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Professor Clive G. Wilson

Oral delivery of peptides and proteins: Problems and perspectives

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The synthesis of analogues of biologically active peptides using solid or solution phase protein synthesis or recombinant DNA technologies has awoken much interest in these drugs as therapeutic agents of the future. The major problem in realising the potential of these compounds is the difficulty in getting sufficient drug at the required site of action. The drugs often show remarkable specificity and potency when directly applied to the tissue but the problems of polarity, high molecular weight and enzymic degradation limit the oral bioavailability. The delivery by the parenteral route is problematic since this route requires requires more nursing care and dose preparation than for oral dosing. The search for mechanisms to increase the fraction of dose absorbed following oral delivery of peptides and proteins is therefore receiving more attention in pharmaceutical laboratories around the world.

Why peptides?

The appreciation that physiological and pathophysiological processes are modulated by the release of peptide and proteins identified a clear therapeutic target. Banting and Best were able to reverse diabetes in the 1920's by subcutaneous bovine insulin and ever since this time, the advantage of a non-injectable insulin has been obvious but not commercially realisable. This molecule represents a considerable challenge in drug delivery but the partial success with the nasal/buccal delivery of smaller molecules such as DDAVP and oxytocin has continued to arouse interest.

Why oral delivery?

Although a wide variety of routes of administration and delivery systems exist, absorption of the therapeutic agent from the gastrointestinal tract is the most desirable approach. Oral delivery is non invasive, relatively free from complications arising from the need for sterile techniques that occur with parenteral formulations and is easily managed, thus increasing patient compliance. However, the oral route presents a large obstacle for peptide and protein drugs

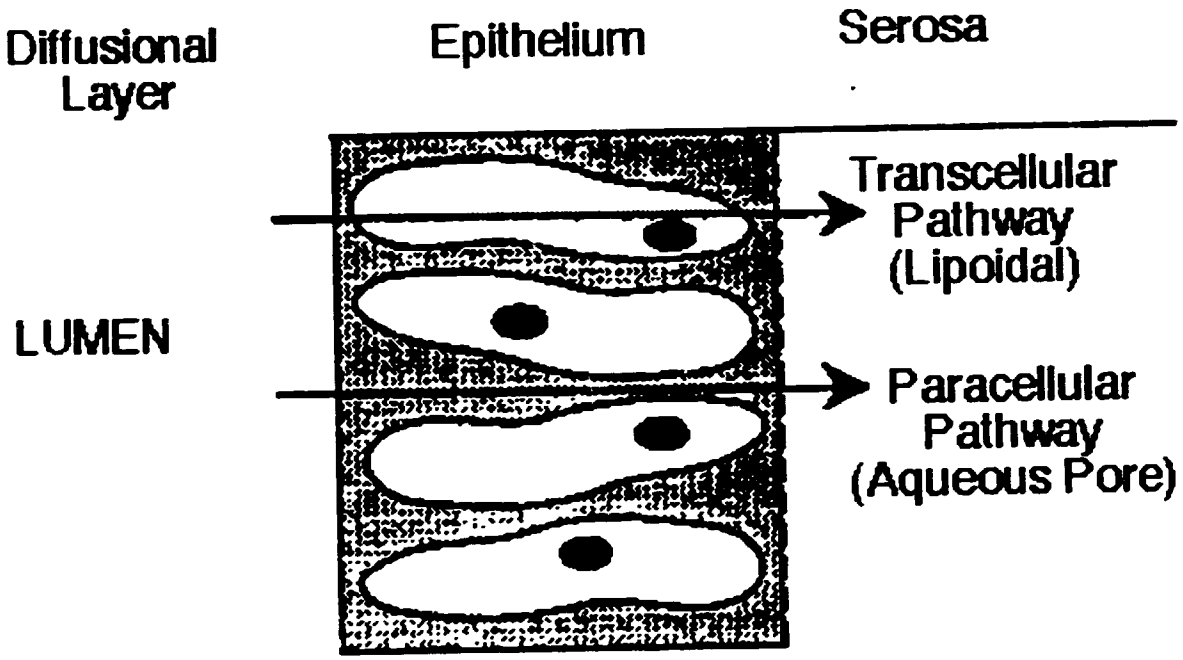

Figure 1. Transcellular and Paracellular pathways

due to the enzymatic and cellular barriers in the gastrointestinal tract .

It is generally believed that the intestinal mucosa is impermeable to macromolecules such as peptides. This is true in terms of nutrition, but is clearly not the case immunologically . Ingested antigens, toxins, and antibodies may pass the mucosal barrier in quantities which may be important in the pathogenesis of local and systemic immunological disease states.

The barriers to peptide absorption:

1. Absorption

The epithelial barrier may be breached through two mechanisms (Figure 1) ; first the *transcellular* movement through the lipid membrane by a diffusional or by an active transport process. Diffusional processes require a partition from the external aqueous phase into the lipid bilayer of the cell membrane and thus lipophilicity and a concentration or electrochemical gradient is prerequisite for solute movement. Active transport against a concentration gradient occurs for amino-acids and some dipeptides but flux decreases markedly with increase in molecular weight.

The second mechanism is via the *paracellular* route, that is to say around the gaps between adjacent cells. The size of the paracellular junctions varies along the gastrointestinal tract; the cells being drawn together by by fusion of membranes at intracellular junctions or through microfilamentous connections between adjacent cells. Experiments with probe molecules shows that the calibre of these tight junctions is variable down the gastrointestinal tract and is relatively low in the stomach and large bowel and most leaky in the small intestine. Generally the apparent pore radius is 7.5 - 8.0 Å in the jejunum. decreasing to 3-3.5 Å in the jejunum and 2 to 2.5 Å in the colon (1). Compounds with molecular sizes greater than these dimensions will therefore be precluded from absorption by the route.

Physicochemical considerations.

Three physicochemical parameters which influence the absorption of peptides must be considered: charge, size and lipophilicity.

Charge

The twenty standard amino acids which make up most peptides have at least an acidic and a basic group attributable to their ionisable carboxylic acid and amino ends. Additionally, five of the twenty standard amino acids have charged side chains. The pKa values of the -COOH groups lie in a small range around 2.2; above pH 3.5 these groups are almost entirely in their carboxylate forms. The NH2 groups all have pKa values near 9.4 and are almost entirely in the ammonium ion form below pH 8.0. The basic amino acids lysine, arginine, and histidine are all positively charged at physiologic pH, