was utilized to trap bacteria in a solidified oil phase. Cocoa butter was used in formulae 16-24 and 31 to hold bacteria inside a solidified oil phase. However, cocoa butter without additives did not protect Lactobacillus bacteria against oxygen flow any better than mineral oil without additives during air drying. When both cysteine and trimagnesium phosphate were added into the solid oil phase (cocoa butter) the cfu/g decreased faster during the first 2 hours (formulae 17, 18, 20, and 22) compared to including only cysteine in the solid oil phase (formula 19) or both cysteine and trimagnesium phosphate in the water phase (formula 21). The outcome was almost the same as occurred when using mineral oil as a vehicle. The undesirable effect of combining trimagnesium phosphate and cysteine in the oil phase was altered by putting cysteine in the solid oil phase and trimagnesium

phosphate in the water phase (formula 21).

Formulae 25 to 27 were the first products freeze dried. They contained Lactinex paste mixed with other protectants (no sodium alginate). The % recovery of viable bacteria was almost the same for these three formulations, suggesting that neither 5% glycerol nor 22% malt extract were necessary or effective protectants to help Lactobacillus bacteria survive during freeze drying when already formulated as Lactinex paste. After dilution of the Lactinex paste with water, the % recovery was less than 10% for freeze dried products except those which had malt extract MSG, and PVP K-30 in the formulation (formulae 28 to 41). The effect of these additives on viability of Lactobacillus bacteria against freeze drying was significant when compared to other additives. The current high solids Lactinex paste prepared by BBL affords excellent protection during drying when compared against diluted products. Diluted products require additives, and dilution is necessary to form calcium alginate beads. Lowering the temperature for vacuum drying, and increasing the drying time, did not increase the survival of Lactobacillus bacteria (formulae 35 and 36).

Among environmental factors affecting the survival of air dried, freeze dried or vacuum dried bacteria, the type and concentration of the drying medium, the residual moisture of the dried product, and oxidation are considered to be the most important to increasing survival rate of microorganisms during and after drying. In 1959, Ota studied the effect of drying medium on preservation of L. bifidus (Bifidobacterium bifidum). He found the best survival of L. bifidus was obtained with sodium glutamate, and next best with skim milk (26). After that, in

1961, Ota and coworkers also found that with 3% sodium glutamate and 3% PVP in the drying medium, the effect to stabilize bacteria against heat was better than other combinations or sodium glutamate alone (26). In formulae 40 and 41 which contained MSG and PVP in alginate beads, the results also show that the combination of additives was the best among the formulations tested. In this combination, both freeze dried and vacuum dried products had more than 25% recovery of viable cells from diluted cells compared to very low recovery for the others.

The sublimation rate of water, when drying a bacterial product, reportedly decrease as the concentration of sodium glutamate increases, and does not decrease with increasing concentration of PVP (26). The protective effect of glycerol on lactic acid bacteria subjected to freeze drying was examined by Valdez et al. (44). They found that the effect of glycerol to protect lactic acid bacteria varied from strain to strain. For L. bulgaricus, the effect of glycerol and sodium glutamate were less than for cysteine as a protectant against freeze drying (45). Bozoglu et al. demonstrated superior survival and less injury for L. acidophilus with 5% glycerol in suspending medium before freeze drying (46). From the results in study-1, it is difficult to draw definite conclusions for glycerol, cysteine, and oil as cryoprotective agents to protect bacteria against freeze drying.

Table II.2 Effect of additives on drying Lactobacillus bacteria trapped inside calcium alginate beads.

FORMULA[a]	MRS PLATE COUNT (cfu/g)				RECOVERY[b] %	
No.	WET	AIR DRY (hr)	FREEZE DRY	VACUUM DRY	FD	VD
1		$<10^4$ (12)	(Note: Freeze Dry and % Recovery			
2	$5.0X10^5$	$7.2X10^5$ (1)	data begin with formula 25;			
		$<10^4$ (2)	Vacuum Dry data begin with			
		$<10^4$ (3)	formula 35.)			
3	$2.9X10^7$	$5.5X10^7$ (1)				
		$4.7X10^7$ (2)				
		$4.0X10^7$ (3)				
4	$3.6X10^7$	$5.4X10^7$ (3)				
5	--	$5.2X10^7$ (1)				
		$4.8X10^7$ (2)				
6	$3.7X10^7$	$6.2X10^7$ (1)				
		$3.4X10^7$ (3)				
7	$1.9X10^6$	$7.0X10^5$ (1)				
		$6.9X10^5$ (2)				
		$1.3X10^5$ (3)				
8	$3.8X10^6$	$8.8X10^6$ (1)				
9	$6.5X10^6$	$5.4X10^5$ (2)				
		$6.3X10^5$ (3)				
10	$5.3X10^6$	$4.0X10^6$ (1)				
		$7.4X10^6$ (2)				
		$2.6X10^6$ (3)				
11	$6.0X10^7$	$2.0X10^7$ (1)				
		$1.8X10^7$ (2)				
12	$1.9X10^7$	$1.3X10^7$ (1)				
		$1.8X10^7$ (2)				
		$2.1X10^7$ (3)				

Table II.2 (continued)

FORMULA[a]	MRS PLATE COUNT (cfu/g)				RECOVERY[b] %	
No.	WET	AIR DRY (hr)	FREEZE DRY	VACUUM DRY	FD	VD
13	$8.1X10^6$	$1.3X10^7$ (1)				
		$1.4X10^7$ (2)				
14	$1.2X10^7$	$1.7X10^7$ (1)				
		$4.9X10^7$ (2)				
15	$3.2X10^7$	$1.4X10^7$ (1)				
		$1.2X10^6$ (2)				
16	$4.5X10^6$	$4.7X10^6$ (1)				
		$2.3X10^6$ (2)				
		$<10^4$ (3)				
17	$5.5X10^6$	$2.0X10^6$ (1)				
		$1.6X10^6$ (2)				
		$<10^4$ (3)				
18	$1.4X10^7$	$9.4X10^6$ (1)				
		$7.3X10^6$ (2)				
		$1.5X10^5$ (3)				
19	$2.0X10^7$	$1.6X10^7$ (3)				
		$1.2X10^6$ (5)				
20	$1.4X10^7$	$4.8X10^6$ (1)				
		$1.4X10^6$ (2)				
		$8.2X10^5$ (3)				
21	$1.5X10^7$	$1.7X10^7$ (1)				
		$1.5X10^7$ (2)				
		$1.1X10^6$ (3)				
22	$1.2X10^7$	$1.4X10^7$ (1)				
		$7.4X10^6$ (2)				
		$4.9X10^6$ (3)				
23	$1.5X10^7$	$1.2X10^7$ (1)				
		$6.7X10^6$ (2)				

Table II.2 (continued)

FORMULA[a]	MRS PLATE COUNT (cfu/g)				RECOVERY[b] %	
No.	WET	AIR DRY (hr)	FREEZE DRY	VACUUM DRY	FD	VD
24	$2.8X10^7$	$2.0X10^7$ (1) $1.8X10^7$ (2)				
25	$9.6X10^8$	--	$2.4X10^8$	--	25	
26	$9.1X10^8$	--	$2.6X10^8$	--	29	
27	$7.5X10^8$	--	$1.9X10^8$	--	25	
28	$2.3X10^7$	--	$4.6X10^6$	--	0	
29	$3.3X10^7$	--	$2.0X10^5$	--	0	
30	$5.4X10^7$	--	$3.1X10^7$	--	2	
31	$4.5X10^7$	--	$1.3X10^7$	--	8	
32	$7.3X10^7$	--	--	--		
33	$8.2X10^7$	--	$1.9X10^5$	--	0	
34	$5.5X10^7$	--	$3.7X10^6$	--	0	
35	$2.3X10^7$	--	--	$<10^4$		0
36	$2.9X10^7$	--	--	$<10^4$		0
37	$2.1X10^7$	$<10^4$	$2.2X10^6$	$<10^4$	2	0
38	$3.6X10^7$	$1.2X10^5$ (6)	$3.9X10^5$	$<10^4$	0	0
39	$4.5X10^8$		$2.6X10^8$	$2.9X10^7$	58	6
40	$5.4X10^7$	$4.5X10^6$ (4)	$9.4X10^7$	$8.1X10^7$	50	43
41	$6.2X10^7$	$3.5X10^6$ (4)	$7.6X10^7$	$8.0X10^7$	28	29

WET: Samples were collected as soon as the calcium alginate beads formed.

FD: freeze drying

VD: vacuum drying

a: see TABLE 1.

Table II.2 (continued)

b: The percentage of recovery was calculated base on the ratio of cfu/g in freeze dried or vacuum dried product vs "theoretical Lactobacillus cfu/g after drying in the formulation". In this calculation, the water content before and after freeze drying or vacuum drying was assumed to be 100% and 0% remaining, respectively. For example, in formula 30 the total solid content was (4.8 + 3 + 1.5 + 10X1.25 + 2 + 40X0.83) = 55.5 g, and the total weight (including wt. from water) was 155.5 g. The value in column "WET" ($5.4X10^7$) X 155.5/55.5 = $15.1X10^7$/g represented the cfu/g which should be obtained after drying, theoretically. The ratio of cfu/g from dried product vs the theoretical cfu/g would be % recovery. That means the % recovery of freeze dried product in formula 30 was 2%.

Study-2

Survival results for Lactobacillus bacteria grown in our laboratory in evaporated milk and dried by freeze drying or vacuum drying are given in Table II.3. The cfu/g of lactobacilli grown in flasks with pH adjusted after fermention were similar to those grown in a computer controlled fermentor with external continuous neutralization using ammonium hydroxide during fermentation (formula ingredients are given in the Materials and Methods section, study 2, for Table II.3 data). In study-2, the evaporated milk cultures reached pH $\leq$ 5 after fermentation in flasks (formulae 1a-2f), and were neutralized to pH 6.2-6.6 prior to formulation and drying. This was to help prevent acid injury of lactobacilli after formation into calcium alginate beads and either freeze drying or vacuum drying.

It was expected that continuous neutralization during computer controlled fermentation would produce Lactobacillus numbers higher than without neutralization. The essentially equivalent cfu/g, independent of fermentation method, (see "WET" column in Table II.3) was surprizing. It was observed by Peri and Pompei that yogurt spray dried after neutralization gave ten times higher survival than unneutralized yogurt (47). Others have also suggested neutralization or pH adjustment of fermented milk before spray or vacuum drying (31, 48, 49, 50). Approximately 10^9 cfu/g of viable cells were reported in spray dried cultures of L. acidophilus and L. helveticus with pH adjusted to 6.5 followed by addition of ascorbic acid and monosodium glutamate prior to drying (31).

It is interesting to note that a protective effect against freeze

drying occurred with longer soaking of calcium alginate beads in calcium chloride solution (1a, 1d, and 3-33° in Table II.3). This difference was minimized when 20% whey powder was included in the formulation (formula 1b, Table II.3). This "high solids" product (formula 1b) gave excellent survival which is consistent with the findings of study 1. It was, however, impossible to form alginate beads from this high solids mixture. Wright and Klaenhammer demonstrated that the presence of calcium in MRS medium protected L. acidophilus NCFM and L. bulgaricus from freezing death and injury (51). The specific role of calcium in protecting Lactobacillus bacteria against freeze drying is unknown. Fontana et al. observed that the accumulation of divalent cations appeared necessary for the peptidoglycan of Klebsiella pneumoniae to acquire sufficient rigidity for shape determination and cell protection (52). Increased survival of L. bulgaricus was accompanied by a morphological transition of the bacterial cells to bacilloid state (53). Hurst and Stubbs found that growth initiation of Streptococcus lactis involved structural and permeability changes requiring calcium (54).

With mass balance of major protectants (monosodium glutamate, PVP K-30, and malt extract) obtained by placing the protectants in both the sodium alginate solution and in the calcium chloride solution, the survival of lactobacilli increased (4a-37° to 4b-33°, Table II.3). Also, lactobacilli fermented at 33° C with continuous neutralization, survived better during freeze drying compared to when fermented at 37° C (4a and 4b, Table II.3). Pretreatment with 3 hours of air drying before freeze drying was fatal to the bacteria even though additives were in the formulations, plus a mass balance of additives in each solution was

used (4c, Table II.3). Beads which are air dried shrink and become hard, which is very helpful for coating. Freeze dried beads are porous and fragile, and very difficult to spray coat. Thus, the failure of the bacteria in beads to survive air drying followed by freeze drying blocked one avenue of producing beads easy to spray coat.

The percentage recovery was usually higher for freeze drying compared to vacuum drying (Tables II.2 and II.3). L. acidophilus cells surviving freeze drying, and reportedly became sensitive during vacuum drying to oxgall and lysozyme, probably from damage to the cell wall (21). The dried cells, especially from vacuum drying, also became sensitive to NaCl and permeable to orthonitrophenol beta-galactoside from damage to the cytoplasmic membrane (21). Brennan et al. also found that L. acidophilus strains were susceptible to drying, with vacuum drying being more lethal than freeze drying (19). In the current study, a vacuum dried product was prefered because the resultant beads were smaller, harder and less porous, making them easier to coat.

Table II.4 summarizes the effects of additives as a % of fermentation medium (same data as Tables II.2 and II.3) on Lactobacillus bacteria survival after freeze drying or vacuum drying. When the initial bacteria loading was 10^7 cfu/g, and the product contained 2.2% malt extract survival was greater than 25% (formulae 40 and 41 in Table II.4). Reduction of PVP to 1.1%, and omitting oil and $Mg_3(PO_4)_2$ from formula 40, resulted in a decreased protection of about 2-fold. However, the actual number of bacteria per gram in dried calcium alginate beads was almost the same due to the decreased dilution effect with less additives. For direct coating, beads without oil are

preferred as the oil has a tendency to "leak" from the beads making them difficult, or impossible to coat.

When the loading was increased to 10^8 cfu/g in alginate beads, addition of 2% MSG and PVP did not help protect Lactobacillus bacteria against freeze drying or vacuum drying (1a, 1d, and 2d in Table II.4). This result is consistent with Obayashi et al. They found the optimal concentration of sodium glutamate was dependent on bacterial concentration. The higher the concentration of bacterial cells used, the higher the concentration of sodium glutamate necessary (26). When Smittle et al. studied the death of L. bulgaricus resulting from liquid nitrogen freezing, they also observed a better protection (73% killed) from 4% monosodium glutamate for culture with 10^9 cfu/ml compared to when 2% sodium glutamate had been used (92% death during frozen storage) (55).

Adding whey powder into the alginate bacterial mixture did increase the survival of lactobacilli to above 45 % after freeze drying (1b, 1c, 2b, and 2e in Table II.4). This may be attributed to a "shielding effect" of whey powder for the microorganisms, which reduced the interfacial area between living cells and water molecules. In 1979, Espina and Packard reported that recovery of spray dried L. acidophilus was related to solids content in the milk and outlet air temperature for the drier (56). Results obtained in this study consistently suggest that the highest recovery was from relatively high solids loading prior to drying (Table II.4). None of the ingredients (2% malt extract, 7% oil or 1% trimagnesium phosphate) protected L. acidophilus and L. bulgaricus against freeze drying and vacuum drying when used alone or in

partial combination (Table II.4) in dilute cultures. When used together with PVP and MSG, the solids content was increased and viable cell recovery was good, but not superior to using whey to increase the solids.

Table II.3 Survival of lactobacilli (grown in evaporated milk) after drying.

FORMULA No.[a]	MRS PLATE COUNT cfu/g				%RECOVERY[b]	
	WET	WET BEADS (hr)	DRY BEADS			
			F.D.[c]	V.D.[d]	F.D.	V.D.
1a	$6.3X10^8$	$5.4X10^8$ (1)	$7.1X10^7$	$<10^6$	3	0
		$9.7X10^8$ (12)	$2.5X10^8$	$<10^6$	10	0
1b	$4.6X10^8$	$6.2X10^8$ (1)	$9.4X10^8$	$2.1X10^8$	78	18
		$6.5X10^8$ (12)	$8.8X10^8$	$4.0X10^7$	73	3
1c	$2.6X10^8$	--	$3.7X10^8$	$2.9X10^8$	47	37
1d	$5.4X10^8$	$7.7X10^8$ (1)	$3.3X10^7$	$<10^6$	2	0
		$6.3X10^8$ (12)	$2.9X10^8$	$<10^6$	16	0
2a	$5.2X10^8$	$3.0X10^8$ (10)	$<10^6$	$<10^6$	0	0
2b	$9.4X10^7$	--	$8.0X10^7$	$2.9X10^7$	53	18
2c	$4.9X10^8$	$2.6X10^8$ (10)	$<10^6$	$<10^6$	0	0
2d	$2.1X10^8$	$4.1X10^8$ (10)	$<10^6$	$<10^6$	0	0
2e	$1.3X10^8$	--	$1.1X10^8$	$1.9X10^7$	52	9
2f	$1.9X10^8$	$3.0X10^8$ (10)	$<10^6$	$<10^6$	0	0
3-33°	$1.7X10^8$	$6.5X10^8$ (1)	$1.8X10^8$	--	19	
		$5.6X10^8$ (24)	$2.8X10^8$	--	29	
3-37°	$3.0X10^8$	$2.9X10^8$ (1)	--	$<10^6$		0
		$3.3X10^8$ (24)	--	$<10^6$		0
4a-37°	$4.6X10^8$	$1.1X10^9$ (NO)	$1.2X10^8$	$<10^6$	5	0
		$1.5X10^9$ (YES)	$6.3X10^8$	$<10^6$	24	0
4a-33°	$1.8X10^8$	$5.8X10^8$ (NO)	$4.2X10^8$	$1.2X10^8$	43	12[e]
		$8.4X10^8$ (YES)	$4.2X10^8$	$1.5X10^8$	43	15[e]
4b-37°	$4.5X10^8$	$1.5X10^9$ (NO)	$<10^6$	$<10^6$	0	0
		$1.5X10^9$ (YES)	$1.3X10^8$	$2.9X10^7$	6	1
4b-33°	$1.9X10^8$	$2.3X10^8$ (NO)	$4.9X10^7$	$<10^6$	5	0
		$2.8X10^8$ (YES)	$2.1X10^8$	$<10^6$	21	0
4c-37°	$3.9X10^8$	$1.1X10^9$ (YES)	$5.8X10^8$	$2.0X10^7$	39	1
		$2.1X10^9$ (AIR)	$<10^6$	$1.1X10^7$	0	1

Table II.3 (continued)

FORMULA No.[a]	MRS PLATE COUNT cfu/g				%RECOVERY[b]	
	WET	WET BEADS (hr)	DRY BEADS			
			F.D.[c]	V.D.[d]	F.D.	V.D.
4c-33°	$1.6X10^8$	$1.3X10^9$ (YES)	$6.6X10^7$	$5.7X10^8$	11	93[f]
		$1.2X10^9$ (AIR)	$<10^6$	$1.5X10^8$	0	25[g]

33°: 33° C fermented milk culture

37°: 37° C fermented milk culture

a: see STUDY 2, see Materials and Methods section.

b: see footnote b in TABLE 2

c: freeze dried calcium alginate product

d: vacuum dried calcium alginate product

e: moisture content 25%

f: moisture content 22%

g: moisture content 12%

NO: no MSG or PVP K-30 in the calcium chloride solution

YES: had MSG and PVP K-30 (and Malt for 4c) in calcium chloride solution

AIR: the wet calcium alinate beads were dried in the hood at room temperature for 3 hr before freeze drying or vacuum drying.

Table II.4 Summary comparison of additives effects on Lactobacillus survival.

FORMULA No.	WET cfu/g	MSG %	PVP %	Mg %	WHEY %	OIL %	MALT %	H_2O %	%RECOVERY[a] F.D.	V.D.
STUDY-1										
40	$5.4X10^7$	2.2	2.2	1.1		6.1	2.2	73	50	43
41	$6.2X10^7$	2.2	1.1				2.2	75	28	29
STUDY-2										
1a	$6.3X10^8$	2	2.7					75	3	0
1b	$4.6X10^8$	1.6	2.7		20			60	78	18
1c	$2.6X10^8$	1.3	2.7		33			50	47	37
1d	$5.4X10^8$	1.8	2.7			7.6		69	2	0
2a	$5.2X10^8$	1.8		1.3				77	0	0
2b	$9.4X10^7$	0.9		0.7	50			38	53	18
2c	$4.9X10^8$	1.6		1.2		7.7		71	0	0
2d	$2.1X10^8$	1.8	2	1.3				77	0	0
2e	$1.3X10^8$	0.9	1	0.7	50			38	52	9
2f	$1.9X10^8$	1.6	1.8	1.2		7.7		71	0	0
3-33°	$1.7X10^8$	1.6	1.2	0.8				82	19	ND
3-37°	$3.0X10^8$	1.6	1.2	0.8				82	ND	0

Table II.4 (continued)

FORMULA No.	WET cfu/g	MSG %	PVP %	Mg %	WHEY %	OIL %	MALT %	H_2O %	%RECOVERY[a] F.D.	V.D.
4a-37°	$4.6X10^8$	1.4	1.4	1				82	5	0
4a-33°	$1.8X10^8$	1.4	1.4	1				82	43	12[b]
4b-37°	$4.5X10^8$	1.4	1.4	1			1.8	82	0	0
4b-33°	$1.9X10^8$	1.4	1.4	1			1.8	82	5	0

see footnotes in table 1 for abbreviations

a: The % recovery shown in this table is the number for the shortest soaking time tested in calcium chloride solution, and without mass balance additives in the calcium chloride solution.

b: moisture content 25%

33°: 33° C fermented mlik culture

37°: 37° C fermented milk culture

ND: not determined

Study-3

Results of study-3 are shown in Tables II.5 to II.8. See Materials and Methods for formulations used in study-3. Formulae 1-3 in Table II.5 used evaporated milk as the growth medium and the pH was adjusted to between 6 and 7 before making calcium alginate beads. The hardening agent (calcium chloride solution) used in study-3 contained the same percentage of MSG, PVP, and malt extract (ie., mass balanced additives) as the alginate bacterial mixture. Initial growth and loading of bacteria in wet calcium alginate beads provided 10^9 cfu/g (formulae 1-3) which was higher than for previous efforts. After freeze drying or vacuum drying, cells were maintained above 10^9 cfu/g for formulae 1 and 2. The percentage of MSG and PVP used in formula 3 was only half the amount used in formulae 1 and 2, and the recovery of viable bacteria decreased almost 3-fold during freeze drying (Table II.5). Because of the high moisture content in vacuum dried products from formulae 1-2, comparison of Lactobacillus survival was not meaningful.

Table II.6 shows coating freeze dried calcium alginate beads resulted in a rapid decrease in cfu/g between the "none" and "sealing coat" (1, 2, and 3-FD). The decline in viable bacterial number slowed after applying the sealing coat to freeze dried beads. However, none of these enteric coated freeze dried beads gave good bacterial survival after treating with simulated gastric fluid for 2 hr. Eudragit L30D (coating of formula 1-FD, Table II.6) gave better protection against low pH solution (pH = 1.5) compared to Aquateric (coating of formula 2-FD, Table II.6), based on the percent weight-by-weight coating. For vacuum dried products, cfu/g were more stable during spray coating compared to

freeze dried products (3-VD, Table II.6). There was only 50% kill of bacteria from product soaked for 2 hr in simulated gastric fluid treatment for vacuum dried beads when the Eudragit L30D coating was higher than 9% (3-VD, Table II.6).

It is shown in Table II.7 that Lactinex granules contain almost equal numbers of Lactobacillus bacteria distributed within "powder form" and "granule form". When Lactinex granules were coated with Eudragit L30D, the number of bacteria per gram did not decrease without having a sealing coat. The resistance of enteric coated Lactinex granules to low pH solution challenge (simulated gastric fluid treatment) increased with increase of Eudragit L30D loading (Table II.7). After coating the granules with 15% Eudragit L30D, a desired survivalfor Lactobacillus bacteria against simulated gastric fluid challenge was observed.

The effect of storage conditions on enteric coated Lactobacillus granules is shown in Table II.8. Lactobacillus viability was maintained stable in dessicated conditions at room temperature for 60 days. Without dessication, the counts of viable bacteria decreased 100-fold in 19 days storage at room temperature (from 10^7 cfu/g to 10^5 cfu/g, Table II.8). Refrigerated products gave the highest and most stable counts, which indicated that temperature and moisture were the major factors affecting the survival of Lactobacillus bacteria during storage.

Table II.5 Viability of Lactobacillus bacteria after drying (study-3) and prior to spray coating.

FORMULA	MRS PLATE COUNT (cfu/g)			% RECOVERY[a]	
No.	WET BEADS (HR)	DRY BEADS			
		F.D.	V.D.	F.D.	V.D.
1	$1.0X10^9$ (0)				
	$1.6X10^9$ (1)	$1.3X10^9$	$1.7X10^9$	33	44[b]
2	$1.0X10^9$ (0)				
	$1.3X10^9$ (1)	$1.3X10^9$	$1.8X10^9$	26	36[c]
3	$1.0X10^9$ (0)				
	$1.4X10^9$ (2)	$4.6X10^8$	$2.1X10^8$	8	4

a: See footnote b in Table II.2 for calculation. The cfu/g for "wet" is represented by the number in column "wet beads" at time 0 hr.

b: moisture content 26%

c: moisture content 23%

Table II.6 Enteric coating effect on Lactobacillus survival in low pH solution (study-3).

FORMULA[a]	% COATING	COUNTS, cfu/g; (hr[b])	% SURVIVAL[c]
1-FD[d]	none	$1.3X10^9$ (0)	100
	sealing coat	$2.2X10^8$ (0)	17
	3%	$3.1X10^8$ (0)	25
	6%	$1.9X10^8$ (0)	16
	9%	$2.2X10^8$ (0)	19
		$6.8X10^6$ (2)	0
	12%	$1.8X10^8$ (0)	13
		$1.1X10^7$ (2)	1
2-FD	none	$1.3X10^9$ (0)	100
	sealing coat	$4.3X10^8$ (0)	34
	10%	$3.9X10^8$ (0)	34
	15%	$2.9X10^8$ (0)	27
	20%	$2.2X10^8$ (0)	22
		$<10^5$ (2)	0
	25%	$2.5X10^8$ (0)	27
		$<10^5$ (2)	0
3-VD[d]	none	$2.1X10^8$ (0)	100
	sealing coat	$4.2X10^8$ (0)	206
	3%	$1.8X10^8$ (0)	91
	6%	$2.1X10^8$ (0)	110
		$1.0X10^8$ (1)	52
		$2.4X10^7$ (2)	13
	9%	$7.7X10^7$ (0)	42
		$5.2X10^7$ (1)	28
		$3.7X10^7$ (2)	20
	12%	$1.2X10^8$ (0)	67
		$5.0X10^7$ (1)	28
		$2.4X10^7$ (2)	13

Table II.6 (continued)

FORMULA[a]	% COATING	COUNTS, cfu/g; (hr[b])	% SURVIVAL[c]
	20%	$1.3X10^8$ (0)	80
		$1.3X10^8$ (1)	80
		$7.3X10^7$ (2)	45
3-FD	none	$4.6X10^8$ (0)	100
	sealing coat	$2.5X10^8$ (0)	58
	5%	$1.3X10^8$ (0)	31
	10%	$7.5X10^7$ (0)	19
	15%	$1.1X10^8$ (0)	29
		$1.6X10^7$ (1)	4
		$5.4X10^6$ (2)	1
	20%	$1.1X10^8$ (0)	31
		$5.2X10^6$ (1)	1
		$3.0X10^6$ (2)	0

a: see: STUDY 3.

b: simulated gastric fluid treatment time

c: the % survival was calculated by compared cfu/g of coated dosage form to its uncoated dosage form. For example: the cfu/g in formula 4 was $2.0X10^7$ for "none" and $2.1X10^7$ for "sealing coat" which was a 2 % seal coat, thus the actual bacteria number per gram in the seal coated tablets should be $2.1X10^7/0.98$, and the % recovery was $(2.1/0.98)/2 = 107$ %

d: "FD" means freeze dried product and "VD" means vacuum dried product

Table II.7 Survival of Lactobacillus bacteria in Eudragit L30D coated Lactinex granules (Lot: 1411).

SAMPLE	GASTRIC (2 hr)	%R	INTESTINE
POWDER	--		$2.9X10^8$
GRANULE	--		$1.9X10^8$
MIXTURE	--		$7.9X10^7$
5% EU	$2.0X10^6$	2%	$9.8X10^7$
7.5% EU	$1.5X10^7$	13%	$1.2X10^8$
10% EU	$4.6X10^7$	33%	$1.4X10^8$
12.5% EU	$7.8X10^7$	65%	$1.2X10^8$
15% EU	$1.1X10^8$	100%	$1.1X10^8$
	$1.8X10^8$	86%	$2.1X10^8$
25% EU	$2.0X10^8$	133%	$1.5X10^8$
35% EU	$1.7X10^8$	71%	$2.4X10^8$

Gastric acid test (GF): Samples were dissolved in 500 ml simulated gastric fluid [(7 ml 37% HCl + 2 g NaCl)/liter, pH = 1.3 ± .1] for 2 hours, using USP paddle method at 37° C and 50 rpm. Then, remainder of granules were transfered to intestinal fluid, dissolved, and counted.

Intestinal fluid survival test (IF): 100 ml isotonic simulated intestinal fluid [(6.8 g KH_2PO_4 + 200 ml 0.2N NaOH + 2 g NaCl)/liter, pH=7.4±.1] in 100 ml culture bottle was used to dissolve samples. Samples were magnetically stirred for 0.5 hour at 30° C.

plate counts: MRS agar plate, incubated at 37° C for 48 hours.

%R: Percent recovery = (cfu/g after GF 2 hr) / (cfu/g in IF)

POWDER: Lactinex granules which passed through mesh No. 28 sieve.

GRANULE: Lactinex granules which were retained on mesh No. 28 sieve.

Table II.8 Effect of storage conditions on Lactobacillus bacteria survival in Eudragit L30D enteric coated Lactinex granules (Lot: 1411).

condition	Refrigerator	Room Temp.	Desiccator at Room Temp.
at day 0			
0% EU	$2.0X10^8$	$2.5X10^8$	$2.0X10^8$
15% EU	$2.1X10^8$	$2.1X10^8$	$2.1X10^8$
25% EU	$1.5X10^8$	$1.5X10^8$	$1.5X10^8$
35% EU	$2.4X10^8$	$2.4X10^8$	$2.4X10^8$
at day 15			
0% EU	$3.3X10^8$	$7.8X10^7$	$1.8X10^8$
	(13.5%)	(10.4%)	(7.8%)
15% EU	$2.7X10^8$	$7.4X10^7$	$1.1X10^8$
	(11.8%)	(10.1%)	(8.1%)
25% EU	$2.1X10^8$	--	$1.3X10^8$
	(10.7%)		(7.6%)
35% EU	$1.3X10^8$	$5.6X10^7$	$1.3X10^8$
	(10.3%)	(10.6%)	(7.6%)
at day 34			
0% EU	$1.4X10^8$	$1.1X10^5$	$8.8X10^7$
15% EU	$3.3X10^8$	$1.8X10^5$	$4.4X10^7$
25% EU	$2.1X10^8$	—	$9.5X10^7$
35% EU	$1.3X10^8$	$3.2X10^5$	$8.4X10^7$
		(10.5%)	(9.3%)

Table II.8 (continued)

condition	Refrigerator	Room Temp.	Desiccator at Room Temp.
at day 60			
0% EU	--	$<10^4$	--
15% EU	$1.8X10^8$	$<10^4$	$4.7X10^7$
25% EU	$2.7X10^8$	--	$4.6X10^7$
35% EU	$2.8X10^8$	$<10^4$	$8.9X10^7$

Number in parenthesis is the moisture content of product determined by heating testing at 135° C in an oven for 2 days.

Dissolving solution: isotonic simulated intestinal fluid, pH=7.3, stirred at room temp. for 30 minutes.

Desiccator: Using "DRIERITE" (anhydrous $CaSO_4$) as desiccant.

Temp.: Temperature

Study-4

The low cfu/g for formula "1" in study-4 was because the pH of fermented MRS medium culture was not adjusted before concentration (Table II.9). Using mini-tablets to trap bacteria gave a good percentage survival with excellent hardness of dried product after vacuum drying. This is much different from the freeze dried product which had a very fragile structure (formulae 1 and 2 in study-3). The degree of hardness in the dried product influences results of spray coating. Particles with weak and porous structures can not tolerate the high oxygen flow and air pressure which crushes particles during spray coating. Although this formula gave a complete protection with only 5% Eudragit L30D coating, ealier data suggests that lower starting numbers (cfu/g) in the formulation are easier to protect.

Data in Tables II.10 and II.11 are confounded somewhat by the discovery that release of Lactobacillus from mini-tablets is less than complete in 100 ml of intestinal fluid, resulting in some counts which are underestimates of the true number of viable cells. It can be seen that about 10^9 cfu/ml were produced during fermentation, providing $2.5X10^8$ cfu/g after addition of large quantitites of tabletting binder and filler. The loss of Lactobacillus bacteria was negligible when taking the influence of dilution into account. By comparing the data in Tables II.11 and II.12, only 10% of bacteria (about $2X10^7$ cfu/g) were released into intestinal fluid from vacuum dried mini-tablets.

The finding of almost 100% recovery of bacteria loading in the dried mini-tablets was quite encouraging (Tables II.11 and II.12). Most promising is discovery that the enteric coating process did not damage

the Lactobacillus in mini-tablets, and complete recovery of bacteria from coated mini-tablets after 2 hours acid solution treatment (pH = 1.4). This compares favorably to the nearly 100% kill in traditional Lactinex tablets in 15 minutes when treated with simulated gastric fluid, as well as rapid bacterial death for uncoated mini-tablets. It is interesting to note that the moisture content in those dried mini-tablets was similar to the commercial Lactinex product (between 3 to 5%), except the one using whey permeate as medium (Tables II.12 and II.13). Increasing the vacuum drying time from 2 hr to 5 hr did not significantly change the survival counts of Lactobacillus or the moisture content (Table II.12).

Efforts to concentrate fermented evaporated milk medium do not result in greater than a doubling of cell numbers because of the higher solids (> 20%) initially present. Time and pressure are the main concerns in using the Minitan ultrafiltration system. Selecting a medium which favors Lactobacillus bacterial growth, but contains less solid (proteins) could be a potential way to increase the number of bacteria counts after concentration. Whey permeate was chosen as a growth medium. After fermentation and concentration, the major residues from concentrated medium are bacterial cells. Table II.13 results suggests that by switching the growth medium to whey permeate, a 10-fold increase of viable bacteria cells was collected in concentrated medium. This is the first time a 10^{10} cfu/ml counts for Lactobacillus were obtained in this research. Another encouraging result is that the viable cell counts remained 10^{8} cfu/g after 2 hours acid treatment.

The stability of Lactobacillus mini-tablet products are shown in

Table II.14. Product from formula "2" was more stable at room temperature compared with product from formula "4" in study-4. The Lactobacillus bacteria were maintained stable in product "2" after storaging 50 days in a glass bottle with screw cap at room temperature, compared to product "4" which had almost 100% kill after storaging in a plastic container for 54 days (Table II.14). Reasons for product "4" better stability could be attributed to the lower initial bacterial loading and lower moisture content inside the product.

Table II.9 Survival of Lactobacillus bacteria in enteric coated mini-tablet using MRS as growth medium (study-4).

SAMPLE	PLATE COUNTS GASTRIC FLUID TREATMENT (hr)	% SURVIVAL
Wet dough	$3.6X10^7$ (0)	
Vacuum dried	$2.0X10^7$ (0)	100
ENTERIC COATED PRODUCT		
Sealing coat	$2.1X10^7$ (0)	107
	$1.3X10^7$ (2)	66
5%	$4.9X10^7$ (0)	263
	$2.9X10^7$ (2)	156
10%	$5.0X10^7$ (0)	283
	$2.9X10^7$ (2)	164
15%	$4.1X10^7$ (0)	246
	$3.0X10^7$ (2)	180

Table II.10 Lactobacillus counts at each stage of preparation in formula "2" (study-4) using evaporated milk as growth medium.

SAMPLE	COUNTS	COUNTING METHOD
inoculum	$2.7X10^8$/ml	(a)
after fermentation	$1.0X10^9$/ml	(a)
after concentration	$2.4X10^9$/ml	(a)
formulation dough	$2.5X10^8$/g	(a)
after drying	$2.5X10^6$/g	(a) underestimate
mini-tablets	$5.6X10^6$/g	(b) underestimate
	$1.4X10^7$/g	(c)
after sealing coat	$4.0X10^6$/g	(a) underestimate
after enteric coat	$4.4X10^6$/g	(a) underestimate
Immediately after 2 hr simulated gastric fluid treatment:		
sealing coat	$2.0X10^7$/g	(a) underestimate
enteric coat	$4.4X10^7$/g	(a) underestimate

a) Product was stored in 100 ml of simulated intestinal fluid at 4° C for 24 hr, followed by treatment in a stomacher, and counting. It was discovered that this method underestimates the true number for finished mini-tablets.

b) Product was stored in 100 ml simulated intestinal fluid at room temperature for 5 hours before stomaching and counting. This method also underestimates viable cells in mini-tablets.

c) Product was stored in 1000 ml simulated intestinal fluid at room temperature for 5 hours prior to stomaching and counting. Based on dissolution data collected later, these counts may be correct or may still be an underestimate.

Table II.11 Dissolution of mini-tablets from formula "2" in study-4 (in 1000 ml simulated intestinal fluid, basket method, 75 rpm, 37° C).

SAMPLE	TIME (min)	COUNTS
1. Following 2 hr pretreatment of simulated gastric fluid		
enteric coated	30 min	$<10^5$
mini-tablets	60 min	$2.0X10^5/g$
	120 min	$6.7X10^6/g$
	150 min	$3.6X10^7/g$
Without gastric fluid pretreatment		
dried mini-tablets with		
no coating	60 min	$3.7X10^6/g$
	180 min	$1.4X10^7/g$
	300 min	$2.7X10^7/g$
sealing coat	60 min	$9.2X10^6/g$
	180 min	$4.1X10^7/g$
	300 min	$5.1X10^7/g$
enteric coat[a]	60 min	$3.7X10^6/g$
	180 min	$1.2X10^7/g$
	300 min	$2.6X10^7/g$

a: Tablets are sealing coated first, and then enteric coated.

Table II.12 Lactobacillus counts at each stage of preparation in formula "3" (study-4) using evaporated milk as growth medium.

SAMPLE	COUNTS		MOISTURE CONTENT[a]
	before[b]	after[c]	
Fermented milk	$5.5X10^8$/ml		
Concentrated milk	$1.0X10^9$/ml		
Dough	$3.1X10^8$/g		
Mini-Tablets			
2 hr vacuum dried	$2.0X10^7$/g	$1.6X10^7$/g	4.4 %
3 hr vacuum dried	$1.4X10^7$/g	$2.4X10^7$/g	4.5 %
4 hr vacuum dried	$2.5X10^7$/g	$2.1X10^7$/g	3.9 %
5 hr vacuum dried	$1.8X10^7$/g	$1.4X10^7$/g	3.3 %

a: The moisture content was determined by Oregon Freeze Dry Inc., using high vacuum dry method. Samples did not vacuum seal and were leave on rroom temperature before testing.

b: "Before acid pretreatment", only treated with 1000 ml simulated intestinal fluid.

c: Only enteric coated mini-tablets were tested for the survival of bacteria "after" 2 hours acid pretreatment (pH = 1.4).

Table II.13 Survival of Lactobacillus bacteria in mini-tablets using whey permeate as growth medium (study-4).

SAMPLE	COUNTS	MOISTURE CONTENT[a]
Fermented medium	$1.6X10^9$ /ml	
Concentrated medium	$1.2X10^{10}$ /ml	
Dough	$6.0X10^8$ /g	
Mini-Tablets		
3 hr vacuum dried	$9.3X10^7$ /g	1.6 %[c]
5 % Eudragit coated	$7.7X10^7$ /g	
2 hr acid treated[b]	$1.0X10^8$ /g	
Lactinex		
Tablets (Lot 538)		4.7 %
Granules (Lot 1404)		4.2 %

a: The moisture content was determined by Oregon Freeze Dry Inc.

b: Only the enteric coated mini-tablets were treated with acid solution (pH = 1.4).

c: Sample was vacuum sealed immediately after vacuum drying.

Table II.14 Stability (shelf-life) test of 5% Eudragit L30D coated Lactobacillus mini-tablet in formulae "2" and "4" (study-4).

FORMULA No.	DAY[1]	COUNTS
2	0	$2.6X10^7$
	50	$2.3X10^7$
	104	$1.2X10^6$, $(2.1X10^6)^2$ (moisture content 7%)
4	0	$7.7X10^7$
	27	$7.0X10^7$, $(1.5X10^8)^2$
	56	$>10^5$, $(1.3X10^7)^2$ (moisture content 9.8%)

1: At room temperature.

2: Plate counts for samples in refrigerator.

Container:

Glass bottle with cap for product "2".

Plastic container (FLEX-N-POP) for product "4".

CONCLUSIONS

1. Using monosodium glutamate (MSG) combined with polyvinyl pyrrolidone (PVP) and malt extract improved Lactobacillus bacteria survival during freeze drying and vacuum drying. Concentration of malt extract, MSG, and PVP necessary in the drying medium depended on the concentration of bacteria; about 2% for 10^7 cfu/ml of lactobacilli, but more for higher bacterial concentrations.

2. With external continuous neutralization using NH_4OH, Lactobacillus bacteria could grow to 10^9 cfu/ml at both 33° C and 37° C in evaporated milk. Bacteria grown at 33° C showed better resistance to the stress of drying than bacteria grown at 37° C. After adjusting pH in fermented milk culture, the bacterial number can be maintained at 10^9 cfu/g of wet calcium alginate beads.

3. Whey powder is very useful as a protectant for lactobacilli. The highest recovery of bacteria from freeze drying was from the high solids content products including those with whey powder loaded into the product.

4. The structure of dried calcium alginate beads was more fragile for freeze dried products compared to vacuum dried products. Thus the degree of difficulty in coating freeze dried beads was greater.

5. By increasing the hardening time of calcium alginate beads in calcium chloride solution containing a mass balance of major protectants, the survival of Lactobacillus bacteria was higher than when compared to use of calcium chloride solution without mass balance.

6. Survival of Lactobacillus bacteria after drying was generally higher from freeze dried products compared to vacuum dried products. However, after pretreatment with simulated gastric fluid (pH = 1.5) for 2 hours, only the coated products from vacuum drying showed promising results. The best enteric coat tested was Eudragit L30D.

7. L. acidophilus and L. bulgaricus were fully protected against gastric pH after formatting the bacteria inside mini-tablets which were coated with the enteric coating polymer, Eudragit L30D.

8. Enteric coated Lactobacillus granules (Lactinex) provided stable bacteria counts in refrigeration conditions, and in dessication conditions at room temperature. These bacterial formulations survived gastric acid treatment for two hours.

REFERENCES

1. Draser, B. S. and M. L. Hill. 1974. Human intestinal flora. Academic Press. New York. pp. 36.

2. Sandine, W. E., K. S. Muralidhara, P. R. Elliker and D. C. England. 1972.Lactic acid bacteria in food and health: a review with special reference to enteropathogenic Escherichia coli as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Food Prot. 35:691.

3. Bibel, D. J. 1988. Elie Metchnikoff's bacillus of long life. ASM News. 54:661.

4. Luckey, T. D. 1984. Perspectives in intestinal microecology. Microecol. Ther. 14:243.

5. Alm, L. 1983. Survival rate of salmonella and shigella in fermented milk products with and without added human gastric juice. An in vitro study. Prog. Food Nutr. Sci. 7:19.

6. Merson, M. H., G. K. Morris, D. A. Sack et al. 1976. Traveler's diarrhea in Mexico. New England J. Med. 249:1299.

7. Shore, E. G., A. G. Dean, K. J. Holik, and B. R. Davis. 1974. Enterotoxinproducing Escherichia coli and diarrheal disease in adult travelers: a prospective study. J. Infect. Dis. 129:577.

8. Alm, L., D. Humble, E. Ryd-Kjellen, and G. Setterberg. 1983. The effect of acidophilus milk in the treatment of constipation in hospitalized geriatric patients. Nutrition and the intestinal flora. pp.131 in Symp. Swed. Nutr. Found. 15.

9. Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by Lactobacillus acidophilus. Appl. Environ. Microbiol. 49:377.

10. Lin, S. Y., J. W. Ayres, W. Winkler, Jr., and W. E. Sandine. 1989. Lactobacillus effects on cholesterol: in vitro and in vivo results. J. Dairy Sci. 72:2884.

11. Welch, C. 1987. Nutritional and therapeutic aspects of Lactobacillus acidophilus in dairy products. Cultured Dairy Products J. May:24.

12. Reddy, K. P., K. M. Shahani, and S. M. Kulkarni. 1976. B-complex vitamins in cultured and acidified yogurt. J. Dairy Sci. 59:191.

13. Perdigon, G., M. E. Macias, S. Alvarez, G. Oliver, and A. P. Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53:404.

14. Gilliland, S. E., M. L. Speck, G. F. Nauyok, Jr., and F. G. Giesbrecht. 1978. Influence of consuming nonfermented milk containing Lactobacillius acidophilus on fecal flora of healthy males. J. Dairy Sci. 61:1.

15. Pozo-Olaro, J. D., J. H. Warram, R. G. Gomez, and M. G. Cavazos. 1978. Effect of a lactobacilli preparation on traveller's diarrhea. A randomized, double blind clinical trial. Gastroenterology 74:829.

16. Lidbeck, A., J. Gustafsson, and C. E. Nord. 1987. Impact of Lactobacillus supplements on the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. 19:531.

17. Goldin, B. R., L. Swenson, J. Dwyer, M. Sexton, and S. L. Gorbach. 1980. Effect of diet and Lactobacillus acidophillus supplements on human fecal bacterial enzymes. JNCI 64:255.

18. Hussi, E., T. A. Miettinen, A. Ollus, E. Kostiainen, C. Ehnholm, B. Haglund, J. K. Huttunen, and V. Manninen. 1981. Lack of serum cholesterol-lowering effect of skimmed milk and butter milk under controlled conditions. Atherosclerosis 39:267.

19. Gilliland, S. E. and M. L. Speck. 1977. Enumeration and identity of lactobacilli in dietary products. J. Food Prot. 40:760.

20. Brennan M., B. Wanismail, and B. Ray. 1983. Prevalence of viable lactobacillus acidophilus in dried commercial products. J. Food Prot. 46:887.

21. Brennan, M., B. Wanismail, M. C. Johnson, and B. Ray. 1986. Cellular damage in dried Lactobacillus acidophilus. J. Food Prot. 49:47.

22. Porubcan, R. S., and R. Sellars. 1979. in Microbial Technology. Academic Press. New York. vol. 1, pp.59.

23. Marth, E. H. 1973. in Low Temperature Preservation of Foods and Living Material. Mercel Dekker Inc. New York. pp.386.

24. Brown, J. P. 1977. Role of gut bacterial flora in nutrition and health: a review of recent advances in bacteriological techniques. CRC Crit. Rev. Food Sci. Nutr. 8:229.

25. Sandine, W. E. 1979. Roles of Lactobacillus in the intestinal tract. J. Food Prot. 42:259.

26. Obayashi, Y., S. Ota, and S. Arai. 1961. Some factors affecting preservability of freeze-dried bacteria. J. Hyg. Camb. 59:77.

27. Valdez, G. F., G. Giori, A. P. Ruiz-Holgado, and G. Oliver. 1985. Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. Appl. Environ. Microbiol. 49:413.

28. Webb, S. J. 1960. Factors affecting the viability of air-borne bacteria. III. The role of bonded water and protein structure in the death of air-borne cells. Can. J. Microbiol. 6:89.

29. Fry, R. M. 1966. Freezing and drying of bacteria. in Cryobiology. Academic Press, London. pp.665.

30. Scott, W. J. 1958. The effect of residual water on the survival of dried bacteria during storage. J. Gen. Microbiol. 19:624.

31. Porubcan, R. S. and R. L. Sellars. 1975. Stabilized dry cultures of lactic acid-producing bacteria. U.S. Patent No.3,897,307.

32. Kim, H. S., B. J. Kamara, I. C. Good, and G. L. Enders, Jr. 1988. Method for the preparation of stabile microencapsulated lactic acid bacteria. J. Indust. Microbiol. 3:253.

33. Brodelius, P. and K. Mosbach. 1982. Immobilized plant cells. in Advances in Applied Microbiology. Academic Press, New York. pp.1.

34. Casson, D. and A. N. Emery. 1987. On the elimination of artefactual effects in assessing the structure of calcium alginate cell immobilization gels. Enzyme Microb. Technol. 9:102.

35. Scott, C. D. 1987. Immobilized cells: a review of recent literature. Enzyme Microb. Technol. 9:66.

36. Robins-Browne, R. M. and M. M. Levine. 1981. The fate of ingested lactobacilli in the proximal small intestine. Am. J. Clin. Nutr. 34:514.

37. Pettersson, L., W. Graf, and U. Sewelin. 1983. Survival of Lactobacillus acidophilus NCDO 1748 in the human gastrointestinal tract. XV Symp. Swed. Nutr. Found. pp.127.

38. Kleeman, E. G. and T. R. Klaenhammer. 1982. Adherence of Lactobacillus species to human fetal intestinal cells. J. Dairy Sci. 65:2063.

39. Conway, P. L., S. L. Gorbach, and B. R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1.

40. Gorbach, S. L. and B. R. Goldin. Lactobacillus acidophilus strains of bacteria and compositions thereof. Eur. Patent Application No. 86302836.1

41. Nilsson, I., S. Ohlson, L. Haggstrom, N. Molin, and K. Mosbach. 1980. Denitrification of water using immobilized Pseudomonas denitrificans cells. Eur. J. Appl. Mocrobiol. Biotechnol. 10:261.

42. Grade, V. L., B. Thomasset, and J. N. Barbotin. 1981. Electron microscopic evidence of an immobilized living cell system. Enzyme Microb. Technol. 3:216.

43. Linco, P., S. L. Stenroos, and Y. Y. Linco. 1984. Applications of immobilized lactic acid bacteria. Ann. New York Acad. Sci. 434:406.

44. Valdez, G. F., G. S. Giori, A. A. Ruiz-Holgado, and G. Oliver. 1983. Protective effect of adonitol on lactic acid bacteria subjected to freeze-drying. Appl. Environ. Microbiol. 45:302.

45. Valdez, G. F. G. S. Giori, A. P. Ruiz-Holgado, and G. Oliver. 1983. Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. Cryobiology 20:560.

46. Bozoglu, T. F. and G. C. Gurakan. 1989. Freeze-drying injury of Lactobacillus acidophilus. J. Food Prot. 52:259.

47. Peri, C. and C. Pompei. 1977. Optimum survival conditions for lactic acid bacteria in spray-dried yoghurt. Dairy Sci. Abstr. 37:7495.

48. House-Shokuhin-Kogyo, K. K. 1981. Powdered milk production. Dairy Sci. Abstr. 43:5838.

49. Prajapati, J. B., R. K. Shah, and J. M. Dave. 1987. Survival of Lactobacillus acidophilus in blended - spray dried acidophilus preparations. Aust. J. Dairy Technol. March/June:17.

50. Pettersson, H.-E. 1976. Preservation of mixed species lactic starter concentrates by freezing and lyophilization methods. Dairy Sci. Abstr. 38:4404.

51. Wright, C. T. and T. R. Klaenhammer. 1981. Calcium-induced alteration of cellular morphology affecting the resistance of Lactobacillus acidophilus to freezing. Appl. Environ. Microbiol. 41:807.

52. Fontana, R., P. Canepari, and G. Satta. 1979. Alternations in peptidoglycan chemical composition associated with rod-to-sphere transition in a conditional mutant of Klebsiella pneumoniae. J. Bacteriol. 139:1028.

53. Wright, C. T. and T. R. Klaenhammer. 1983. Survival of Lactobacillus bulgaricus during freezing and freeze-drying after growth in the presence of calcium. J. Food Sci. 48:773.

54. Hurst, A. and J. M. Stubbs. 1969. Electron microscopic study of membranes and walls of bacteria and changes occurring during growth initiation. J. Bacteriol. 97:1466.

55. Smittle, R. S., S. E. Gilliland, and M. L. Speck. 1972. Death of Lactobacillus bulgaricus resulting from liquid nitrogen freezing. Appl. Microbiol. 24:551.

56. Espina, F. and V. S. Packard. 1979. Survival of Lactobacillus acidophilus in a spray-drying process. J. Food Prot. 42:149.

CHAPTER III

CALCIUM ALGINATE GELS IN ANTI-INFLAMMATORY DRUG DELIVERY

SELECTIVE REVIEW OF ALGINATE GELS IN DRUG DELIVERY SYSTEMS

Alginic acids are naturally occurring substances found only in the brown seaweeds. Of the many species of brown seaweeds known, only a few are sufficiently abundant or are suitable for commercial extraction of alginates. The most widely used are species of Laminaria, Macrocystis and Eklonia (1). Alginic acid of these species varies from 14 to 40% of the dry solids. Mannuronic and guluronic acids are the two constituents of alginic acid. Alginic acid salts formed with most di- and polyvalent metals are insoluble while those of alkali metals and many organic bases are soluble in water. Most water-soluble alginates are also precipitated by addition of water-miscible organic solvents such as alcohols and ketones. The difference in solubility, depending on the salt form, is extremely useful in drug product formulation. The amount of divalent ion necessary to obtain precipitation of alginates increases in the order Pb<Cu<Ba<Ca< Zn,Co,Ni<Mn (2). The most common application of alginate precipitation in drug product formulation is based on insolubilization of alginates by addition of a calcium salt. Ion exchange properties of alginates depend on the chemical composition of the alginate. Alginates rich in mannuronic acid such as those from Laminaria digitata have a lower affinity for calcium in a sodium-calcium ion exchange reaction than alginates rich in guluronic acid units, such as the alginate from Laminaria hyperborea (1, 2).

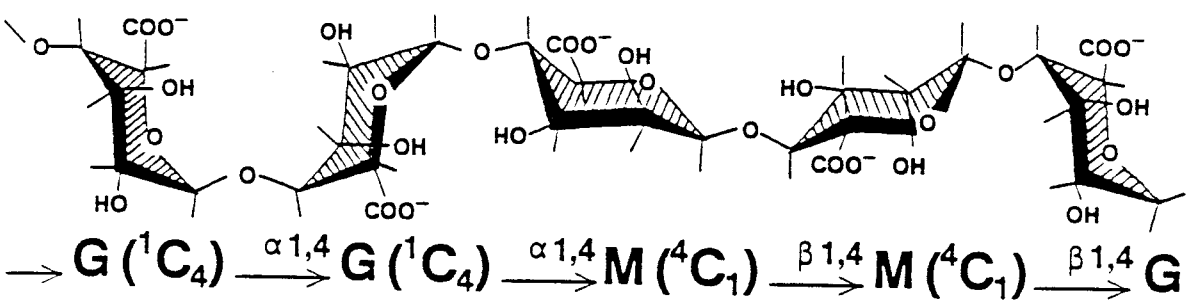
Literature reviewed for this article was selected in a relatively narrow range. Primarily, the search was for information related to a project requiring formulation of alginates into controlled release beads

containing live cells or therapeutical drugs for oral administration. Thus, topics included are alginate cross-linking, enzymes and cell entrapment in alginates, and diffusion in alginate-drug formulations utilizing cross-linked alginates.

PHYSICAL PROPERTIES OF SODIUM/CALCIUM ALGINATE GELS

Effect of Alginate Structure and Composition.

It is well-established that regular, crystalline, intermolecular packing typical of polysaccharide chains in the condensed phase may persist under conditions of extensive hydration, as sterically regular, co-operative, interchain "junction zones" in solutions, gels, and biological tissues (3). Sodium alginate is a binary copolymer of D-mannuronate and L-guluronate residues arranged in a blockwise pattern along a linear chain. Chemically, alginate is a (1 to 4)-link, linear, block copolymer of beta-D-mannuronate and its C-5 epimer alpha-L-guluronate, with residues arranged in homopolymeric sequences of both types, and in regions which approximate to a disaccharide repeating-structure (4, 5).



Studies of chain-length dependence of polyguluronate calcium-binding activity shows the onset of co-operative binding above a degree of polymerization of about 20 residues (6). Physical properties of alginates depend not only upon the uronate composition i.e., the mannuronate/guluronate ratio, but also upon the monomer sequence distribution in the copolymer (7). Alginate can be separated into three fractions, one containing >80% of mannuronate, the second of >80% guluronate, and the third a blockwise arrangement of the uronates along the chains, with blocks of continuous mannuronate and guluronate residues separated by blocks approaching an alternating pattern (7). From microscopic visualization (SEM) of metal alginate, the alginate fine structure is found to be a regularly arranged network having many host spaces in its fiber net. In acid solution such a fiber net developed a rounded, buckled and twisted form as much as possible and finally changed to a porous cluster form (8). Alginate forms three types of packing in acid solution : cage or channel form where the guest position is surrounded by an alginate fiber net; round hole type where dimer alginate fiber makes a ring and rounded cylindrical space; or a form which depends on the so-called helical cylindrical chain of alginic acid (8). In general, the binding properties of guluronic acid-rich alginates are found to be more effective than mannuronate acid-rich alginates. Degraded sodium alginates bind strontium more strongly than undegraded ones (8).

The level of bound calcium in alginate chains resistant to displacement by swamping concentrations of sodium ions is equivalent to half the stoichiometric requirement of poly-L-guluronate chain-sequences

(9). Ion-exchange studies of the preferential binding of calcium in competition with magnesium for an alginate containing 90% L-guluronate residues, show strong selectivity for calcium ions up to about 40-45% of the stoichiometric requirement of the entire molecule, beyond which the calcium selectivity coefficient drops off rapidly (10).

Calcium alginate beads appear to be lost via the peeling off of a thin outer layer which appears to have a different structure from underlying calcium alginate (11). The presence of this layer is described as "a membrane-like coating", which is mechanically weaker than the underlying calcium alginate (12). The structure of calcium alginate inside the beads is solid and not sponge-like: a sponge-like structure could be the result of dehydration of incompletely fixed beads (11).

Effect of Calcium Chloride and Sodium Alginate Concentrations.

When a droplet of sodium alginate solution contacts calcium chloride solution, gelation occurs instantly on the surface of the droplet, which forms an almost spherical bead. Although the intrinsic rate of curing appears likely to be extremely fast, the actual rate of complete spherical gel formation is primarily controlled by penetration of calcium ions into the interior of the droplet, and therefore is dependent on the alginate solution droplet size. Beads of sodium alginate shrink when dropped into calcium chloride solution. Contracting cross-linking alginate polymer squeezes water out of the interior, traversing the already cured outer part of the bead to the bulk solution. Because small beads shrink proportionally more than larger beads, small beads become more dense than larger beads (13).

Weight changes of beads produced using various initial Na-alginate concentrations (1,2,3,and 4% w/v) occur mostly in the first several hours but take about 70 h to reach constant weight in 0.1 M calcium chloride solution (14). The production of a fully-cured state depends on the bulk calcium chloride concentration, and a threshold bulk calcium chloride is required for an initial gel to form. The weight of beads decreases with increasing calcium chloride concentration and appears to become constant at calcium chloride concentrations above 0.08 M for Na-alginate solution from 1 to 4% w/v (14).

Diffusion characteristics of hemoglobin from 1% w/v calcium alginate gels are not affected by a variation of calcium chloride concentration from 0.125 to 0.5 M (15). The effective diffusion coefficient of glucose in calcium alginate gel determined at 35^0 C is at least 24% less than in water. Increasing the concentration of either the calcium chloride or sodium alginate component used in calcium alginate formation substantially decreases the diffusion coefficient, but the effect of sodium alginate is the greater of the two (14, 15, 16). Diffusion characteristics of a low-molecular-weight entrapped drug are not affected by calcium ion or gel concentrations. In the case of high-molecular-weight compounds the diffusion characteristics are significantly affected by alginate gel concentration, but not calcium ion concentration (15).

Effect of Bead Diameter and Gelation Method.

Bead dimensions up to 5 mm in diameter provide little or no diffusional resistance to low-molecular-weight compounds but the release and diffusion of high-molecular-weight compounds is greatly restricted

when diameters are increased from 2 mm to 5 mm (15). When increasing cell loading in alginate beads, the rate of conversion of methanol into formaldehyde by immobilized Hansenula polymorpha cells becomes increasingly limited by diffusion of oxygen from the liquid through the gel matrix (17). The magnitude of apparent K_m and V_{max} values for cell-bound invertase activity (Saccharomyces pastorianus) immobilized in spherical agar pellets depended on both pellet size and intrapellet concentration of cell-bound enzyme (18). Reduction of invertase activity in immobilized cells is due to a combination of diffusion hindrances from the cells themselves and from the gel network (19). Respiration kinetics of cells (Daucus carota) immobilized by entrapment in calcium alginate show that as cell loading and diameter of the bead increase the slope of the Lineweaver-Burk plots becomes steeper, indicating increasing limitation of the respiration rate by diffusion of oxygen (20, 21). Critical diameters and cell loadings for good diffusion characteristics allow the diameter of beads to be as high as 1.40 mm for 50% cell loading up to 3.80 mm for 5% loading (21).

Gelling method (internal/external) and particle shape (sphere, cylinder, slab) have no effect on enzyme activity if specific surface area is the same for a fixed alginate/yeast ratio (19). Internal gelation involves blending the material-containing alginate solution with a suspension of calcium citrate and D-gulcono-1,5-lactose (22). Generally, light transmission through a Na-alginate solution is found to be better than through Ca-alginate gel (23). Swelling of dried gel particles prepared from fully-cured hydrogels is of interest: particles remain unchanged in distilled water or acidic medium (pH 1.5 KCl-HCl)

but swell rather rapidly in pH 7.0 phosphate buffer to a size greater than their original size before being dried (14).

APPLICATIONS OF SODIUM/CALCIUM ALGINATE GELS

Tablet and Alginate Gels.

Alginic acid, sodium alginate, and calcium alginate are well-established tabletting excipients. Both alginic acid and calcium alginate are insoluble in water but absorb several times their weight of water. Alginic acid cannot be used to formulate acid-sensitive drugs. The water soluble sodium alginate is sometimes employed as a tablet binder. When starch or micro-crystalline cellulose was added to sulphanilamide granules without any other excipients in the formulation, the dissolution rate of granules was retarded. In contrast, the inclusion of 1% w/w calcium alginate enhanced dissolution of the granules (24). At 1% concentration of either sodium or calcium alginate, there was practically no effect on the granule dissolution rate and at 5% sodium alginate there was generally a retardation while at the same concentration of calcium alginate there was an improvement in dissolution (25).

Strongly swelling alginate can "waterproof" the tablet by its rapid swelling on the tablet's surface. The wetted alginate can form a viscous gel on the tablet surface producing an adhesive surface relatively impervious to liquid entry (24). In tablets containing microcrystalline cellulose and sulphonamide, surfactant improved liquid penetration by improving the wettability of the tablet interior facilitating liquid access. For sulphanilamide, tablets formulated with

1% w/w calcium alginate demonstrated a much slower water uptake pattern than without calcium alginate. Formulations with surfactants had slower initial water uptake rate but showed greater capacity of water uptake (24).

Biocompatible Membrane.

Several methods have been developed for microencapsulating biologically active molecules, tissue, and cells so they remain viable and in a protected state within a semipermeable membrane which permits passage of low molecular weight substances, such as nutrients and oxygen, but not of cells and high molecular weight proteins. Many hydrogels have gained general acceptance as being biocompatible materials since, as implants, they are tolerated by the body fluids. The soft pliable consistency (high compliance) of most hydrogels may also contribute to their biocompatibility by reducing frictional irritation to surrounding tissues (26). Calcium alginate has been studied extensively as a means of immobilizing fragile cells in an ionically cross linked polysaccharide gel matrix.

Protoplasts, which are obtained by removal of plant cell walls, have been mechanically stabilized by immobilization in calcium alginate gels (27). Clotting of milk by immobilized Silybum marianum protoplasts in calcium alginate beads was higher than other natural polysaccharides (agar and Cellulose acetate) and synthetic (polyacrylamide) gels (28). Lysis experiments with protoplasts (Daucus carota Ca68) immobilized in calcium alginate beads showed that with 50 mM calcium concentration, sorbital concentration had a very small affect, but viability of the protoplasts decreased with decreasing sorbital concentration (29) at

lower calcium concentration (10 or 25 mM).

Adipocytes were entrapped in either calcium alginate or agarose and subsequently tested for their capability to incorporate [3-^{3}H] glucose into lipids following insulin stimulation as well as for their ability to release free fatty acids by noradrenaline stimulation. It was found that, at the outset, the immobilized adipocyte preparation showed a higher basal incorporation than free cells which was more pronounced with cells immobilized in calcium alginate (30).

Animal experiments have established that islet transplantation can prevent or reverse the retinal and renal complications associated with diabetes (31, 32). To prevent rejection by host antibodies, it has been suggested that these cells be immobilized in a durable, semipermeable tissue compatible matrix. Islets were microencapsulated in a biocompatible alginate-polylysine-polyethyleneimine membrane and implanted into Wister rats with streptozotocin-induced diabetes. The results on implantation of microencapsulated islets compared to suitable controls showed that the unencapsulated islets survived for 6 to 8 days as anticipated. Rats receiving microencapsulated islets were normoglycemic for almost 3 weeks; the symptoms of diabetes such as polyuria and polydypsia were markedly reduced (33). The microencapsulated islets remained morphologically and functionally intact for as long as 4 months in culture <u>in vitro</u> at 37° C (33). Prolonged survival of islets allografts was achieved by encapsulating individual islets in alginate-polylysine-alginate membranes. A single intraperitoneal transplant of encapsulated islets reversed the diabetic state for up to 1 year. In contrast, a single injection of

unencapsulated islets was effective for less than 2 weeks (34). Human erythrocytes which had been encapsulated in calcium alginate beads were coated with a water-insoluble cationic polyacrylate, Eudragit RL. The alginate/Eudragit beads could withstand greater compressive forces before mechanical failure than uncoated beads. Encapsulated erythrocytes continued to consume glucose and reversibly bind oxygen even after 21 days storage at 4° C (35).

Sustained Release Drug Delivery.

Controlled release formulations prepared by utilization of biopolymers are of special interest because of the possible biological degradation of the matrices after the duration of action is complete. Also, there is a relatively low cost of raw materials. There is a growing realization that conventional application of agricultural chemicals is often wasteful, and can cause toxicity problems in nontarget species. Delivery from controlled release formulations is a promising concept, and many advances have been made in recent years. Alginate gels were selected as matrices for studying the utility of controlled release in certain agricultural applications because of the biodegradability of these gels and the ease of incorporation of pesticides using an aqueous system at ambient temperature.

Different herbicides were entrapped in sodium alginate and added dropwise into calcium chloride to form calcium alginate beads. The hydrated alginate beads containing desmetryn showed a 99.9% release of the herbicide in 1.8-2 days while the dried beads had a 99.3% release in 9.75 to 10.0 days (36, 37). Diquat dibromide can be incorporated into the alginate and slowly released into water close to plants. Plants

treated with the diquat/alginate formulation in a river were completely destroyed above river bed level and no regrowth appeared during the period of the experiment; cut weeds had regrown to about half their original size during the same period (38). The herbicides 2.4-D[(2,4-dichlorophenoxy)acetic acid] and dichlobenil (2.6-dichlorobenzonitrile) were individually incorporated into 1.6-2.8 mm alginate gel beads using calcium chloride or barium chloride as gellants. Hydrated beads released the relatively water soluble 2,4-D as fast as unformulated material dissolved. About 96% of the 2,4-D was released into in solution in 2 days, but dried beads gave slower release, about 4-6 days for a calcium gelled sample and 14 days for a barium gelled sample (39). Unformulated dichlobenil dissolved completely in 41 days while hydrated beads exhausted their herbicide content in about 90 days for both calcium and barium gelled beads. Dried calcium gelled beads released 84% and barium gelled beads released only 59% of their dichlobenil content into solution in 150 days (39). Release from the calcium alginate gels was nonlinear; a high initial release rate diminishes with time. Barium alginate gels produced a linear or zero-order release throughout the 150 days duration (40).

A mixture of carbendazim and triadimefon in an aqueous gel formed from sodium alginate is an alternative to conventional sealants for the treatment of pruning wounds on apple and plum trees. Callus tissue formation was not impaired and treatment did not affect the natural microbial population of the wood. An inhibitory dose of each fungicide was established beneath the flush wound surface and maintained for at least 100 days irrespective of time of treatment (41). Fungicide

movement and persistence were similar with sodium alginate and xanthan gels but movement was reduced and persistence increased with the esterified material (42).

The time during which an orally administered drug delivery system resides in the stomach may have an important influence on drug absorption. Sodium alginate gel systems have been evaluated as sustained release oral drug delivery systems with a potential for prolonged gastric residence. A sodium alginate sustained release floating gel system was studied. Different compositions of sodium alginate, calcium phosphate, sodium bicarbonate, lactose and a drug (chlorpheniramine maleate, caffeine or sodium salicylate) were mixed and hand-filled into No. 1 hard gelatin capsules. Drug release was found to be linear with the square root of time and inversely proportional to the alginate content in 0.1 N HCl dissolution fluid. Release was also drug dependent with the cationic drug chlorpheniramine maleate having a slower release rate than the anionic drug sodium salicylate (43). Tablets made from compressing mixtures of sodium alginate, sodium-calcium alginate and at least one active ingredient showed a better sustained release profile in distilled water compared to tablets which had calcium hydrogen phosphate instead of sodium-calcium alginate (44). The total amount of sulphamethoxazole entrapped in calcium alginate beads released in a dissolution test (0.1N HCl) did not vary with sodium alginate concentrations (from 1.0 to 2.0% w/v). The times to release of 50% of sulphamethoxazole content were 120, 160, and 200 minutes for 1, 1.6, and 2 w/v of sodium alginate solutions respectively. The release rate of sulphamethoxazole from 1.6% w/v sodium alginate beads in 0.1 N

HCl dissolution fluid was found to decrease with increasing calcium chloride concentration (45). Phenformin HCl was entrapped in polyacrylamide beads or polyacrylamide beads cross linked with calcium alginate. Retardation of drug dissolution was not much affected by cross linking with calcium alginate in 0.1 N HCl solution, but in phosphate buffer solution (pH=7.2) the effect of calcium alginate concentration was highly efficient for retarding drug dissolution (46).

Sodium alginate has also been used as a delivery system for oils and oil soluble substances. Vitamin oil or vegetable oil was added to 2% sodium alginate solution and mixed in a homogenizer. The emulsion was fed through a capillary into 2.5% calcium chloride in methanol to form spherical micro-beads. The oil-in-water multicompartmentalized, mechanically stable microcapsules could protect vitamins from oxidative degradation and can be handled as solids (47). When sodium alginate was incorporated in a chewing gum composition, the release rate of insolubolized active ingredient (like peppermint oil) was prolonged and controlled (48). A water-in-oil emulsion could also be made by mixing calcium alginate gel with an oil phase which was either self-stabilizing or contained water-in-oil emulsion stabilizers (49).

Many techniques for drug encapsulation are becoming well developed and are now generally available to researchers. While the study of new approaches and materials will undoubtedly continue, the specialized use of alginate delivery system for drugs should continue to represent an important area of research.

INTRODUCTION

Sustained Release Dosage Form Design

Ibuprofen is a nonsteroidal anti-inflammatory agent (NSAID) which has pharmacologic actions similar to those of other NSAID's such as aspirin, phenylbutazone, and indomethacin. Ibuprofen is used extensively for treatment of acute and chronic rheumatoid arthritis, osteoarthritis, and mild to moderate pain (50, 51). Ibuprofen is practically insoluble in water and very soluble in alcohol, with an apparent pKa of 4.8. Approximately 80% of an oral dose of ibuprofen is absorbed from the GI tract. The most frequent adverse effects reported in ibuprofen treated patients involve irritation of the GI tract (52). The plasma half-life of the drug has been reported to be 1.5 to 3 hours (53, 54, 55). Because of ibuprofen's short elimination half-life , adverse effects on the GI tract, and poor water solubility, it is challenging to design a sustained release dosage form which can deliver suitable amounts of completely bioavailable active ingredient, reduce side effects on the GI tract, and reduce dosing frequency to increase the duration of effect and compliance.

Bioavailability of drugs from sustained release dosage forms is dependent on a variety of factors including drug release rate, gastric emptying, intestinal residence time, and sites of drug absorption. It was shown that particles with diameters less than 5 mm can pass through the stomach in one hour, and had a mean residence time of 3-5 hours in the small intestine (56).

Colonic Drug Delivery

Sulfasalazine.

Ulcerative colitis and Crohn's disease are chronic idiopathic inflammatory conditions that tend to affect people during their most productive years. Ulcerative colitis affects the distal colonic mucosa and may extend a variable distance proximally, or be limited to the rectum. Crohn's disease is a transmural, often granulomatous and segmental process that often affects the small bowel as well as the colon (57).

In the late 1930s, Dr. Nana Svartz, a Scandinavian rheumatologist, introduced sulfasalazine for treatment of rheumatoid arthritis. Known to respond to aspirin, rheumatoid arthritis was thought to be of bacterial origin. It seemed sensible, therefore, to have an agent that combined one of the first sulfonamides, sulfapyridine, with an aspirin analog, 5-aminosalicylate acid (5-ASA). Sulfasalazine proved to efficacious in treating rheumatologic disorders (58), and sulfasalazine was also found to be an effective agent in treatment of patients with colitis. Today, sulfasalazine is the most widely prescribed agent for treatment of inflammatory bowel disease in the United States (59).

About one quarter to a third of the digested compound is absorbed in the small bowel with detectable blood levels achieved within one to two hours after intake (60, 61). The rest is split by bacterial azo-reductases in the distal small intestine and colon into 5-aminosalicylic acid and sulfapyridine (62). Most of the sulfapyridine metabolite is absorbed from the colon, partially metabolized by the liver, and excreted in the urine (63, 64). 5-ASA is apparently responsible for

most of the drug's local therapeutic effects, whereas sulfapyridine is thought to cause most of its toxic effects (65, 66). The efficacy of sulfasalazine in treatment of ulcerative colitis was firmly established through a randomized controlled study reported in 1962 and confirmed in 1964. Effectiveness of sulfasalazine therapy for Crohn's disease involving the colon has also been established in several studies (57, 67). The mechanism of action of sulfasalazine is multifactorial and may exceed the effects of the 5-ASA component. The drug's interference with prostaglandin synthesis by inhibition of cyclo-oxygenase does not account for its efficacy, because other potent inhibitors of this enzyme, such as ibuprofen and indomethacin, have failed to alleviate inflammatory bowel disease (59, 68).

Side effects are common, particularly in patients who have slow acetylation, and usually occur during the first few weeks of therapy. These effects regularly preclude use of the drug (67, 69, 70, 71). Some side effects, such as headache and gastrointestinal distress, are dose-related and may be minimized if an enteric coated preparation is used.

5-Amino Salicylic Acid (5-ASA).

As noted earlier, investigation of sulfasalazine's metabolism and distribution of its metabolites suggested that 5-ASA is the therapeutically active portion of the drug. This led to interest in development of delivery of 5-ASA alone to active disease sites. When 5-ASA is given orally, most is absorbed from the proximal small bowel and inactivated by hepatic metabolism (65). In cases of distal ulcerative colitis and proctitis it has been effective in the form of an enema or suppository (72, 73, 74, 75). However, suppository and enema forms of

5-ASA are inconvenient for some patients and not likely to benefit most patients with Crohn's disease involving the ileum and ascending colon. It may also be difficult for patients to use enemas or suppositories as maintenance medication. These factors have led to development of new oral forms of 5-ASA.

Some oral preparations of 5-ASA have been coated with an acrylic resin or compressed with sodium bicarbonate, surrounded by ethylcellulose, and coated with resin (76, 77, 78, 79, 80, 81). The coating prevents breakdown in the stomach and dissolves at alkaline pH because of its pK_as (3, 6, and 13.9); the buffer promotes fine dispersion of the drug. The 5-ASA appears to be delivered intraluminally to the terminal ileum and the colon. These products have been found to be as effective as sulfasalazine in treatment of mild to moderate ulcerative colitis, and in many studies they have been able to sustain remissions as well as sulfasalazine but with fewer side effects (82, 83, 84). Oral 5-ASA coated with ethylcellulose can allow continuous drug release through the GI tract, with only about 25% of 5-ASA carried to the colon (85, 86). After liberation of 5-ASA in the colon from slow release oral preparations of 5-ASA, 5-ASA is only partly absorbed. A major part of this 5-ASA is presystemically eliminated in N-acetylated form during its first-pass through the intestinal mucosa and liver (87). Mucosal N-acetylation was rapid, cofactor- and pH-dependent, and could be enriched in the cytosolic fraction (88).

None of the currently known oral dosage forms are designed to avoid major drug release in either the stomach or upper intestine (or both). The objectives of this study were to identify the dissolution

characteristics of drug from calcium alginate beads used as a drug delivery system for ibuprofen, sulfasalazine, or 5-aminosalicylic acid, especially when beads are coated with polymer of ethlycellulose base (Aquacoat) and/or acrylic resin base (Eudragit L30D). Knowledge of such characteristics may lead to development of a colonic site-specific drug delivery system.

MATERIALS AND METHODS

Materials

Alginic acid sodium salt, low viscosity (mostly munnuronic acid, Sigma), Calcium chloride dihydrate (Mallinckrokt), Hydroxypropyl cellulose (Klucel, Aqualon), Polyvinylpyrrolidone K-30 (PVP K-30, EM Science), Ibuprofen powder (Upjohn Co.), Motrin SR800 (Upjohn Co.), Sulfasalazine (Sigma), Azufidine IR (Pharmacia), Azufidine EN (Pharmacia), 5-Aminosalicylic acid (Sigma), Aquacoat ECD-30 (FMC Co.), Eudragit L30D (Rohm Pharma, GMBH), Triethyl citrate (Aldrich), Dibutyl sebacate (Sigma), Hydroxypropylmethylcellulose phthalate (HP-55S, Shin-Etsu Chemical Co.).

Methods

Ibuprofen Bead Formation.

Sodium alginate solution (usually 2 g/100 ml in deionized water but studied from 1.5 to 3.5 g/100 ml) was mixed with ibuprofen powder (variable from 10 to 50 g) in a commercially available blender for 3 minutes. This ibuprofen/alginate was stirred with a magnetic stirring bar in a beaker, and then the ibuprofen/alginate mixture was pumped through a No. 21 gauge hypodermic needle attached to a disposable plastic syringe with piston to produce drops. The droplets were allowed to fall into 0.5 M calcium chloride solution with stirring to form ibuprofen/alginate beads. A harvard apparatus diffusion/withdrawal pump was used to push the piston through the syringe. Beads were collected on a No.1 filter paper and dried in an air-flow oven (Thelco Model 28,

Precision Sci.) at 60° C for 2 days. Dried beads were sieved, and only beads with diameters 0.97 to 3.33 mm were used. The compositions, including concentration of alginate and ibuprofen, and the diameter range of beads for different formulations are given in Table III.1.

Besides producing ibuprofen/alginate beads, ibuprofen/alginate granules were also made using a high pressure, paint spray gun. 20% w/v ibuprofen suspended in 2% w/v sodium alginate solution was poured into the spray gun container, and sprayed into a basin containing 0.5 M calcium chloride. The granules were collected on a No. 1 filter paper and dried at 60° C for 1 day. These dried ibuprofen/alginate granules were filled into a No. 0 soft gelatin capsule for compatibility tests.

Coating of Ibuprofen Beads.

ibuprofen beads produced from 2% w/v sodium alginate and 20% w/v ibuprofen were spray coated with varied percentages of Aquacoat (see Table III.1). The coating solutions were plasticized with 15% w/w each of triethyl citrate and dibutyl sebacate (total plasticizer 30%). The spray coater was an Aerocoat spray coater using a modified Lab-Line/P.R.La High Speed Fluid Bed Dryer (Lab Line Instrument Inc.). Coating chamber, wurster column, nozzle (inner diameter 0.8 mm), and tubes were rinsed with 75% alcohol, and air dried prior to use. 70 grams of dried ibuprofen/alginate beads were added to the coating chamber and prewarmed to 30° C for 30 minutes. The coating solution was continuously stirred and pumped via a peristaltic pump (Gilson) at a rate equivalent to 6 ml/min to the coating chamber. The temperature setting was 30° C and the atomizing air was maintained between 10-15 psi.

Some Auqacoat coated ibuprofen/alginate beads were mixed with 1% w/w magnesium stearate (as a lubricant) and 20% w/w Avicel PH 102 (as a binder and disintegrant). Tablets were prepared manually by compressing these mixtures using a 2811 Pellet Press (0.5 inches Diameter punch and Die, Parr Instrument Co.), to produce a hardness of about 6 on the Strong-Cobb hardness tester.

Dissolution of Ibuprofen/Alginate Products.

Three dissolution methods were used to study the release rate of ibuprofen from these ibuprofen/alginate products.

1. Paddle method: 1 g of ibuprofen/alginate beads were placed in a U.S.P. dissolution flask and agitated using a paddle rotating at 75 rpm at 37° C. The dissolution solution was simulated intestinal fluid, pH 7.4 ± 0.5, and 3 ml of solution was withdrawn periodically and assayed spectrophotometrically at 221 nm wavelength. Each formulation was dissolved in triplicate. Reference solution for total dissolution was prepared by dissolving 0.1 g of beads in the same solution. Beads were disintegrated in a high speed chopper containing 100 ml of dissolution medium. The ibuprofen suspension was magnetic stirred to release and dissolve drug from the disintegrated ibuprofen/alginate beads.
2. Side basket method: 1 g of sample was placed in a laboratory-made basket (see Figure III.1). The basket was positioned beside the wall of the flask, between wall and paddle, approximately 6.5 cm above dissolution flask base. Other conditions remained the same as for the paddle method.

3. Basket method: Basket method used in this study was the U.S.P. standard basket method. The temperature setting, rotating speed, and sampling methods were the same as for the paddle method.

Table III.1 Composition of ibuprofen/alginate beads and spray coating solution.

Formulation	Weight of each ingredient (g)			Bead diameter (mm)
	Drug	Na-Alginate	Water	
1	5	2.0	130	0.97 - 1.40
2	10	2.0	130	1.40 - 1.65
3	20	2.0	130	1.65 - 2.36
4	30	2.0	130	1.65 - 2.36
5	40	2.0	130	1.65 - 2.36
6	50	2.0	130	2.36 - 3.33
7	10	1.5	100	1.65 - 2.36
8	10	2.0	100	1.65 - 2.36
9	10	2.5	100	1.65 - 2.36
10	10	3.0	100	1.65 - 2.36
11	10	3.5	100	1.65 - 2.36
12	10	4.0	100	1.65 - 2.36

Composition of coating solution

% coating	wt of Beads[a] (g)	Aquacoat (g)	TEC (g)	DBS (g)	Water (g)
2	120	5.6	0.36	0.36	5.68
3	85	6.0	0.38	0.38	5.99
4	70	6.5	0.42	0.42	6.68
5	80	9.3	0.60	0.60	9.47
6	70	9.8	0.63	0.63	9.94
8	70	13.0	0.84	0.84	13.23

a: Only beads produced from 2% w/v sodium alginate and 20 g w/v ibuprofen were used for spray coating.

Figure III.1 Picture of side basket used in dissolution study of ibuprofen/alginate beads.

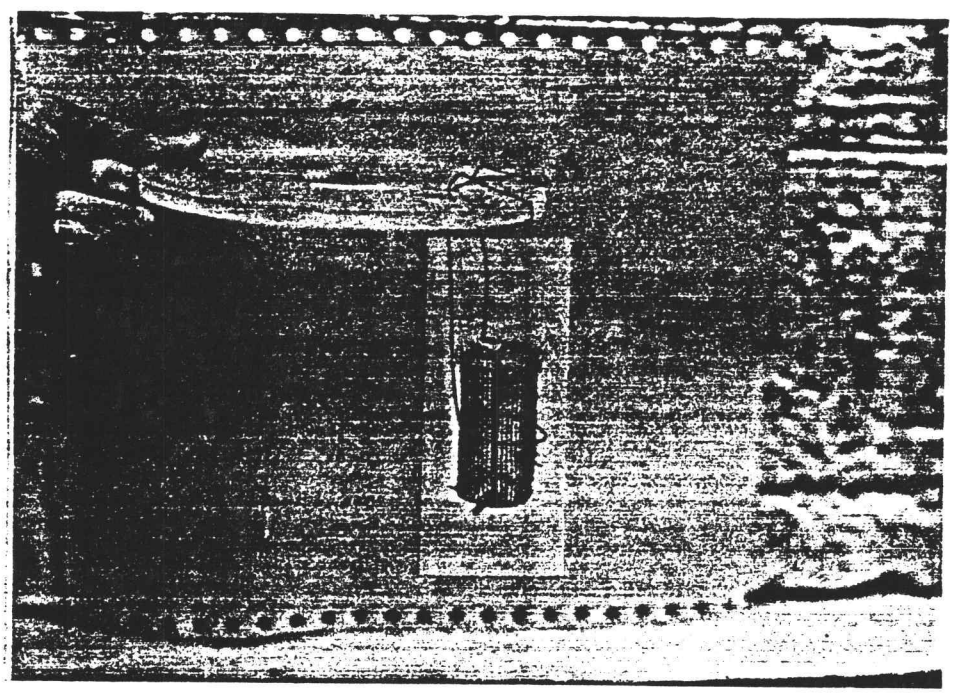


Figure III.1

Sulfasalazine (SS) bead formatiom.

Sodium alginate solution (2 or 4% w/v) was mixed with SS (13 or 18% w/v) in a blender for 3 minutes. The mixture was pumped through a lab-made device containing 6 hypodermic needle outlets to produce drops. This device was attached to a harvard infusion pump, and the drops were allowed to fall into 0.5 M calcium chloride solution to form SS/alginate beads. Beads were dried in a air-flow oven at 32° C for 2 days. Only dried beads with diameters 1.5 to 2 mm were used in this study. Some of the dried SS/alginate beads were spray coated with varying percentages of Aquacoat plasticized with 15% dibutyl sebacate and 15% triethyl citrate (Table III.2). Coated beads from formulae 2, 3, 5, and 6 were also placed in a No. 0 gelatin capsule which was enteric coated with HP-55S. Half of the capsule was hand-dipped into a mixture of 14 g hydroxypropyl methylcellulose phthalate (HP-55S), 1.5 g dibutyl phthalate, 25 ml methylchloride, and 60 ml methanol. The capsule was placed on a capsule filler, and dried using a hair dryer. After drying, the other half of the capsule was coated using the same procedure. Beads from formula 10 were directly enteric coated with Eudragit L30D, using the same percentage of plasticizers as for Aquacoat. Coating conditions were: fluid inlet = 3 ml/min, atomizing air = 15-20 psi, Temp = 30° C, blower = 5, and nozzle diameter = 0.8 mm.

Coated SS/alginate beads were allowed to dissolve in a U.S.P. dissolution apparatus using the paddle method at 50 rpm and 37° C. Beads were initially tested only in simulated intestinal fluid at pH = 7.5 $\pm$ 0.1, since neither sulfasalazine nor calcium alginate are soluble in gastric fluid. However, some of the coated beads were pretreated in

simulated gastric fluid at pH = 1.5 ± 0.1 for 2 hours before transfer to intestinal fluid. Each formulation was dissolved in triplicate and assayed spectrophotometrically at 240 nm.

5-Amino Salicylic Acid (5-ASA) bead formation.

3% sodium alginate solution was pumped through a device to produce drops which were allowed to fall into 0.5 M calcium chloride solution, which produced "blank" calcium alginate beads. This device contained 24 hypodermic needle outlets and an air pump to supply air pressure up to 20 psi. Calcium alginate beads were oven dried at 55° C for 2 days before use. 16% w/v 5-ASA was suspended in 95% ethanol solution which contained 1.6% klucel and PVP-K30 as binders. The 5-ASA suspension was spray coated on the dried "blank" calcium alginate beads at 30° C in the dark. These 5-ASA/alginate beads were sieved and only beads with diameters 1.5 to 2 mm were used for further coating with Aquacoat and/or Eudragit L30D, plasticized with 15% w/w dibutyl sebacate and triethyl citrate (Table III.3). The coating conditions were: fluid inlet = 4 ml/min, atomizing air = 10-15 psi, Temp = 30° C, blower = 5, and nozzle size = 1.2 mm for drug loading and 0.8 mm for polymer coating.

Coated 5-ASA/alginate beads were allowed to dissolve in a U.S.P. dissolution apparatus using the paddle method at 50 rpm and 37° C. Each formulation was pretreated with simulated gastric fluid (pH = 1.4) before challenge with simulated intestinal fluid (pH = 7.4) and assayed at 225 nm.

Table III.2 Composition of coated sulfasalazine/alginate beads.

FORMULA	CODE	ALGINATE w/v	SSA w/v	AQUACOAT w/w
1	Na2 AQ0	2 %	18 %	0 %
2	Na2 AQ2	2 %	18 %	2 %
3	Na2 AQ6	2 %	18 %	6 %
4	Na2 AQ8	2 %	18 %	8 %
5	Na4 AQ0	4 %	18 %	0 %
6	Na4 AQ3	4 %	18 %	3 %
7	Na4 AQ6	4 %	18 %	6 %
8	Na4 AQ8	4 %	18 %	8 %
9	Na2 AQ0 EU0	2 %	13 %	0 %
10	Na2 AQ3 EU3	2 %	13 %	3 %[a]

a: This formulation has 3% Eudragit L30D coated on the outside surface of beads.

Table III.3 Formulation of coated 5-ASA/alginate beads.

FORMULA	CODE[a]	AQUACOAT w/w	EUDRAGIT w/w
1	EU2	0 %	2 %
2	EU4	0 %	4 %
3	EU6	0 %	6 %
4	EU8	0 %	8 %
5	AQ4	4 %	0 %
6	AQ4 EU2	4 %	2 %
7	AQ4 EU4	4 %	4 %
8	AQ4 EU6	4 %	6 %
9	EU6 AQ2	2 %	6 %
10	EU6 AQ4	4 %	6 %
11	EU6 AQ6	6 %	6 %

a: The order of polymer coated on 5-ASA/alginate beads is indicated by "CODE". For example, AQ4 EU2 means the inner coat is 4% Aquacoat and the outer coat is 2% Eudragit L30D.

RESULTS AND DISCUSSION

The solubility of 2% w/v wet calcium alginate beads in different dissolution media was compared. Beads did not dissolve in 1 M $CaCl_2$, $MgCl_2$, KCl, or NaCl solution. For 6.8 g KH_2PO_4 in 810 ml deionized water, beads dissolved when the pH of solution was 7, 8, or 10 (pH was adjusted using 0.2 N NaOH); but not at pH = 2 or 4 (pH was adjusted using concentrated HCl). Increasing the concentration of KH_2PO_4 to 12 g in 810 ml water did not substantially increase the dissolution rate of calcium alginate beads. The same phenomenon existed when using NaH_2PO_4 or H_3PO_4 instead of KH_2PO_4, except a visible precipitation occurred in H_3PO_4 solution. The average diameter of wet calcium alginate beads (2% w/v sodium alginate dripped into 0.5 M calcium chloride) made using No. 21 G hypodermic needles was 0.262 cm, which shrank to 0.199 cm after 3 hours drying in a air-flow hood at room temperature. The volume was reduced about 35% during 3 hours air drying. The size of these beads did not changed with longer drying time at the same conditions.

Sustained Release Dosage Form Design

Because ibuprofen is an acidic compound (pK_a 4.8) with a very low solubility in acidic medium and is not expected to dissolve in the stomach, release of drug from the beads was initially studied only in simulated intestinal fluid. Dissolution of ibuprofen was reported to be as low as 5% at pH 2, which may be attributed to aggregation or agglomeration of drug particles (89, 90). Figure III.2a shows dissolution data for ibuprofen/alginate beads containing variable

amounts of ibuprofen, when using the U.S.P. paddle method for dissolution. Beads loaded with 5 g ibuprofen/100 ml sodium alginate had the highest release rate for percentage of drug loaded vs time. Beads loaded with 10, 20, 30, 40, or 50 g drug showed a similar release rate in simulated intestinal fluid. Thus the rate of percentage of drug release from calcium/alginate beads did not vary substantially with ibuprofen loading when the loading was higher than 10 g. However, the actual amount of drug released was quite different with variable loading since the same weight of beads contained variable amounts of drug. After switching the dissolution method to the U.S.P. basket method, a difference of release rate in these formulations became more apparent (Figure III.2b). The basket method gave a slower release rate, and thus became more discriminating, compared to the paddle method. This finding has been reported for tolbutamide products and is attributed to hydrodynamic flow rate variations between the two dissolution methods (91).

With higher loading (50 g of ibuprofen suspended in 2% w/v sodium alginate solution), the bead shape remained spherical but with larger diameters. The size of dried beads depended primarily on drug content, and on the method of drying (92). The size of beads plays an important part in determining release rate of drug. Beads loaded with the lowest concentration of ibuprofen (5 g) have the smallest diameter range, and fastest dissolution rate. This may be due to smaller beads having a greater total surface area to facilitate dissolution. From Table III.4, formulations with lower drug concentration in beads showed a lower percentage of drug recovery compared to those with higher drug

concentrations. This is reasonable because a small quantity of drug lost during bead production would count for a larger percentage of total drug content when drug concentration is low. It is interesting to note that the percent of ibuprofen unreleased in dissolution tests increased with increasing percentage of ibuprofen loading, and then reached a constant percentage (Table III.4).

The amount of sodium alginate loading plays an important part in determining the shape of beads. When the amount of alginate loading exceeded 3.0% w/v, beads became tear-shaped. 1.5 to 2.5% w/v loading of sodium alginate gave reproducible spherical beads. Figures III.3a and III.3b show release rate of ibuprofen from ibuprofen/alginate beads with different percentages of sodium alginate. Varying sodium alginate loading did not influence the dissolution pattern significantly. The total percents released of ibuprofen were all above 80% for sodium alginate content from 1.5 to 4% w/v in both dissolution tests (paddle and basket methods). However, none of these non-coated ibuprofen/alginate beads gave a desirable dissolution pattern to develop a sustained release dosage form for ibuprofen.

The dissolution of ibuprofen/alginate beads coated with different percentages of Aquacoat are illustrated in Figures III.4a, III.4b, and III.4c. Basically, there were no differences in dissolution patterns between paddle and side-basket methods (Figures III.4a and III.4b). The water content in the beads were about 8.7, 8.7, 9.1, and 8.5% in 0, 2, 4, and 6% coated beads, respectively. The results in basket dissolution gave a significantly delayed release of ibuprofen from 2 and 4% Aquacoat coated beads compared to the other two dissolution methods (Figure

III.4c). It is expected that the time for 50% drug to dissolve will be extended when using U.S.P. basket method compared with U.S.P. paddle method at the same rotation speeds and dissolution conditions. These results indicated that beads coated with different percentages of Aquacoat plasticized with dibutyl sebacate and triethyl citrate exhibited an inversely proportional decrease in release rate as percentage coating increased. Aquacoat, and not calcium alginate, served as a rate controlling barrier in these formulations. Dissolution from tablets compressed from beads was very slow because the compressed tablets did not disintegrate until about 12 hours after the dissolution test began. However, if slight agitation was applied to the tablet, rapid disintegration of tablet into beads occurred. Granules of ibuprofen/alginate prepared by air compressor and spray gun were very fine. The density of the granules was very low which made it impossible to fill more than 300 mg of granules in a No. 0 gelatin capsule.

These formulations did not give a dosage form with constant release of ibuprofen equivalent to an ibuprofen sustained release caplet (SR 800, Upjohn). Assuming the release pattern from 10 to 80% to be approximately linear, a release rate can be obtained from the dissolution slope using simple linear regression. The times to 50% release were 1, 9, 14, and 17 hours for 0, 2, 4, and 6% Aquacoat coated beads using the basket method, respectively (Figure III.4c). Lag times for dissolution existed for these 2, 4, and 6% Aquacoat coated beads, which were 4, 6, and 10 hr, respectively. A combination of different percentage coated beads (40% w/w of 0% coated, 30% of 2% coated, and 30% of 4% coated, with total 1 g beads) was utilized to mimic the release

pattern of SR 800. Results in Figure III.5 indicated a possibility of formulating a sustained release dosage form for ibuprofen using the coated calcium alginate gel technique. This combination showed three different release rates of 40.8 mg/hr for 0 to 2 hr, 101 mg/hr for 2 to 6 hr, and 21 mg/hr for 6 to 12 hr. Figure III.6 shows a computer simulation using MAXSIM to predict the in vivo serum plasma concentration of ibuprofen if a 70 kg adult is dosed with 1 g of this combination every 12 hr. The pharmacokinetic parameters chosen for this simulation were F = 0.82 (bioavailability), Vd = 10.5 liters/ 70 kg (volume of distribution), and Kel = 0.3467/hr (elimination rate constant, $t_{1/2}$ = 2 hr) for a normal subject (92, 93). Because the absorption of ibuprofen in the intestinal tract is faster than the release of ibuprofen from coated beads, the equation used to estimate serum plasma concentration was reduced from a one-compartment open model, $C = C_o (e^{-Kel*t} - e^{-Ka*t})$ to an IV infusion-like equation, $C = K_r (1 - e^{-Kel*t})/Vd*Kel$ (93, 94). Simulation results show that concentration increased to 10 mcg/ml in 3 hr with a peak close to 23 mcg/ml at 6 hr, and maintained plasma concentration above 10 mcg/ml to 24 hr.

Correlation of ibuprofen serum concentration with the anatomical position of sustained release dosage form has indicated that (under fasting conditions) tablets eroded during their transit through the entire GI tract. The major site of drug absorption from this dosage form was the large bowel under fasting conditions (95). Differences in GI transit between fed and fasted conditions had little effect on ibuprofen bioavailability (96). This means that if Aquacoat coated ibuprofen/alginate beads can pass through the stomach intact,

combinations of varying percentages of coated beads could be a useful oral sustained release dosage form for ibuprofen.

Table III.4 Variation of ibuprofen content in ibuprofen/alginate beads at different stages of preparation.

Treatment No.	1	2	3	4	5	6
Ibuprofen loading (g)	5	10	20	30	40	50
Alginate loading (g)	2	2	2	2	2	2
Total solid (g/100ml)	7	12	22	32	42	52
Ideal drug content (dried weight, mg/g)	657	767	836	862	876	884
Actual drug content[a] (mg/g)	532	718	820	850	860	881
Drug content/g bead released in dissolution test	478	646	660	680	688	715
Percent recovery[b] (in dried beads)	81	94	98	99	98	100
Percent unreleased[c] in dissolution test	8	10	19	20	19	19

a: Actual drug content was determined by powdering 0.1 g beads in simulated intestinal fluid with a high speed chopper, and then homogenized, and assayed at 221 nm.

b: The percentage recovery of ibuprofen in oven dried beads was calculated by dividing the actual drug content by the ideal drug content.

c: The percentage ibuprofen unreleased was calculated by the difference between drug content after dissolution compared to the actual drug content.

Figure III.2a Effect of ibuprofen loading on release of ibuprofen from ibuprofen/alginate beads (paddle method) in simulated intestinal fluid.

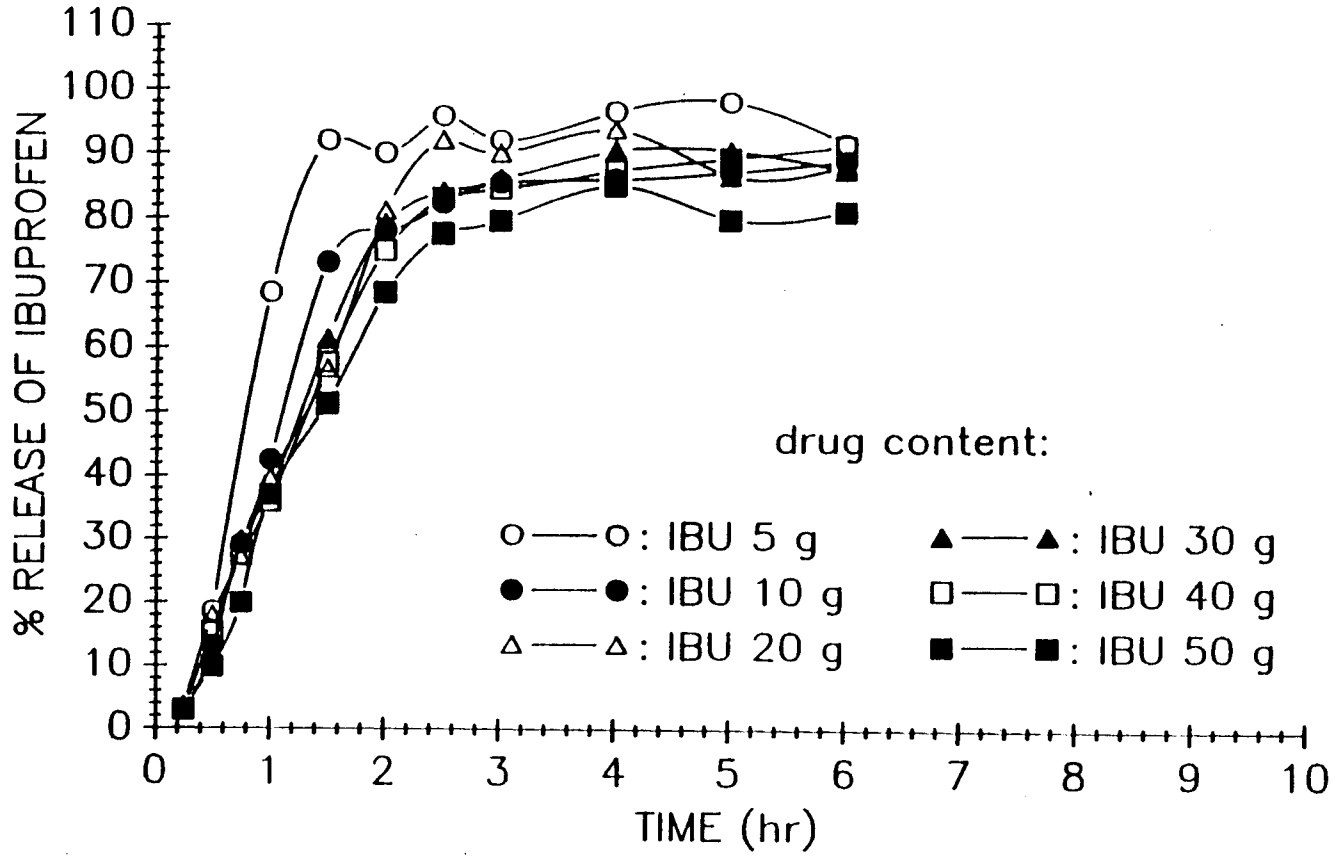


Figure III.2a

Figure III.2b Effect of ibuprofen loading on release of ibuprofen from ibuprofen/alginate beads (basket method) in simulated intestinal fluid.

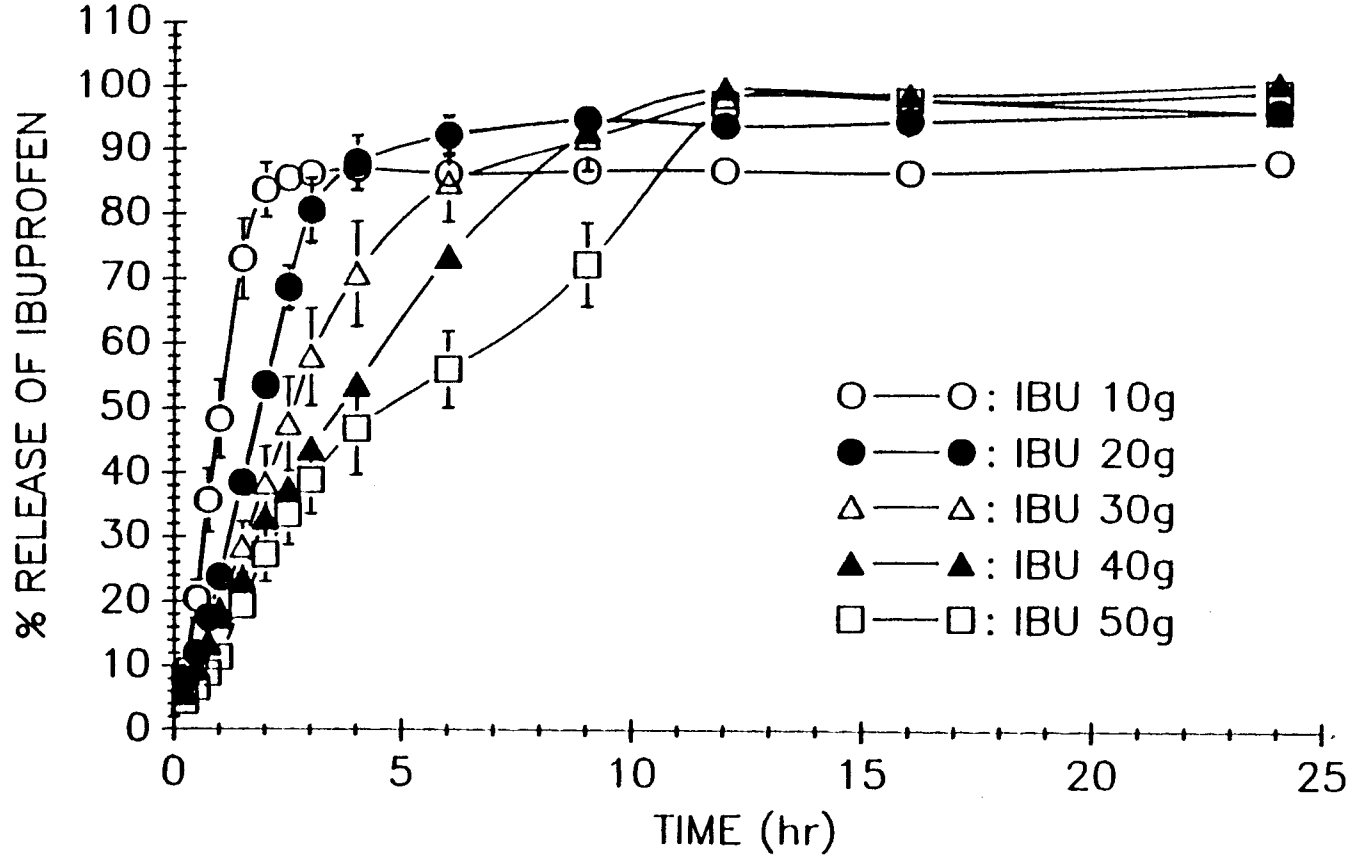


Figure III.2b

Figure III.3a Effect of Na-alginate loading on release of ibuprofen from ibuprofen/alginate beads (paddle method) in simulated intestinal fluid.

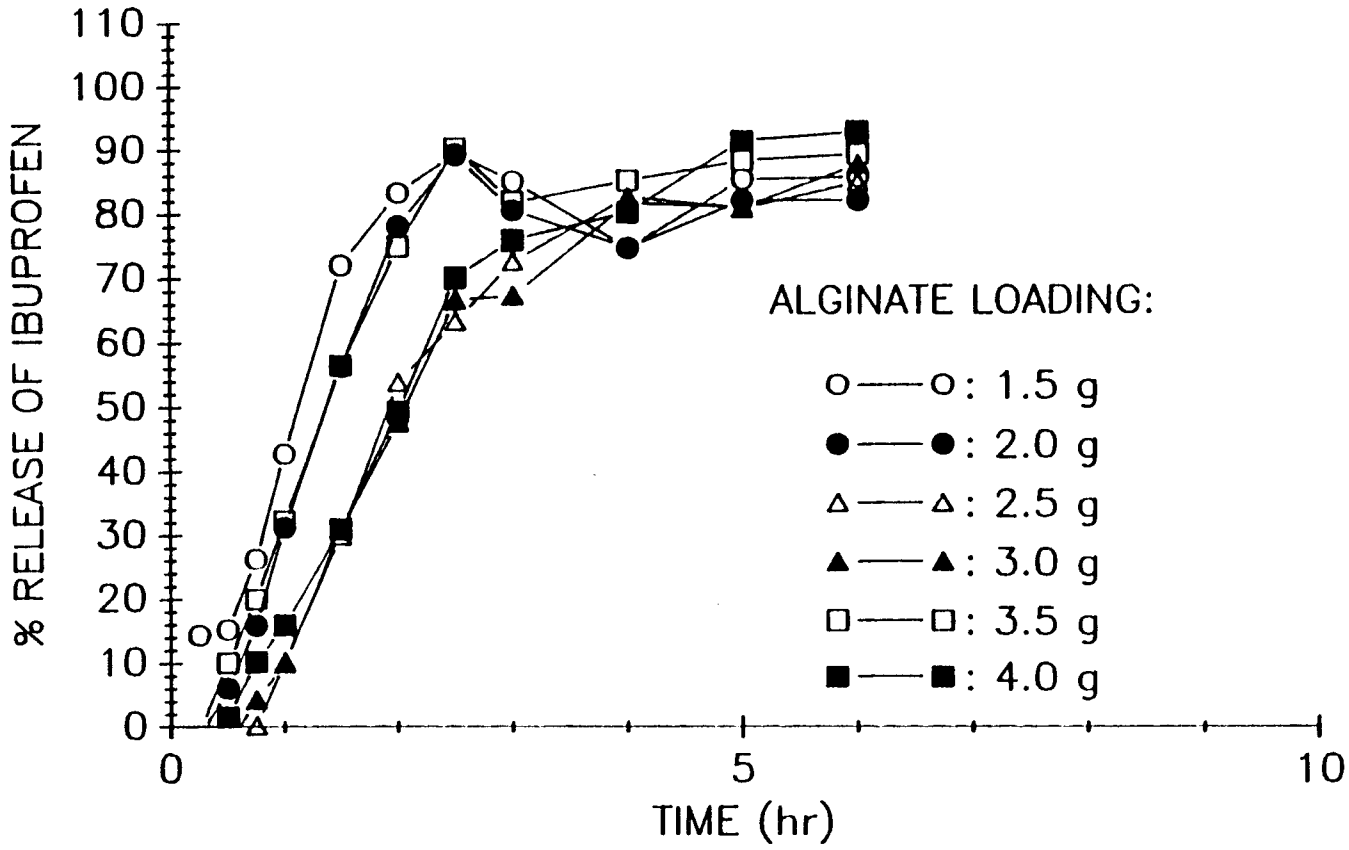


Figure III.3a

Figure III.3b Effect of Na-alginate loading on release of ibuprofen from ibuprofen/alginate beads (basket method) in simulated intestinal fluid.

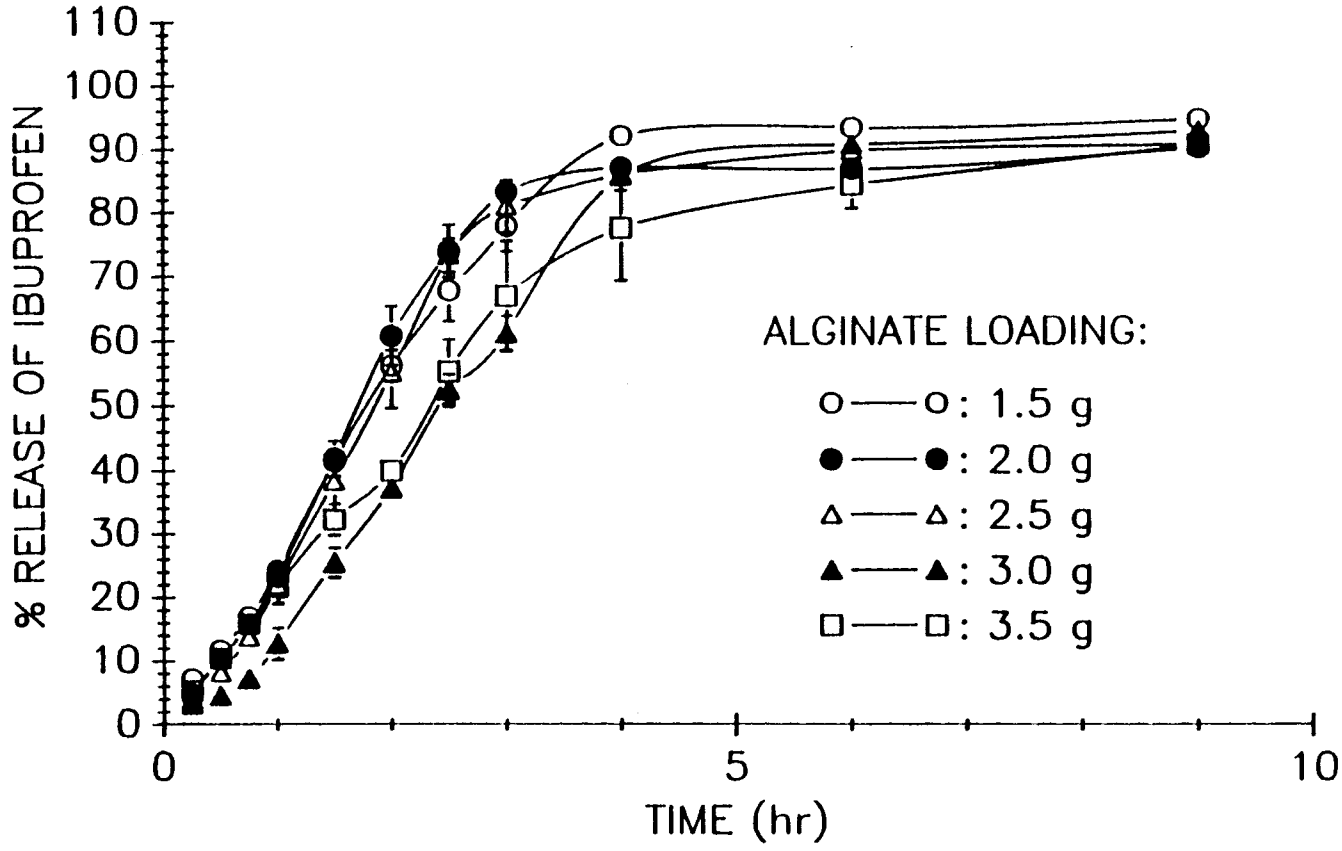


Figure III.3b

Figure III.4a The release pattern of ibuprofen from Aquacoat coated ibuprofen/alginate beads (paddle method) in simulated intestinal fluid.

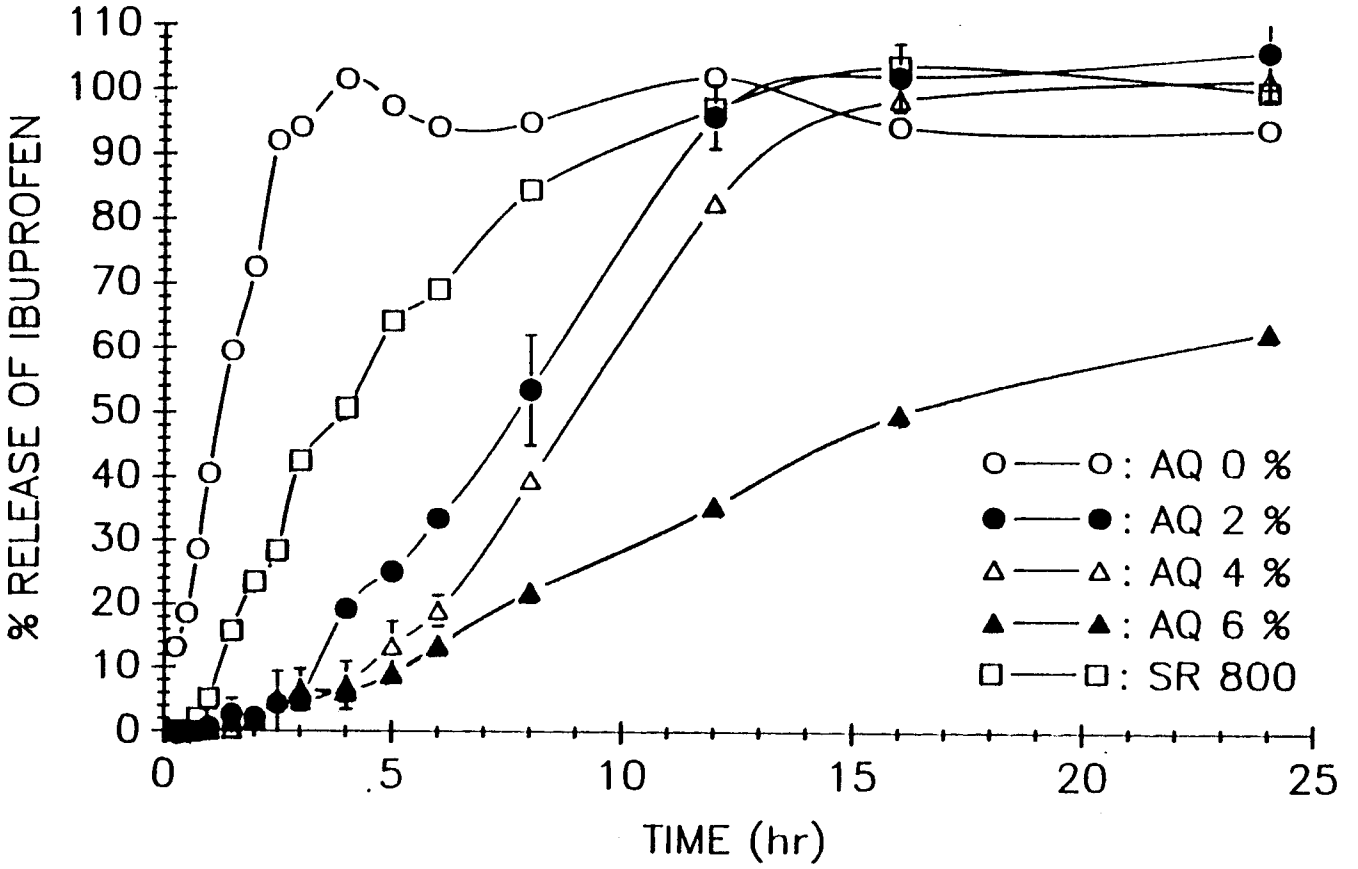


Figure III.4a

Figure III.4b The release pattern of ibuprofen from Aquacoat coated ibuprofen/alginate beads (side basket method) in simulated intestinal fluid.

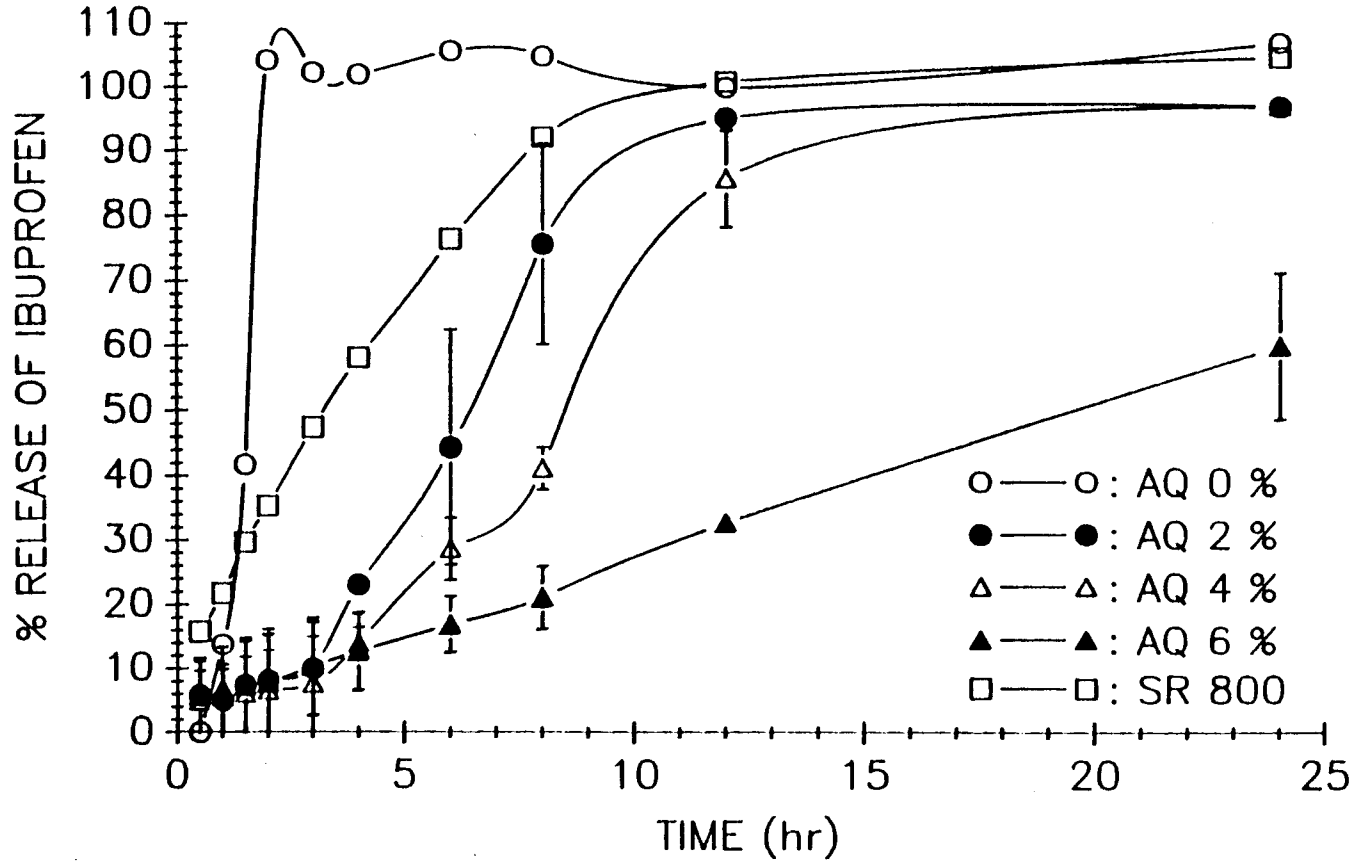


Figure III.4b

Figure III.4c The release pattern of ibuprofen from Aquacoat coated ibuprofen/alginate beads (basket method) in simulated intestinal fluid.

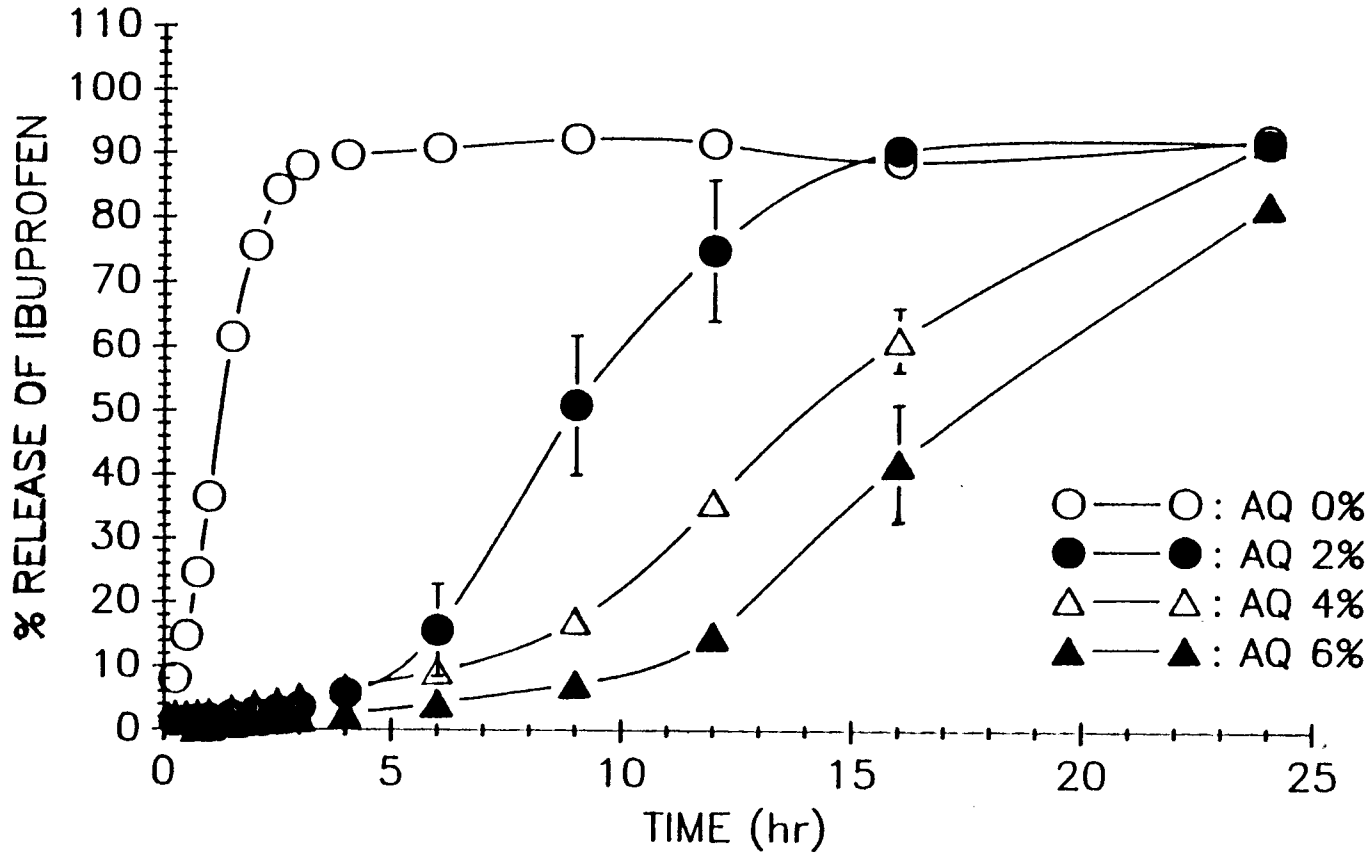


Figure III.4c

Figure III.5 Dissolution test of a mixture of ibuprofen/alginate beads coated with different percentages of Aquacoat (basket method) in simulated intestinal fluid.

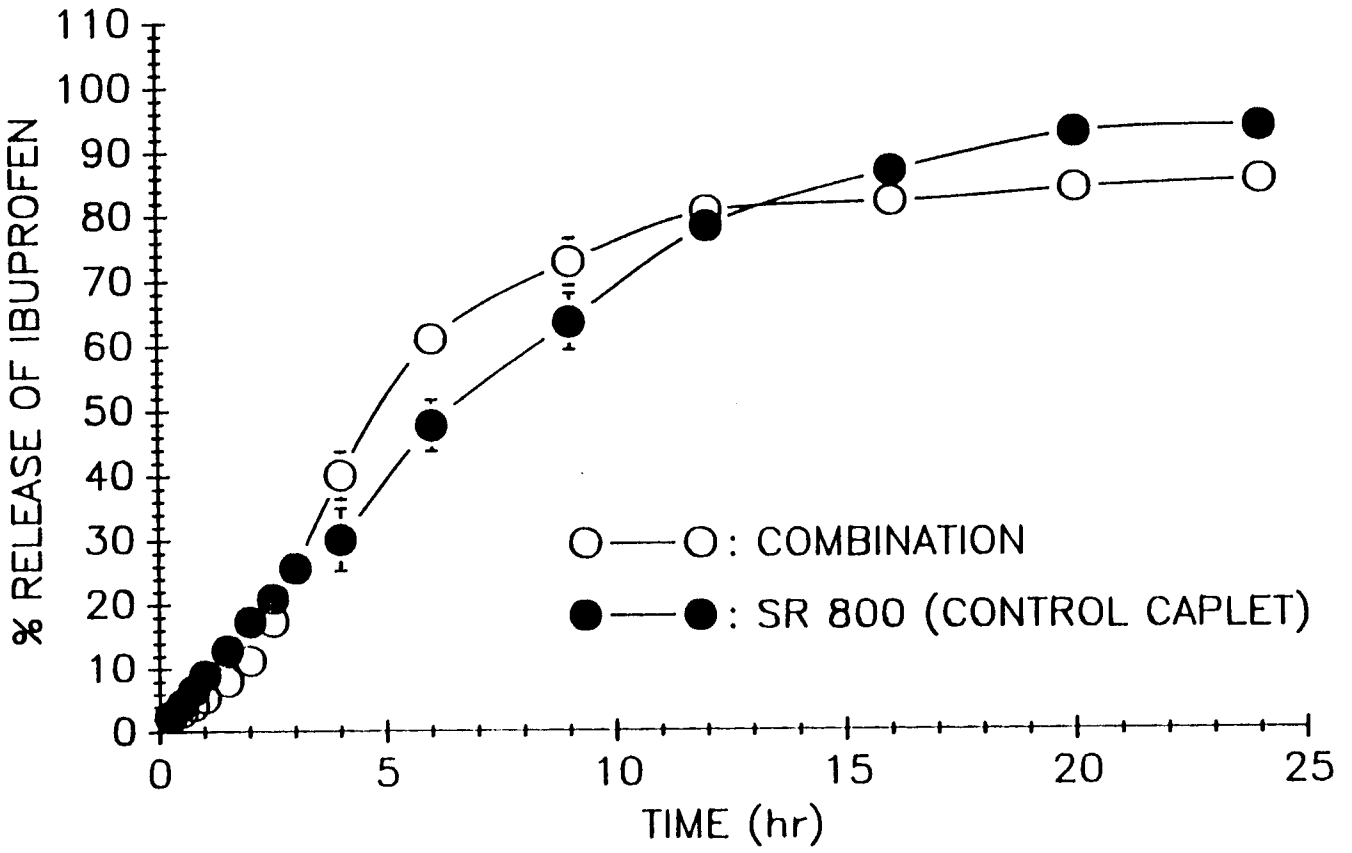

% RELEASE OF IBUPROFEN
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
TIME (hr)
O—O : COMBINATION
●—● : SR 800 (CONTROL CAPLET)

Figure III.5

Figure III.6 Computer simulation data of ibuprofen plasma concentration from a mixture of Aquacoat coated ibuprofen/alginate beads.

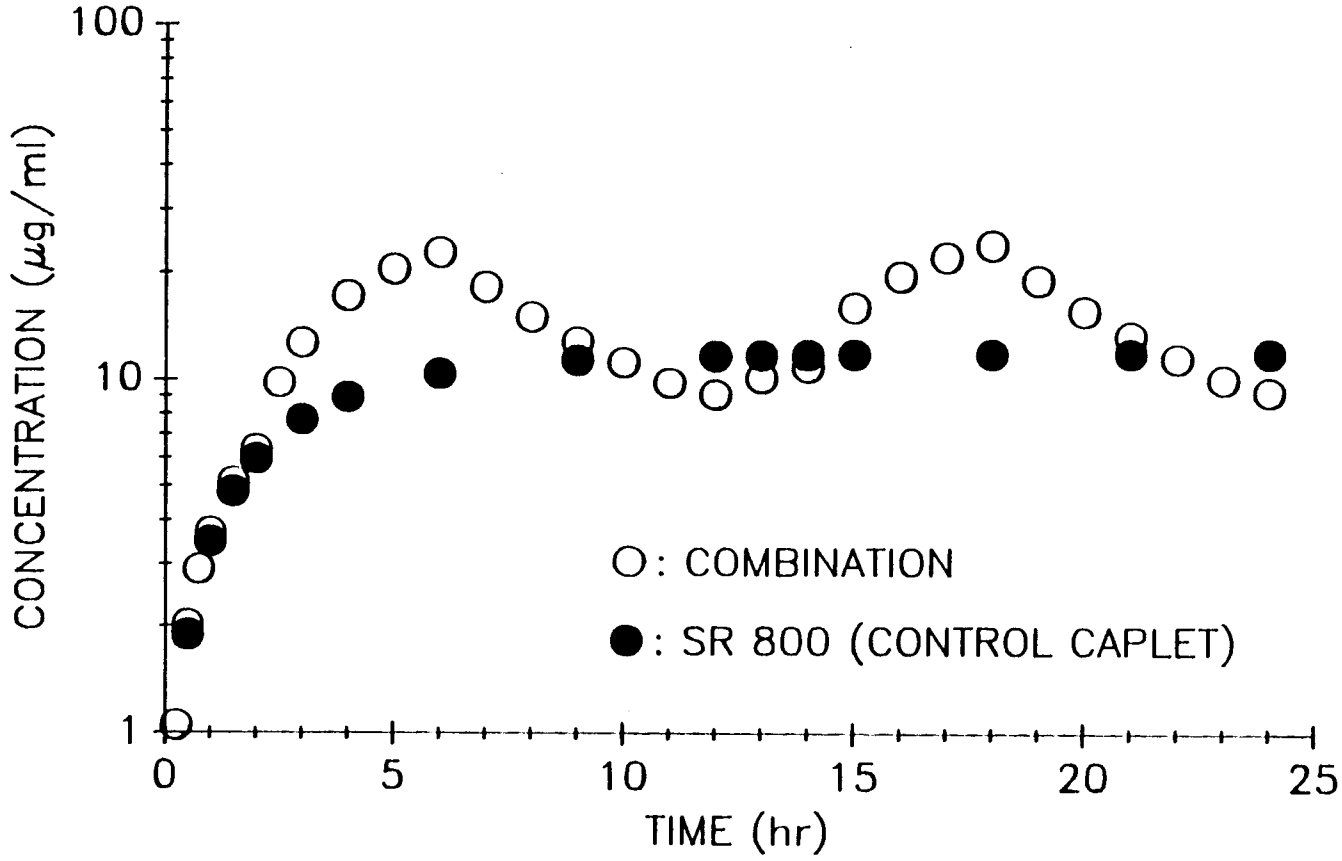

100
10
1
CONCENTRATION (μg/ml)
O: COMBINATION
●: SR 800 (CONTROL CAPLET)
0
5
10
15
20
25
TIME (hr)

Figure III.6

Colonic Drug Delivery

Sulfasalazine (SS).

The SS/alginate beads with 2 or 4% sodium alginate provided dissolution profiles similar to a commercially available enteric coated tablet, Azulfidine EN, for treatment of ulcerative colitis patients (Figure III.7). After 2 hours acid pretreatment, about 90% of drug was released in 1 hour after switching to simulated intestinal fluid. Because sulfasalazine and calcium alginate do not dissolve in simulated gastric fluid, dissolution testing of Aquacoat coated SS/alginate beads was initially conducted in simulated intestinal fluid. After coating with Aquacoat at different percentages, 2 to 8%, different dissolution patterns occurred for both SS/alginate beads with 2 and 4% sodium alginate loading (Figures III.8a and III.8b). An unexpected delayed "burst" or sudden release of SS from Aquacoat coated beads was obtained in intestinal fluid treatment. The lag time for 6 or 8% Aquacoat coated SS/alginate beads (both 2 and 4% sodium alginate) was about 6 hours in simulated intestinal fluid, which might suggest that drug could be delivered to colon before dissolving. Beads containing 4% sodium alginate showed a faster release pattern with 6 or 8% Aquacoat coats compared to beads with 2% sodium alginate. However, the release rate of SS from coated SS/alginate beads was similar to SS/alginate without coating when beads were pretreated in simulated gastric fluid for 2 hours (Figures III.8c and III.8d).

Some Aquacoat coated SS/alginate beads were filled into No. 0 gelatin capsules, which were then enteric coated with HP-55S (hand-dipping). These enteric coated gelatin capsules were maintained intact

after 2 hours simulated gastric fluid treatment, and then dissolved in 1 hour when switched to simulated intestinal fluid (Figure III.9a). Delayed release of SS from coated SS/alginate beads did occur for these enteric encapsulated beads. The lag time for SS to leave the encapsulated beads in simulated intestinal fluid from 2 hours acid pretreatment was similar to the release of SS from (non enteric coated) without gastric fluid pretreatment. However, the dissolution patterns became somewhat more sustained release rather than exhibiting such a pronounced "burst" release. Visual inspection during dissolution revealed that the enteric coated gelatin capsule dissolved in intestinal fluid, but appeared to "glue" or hold the beads together during dissolution. This may be the cause of the apparent sustaining effect. The data show it is possible to obtain the major portion of drug release between 5 to 20 hours post dosing which is very useful for colonic drug delivery.

Beads coated with Eudragit L30D on the outer surface also showed a very sustained release of SS (Figure III.9b). The effect of directly enteric coating over the sustaining coat on dissolution rate of SS from Aquacoat coated beads was quite unexpected compared with beads protected by enteric coated capsules. Under scanning electron microscope examination, the beads without Eudragit L30D showed a surface with cracks (Figure III.10a). These cracks were sealed after applying a layer of enteric coating polymer on top of Aquacoat (Figure III.10b). It is possible that the "burst" effect of these Aquacoat coated SS/alginate beads is related to these cracks found on the bead's surface, and the swelling ability of calcium alginate in simulated

intestinal fluid. It seems that acid pretreatment changed calcium alginate swelling properties, although calcium alginate does not dissolve in acid solution.

Figure III.7 Effect of alginate loading on percent release of sulfasalazine (with 2 hours simulated gastric fluid treatment).

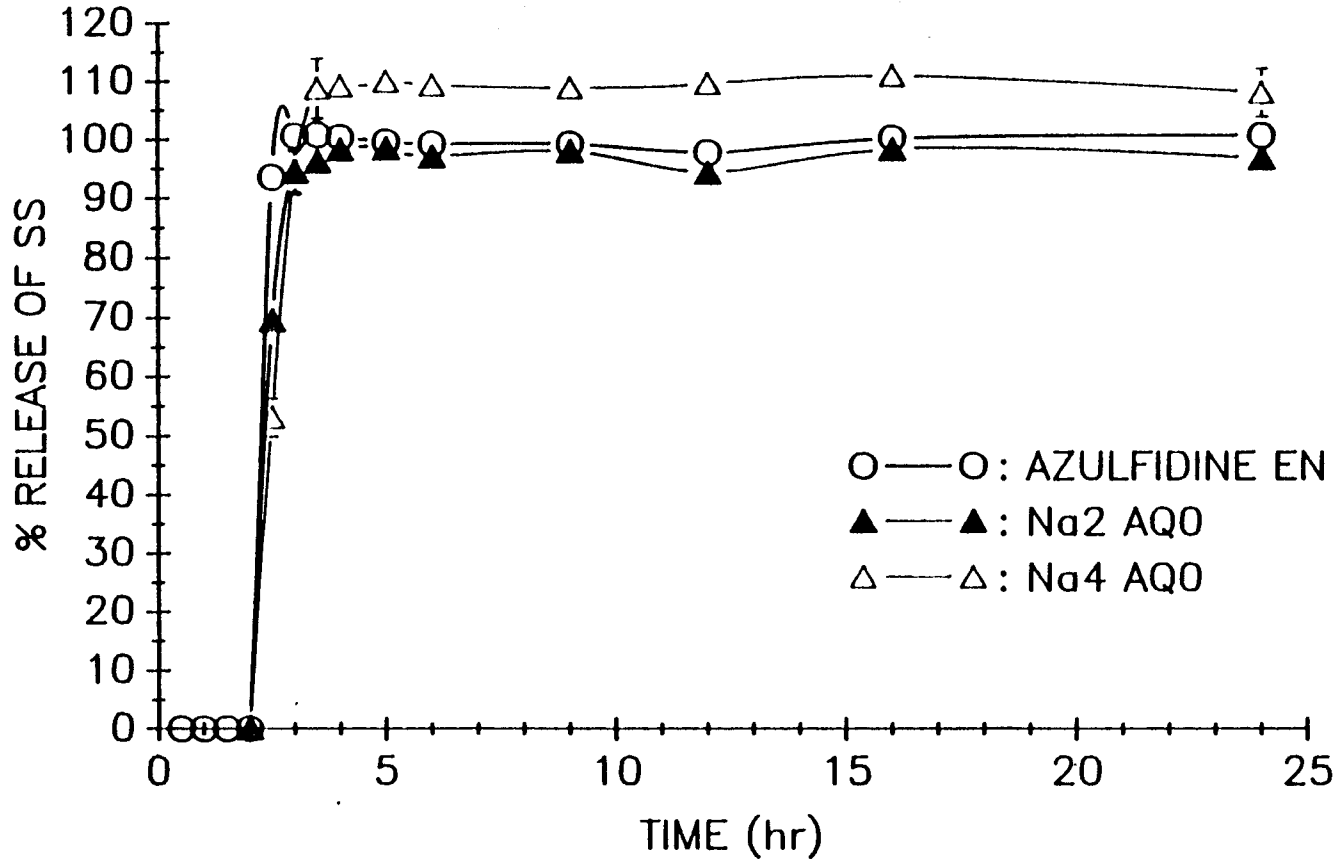

120
110
100
90
80
70
60
50
40
30
20
10
0
% RELEASE OF SS
0
5
10
15
20
25
TIME (hr)
O—O: AZULFIDINE EN
▲—▲: Na2 AQO
△—△: Na4 AQO

Figure III.7

Figure III.8a Effect of Aquacoat coating on percent release of sulfasalazine from beads which contained 2% alginate (without simulated gastric fluid treatment).

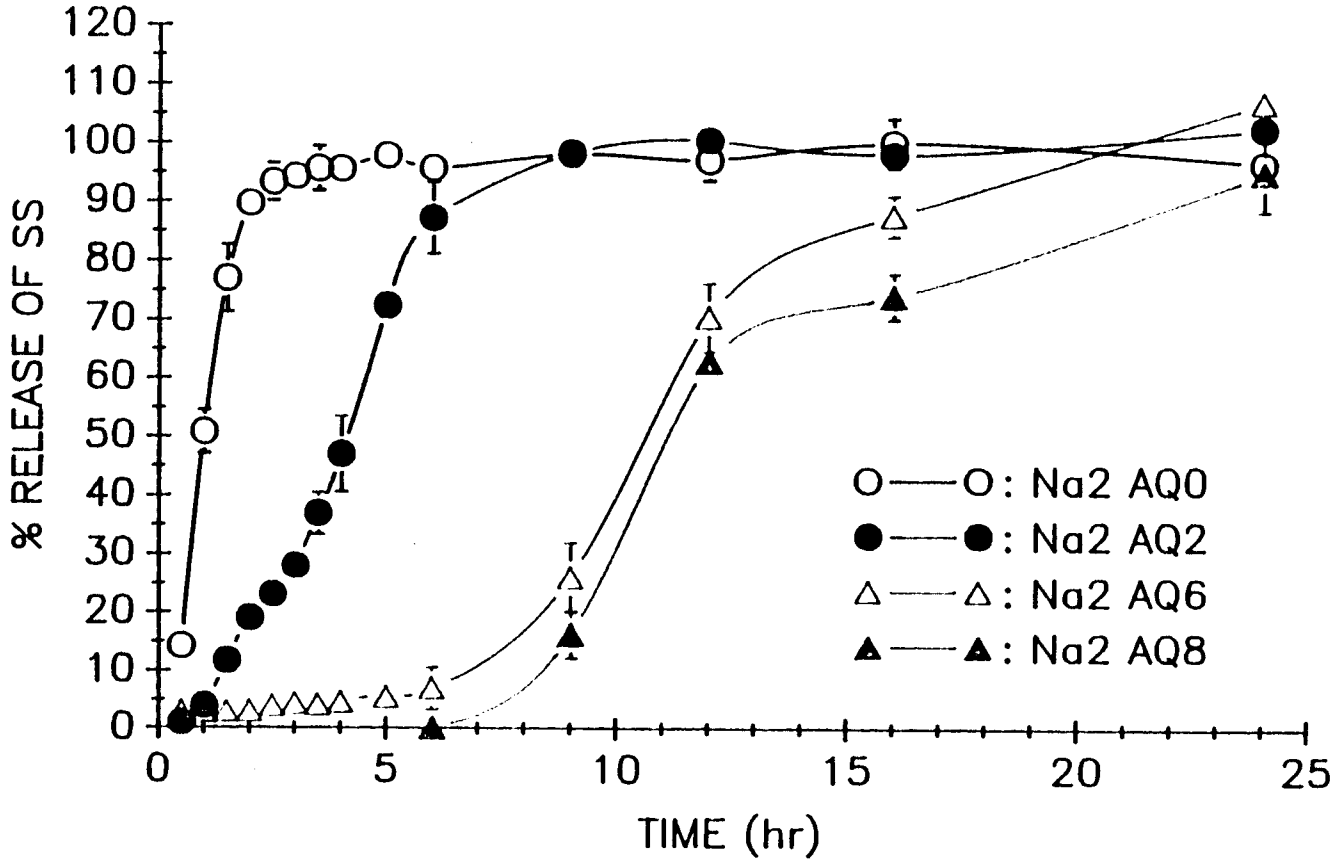


Figure III.8a

Figure III.8b Effect of Aquacoat coating on percent release of sulfasalazine from beads which contained 4% alginate (without simulated gastric fluid treatment).

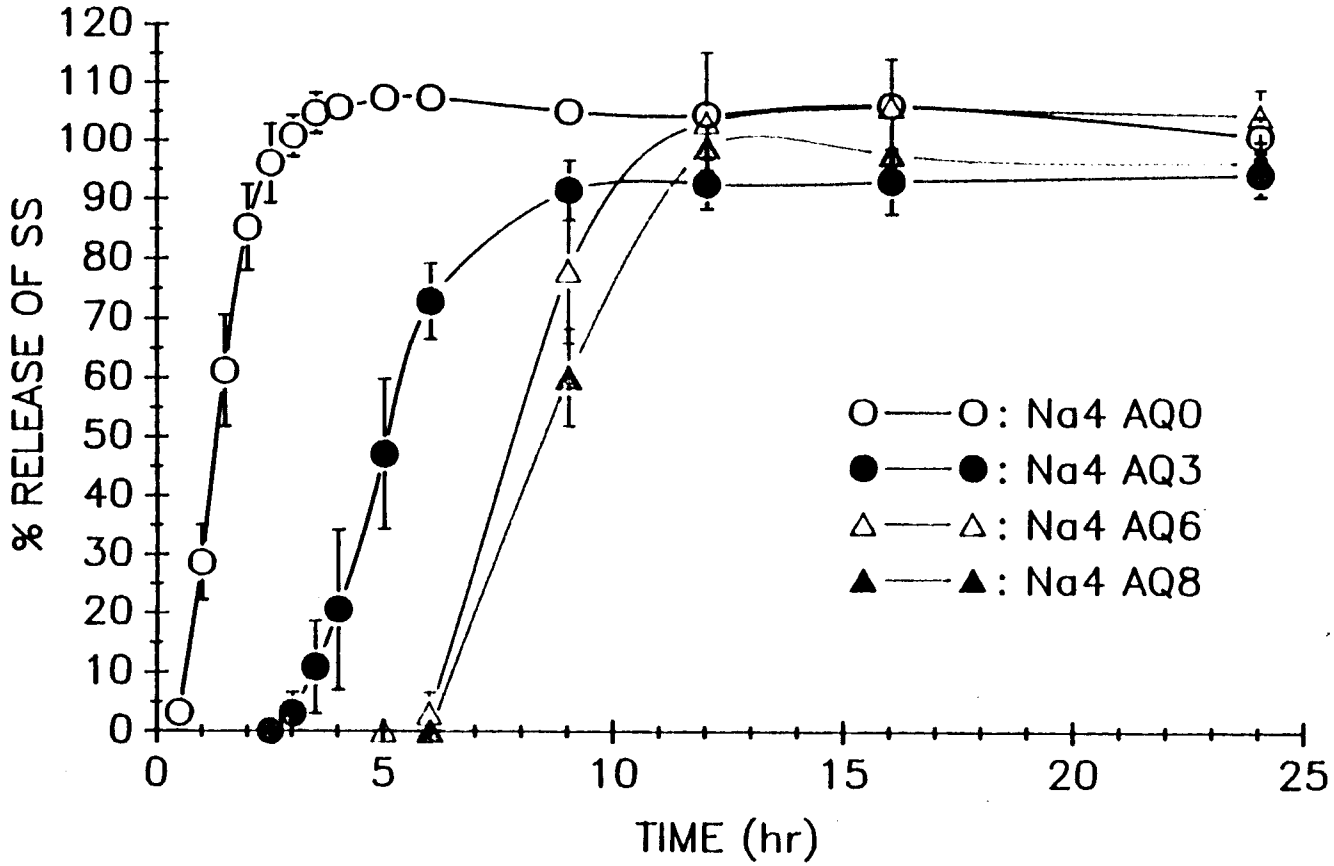


Figure III.8b

Figure III.8c Effect of Aquacoat coating on percent release of sulfasalazine from beads which contained 2% alginate (with 2 hours simulated gastric fluid treatment).

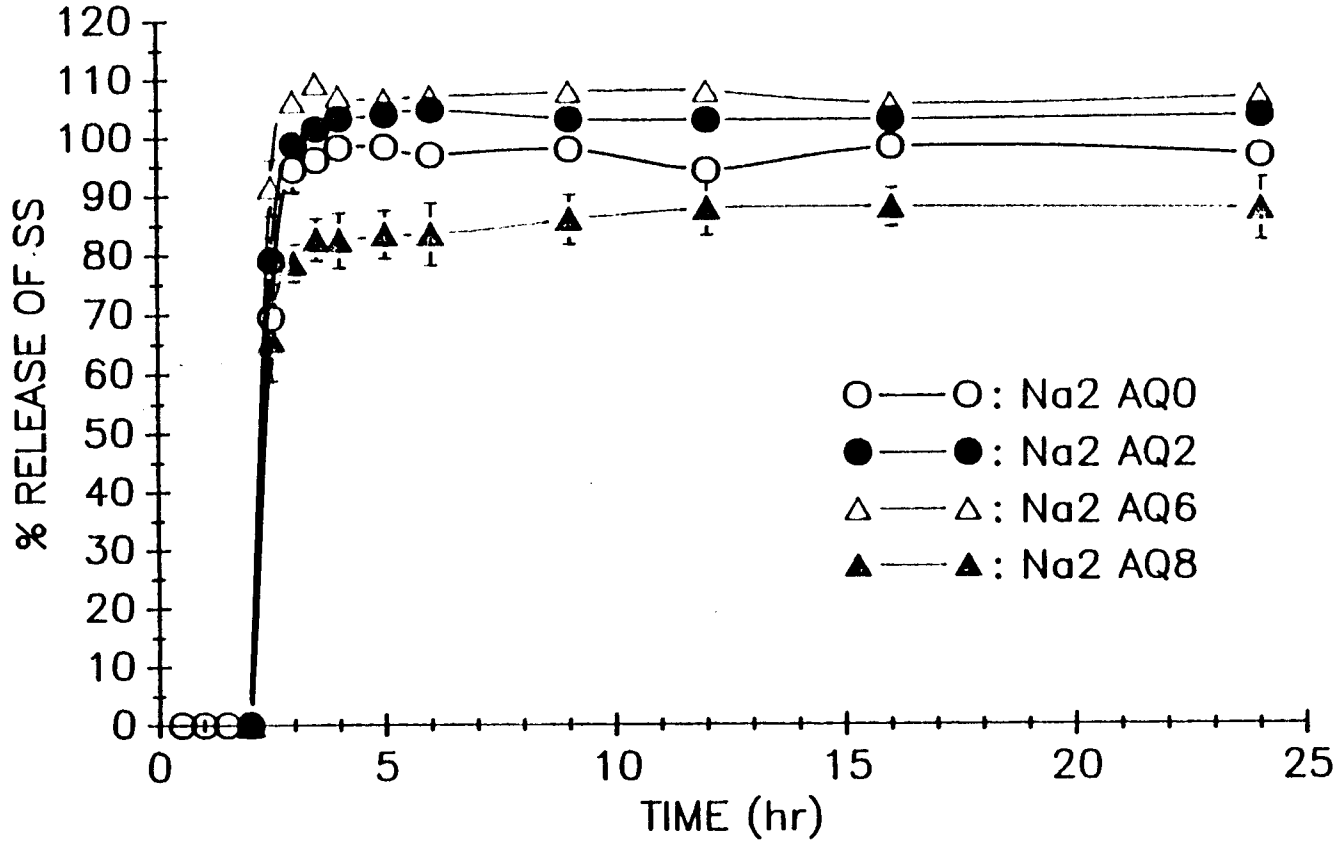


Figure III.8c

Figure III.8d Effect of Aquacoat coating on percent release of sulfasalazine from beads which contained 4% alginate (with 2 hours simulated gastric fluid treatment).

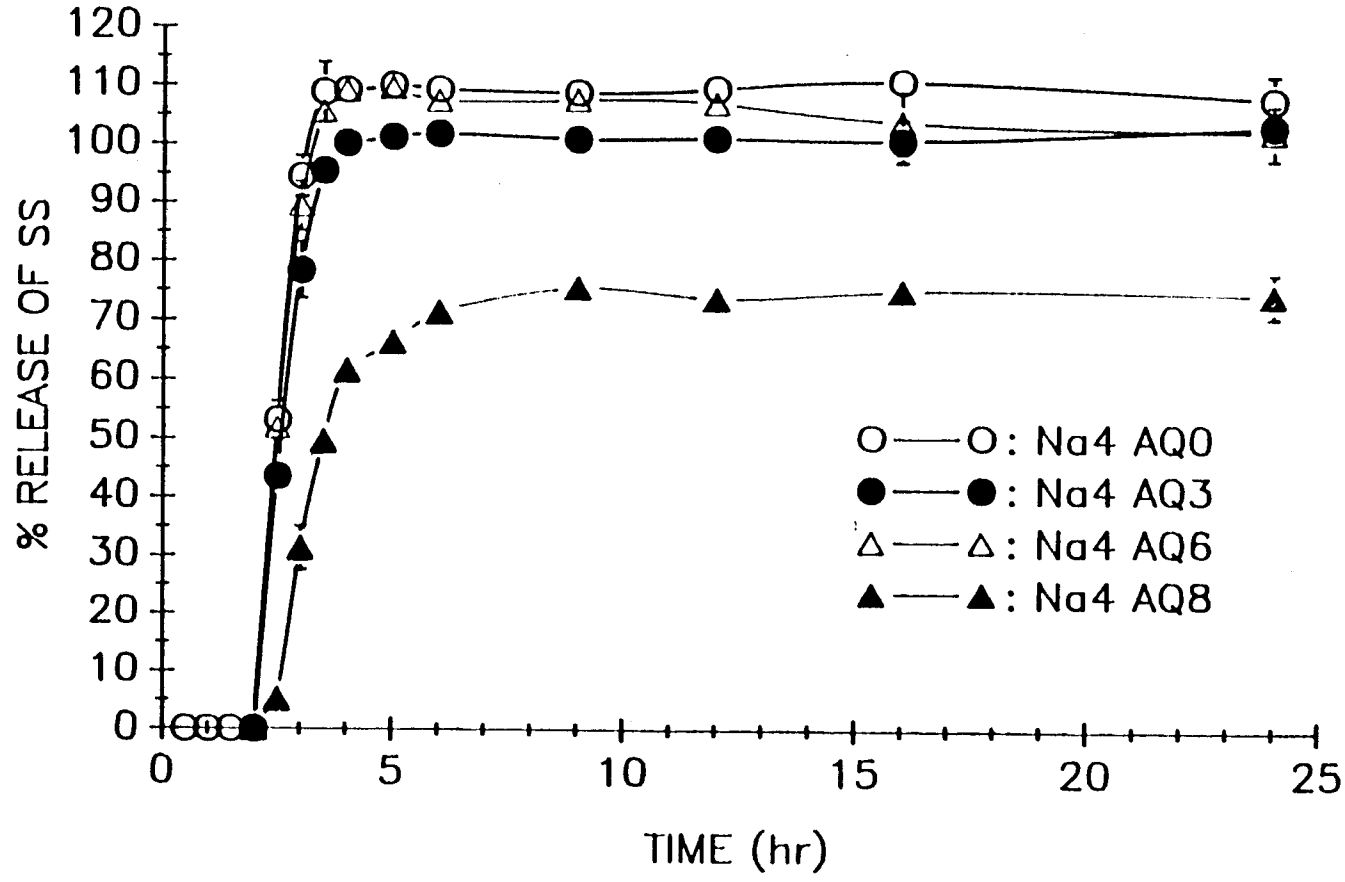


Figure III.8d

Figure III.9a Influence of HP-55S enteric coated capsule on percent release of sulfasalazine from Aquacoat coated SS/alginate beads (with 2 hours simulated gastric fluid treatment).

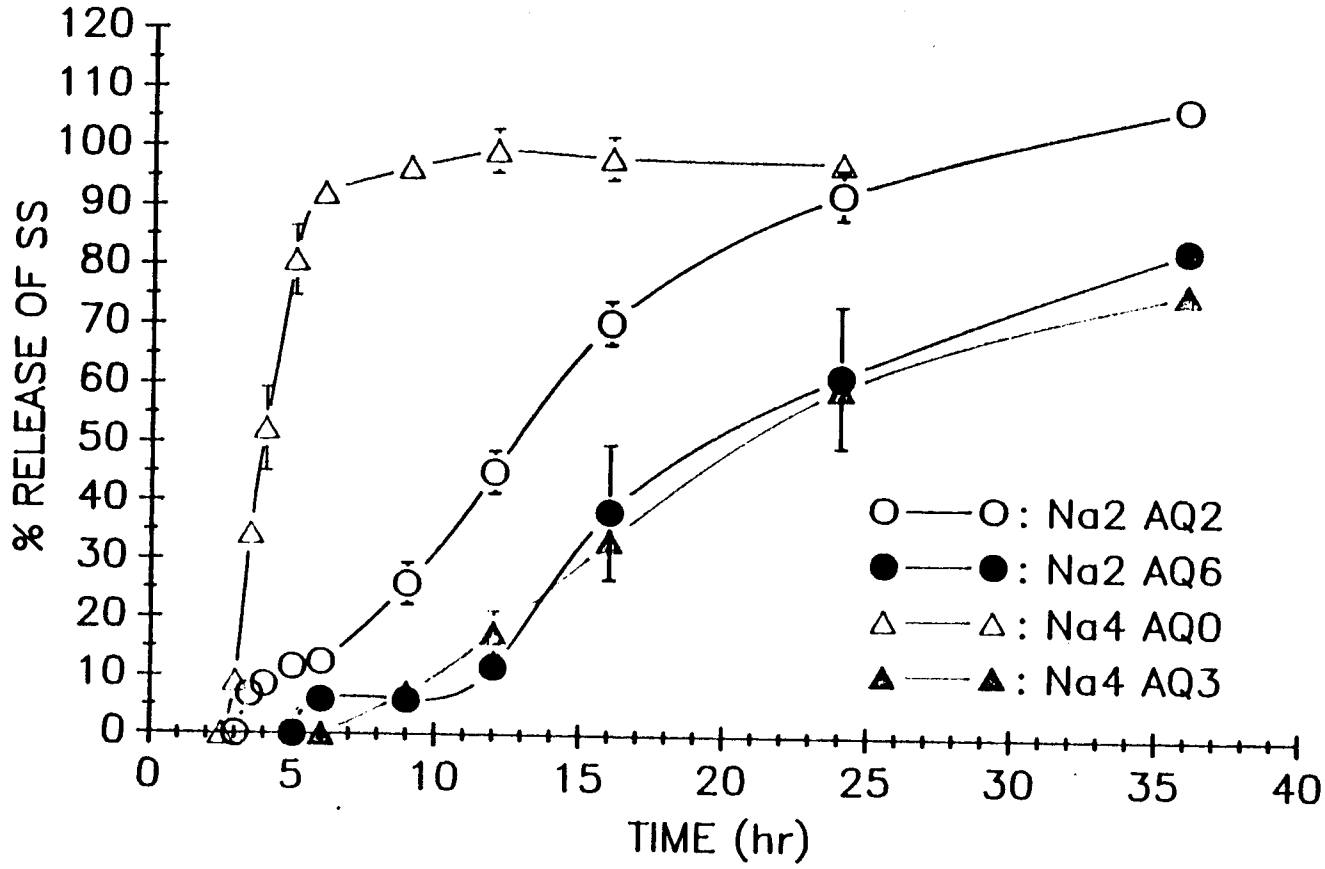


Figure III.9a

Figure III.9b Effect of Eudragit L30D enteric coating on percent release of sulfasalazine from Aquacoat coated SS/alginate beads (with 2 hours simulated gastric fluid treatment).

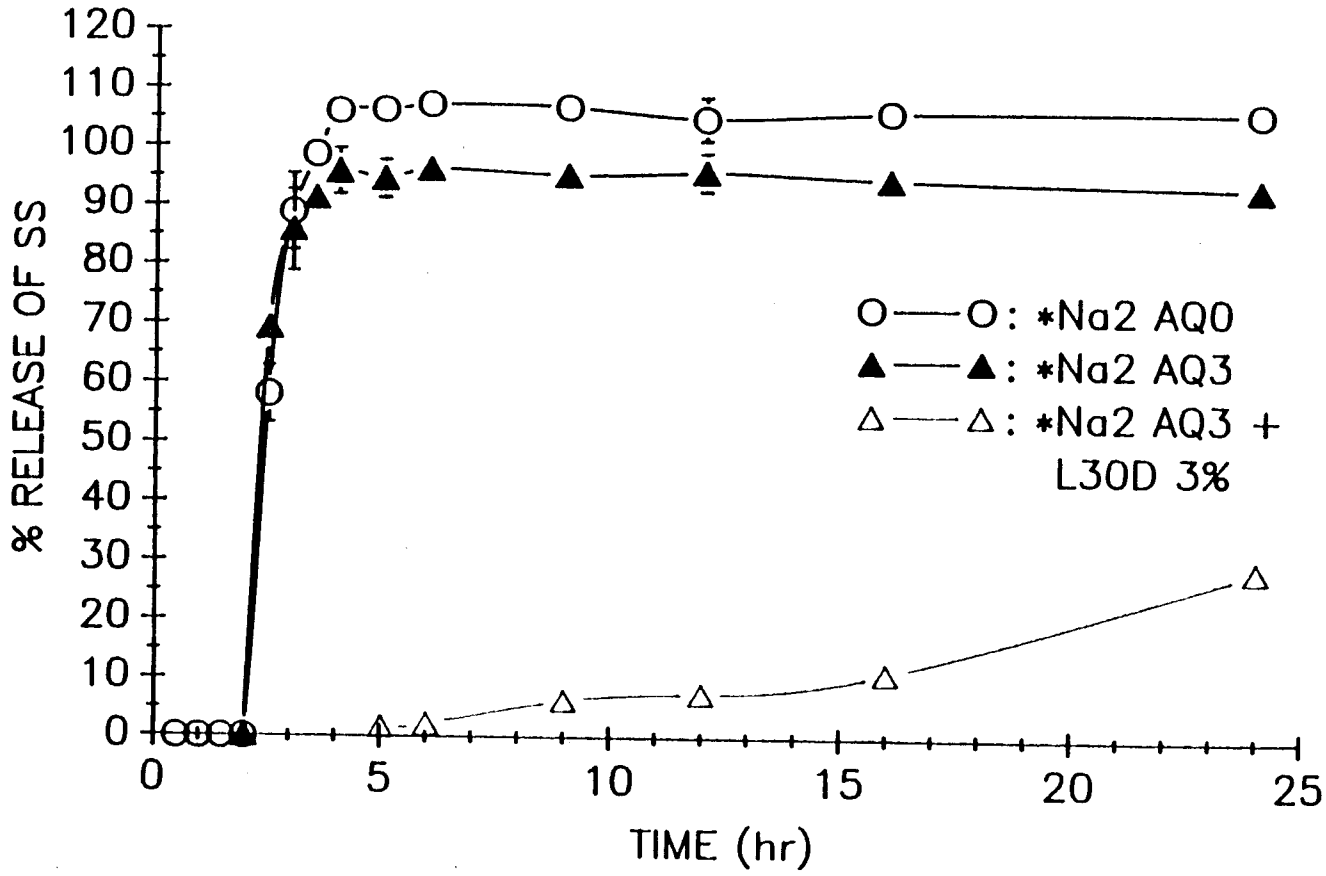


Figure III.9b

Figure III.10a SEM pictures of "cross-section" (top) and "surface" (bottom) of 6% Aquacoat coated SS/alginate beads.

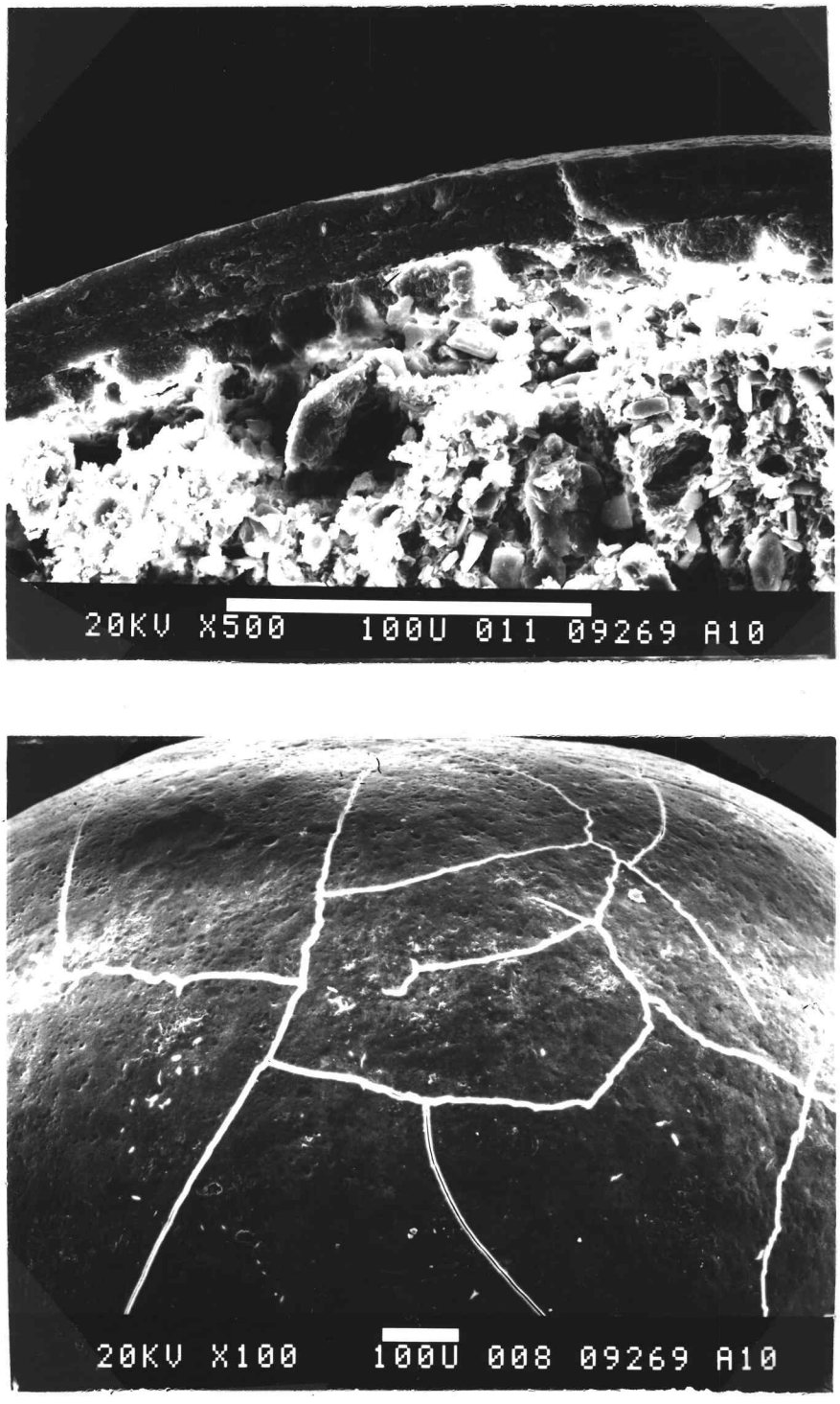
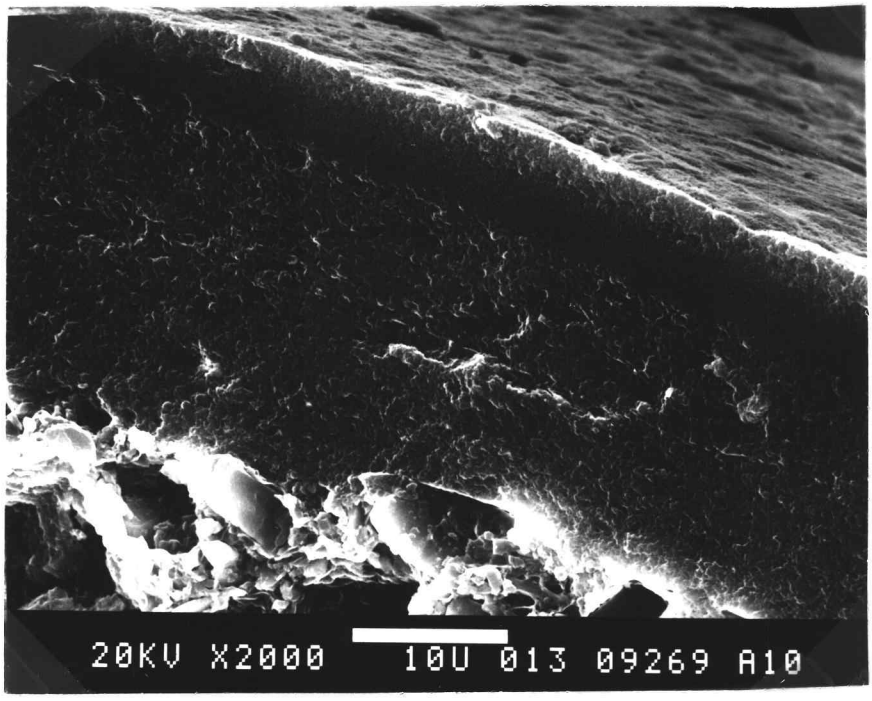


Figure III.10a

Figure III.10b SEM pictures of "cross-section" (top) and "surface" (bottom) of 6% Aquacoat and 3% Eudragit L30D coated SS/alginate beads.



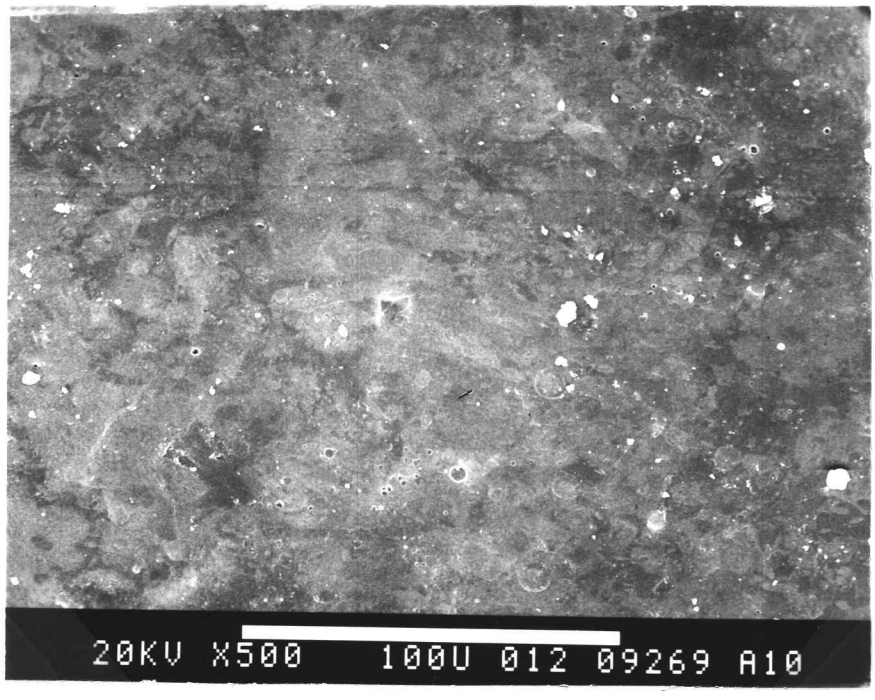


Figure III.10b

5-Aminosalicylic Acid (5-ASA).

5-ASA has a solubility of about 1 mg/ml in water with pK_a values of 3, 6, and 13.9. Classically, sulfasalazine is assayed spectrophotometrically, while 5-aminosalicylic acid is assayed spectrofluorimetrically (97). It was necessary to use a rapid and stable assay of 5-ASA for *in vitro* study. Figure III.11 shows the standard curve and reproducibility of 5-ASA in phosphate buffer solution (simulated intestinal fluid). Using a UV spectrophotometer to detect 5-ASA concentration in phosphate buffer solution (pH = 7.4) at wavelength 225 nm gave a reliable and consistent reading up to 9 days at room temperature. Because 5-ASA is sensitive to light in the presence of water, it is not clear at this time whether there exists a light degraded form of 5-ASA in the standard solution which also shares the same intensity of absorbance at 225 nm.

After coating 5-ASA/alginate beads with different percentages of Eudragit L30D, only beads coated with 6% or more Eudragit L30D provided protection of 5-ASA/alginate beads during 2 hours acid pretreatment (Figure III.12). This suggests that a minimum 6% w/w coating of Eudragit L30D on these 5-ASA/alginate beads is required to obtain an enteric coating effect. The difference of these 5-ASA/alginate beads compared to ibuprofen/alginate beads or SS/alginate beads is that the drug quite unexpectedly did not form a drug/alginate matrix. Because 5-ASA did not form spherical beads with calcium alginate after drying, 5-ASA was loaded directly on the top of dried calcium alginate beads. This could delay the time for the calcium alginate gel to contact water. Since transit time is about 3 to 5 hours for an oral dosage form (with

diameters 2 to 5 mm) to reach the colon, a delayed release of 5-ASA would be more suitable than just an enteric coat. By coating another layer of polymer (Aquacoat) on the surface of 6% Eudragit coated beads, dissolution patterns were changed dramatically (Figure III.13). As expected, increasing the thickness of Aquacoat coating decreased the release rate of 5-ASA from coated beads.

Reversing the coating order but maintaining the same percentages of polymer loading revealed a different type of dissolution pattern for 5-ASA/alginate beads coated with 6% Eudragit L30D and 4% Aquacoat (Figures III.13 and III.14). 4% Aquacoat coating did not prevent dissolving of 5-ASA in acid solution (Figure III.14). After applying 4% or more Eudragit on these 4% Aquacoat coated beads, a protection against 2 hours acid treatment was established (Figure III.14). A more desirable sustained release dosage form for 5-ASA was obtained using formulae 7 and 8 in Table III.3 (also see Figure III.14).

The influence of acid pretreatment time on dissolution of these coated 5-ASA/alginate beads is shown in Figures III.15a and III.15b. With longer time of acid fluid treatment, 5-ASA tended to release faster after switching to simulated intestinal fluid. The difference in percent release between 0.5 hours and 2 hours acid treatment was not significant, which indicated a study with longer acid treatment should be conduct to verify the possibility of acid influence. The stability of two 5-ASA products, formulae 10 and 11 (see Table III.3), was studied for 2 weeks. These products were kept in screw-lock plastic containers at room temperature, and stored in a drawer. Dissolution patterns did not change during these 2 weeks storage, both products maintained almost

the same release profile at day 14 compared to day 1 (Figure III.16). However, these coated 5-ASA beads had a cracked surface after 3 months storage at the same conditions, which did not occur during the first 2 weeks of study. Some of these products were kept in a desiccator from the beginning, and there were no visible cracks on the surface after 3 months storage.

Figure III.11 Standard curve of 5-aminosalicylic acid in phosphate buffer solution (at wavelength 225 nm).

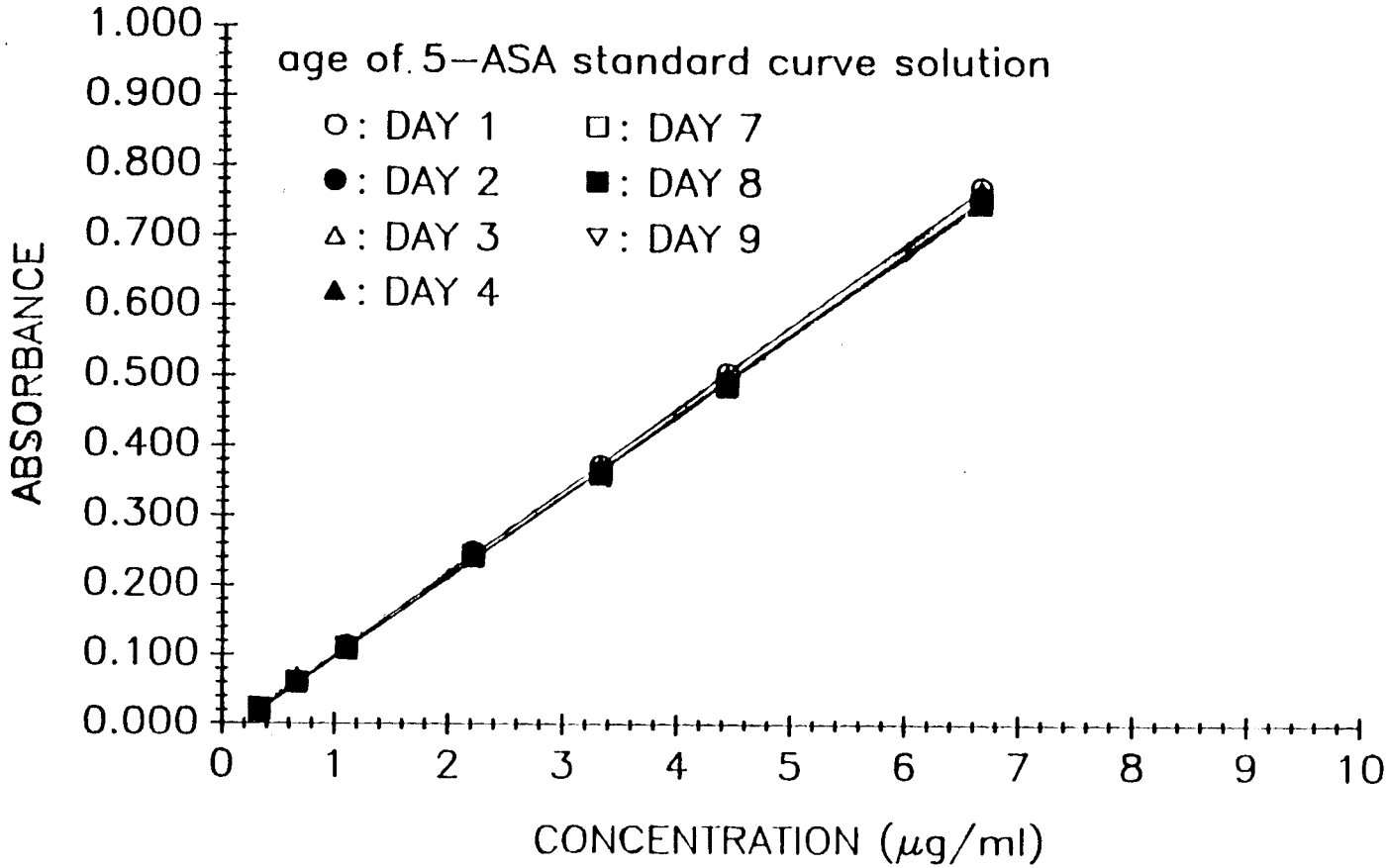


Figure III.11

Figure III.12 Effect of Eudragit L30D coating on percent release of 5-aminosalicylic acid from 5-ASA/alginate beads (with 2 hours simulated gastric fluid treatment).

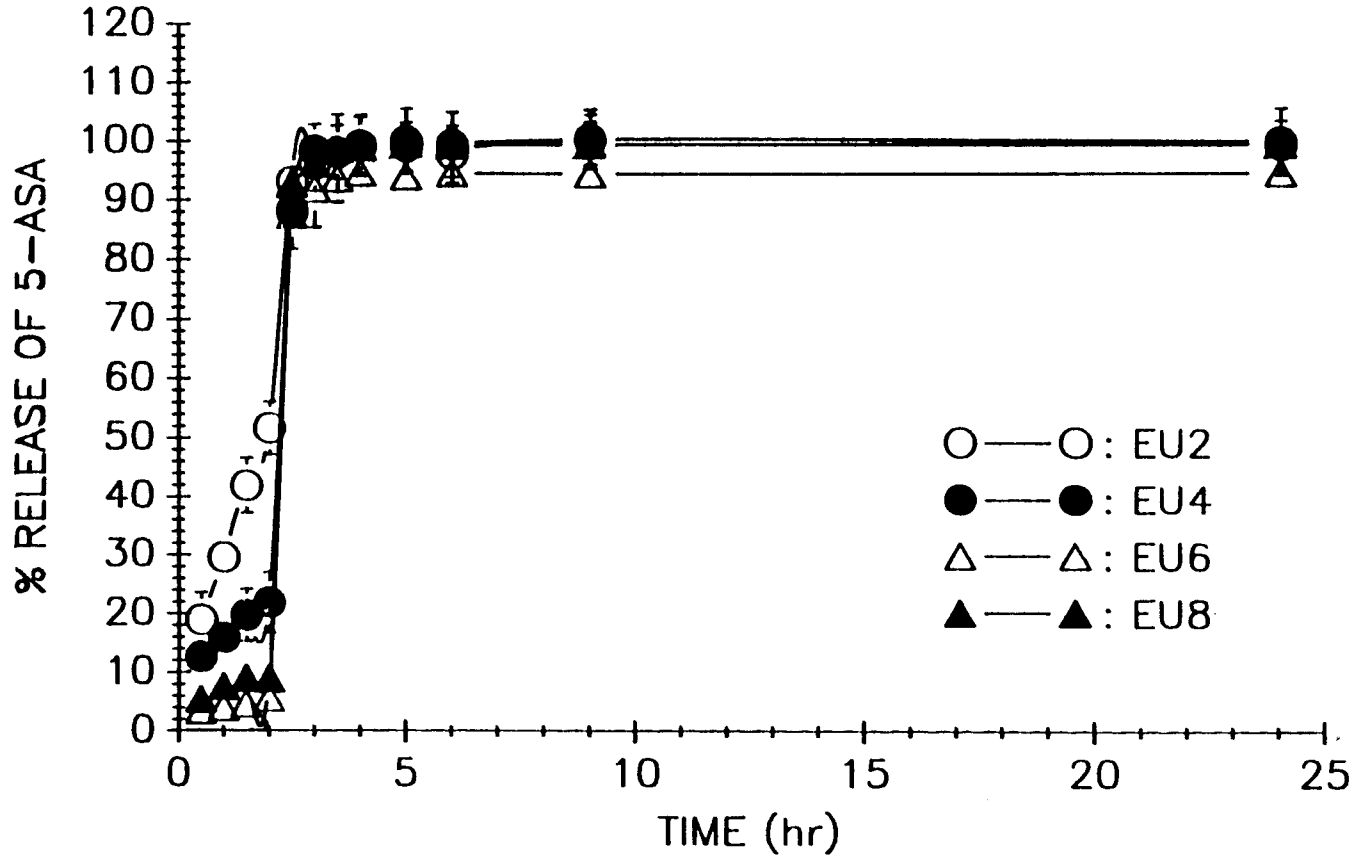


Figure III.12

Figure III.13 Effect of Aquacoat coating on Eudragit L30D coated 5-ASA/alginate beads (with 2 hours simulated gastric fluid treatment).

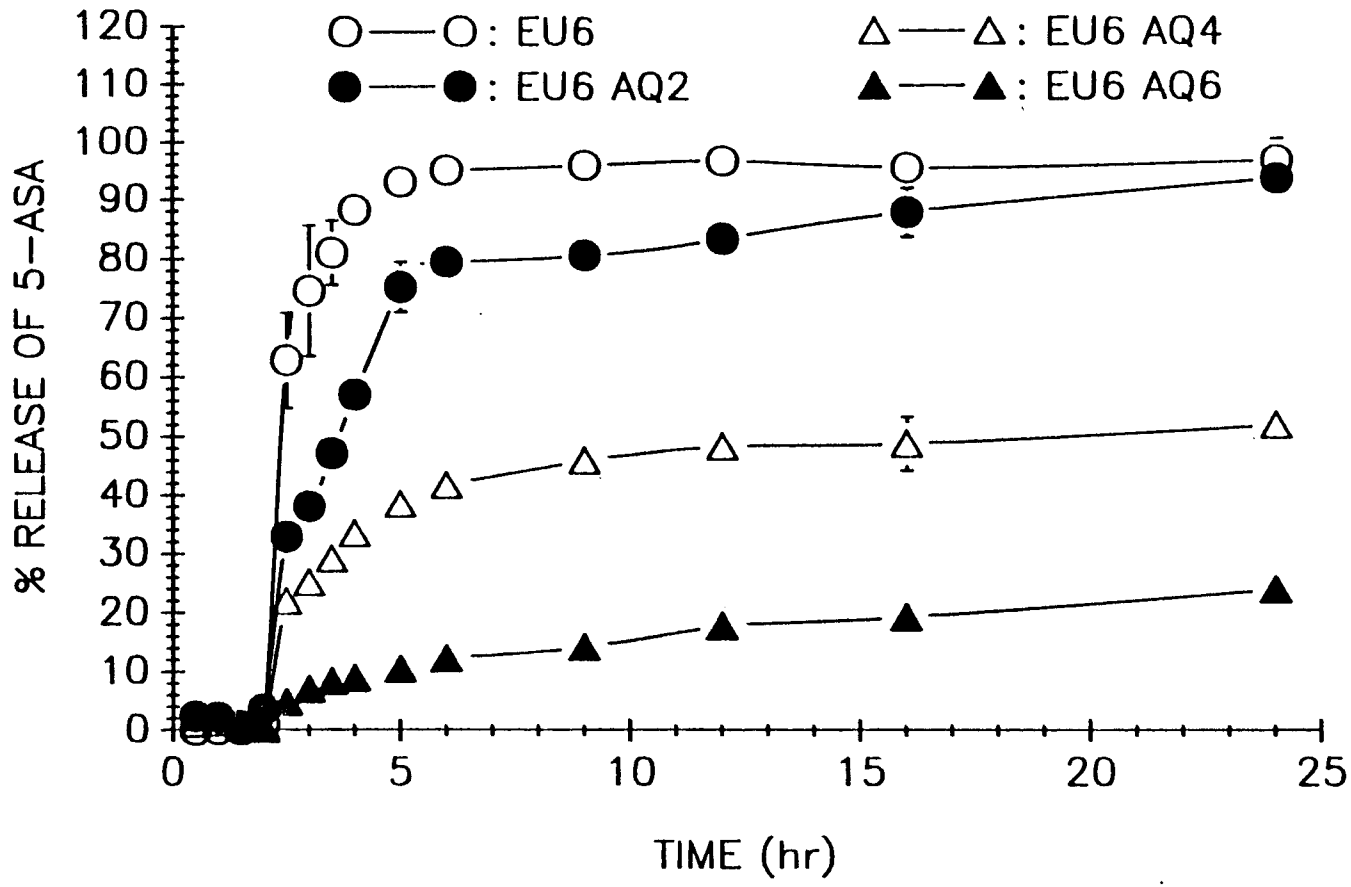


Figure III.13

Figure III.14 Dissolution of 5-ASA/alginate beads coated with 4% Aquacoat and variable percentages of Eudragit L30D (with 2 hours simulated gastric fluid treatment).

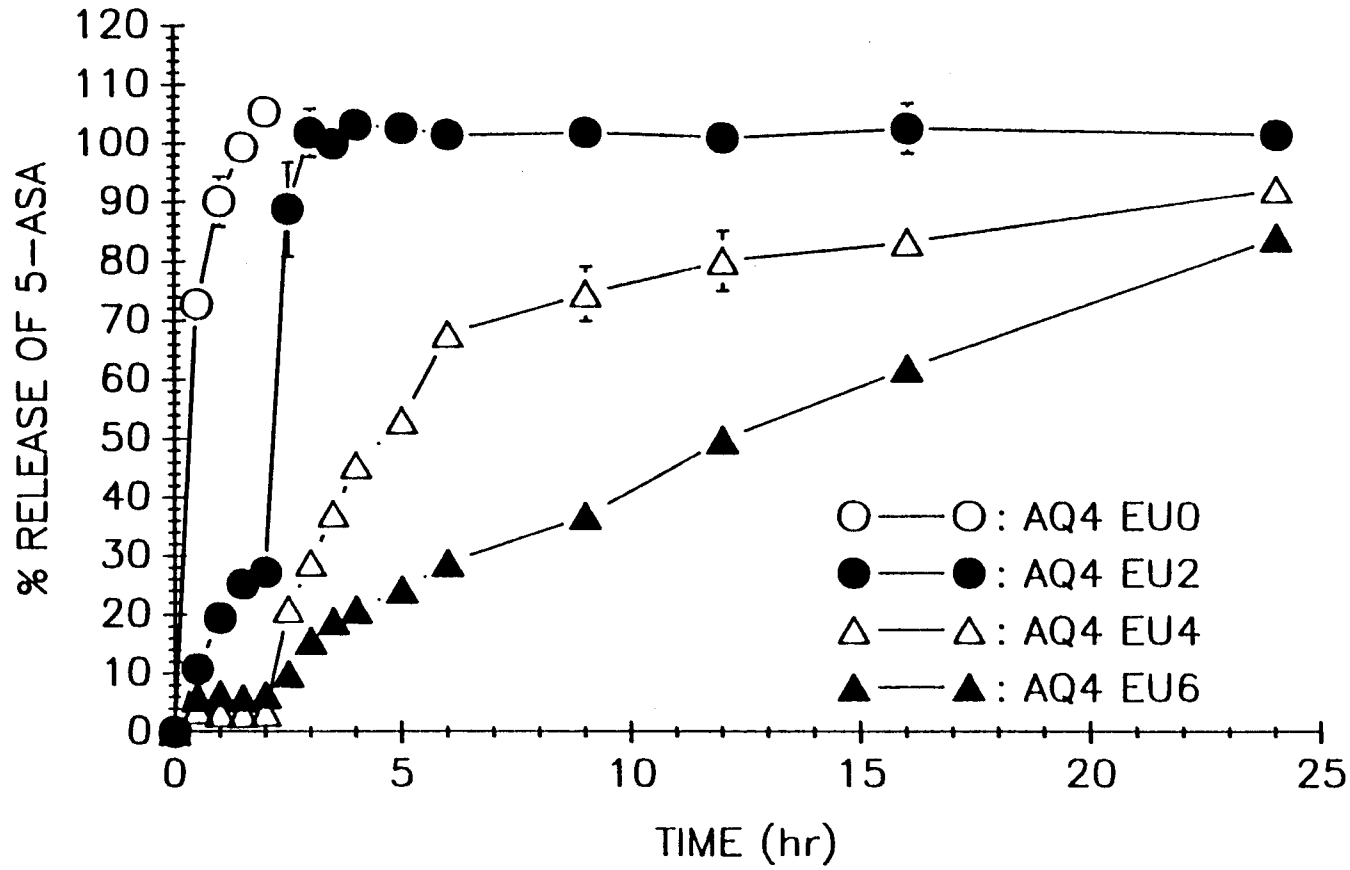


Figure III.14

Figure III.15a Influence of acid pretreatment time (GF) on dissolution of 6% Eudragit L30D and 2% Aquacoat coated 5-ASA/alginate beads.

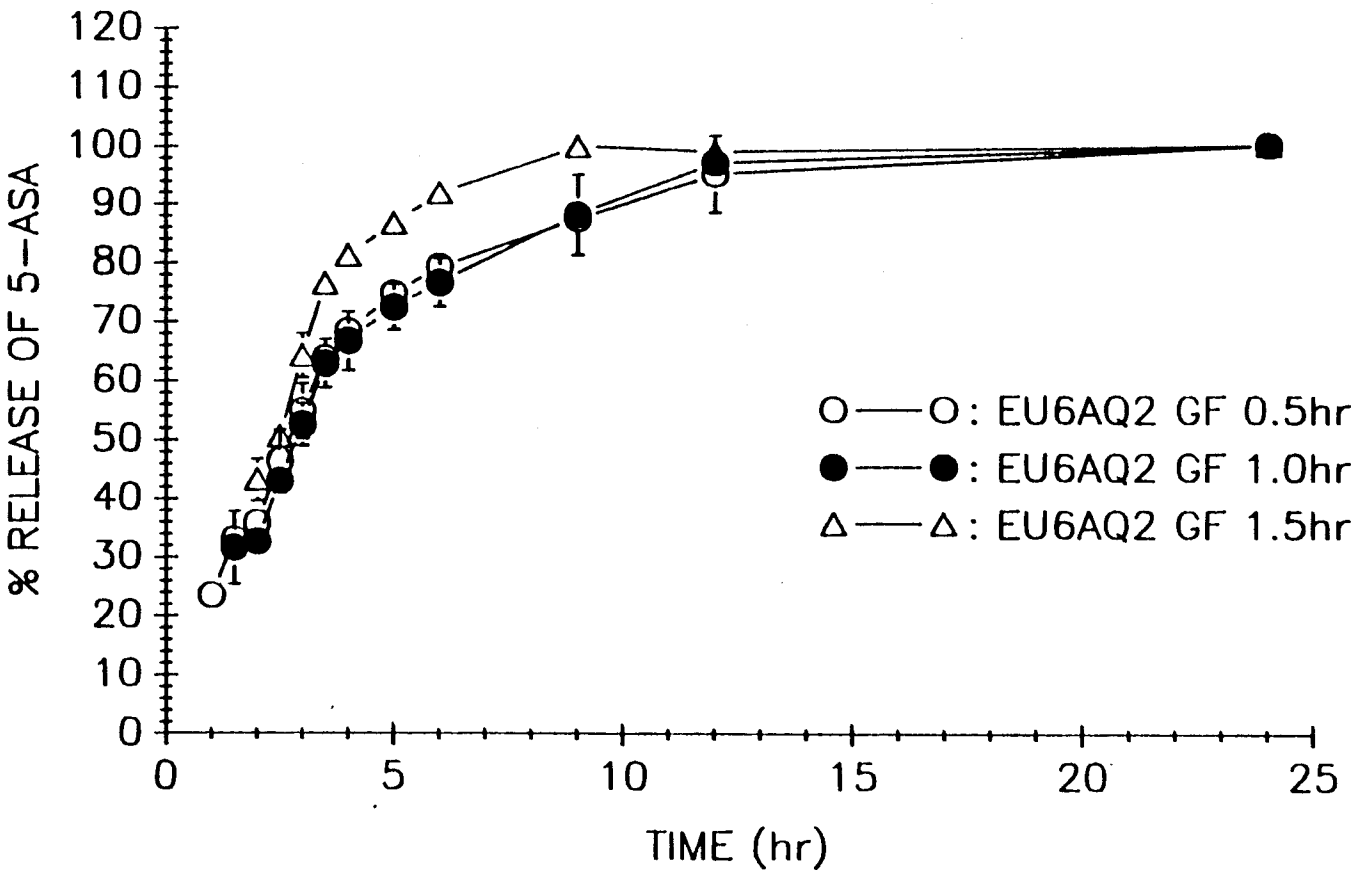


Figure III.15a

Figure III.15b Influence of acid pretreatment time (GF) on dissolution of 6% Eudragit L30D and 4% Aquacoat coated 5-ASA/alginate beads.

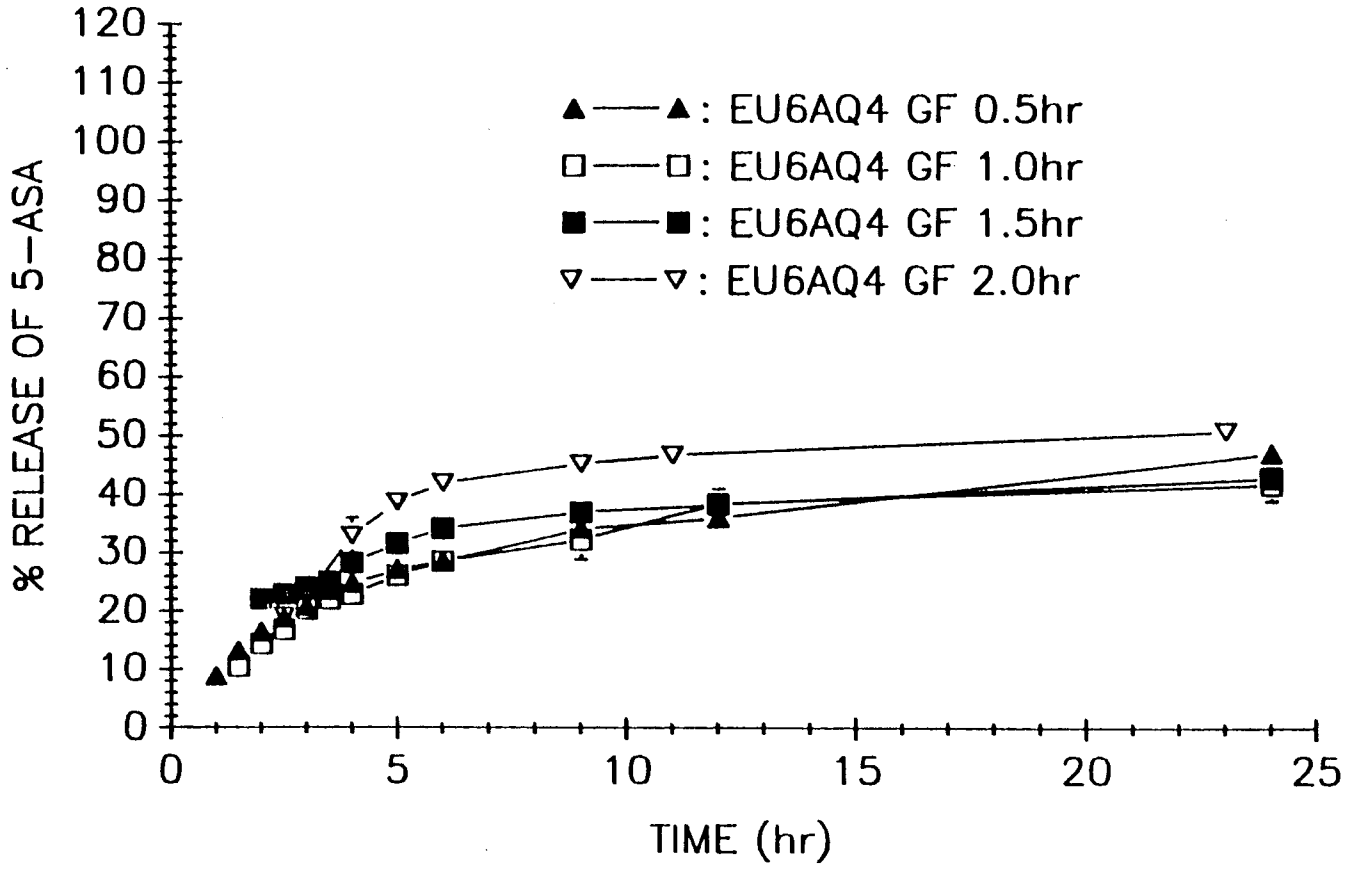


Figure III.15b

Figure III.16 Stability test of Eudragit L30D and Aquacoat coated 5-ASA/alginate beads (stored at room temperature).

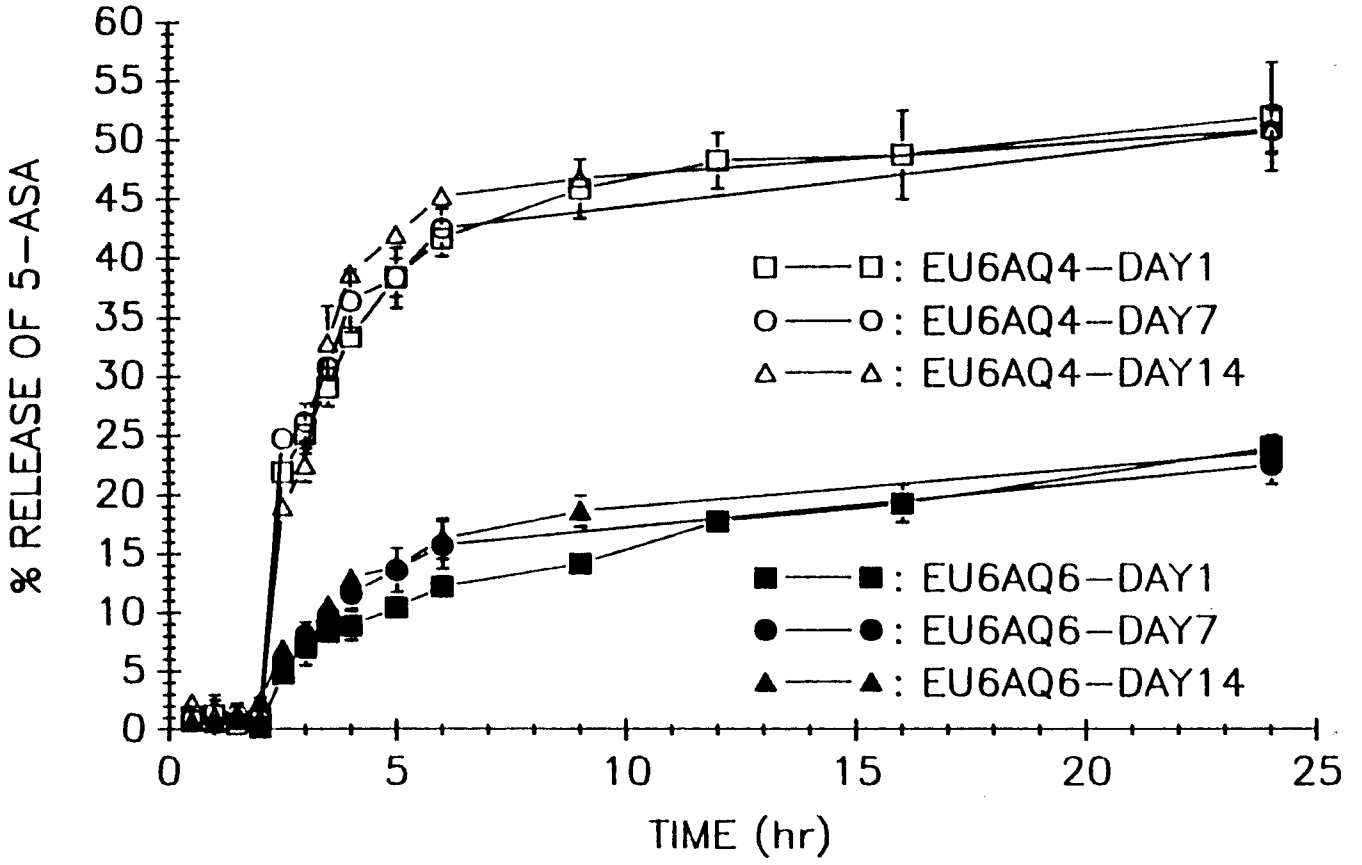


Figure III,16

Inflammation of the colon is commonly treated topically by enema administration. The spreading of enema solutions within the large intestine is highly variable, even in healthy subjects (98). Preparations often fail to reach even the transverse colon (98). Selective delivery of orally administered topically active antiinflammatory drugs to the terminal ileum and ascending colon could be potentially useful for patients with inflammatory bowel disease involving these sites. Because the therapeutic aim in ulcerative colitis is to release 5-ASA for a topical effect on inflamed colonic mucosa, it is important that the preparation achieves topical delivery. In an aqueous solution, the release of 5-ASA take place in both acid and alkaline pH. 5-ASA appears to be both rapidly absorbed by the small intestine and mainly converted within one hour to the acetyl derivative which is largely excreted within about 12 hours (99). Thus, when taken by mouth, 5-ASA does not reach the colon or the lower small bowel in appreciable amounts, unless it is given as a controlled release dosage form.

The use of controlled release systems, such as osmotic devices and coated multi-particles preparations, may facilitate drug delivery to the colon (100). Such systems could be designed to protect the active compound from the acidic environment of the stomach, reduce the dose amount, minimize systemic absorption from the small intestine, and modulate drug release from the preparation (101). Several forms of oral 5-ASA have been developed to overcome this problem. One of these preparations contains 5-ASA in microgranules, coated with a semipermeable membrane of ethyl cellulose, and was designed to release

drug along the GI tract independently of bacterial flora, pH of GI tract, and intestinal transit time (81, 85). These microgranules fall apart in the stomach, and the granules are then dissolved at a certain rate, which is little influenced by pH below 6, but which is released at a faster rate at pH 7.5 (85). Because about 65% of drug administered was excreted in the ileostomy bag, this formulation is suitable for treatment of disease in the jejunum and ileum rather for drug delivery to the colon (81). By mixing 5-ASA powder with buffering sodium carbonate and glycerine, a compressed tablet with this mixture was coated with methacrylic acid copolymer Eudragit L100 (80, 85). The dosage form released 5-ASA largely in the proximal small bowel or even higher when taken with a meal, which indicated delivery of 5-ASA to the colon is not reliable.

Another enteric coating polymer was used for delayed release of 5-ASA. Acrylic acid polymer Eudragit S-100 was selected to cover 5-ASA tablets. This acrylic based resin disintegrates at pH above 7, the prevailing pH of the distal small intestine and colon (79). Radiological evidence showed that with an acrylic coating of Eudragit S-100 (120 um in thickness) most of the dose remained intact after oral ingestion until it reached the ascending colon (100). This suggested that delivery of 5-ASA to the small intestine was doubtable with this design. Osalazine is a new compound developed for the treatment of ulcerative colitis. It consists of two salicylate radicals linked by an azo bond, and is split in the colon by bacterial azoreductase (like sulfasalazine) (102). Because it requires a certain bacterial concentration at the site of action, which is lower in the small

intestinal than higher up in the colon, olsalazine can hardly be the best way to deliver 5-ASA to ileum.

Only slow-release forms of 5-ASA have been tested in the treatment of Crohn's disease of the ileum, where sulfasalazine and other preparations have been of less benefit (85). However, the effect of using slow-release preparations to treat Crohn's disease patients did not result in a significant improvement in the Crohn's disease activity index, or sedimentation rate compared with sulfasalazine (68, 85, 103). Thus, a new slow-release dosage form which can concentrate active drug in the ileum could become the preferred oral agent in treating patients with Crohn's disease of the small intestine. It has been shown that transit through the small intestine in healthy subjects is much more consistent than gastric emptying with mean small intestine transit time around 3 to 4 hours. Small intestine transit time does not appear to be influenced by the physical state, or size of the dosage form, nor by the presence of food in the stomach (56, 104, 105). However, gastric emptying, time varies according to feeding conditions for different physical forms. Solutions and small pellets (less than 5 mm in size) are not greatly affected by the digestive state of the individual (104, 105).

By combining calcium alginate loaded with drug and both enteric coating and sustained release coating, the delivery system can not only pass through the stomach more uniformly (independly of the presence of food), but also avoid being dissolved in a stomach full with food. Because of the consistency of small intestinal transit times, a lag time of two to three hours could ensure the deposition of drug near the

ileum. The lag time for drug dissolution can now be controlled by altering the composition of calcium alginate, drug loading, enteric coating thickness, and sustained release coating thickness. After entering the intestinal tract, the rate of enteric coated film dissolution can be regulated by the amount of sustained release coated film. When calcium alginate gel swells sufficiently to exceed the strength of sustaining film, the release of drug is facilitated. Thus, this formulation provides an oral dosage form design for targeted delivery of drug to any desired site in the gastrointestinal tract.

REFERENCES

1. McDowell, R. H. 1968. Properties of Alginates. Alginate Industries Limited, London. pp. 1-16.

2. Muzzarelli, R. A. A. 1972. Natural Chelating Polymers: Alginic acid, Chitin and Chitosan. Pergamon Press. pp. 23-80.

3. Rees, D. A. and E. J. Welsh. 1977. Secondary and tertiary structure of polysaccharides in solutions and gels. Angrew. Chem. 16:228.

4. Rees, D. A. and J. W. B. Sanuel. 1967. The structure of alginic acid. VI. Minor features and structural variations. J. Chem. Soc. C:2295.

5. Haug, A., B. Larsen, and O. Smidsrod. 1966. A study of constitution of alginic acid by partial acid hydrolysis. Acta. Chem. Scand. 20:183.

6. Kohn, R. and B. Larsen. 1972. Preparation of water-soluble glycuronans and their calcium salts, and the determination of calcium ion activity in relation to the degree of polymerization. Acta. Chem. Scand. 26:2455.

7. Grasdalen, H., B. Larsen, and O. Smidsrod. 1979. A P.M.R. study of the composition and sequence of uronate residues in algintaes. Carbohydr. Res. 68:23.

8. Takahashi, Y. 1984. The formation of inclusion compounds of alginic acid in aqueous solution. J. Inclu. Phenom. 2:399.

9. Morris, E. R. D. A. Rees, D. Thom, and J. Boyd. 1978. Chiroptical and stoichiometric evidence of a specific, primary diverisation process in alginate gelation. Carbohydr. Res. 66:145.

10. Smidsroed, O. and A. Haug. 1972. Properties of poly (1,4 - hexuronates) in the gel state. II. Comparison of gels of different chemical composition. Acta. Chem. Scand. 26:79.

11. Kuek, C. and T. M. Armitage. 1985. Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of Aspergillus phoenicus. Enzyme Microb. Technol. 7:121.

12. Veelken, M. and H. Pape. 1984. Production of nikkomycin by immobilized Streptomyces cells - physiological properties. Appl. Microbiol. Biotechnol. 19:146.

13. Robinson, P. K., A. L. Dainty, K. H. Goulding, I. Simpkins ,and M. D. Trevan. 1985. Physiology of alginate - immobilized Chlorella. Enzyme Microb. Technol. 7:212.

14. Yotsuyanagi, T., T. Ohkubo, T. Ohhashi, and K. Ikeda. 1987. Calcium-induced gelation of alginic acid and pH-sensitive reswelling of dried gels. Chem. Pharm. Bull. 35:1555.

15. Kierstan, M., G. Darcy, and J. Reilly. 1982. Studies on the characteristics of alginate gels in relation to their use in separation and immobilization applications. Biotechnol. Bioeng. 24:1507.

16. Itamunoala, G. F. 1987. Effective diffusion coefficients in calcium alginate gel. Biotechnol. Prog. 3:115.

17. Hiemstra, H., L. Dijkhuizen, and W. Harser. 1983. Diffusion of oxygen in alginate gels related to the kinetics of methanol oxidation by immobilized Hansenula polymorpha cells. Eur. J. Appl. Microbiol. Biotechnol. 18:189.

18. Toda, K. and M. Shoda. 1975. Sucrose inversion by immobilized yeast cells in a complete mixing reactor. Biotechnol. Bioeng. 17:481.

19. Johansen, A. and J. M. Flink. 1986. Influence of alginate properties on sucrose inversion by immobillized whole cell invertase. Enzyme Microb. Technol. 8:485.

20. Tramper, J. and A. W. A. de Man. 1986. Characterization of Nitrobacter agilis immobilized in calcium alginate. Enzyme Microb. Technol. 8:472.

21. Hulst, A. C., J. Tramper, P. Brodelius, L. J. C. Eijkenboom, and K. Ch. A. M. Luyben. 1985. Immobilized plant cells: Respiration and oxygen transfer. J. Chem. Tech. Biotechnol. 35B:198.

22. Johansen, A. and J. M. Flink. 1986. Immobilization of yeast cells by internal gelation of alginate. Enzyme Microb. Technol. 8:145.

23. Robinson, P. K., K. H. Goulding, A. L. Mak, and M. D. Trevan. 1986. Factors affecting the growth characteristics of alginate-entrapped Chlorella. Enzyme Microb. Technol. 8:729.

24. Wan, L. S. C. and P. W. S. Heng. 1985. Liquid penetration into tablets containing surfactants. Chem. Pharm. Bull. 33:2569.

25. Wan, L. S. C. and P. W. S. Heng. 1987. Influence of surfactant on the properties of granules and tablets containing sodium alginate. Pharm. Acta. Helv. 62:169.

26. Goosen, M. F. A., G. M. O'Shea, H. M. Gharpetian, S. Chou, and A. M. Sun. 1985. Optimization of microencapsulation parameters: Semipermeable microcapsules as a bioartificial pancreas. Biotechnol. Bioeng. 27:146.

27. Sheurich, P., H. Schnabl, U. Zimmermann, and J. Klein. 1980. Immobilization and mechanical support of individual protoplasts. Biochim. Biophys. Acta. 598:645.

28. Cabral, J. M. S., P. Fevereiro, J. M. Novais, and M. S. S. Pais. 1984. Comparison of immobilization methods for plant cells and protoplasts. Ann. N.Y. Acad. Sci. 434:501.

29. Linse, L. and P. Brodelius. 1984. Immobilization of plant protoplasts. Ann. N. Y. Acad. Sci. 434:487.

30. Nilsson, K. and K. Mosbach. 1980. Preparation of immobilized animal cells. FEBS Letters. 118:145.

31. Gray, B. N. and E. Watkins. 1976. Prevention of Vascular complications of diabetes by pancreatic islet Transplantation. Arch. Surg. 111:254.

32. Mauer, S. M., M. W. Steefes, D. E. R. Sutherland, J. S. Najarian, A. F. Michael, and D. M. Brown. 1975. Study of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes. 24:280.

33. Lim, F. and A. M. Sun. 1980. Microencapsulated islets as bioartificial endocrine pancreas. Science. 210:908.

34. O'shea, G. M., M. F. A. Goosen and A. M. Sun. 1984. Prolonged survival of transplanted of langerhans encapsulated in a biocompatible membrane. Biochim. Biophys. Acta. 804:133.

35. Lanberti, F. V. and M. V. Sefton. 1983. Microencapsulation of erythrocytes in Eudragit-RL coated calcium alginate. Biochim. Biophys. Acta. 759:81.

36. Pfister, G., M. Bahadir, and F. Korte. 1986. Release characteristics of herbicides from Ca alginate gel formulations. J. Controlled Release. 3:229.

37. Bahadir, G. and G. S. U. Munchen. 1987. Safe formulations of agrochemicals. Chemosphere. 16:615.

38. Barrett, P. F. 1978. Some studies on the use of alginates for the placement and controlled release of diquat on submerged aquatic plants. Pestic. Sci. 9:425.

39. Connick, W. J., Jr. 1982. Controllrd release of the herbicides 2,4 - D and dichlobenil from alginate gels. J. Appl. Polym. Sci. 27:3341.

40. Connick, W. J., Jr., J. M. Bradow, W. Wells, K. K. Steward, and T. K. Van. 1984. Preparation and evaluation of controlled release formulations of 2,6 - dichlorobenzonitrile. J. Agric. Food Chem. 32:1199.

41. Clifford, D. R., P. Gendle, and M. E. Holgate. 1987. Gel formulations for the treatment of pruning wounds. I. Carbendazim and triadimefon in sodium alginate. Ann. Appl. Biol. 110:489.

42. Clifford, D. R., P. Gendle, and M. E. Holgate. 1987. Gel formulations for the treatment of pruning wounds. II. Results with differing gel and fungicide components and comparison witn sealant compositions. Ann Appl. Biol. 110:501.

43. Stockwell, A. F. and S. S. Davis. 1986. In vitro evaluation of alginate gel systems as sustained release drug delivery systems. J. Controlled Release. 3:167.

44. Horder, R., M. Banks, and T. M. Hoadley. 1986. Slow release solid preparation. European Patent Application 0,188,040 A1.

45. Badwan, A. A., A. Abumalooh, E. Salam, A. Abukalaf, and O. Jawan. 1985. A sustained release drug delivery system using calcium alginate beads. Drug Dev. Ind. Pharm. 11:239.

46. El-Samaligy, M. and H. A. Mahmoud. 1986. Effect of aqueous phase modifiers on drug release from polyacrylamide microbeads. Pharm. Ind. 48:1070.

47. Lim, F. and R. D. Moss. 1982. A method of encapsulating oils and oil-soluble materials. UK Patent Application GB 2,086,835 A.

48. Yang, R. K. and S. C. Sharma. 1982. A delivery system for an active ingredient and a process for preparation thereof. European Patent Application 0,202,819 A2.

49. Roberts, B. A. 1984. Oleaginous compositions. United States Patent, No. 4,446,165.

50. Adams, S.S., K. F. McCullough, and J. S. Nicholson. 1969. The pharmacological properties of ibuprofen, an anti-inflammatory analgesic and antipyretic agent. Arch. Int. Pharmacodyn. Ther. 178:115.

51. Kantor, T. G. 1979. Ibuprofen. Ann. Intern. Med. 91:877.

52. Lanza, F. L., D. Fakouhi, A. Rubin, R. E. Davis, M. F. Rack, C. Nissen, and S. Deis. 1989. A double-blind placebo-controlled comparison of the efficacy and safety of 50, 100, and 200 ug of misoprostol QID in the prevention of ibuprofen-induced gastric and duodenal mucosal lesions and symptoms. Am. J. Gastroenterol. 84:633.

53. Albert, K. S. and C. M. Gernaat. 1984. Pharmacokinetics of ibuprofen. Am. J. Med. (ibuprofen symposium) pp. 40s.

54. Jamali, F., N. N. Singh, F. M. Pasutto, A. S. Russell, and T. Coutts. 1988. pharmacokinetics of ibuprofen enantiomers in humans following oral administration of tablets with different absorption rates. Pharm. Res. 5:40.

55. Lee, E. J. D., K. William, R. Day, G. Graham, and D. Champion. 1985. Stereoselective disposition of ibuprofen enantiomers in man. Br. J. Pharmac. 19:669.

56. Khosla, R. and S. S. Davis. 1989. Gastric emptying and small and large bowel transit of non-disintegrating tablets in fasted subjects. Int. J. Pharm. 52:1.

57. Van Rosendaal, G. M. A. 1989. Recent advances in pharmacotherapy: Inflammatory bowel disease. CMAJ 141:113.

58. Svartz, N. 1988. Sulfasalazine: II. Some notes on the discovery and development of salazopyrin. Am. J. Gastroenterol. 83:497.

59. Bachrach, W. H. 1988. Sulfasalazine: I. An historical perspective. Am. J. Gastroenterol. 83:487.

60. Schroder H. and D. E. S. Campbell. 1972. Absorption, metabolism, and excretion of salicylazosulfapyridine in man. Clin. Pharmcol. Ther. 13:539.

61. Das, K. M., J. R. Chowdhury, B. Zapp, and J. W. Fara. 1979. Small bowel absorption of sulfasalazine and its hepatic metabolism in human beings, cats, and rats. Gastroenterology 77:280.

62. Dull, B. J., K. Salata, and P. Goldman. 1987. Role of the intestinal flora in the acetylation of sulfasalazine metabolites. Bichem. Pharmacol. 36:3772.

63. Peppercorn, M. A. and P. Goldman. 1972. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J. Pharmacol. Exp. Ther. 181:555.

64. Peppercon, M. A. and P. Goldman. 1973. Distribution studies of salicylazosulfapyridine and its metabilites. Gastroenterology 64:240.

65. Peppercon, M. A. 1987. Sulfasalazine and related new drugs. Ther. Rev. 27:260.

66. Khan, A. K. A., J. Piris, and S. C. Truelove. 1977. An experiment to determine the active therapeutic moiety of sulphasalazine. Lancet (II):892.

67. Klotz, U., K. Maier, C. Fischer, and K. Heinkel. 1980. Therapeutic efficacy of sulfasalazine and its metabolites in patients with ulcerative colitis and Crohn's disease. New Eng. J. Med. 303:1499.

68. Peppercon, M. A. 1990. Advances in drug therapy for inflammatory bowel disease. Ann. Inter. Med. 112:50.

69. Franklin, J. L. and I. H. Rosenberg. 1988. Sulfasalazine inhibits folate absorption. Nutr. Rev. 46:320.

70. Das, K. M., M. A. Eastwood, J. P. A. McManus, and W. Sircus. 1973. Adverse reactions during salicylazosulfapyridine therapy and the relation with drug metabolism and acetylator phenotype. New Eng. J. Med. 289:491.

71. Hawkey, C. J. 1986. Salicylates for the sulfa-sensitive patient with ulcerative colitis?. Gastroenterology 90:1082.

72. Campieri, M., G. A. Lanfranchi, G. Bazzocchi, C. Brignola, F. Sarti, G. Franzin, A. Battocchia, G. Labo, and P. R. D. Monte. 1981. Treatment of ulcerative colitis with high-dose 5-aminosalicylic acid enemas. Lancet (II):270.

73. Sutherland L. R., F. Martin, S. Greer, M. Robinson, N. Greenberger, F. Saibil, T. Martin, J. Sparr, E. Prokipchuk, and L. Borgen. 1987. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and protitis. Gastroenterology 92:1894.

74. Guarino, J., M. Chatzinoff, T. Berk, and L. S. Friedman. 1987. 5-Aminosalicylic acid enemas in refractory distal ulcerative colitis: Long-term results. Am. J. Gastroenterol. 82:732.

75. Karp, L. C. and S. R. Targan. 1988. New enema treatments for inflammatory bowel disease. Dig. Dis. Sci. 33:85s.

76. Dew, M. J., R. E. J. Ryder, N. Evans, B. K. Evans, and J. Rhodes. 1983. Colonic release of 5-amino salicylic acid from an oral preparation in active ulcerative colitis. Br. J. Clin. Pharmac. 16:185.

77. Dew, M. J., P. Ebden, N. S. Kidwai, G. Lee, B. K. Evans, and J. Rhodes. 1984. Comparison of the absorption and metabilism of sulphasalazine and acrylic-coated 5-amino salicylic acid in normal subjects and patients with colitis. Br. J. Clin. Pharmac. 17:474.

78. Schroeder, K. W., W. J. Tremaine, and D. M. Ilstrup. 1987. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colits. New Eng. J. Med. 317:1625.

79. Riley, S. A., V. Mani, M. J. Goodman, M. E. Herdm S. Dutt, and L. A. Turnberg. 1988. Comparison of delayed-release 5-aminosalicylic acid (Mesalazine) and sulfasalazine as maintenance treatment for patients with ulcerative colitis. Gastroenterology 94:1383.

80. Klotz, U., K. E. Maier, C. Fischer, and K. H. Bauer. 1985. A new slow-release form of 5-aminosalicylic acid for the oral treatment of inflammatory bowel disease. Arzneim. Forsch. Drug Res. 35:636.

81. Rasmussen, S. N., S. Bondesen, E. F. Hvidberg, S. H. Hansen, V. Binder, S. Halskov, and H. Flachs. 1982. 5-Aminosalicylic acid in a slow-release preparation: Bioavailability, plasma level, and excretion in humans. Gastroenterology 83:1062.

82. Borgen, L., V. Patel, and D. Powell. 1986. A clinical pharmacologic study of 5-aminosalicylic acid oral dosage forms. Gastroenterology 90:1351.

83. Mardini, H. A., D. C. Lindsay, C. M. Deighton, and C. O. Record. 1987. Effect of polymer coating on faecal recovery of ingested 5-aminosalicylic acid in patients with ulcerative colitis. Gut 28:1084.

84. Dew, M. J., A. D. Harries, N. Evans, and J. Rhodes. 1983. Maintenance of remission in ulcerative colitis with 5-amino salicylic acid in high doses by nouth. Br. Med. J. 287:23.

85. Jarnerot, G. 1989. Newer 5-aminosalicylic acid based drugs in Chronic inflammatory bowel disease. Drugs 37:73.

86. Friedman, G. 1986. Sulfasalazine and new analogues. Am. J. Gastroenterol. 81:141.

87. Klotz, U. and K. E. Maier. 1987. Pharmacology and pharmacokinetics of 5-aminosalicylic acid. Dig. Dis. Sci. 32:46s.

88. Allgayer, H., N. O. Ahnfelt, W. Kruis, U. Klotz, K. Frank-Holmberg, H. N. A. Soderberg, and G. Paumgartner. Colonic N-acetylation of 5-aminosalicylic acid in inflammatory bowel disease. Gastroenterology 97:38.

89. Sartnurak, S. 1985. Evaluation of solid dispersed particles for formulation of oral ibuprofen tablets. Ph.D. thesis. Oregon State University pp.60.

90. Bodmeier, R. and O. Paeratakul. 1989. Spherical agglomerates of water-insoluble drugs. J. Pharm. Sci. 78:964.

91. Ayres, J. W., H. Huang, and K. Albert. 1984. Deneric tolbutamide tablet dissolution: Intralot and interlot variation. J. Pharm. Sci. 73:1629.

92. Lockwood, G. F., K. S. Albert, W. R. Gillespie, G. G. Bole, T. M. Harkcom, G. J. Szpunar, and J. G. Wagner. 1983. Pharmacokinetics of ibuprofen in man. I. Free and toatl area/dose relationship. Clin. Pharmacol. Ther. 34:97.

93. Wagner, J. G., K. S. Albert, G. J. Szpunar, and G. F. Lockwood. 1984. Pharmacokinetics of ibuprofen in man IV: Absorption and disposition. J. Pharmacokin. Biopharm. 12:381.

94. Jenkinson, M. L., R. Fitzpatrick, P. J. Streete, and G. N. Volans. 1988. The relationship between plasma ibuprofen concentrations and toxicity in acute ibuprofen overdose. Human Toxicol. 7:319.

95. Parr, A. F., R. M. Beihn, R. M. Franz, G. J. Szpunar, and M. Jay. 1987. Correlation of ibuprofen bioavailability with gastrointestinal transit by scintigraphic monitoring of ^{171}Er-labeled sustained-release tablets. Pharm. Res. 4:486.

96. Borin, M. T., S. Khare, R. M. Beihn, and M. Jay. 1990. The effect of food on gastrointestinal (GI) transit of sustained-release ibuprofen tablets as evaluated by gamma scintigraphy. Pharm. Res. 7:304.

97. Shaw, P. N., L. Aarons, and J. B. Houston. 1980. HPLC analysis of sulphasalazine and its metabolites. J. Pharm. Pharmacol. 32:67p.

98. Wood, E., C. G. Wilson, J. G. Hardy. 1985. The spreading of foam and solution enemas. Int. J. Pharm. 25:191.

99. Myers, B., D. N. W. Evans, J. Rhodes, S. K. Evans, B. R. Hughes, M. G. Lee, A. Richens, and D. Richards. 1987. Metabolism and urinary excretion of 5-amino salicylic acid in healthy volunteers when given intravenously released for absorption at different sites in the gastrointestinal tract. Gut 28:196.

100. Hardy, J. G., C. G. Wilson, and E. Wood. 1985. Drug delivery to the proxinal colon. J. Pharm. Pharmacol. 37:874.

101. Dew, M. J., P. J. Hughes, M. G. Lee, B. K. Evans, and J. Rhodes. 1982. An oral preparation to release drugs in the human colon. Br. J. Clin. Pharmac. 14:405.

102. Sandberg-Gertzen, H., G. Jarnerot, and W. Kraaz. 1986. Azodisal sodium in the treatment of ulcerative colitis. Gastroenterology 90:1024.

103. Rasmussen, S. N., V. Binder, K. Maier, S. Bondesen, C. Fischer, U. Klotz, S. H. Hansen, and E. F. Hvidberg. 1983. Treatment of Crohn's disease with peroral 5-aminosalicylic acid. Gastroenterology 85:1350.

104. Davis, S. S., J. G. Hardy, and J. W. Fara. 1986. Transit of pharmaceutical dosage forms through the small intestine. Gut 27:886.

105. Khosla, A., L. C. Feely, and S. S. Davis. 1989. Gastrointestinal transit of non-disintegrating tablets in fed subjects. Int. J. Pharm. 53:107.

BIBLIOGRAPHY

Adams, S.S., K. F. McCullough, and J. S. Nicholson. 1969. The pharmacological properties of ibuprofen, an anti-inflammatory analgesic and antipyretic agent. Arch. Int. Pharmacodyn. Ther. 178:115.

Albert, K. S. and C. M. Gernaat. 1984. Pharmacokinetics of ibuprofen. Am. J. Med. (ibuprofen symposium) pp. 40s.

Allgayer, H., N. O. Ahnfelt, W. Kruis, U. Klotz, K. Frank-Holmberg, H. N. A. Soderberg, and G. Paumgartner. 1980. Colonic N-acetylation of 5-aminosalicylic acid in inflammatory bowel disease. Gastroenterology 97:38.

Alm, L. 1983. Survival rate of salmonella and shigella in fermented milk products with and without added human gastric juice. An in vitro study. Prog. Food Nutr. Sci. 7:19.

Alm, L., D. Humble, E. Ryd-Kjellen, and G. Setterberg. 1983. The effect of acidophilus milk in the treatment of constipation in hospitalized geriatric patients. Nutrition and the intestinal flora. pp.131 in Symp. Swed. Nutr. Found. 15.

Ayres, J. W., H. Huang, and K. Albert. 1984. Generic tolbutamide tablet dissolution: Intralot and interlot variation. J. Pharm. Sci. 73:1629.

Badwan, A. A., A. Abumalooh, E. Salam, A. Abukalaf, and O. Jawan. 1985. A sustained release drug delivery system using calcium alginate beads. Drug Dev. Ind. Pharm. 11:239.

Bachrach, W. H. 1988. sulfasalazine: I. An historical perspective. Am. J. Gastroenterol. 83:487.

Bahadir, G. and G. S. U. Munchen. 1987. Safe formulations of agrochemicals. Chemosphere. 16:615.

Barrett, P. F. 1978. Some studies on the use of alginates for the placement and controlled release of diquat on submerged aquatic plants. Pestic. Sci. 9:425.

Bibel, D. J. 1988. Elie Metchnikoff's bacillus of long life. ASM News. 54:661.

Bodmeier, R. and O. Paeratakul. 1989. Spherical agglomerates of water-insoluble drugs. J. Pharm. Sci. 78:964.

Borgen, L., V. Patel, and D. Powell. 1986. A clinical pharmacologic study of 5-aminosalicylic acid oral dosage forms. Gastroenterology 90:1351.

Borin, M. T., S. Khare, R. M. Beihn, and M. Jay. 1990. The effect of food on gastrointestinal (GI) transit of sustained-release ibuprofen tablets as evaluated by gamma scintigraphy. Pharm. Res. 7:304.

Bozoglu, T. F. and G. C. Gurakan. 1989. Freeze-drying injury of Lactobacillus acidophilus. J. Food Prot. 52:259.

Brennan, M., B. Wanismail, M. C. Johnson, and B. Ray. 1986. Cellular damage in dried Lactobacillus acidophilus. J. Food Prot. 49:47.

Brennan, M., B. Wanismail, and B. Ray. 1983. Prevalence of viable lactobacillus acidophilus in dried commercial products. J. Food Prot. 46:887.

Brodelius, P. and K. Mosbach. 1982. Immobilized plant cells. in Advances in Applied Microbiology. Academic Press, New York. pp.1.

Brown, J. P. 1977. Role of gut bacterial flora in nutrition and health: a review of recent advances in bacteriological techniques. CRC Crit. Rev. Food Sci. Nutr. 8:229.

Cabral, J. M. S., P. Fevereiro, J. M. Novais, and M. S. S. Pais. 1984. Comparison of immobilization methods for plant cells and protoplasts. Ann. N.Y. Acad. Sci. 434:501.

Campieri, M., G. A. Lanfranchi, G. Bazzocchi, C. Brignola, F. Sarti, G. Franzin, A. Battocchia, G. Labo, and P. R. D. Monte. 1981. Treatment of ulcerative colitis with high-dose 5-aminosalicylic acid enemas. Lancet (II):270.

Casson, D. and A. N. Emery. 1987. On the elimination of artefactual effects in assessing the structure of calcium alginate cell immobilization gels. Enzyme Microb. Technol. 9:102.

Clifford, D. R., P. Gendle, and M. E. Holgate. 1987. Gel formulations for the treatment of pruning wounds. I. Carbendazim and triadimefon in sodium alginate. Ann. Appl. Biol. 110:489.

Clifford, D. R., P. Gendle, and M. E. Holgate. 1987. Gel formulations for the treatment of pruning wounds. II. Results with differing gel and fungicide components and comparison witn sealant compositions. Ann Appl. Biol. 110:501.

Connick, W. J., Jr. 1982. Controllrd release of the herbicides 2,4 - D and dichlobenil from alginate gels. J. Appl. Polym. Sci. 27:3341.

Connick, W. J., Jr., J. M. Bradow, W. Wells, K. K. Steward, and T. K. Van. 1984. Preparation and evaluation of controlled release formulations of 2,6 - dichlorobenzonitrile. J. Agric. Food Chem. 32:1199.

Conway, P. L., S. L. Gorbach, and B. R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1.

Das, K. M., J. R. Chowdhury, B. Zapp, and J. W. Fara. 1979. Small bowel absorption of sulfasalazine and its hepatic metabolism in human beings, cats, and rats. Gastroenterology 77:280.

Das, K. M., M. A. Eastwood, J. P. A. McManus, and W. Sircus. 1973. Adverse reactions during salicylazosulfapyridine therapy and the relation with drug metabolism and acetylator phenotype. New Eng. J. Med. 289:491.

Davis, S. S., J. G. Hardy, and J. W. Fara. 1986. Transit of pharmaceutical dosage forms through the small intestine. Gut 27:886.

Dew, M. J., P. Ebden, N. S. Kidwai, G. Lee, B. K. Evans, and J. Rhodes. 1984. Comparison of the absorption and metabilism of sulphasalazine and acrylic-coated 5-amino salicylic acid in normal subjects and patients with colitis. Br. J. Clin. Pharmac. 17:474.

Dew, M. J., A. D. Harries, N. Evans, and J. Rhodes. 1983. Maintenance of remission in ulcerative colitis with 5-amino salicylic acid in high doses by nouth. Br. Med. J. 287:23.

Dew, M. J., P. J. Hughes, M. G. Lee, B. K. Evans, and J. Rhodes. 1982. An oral preparation to release drugs in the human colon. Br. J. Clin. Pharmac. 14:405.

Dew, M. J., R. E. J. Ryder, N. Evans, B. K. Evans, and J. Rhodes. 1983. Colonic release of 5-amino salicylic acid from an oral preparation in active ulcerative colitis. Br. J. Clin. Pharmac. 16:185.

Draser, B. S. and M. L. Hill. 1974. Human intestinal flora. Academic Press. New York. pp. 36.

Dull, B. J., K. Salata, and P. Goldman. 1987. Role of the intestinal flora in the acetylation of sulfasalazine metabolites. Bichem. Pharmacol. 36:3772.

El-Samaligy, M. and H. A. Mahmoud. 1986. Effect of aqueous phase modifiers on drug release from polyacrylamide microbeads. Pharm. Ind. 48:1070.

Espina, F. and V. S. Packard. 1979. Survival of Lactobacillus acidophilus in a spray-drying process. J. Food Prot. 42:149.

Fontana, R., P. Canepari, and G. Satta. 1979. Alternations in peptidoglycan chemical composition associated with rod-to-sphere transition in a conditional mutant of Klebsiella pneumoniae. J. Bacteriol. 139:1028.

Franklin, J. L. and I. H. Rosenberg. 1988. Sulfasalazine inhibits folate absorption. Nutr. Rev. 46:320.

Friedman, G. 1986. Sulfasalazine and new analogues. Am. J. Gastroenterol. 81:141.

Fry, R. M. 1966. Freezing and drying of bacteria. in Cryobiology. Academic Press, London. pp.665.

Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by Lactobacillus Acidophilus. Appl. Environ. Microbiol. 49:377.

Gilliland, S. E., and M. L. Speck. 1977. Deconjugation of Bile acids by intestinal Lactobacilli. Appl. Environ. Microbiol. 33:15.

Gilliland, S. E. and M. L. Speck. 1977. Enumeration and identity of lactobacilli in dietary products. J. Food Prot. 40:760.

Gilliland, S. E., M. L. Speck, G. F. Nauyok, Jr., and F. G. Giesbrecht. 1978. Influence of consuming nonfermented milk containing Lactobacillius acidophilus on fecal flora of healthy males. J. Dairy Sci. 61:1.

Goldin, B. R., L. Swenson, J. Dwyer, M. Sexton, and S. L. Gorbach. 1980. Effect of diet and Lactobacillus acidophillus supplements on human fecal bacterial enzymes. JNCI 64:255.

Goosen, M. F. A., G. M. O'Shea, H. M. Gharpetian, S. Chou, and A. M. Sun. 1985. Optimization of microencapsulation parameters: Semipermeable microcapsules as a bioartificial pancreas. Biotechnol. Bioeng. 27:146.

Gorbach, S. L. and B. R. Goldin. 1986. Lactobacillus acidophilus strains of bacteria and compositions thereof. Eur. Patent Application No. 86302836.1

Gotto et al. 1984. Recommendations for treatment of Hyperlipidemia in Adults. Am. Heart Associ. 69:1065A.

Grade, V. L., B. Thomasset, and J. N. Barbotin. 1981. Electron microscopic evidence of an immobilized living cell system. Enzyme Microb. Technol. 3:216.

Grasdalen, H., B. Larsen, and O. Smidsrod. 1979. A P.M.R. study of the composition and sequence of uronate residues in algintaes. Carbohydr. Res. 68:23.

Gray, B. N. and E. Watkins. 1976. Prevention of Vascular complications of diabetes by pancreatic islet Transplantation. Arch. Surg. 111:254.

Grunewald, K. K. 1982. Serum cholesterol levels in rats fed skim milk fermented by Lactobacillus Acidophilus. J. Food Sci. 7:2078.

Guarino, J., M. Chatzinoff, T. Berk, and L. S. Friedman. 1987. 5-Aminosalicylic acid enemas in refractory distal ulcerative colitis: Long-term results. Am. J. Gastroenterol. 82:732.

Hardy, J. G., C. G. Wilson, and E. Wood. 1985. Drug delivery to the proxinal colon. J. Pharm. Pharmacol. 37:874.

Harrison, V. C., and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr. 28:1351.

Haug, A., B. Larsen, and O. Smidsrod. 1966. A study of constitution of alginic acid by partial acid hydrolysis. Acta. Chem. Scand. 20:183.

Hawkey, C. J. 1986. Salicylates for the sulfa-sensitive patient with ulcerative colitis?. Gastroenterology. 90:1082.

Hepner, G., R. Fried, S. St. Jeor, L. Fusetti, and R. Morin. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19.

Hiemstra, H., L. Dijkhuizen, and W. Harser. 1983. Diffusion of oxygen in alginate gels related to the kinetics of methanol oxidation by immobilized Hansenula polymorpha cells. Eur. J. Appl. Microbiol. Biotechnol. 18:189.

Horder, R., M. Banks, and T. M. Hoadley. 1986. Slow release solid preparation. European Patent Application 0,188,040 A1.

House-Shokuhin-Kogyo, K. K. 1981. Powdered milk production. Dairy Sci. Abstr. 43:5838.

Hulst, A. C., J. Tramper, P. Brodelius, L. J. C. Eijkenboom, and K. Ch. A. M. Luyben. 1985. Immobilized plant cells: Respiration and oxygen transfer. J. Chem. Tech. Biotechnol. 35B:198.

Hurst, A. and J. M. Stubbs. 1969. Electron microscopic study of membranes and walls of bacteria and changes occurring during growth initiation. J. Bacteriol. 97:1466.

Hussi, E., T. A. Miettinen, A. Ollus, E. Kostiainen, C. Ehnholm, B. Haglund, J. K. Huttunen, and V. Manninen. 1981. Lack of serum cholesterol-lowering effect of skimmed milk and butter milk under controlled conditions. Atherosclerosis 39:267.

Itamunoala, G. F. 1987. Effective diffusion coefficients in calcium alginate gel. Biotechnol. Prog. 3:115.

Jamali, F., N. N. Singh, F. M. Pasutto, A. S. Russell, and T. Coutts. 1988. pharmacokinetics of ibuprofen enantiomers in humans following oral administration of tablets with different absorption rates. Pharm. Res. 5:40.

Jarnerot, G. 1989. Newer 5-aminosalicylic acid based drugs in Chronic inflammatory bowel disease. Drugs 37:73.

Jaspers, D. A., L. W. Massey, and L. O. Luedecke. 1984. Effect of consuming yogurts prepared with three culture strains on human serum lipoproteins. J. Food Sci. 49:1178.

Jenkinson, M. L., R. Fitzpatrick, P. J. Streete, and G. N. Volans. 1988. The relationship between plasma ibuprofen concentrations and toxicity in acute ibuprofen overdose. Human Toxicol. 7:319.

Johansen, A. and J. M. Flink. 1986. Influence of alginate properties on sucrose inversion by immobillized whole cell invertase. Enzyme Microb. Technol. 8:485.

Johansen, A. and J. M. Flink. 1986. Immobilization of yeast cells by internal gelation of alginate. Enzyme Microb. Technol. 8:145.

Kantor, T. G. 1979. Ibuprofen. Ann. Intern. Med. 91:877.

Karp, L. C. and S. R. Targan. 1988. New enema treatments for inflammatory bowel disease. Dig. Dis. Sci. 33:85s.

Khan, A. K. A., J. Piris, and S. C. Truelove. 1977. An experiment to determine the active therapeutic moiety of sulphasalazine. Lancet (II):892.

Khosla, R. and S. S. Davis. 1989. Gastric emptying and small and large bowel transit of non-disintegrating tablets in fasted subjects. Int. J. Pharm. 52:1.

Khosla, A., L. C. Feely, and S. S. Davis. 1989. Gastrointestinal transit of non-disintegrating tablets in fed subjects. Int. J. Pharm. 53:107.

Kierstan, M., G. Darcy, and J. Reilly. 1982. Studies on the characteristics of alginate gels in relation to their use in separation and immobilization applications. Biotechnol. Bioeng. 24:1507.

Kim, H. S., B. J. Kamara, I. C. Good, and G. L. Enders, Jr. 1988. Method for the preparation of stabile microencapsulated lactic acid bacteria. J. Indust. Microbiol. 3:253.

Kleeman, E. G. and T. R. Klaenhammer. 1982. Adherence of Lactobacillus species to human fetal intestinal cells. J. Dairy Sci. 65:2063.

Klotz, U. and K. E. Maier. 1987. Pharmacology and pharmacokinetics of 5-aminosalicylic acid. Dig. Dis. Sci. 32:46s.

Klotz, U., K. E. Maier, C. Fischer, and K. H. Bauer. 1985. A new slow-release form of 5-aminosalicylic acid for the oral treatment of inflammatory bowel disease. Arzneim. Forsch. Drug Res. 35:636.

Klotz, U., K. Maier, C. Fischer, and K. Heinkel. 1980. Therapeutic efficacy of sulfasalazine and its metabolites in patients with ulcerative colitis and Crohn's disease. New Eng. J. Med. 303:1499.

Kohn, R. and B. Larsen. 1972. Preparation of water-soluble glycuronans and their calcium salts, and the determination of calcium ion activity in relation to the degree of polymerization. Acta. Chem. Scand. 26:2455.

Kuek, C. and T. M. Armitage. 1985. Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of Aspergillus phoenicus. Enzyme Microb. Technol. 7:121.

Lanberti, F. V. and M. V. Sefton. 1983. Microencapsulation of erythrocytes in Eudragit-RL coated calcium alginate. Biochim. Biophys. Acta. 759:81.

Lanza, F. L., D. Fakouhi, A. Rubin, R. E. Davis, M. F. Rack, C. Nissen, and S. Deis. 1989. A double-blind placebo-controlled comparison of the efficacy and safety of 50, 100, and 200 ug of misoprostol QID in the prevention of ibuprofen-induced gastric and duodenal mucosal lesions and symptoms. Am. J. Gastroenterol. 84:633.

Lee, E. J. D., K. William, R. Day, G. Graham, and D. Champion. 1985. Stereoselective disposition of ibuprofen enantiomers in man. Br. J. Pharmac. 19:669.

Lidbeck, A., J. Gustafsson, and C. E. Nord. 1987. Impact of Lactobacillus supplements on the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. 19:531.

Lim, F. and R. D. Moss. 1982. A method of encapsulating oils and oil-soluble materials. UK Patent Application GB 2,086,835 A.

Lim, F. and A. M. Sun. 1980. Microencapsulated islets as bioartificial endocrine pancreas. Science. 210:908.

Lin, S. Y., J. W. Ayres, W. Winkler, Jr., and W. E. Sandine. 1989. Lactobacillus effects on cholesterol: In vitro and in vivo results. J. Dairy Sci. 72:2884.

Linco, P., S. L. Stenroos, and Y. Y. Linco. 1984. Applications of immobilized lactic acid bacteria. Ann. New York Acad. Sci. 434:406.

Linse, L. and P. Brodelius. 1984. Immobilization of plant protoplasts. Ann. N. Y. Acad. Sci. 434:487.

Lockwood, G. F., K. S. Albert, W. R. Gillespie, G. G. Bole, T. M. Harkcom, G. J. Szpunar, and J. G. Wagner. 1983. Pharmacokinetics of ibuprofen in man. I. Free and toatl area/dose relationship. Clin. Pharmacol. Ther. 34:97.

Luckey, T. D. 1984. Perspectives in intestinal microecology. Microecol. Ther. 14:243.

Mann, G. V., and C. R. Nair. 1977. A factor in milk which influences cholesteremia in rats. Atherosclerosis 26:363.

Mann, G. V., and A. Spoerry. 1974. Studies of a surfactant and cholesteremia in the Maasai. Am. J. Clin. Nutr. 27:464.

Mardini, H. A., D. C. Lindsay, C. M. Deighton, and C. O. Record. 1987. Effect of polymer coating on faecal recovery of ingested 5-aminosalicylic acid in patients with ulcerative colitis. Gut 28:1084.

Marth, E. H. 1973. in Low Temperature Preservation of Foods and Living Material. Mercel Dekker Inc. New York. pp.386.

Mauer, S. M., M. W. Steefes, D. E. R. Sutherland, J. S. Najarian, A. F. Michael, and D. M. Brown. 1975. Study of the rate of regression of the glomerular lesions in diabetic rats treated with pancreatic islet transplantation. Diabetes. 24:280.

McDowell, R. H. 1968. Properties of Alginates. Alginate Industries Limited, London. pp. 1-16.

Merson, M. H., G. K. Morris, D. A. Sack et al. 1976. Traveler's diarrhea in Mexico. New England J. Med. 249:1299.

Morris, E. R. D. A. Rees, D. Thom, and J. Boyd. 1978. Chiroptical and stoichiometric evidence of a specific, primary diverisation process in alginate gelation. Carbohydr. Res. 66:145.

Mott, G. E., R. W. Moore, H. E. Redmond, and R. Reiser. 1973. Lowering of serum cholesterol by intestinal bacteria in cholesterol-fed piglets. Lipids 8:428.

Muzzarelli, R. A. A. 1972. Natural Chelating Polymers: Alginic acid, Chitin and Chitosan. Pergamon Press. pp. 23-80.

Myers, B., D. N. W. Evans, J. Rhodes, S. K. Evans, B. R. Hughes, M. G. Lee, A. Richens, and D. Richards. 1987. Metabolism and urinary excretion of 5-amino salicylic acid in healthy volunteers when given intravenously released for absorption at different sites in the gastrointestinal tract. Gut 28:196.

Nilsson, I., S. Ohlson, L. Haggstrom, N. Molin, and K. Mosbach. 1980. Denitrification of water using immobilized Pseudomonas denitrificans cells. Eur. J. Appl. Mocrobiol. Biotechnol. 10:261.

Nilsson, K. and K. Mosbach. 1980. Preparation of immobilized animal cells. FEBS Letters. 118:145.

Obayashi, Y., S. Ota, and S. Arai. 1961. Some factors affecting preservability of freeze-dried bacteria. J. Hyg. Camb. 59:77.

O'shea, G. M., M. F. A. Goosen and A. M. Sun. 1984. Prolonged survival of transplanted of langerhans encapsulated in a biocompatible membrane. Biochim. Biophys. Acta. 804:133.

Parr, A. F., R. M. Beihn, R. M. Franz, G. J. Szpunar, and M. Jay. 1987. Correlation of ibuprofen bioavailability with gastrointestinal transit by scintigraphic monitoring of ^{171}Er-labeled sustained-release tablets. Pharm. Res. 4:486.

Peppercon, M. A. 1987. Sulfasalazine and related new drugs. Ther. Rev. 27:260.

Peppercon, M. A. 1990. Advances in drug therapy for inflammatory bowel disease. Ann. Inter. Med. 112:50.

Peppercorn, M. A. and P. Goldman. 1972. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J. pharmacol. Exp. Ther. 181:555.

Peppercon, M. A. and P. Goldman. 1973. Distribution studies of salicylazosulfapyridine and its metabilites. Gastroenterology 64:240.

Perdigon, G., M. E. Macias, S. Alvarez, G. Oliver, and A. P. Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53:404.

Peri, C. and C. Pompei. 1977. Optimum survival conditions for lactic acid bacteria in spray-dried yoghurt. Dairy Sci. Abstr. 37:7495.

Pettersson, H.-E. 1976. Preservation of mixed species lactic starter concentrates by freezing and lyophilization methods. Dairy Sci. Abstr. 38:4404.

Pettersson, L., W. Graf, and U. Sewelin. 1983. Survival of Lactobacillus acidophilus NCDO 1748 in the human gastrointestinal tract. XV Symp. Swed. Nutr. Found. pp.127.

Pfister, G., M. Bahadir, and F. Korte. 1986. Release characteristics of herbicides from Ca alginate gel formulations. J. Controlled Release. 3:229.

Porubcan, R. S. and R. L. Sellars. 1975. Stabilized dry cultures of lactic acid-producing bacteria. U.S. Patent No.3,897,307.

Porubcan, R. S., and R. L. Sellars. 1979. in Microbial Technology. Academic Press. New York. vol. 1, pp.59.

Pozo-Olaro, J. D., J. H. Warram, R. G. Gomez, and M. G. Cavazos. 1978. Effect of a lactobacilli preparation on traveller's diarrhea. A randomized, double blind clinical trial. Gastroenterology 74:829.

Prajapati, J. B., R. K. Shah, and J. M. Dave. 1987. Survival of Lactobacillus acidophilus in blended - spray dried acidophilus preparations. Aust. J. Dairy Technol. March/June:17.

Pulusani, S. R., and D. R. Rao. 1983. Whole body, liver and plasma cholesterol levels in rats fed Thermophilus, Bulgaricus and Acidophilus milks. J. Food Sci. 48:280.

Rasmussen, S. N., V. Binder, K. Maier, S. Bondesen, C. Fischer, U. Klotz, S. H. Hansen, and E. F. Hvidberg. 1983. Treatment of Crohn's disease with peroral 5-aminosalicylic acid. Gastroenterology 85:1350.

Rasmussen, S. N., S. Bondesen, E. F. Hvidberg, S. H. Hansen, V. Binder, S. Halskov, and H. Flachs. 1982. 5-Aminosalicylic acid in a slow-release preparation: Bioavailability, plasma level, and excretion in humans. Gastroenterology 83:1062.

Reddy, K. P., K. M. Shahani, and S. M. Kulkarni. 1976. B-complex vitamins in cultured and acidified yogurt. J. Dairy Sci. 59:191.

Rees, D. A. and J. W. B. Sanuel. 1967. The structure of alginic acid. VI. Minor features and structural variations. J. Chem. Soc. C:2295.

Rees, D. A. and E. J. Welsh. 1977. Secondary and tertiary structure of polysaccharides in solutions and gels. Angrew. Chem. 16:228.

Richardson, T. 1978. The hypocholesterolemic effect of milk - a review. J. Food Protect. 41:226.

Riley, S. A., V. Mani, M. J. Goodman, M. E. Herdm S. Dutt, and L. A. Turnberg. 1988. Comparison of delayed-release 5-aminosalicylic acid (Mesalazine) and sulfasalazine as maintenance treatment for patients with ulcerative colitis. Gastroenterology 94:1383.

Roberts, B. A. 1984. Oleaginous compositions. United States Patent, No. 4,446,165.

Robins-Browne, R. M. and M. M. Levine. 1981. The fate of ingested lactobacilli in the proximal small intestine. Am. J. Clin. Nutr. 34:514.

Robinson, P. K., A. L. Dainty, K. H. Goulding, I. Simpkins ,and M. D. Trevan. 1985. Physiology of alginate - immobilized Chlorella. Enzyme Microb. Technol. 7:212.

Robinson, P. K., K. H. Goulding, A. L. Mak, and M. D. Trevan. 1986. Factors affecting the growth characteristics of alginate-entrapped Chlorella. Enzyme Microb. Technol. 8:729.

Rossouw, J. E., E. M. Burger, P. Van der Vyver, and J. J. Ferreira. 1981. The effect of skim milk, yoghurt and full cream milk on human serum lipids. Am. J. Clin. Nutr. 34:351.

Sandberg-Gertzen, H., G. jarnerot, and W. Kraaz. 1986. Azodisal sodium in the treatment of ulcerative colitis. Gastroenterology 90:1024.

Sandine, W. E. 1979. Roles of Lactobacillus in the intestinal tract. J. Food Prot. 42:259.

Sandine, W. E., K. S. Muralidhara, P. R. Elliker and D. C. England. 1972.Lactic acid bacteria in food and health: a review with special reference to enteropathogenic Escherichia coli as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Food Prot. 35:691.

Sartnurak, S. 1985. Evaluation of solid dispersed particles for formulation of oral ibuprofen tablets. Ph.D. thesis. Oregon State University pp.60.

Schroder H. and D. E. S. Campbell. 1972. Absorption, metabolism, and excretion of salicylazosulfapyridine in man. Clin. Pharmcol. Ther. 13:539.

Schroeder, K. W., W. J. Tremaine, and D. M. Ilstrup. 1987. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colits. New Eng. J. Med. 317:1625.

Scott, C. D. 1987. Immobilized cells: a review of recent literature. Enzyme Microb. Technol. 9:66.

Scott, W. J. 1958. The effect of residual water on the survival of dried bacteria during storage. J. Gen. Microbiol. 19:624.

Shaw, P. N., L. Aarons, and J. B. Houston. 1980. HPLC analysis of sulphasalazine and its metabolites. J. Pharm. Pharmacol. 32:67p.

Sheurich, P., H. Schnabl, U. Zimmermann, and J. Klein. 1980. Immobilization and mechanical support of individual protoplasts. Biochim. Biophys. Acta. 598:645.

Shore, E. G., A. G. Dean, K. J. Holik, and B. R. Davis. 1974. Enterotoxinproducing Escherichia coli and diarrheal disease in adult travelers: a prospective study. J. Infect. Dis. 129:577.

Smidsroed, O. and A. Haug. 1972. Properties of poly (1,4 - hexuronates) in the gel state. II. Comparison of gels of different chemical composition. Acta. Chem. Scand. 26:79.

Smittle, R. S., S. E. Gilliland, and M. L. Speck. 1972. Death of Lactobacillus bulgaricus resulting from liquid nitrogen freezing. Appl. Microbiol. 24:551.

Stockwell, A. F. and S. S. Davis. 1986. In vitro evaluation of alginate gel systems as sustained release drug delivery systems. J. Controlled Release. 3:167.

Sutherland L. R., F. Martin, S. Greer, M. Robinson, N. Greenberger, F. Saibil, T. Martin, J. Sparr, E. Prokipchuk, and L. Borgen. 1987. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and protitis. Gastroenterology 92:1894.

Svartz, N. 1988. Sulfasalazine: II. Some notes on the discovery and development of salazopyrin. Am. J. Gastroenterol. 83:497.

Takahashi, Y. 1984. The formation of inclusion compounds of alginic acid in aqueous solution. J. Inclu. Phenom. 2:399.

Toda, K. and M. Shoda. 1975. Sucrose inversion by immobilized yeast cells in a complete mixing reactor. Biotechnol. Bioeng. 17:481.

Tompson, L. U., D. J. Jenkins, M. V. Amer, R. Reichert, A. Jenkins, and J. Kamulsky. 1982. The effect of fermented and unfermented milks on serum cholesterol. Am. J. Clin. Nutr. 36:1106.

Tramper, J. and A. W. A. de Man. 1986. Characterization of Nitrobacter agilis immobilized in calcium alginate. Enzyme Microb. Technol. 8:472.

Valdez, G. F., G. S. Giori, A. P. Ruiz-Holgado, and G. Oliver. 1983. Protective effect of adonitol on lactic acid bacteria subjected to freeze-drying. Appl. Environ. Microbiol. 45:302.

Valdez, G. F., G. S. Giori, A. P. Ruiz-Holgado, and G. Oliver. 1983. Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. Cryobiology 20:560.

Valdez, G. F., G. S. Giori, A. P. Ruiz-Holgado, and G. Oliver. 1985. Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria. Appl. Environ. Microbiol. 49:413.

Van Rosendaal, G. M. A. 1989. Recent advances in pharmacotherapy: Inflammatory bowel disease. CMAJ 141:113.

Veelken, M. and H. Pape. 1984. Production of nikkomycin by immobilized Streptomyces cells - physiological properties. Appl. Microbiol. Biotechnol. 19:146.

Wan, L. S. C. and P. W. S. Heng. 1985. Liquid penetration into tablets containing surfactants. Chem. Pharm. Bull. 33:2569.

Wan, L. S. C. and P. W. S. Heng. 1987. Influence of surfactant on the properties of granules and tablets containing sodium alginate. Pharm. Acta. Helv. 62:169.

Wagner, J. G., K. S. Albert, G. J. Szpunar, and G. F. Lockwood. 1984. Pharmacokinetics of ibuprofen in man IV: Absorption and disposition. J. Pharmacokin. Biopharm. 12:381.

Ward, P. C., R. D. McCarthy, and A. Kilara. 1982. Isolation of an inhibitor of hepatic cholesterolgenesis from human milk. Atherosclerosis. 41:185.

Webb, S. J. 1960. Factors affecting the viability of air-borne bacteria. III. The role of bonded water and protein structure in the death of air-borne cells. Can. J. Microbiol. 6:89.

Welch, C. 1987. Nutritional and therapeutic aspects of Lactobacillus acidophilus in dairy products. Cultured Dairy Products J. May:24.

Wood, E., C. G. Wilson, J. G. Hardy. 1985. The spreading of foam and solution enemas. Int. J. Pharm. 25:191.

Wostmann, B. S., N. L. Wiech, and E. Kung. 1966. Catabolism and elimination of cholesterol in germfree rats. J. Lipid. Res. 7:77.

Wright, C. T. and T. R. Klaenhammer. 1981. Calcium-induced alteration of cellular morphology affecting the resistance of Lactobacillus acidophilus to freezing. Appl. Environ. Microbiol. 41:807.

Wright, C. T. and T. R. Klaenhammer. 1983. Survival of Lactobacillus bulgaricus during freezing and freeze-drying after growth in the presence of calcium. J. Food Sci. 48:773.

Yang, R. K. and S. C. Sharma. 1982. A delivery system for an active ingredient and a process for preparation thereof. European Patent Application 0,202,819 A2.

Yotsuyanagi, T., T. Ohkubo, T. Ohhashi, and K. Ikeda. 1987. Calcium-induced gelation of alginic acid and pH-sensitive reswelling of dried gels. Chem. Pharm. Bull. 35:1555.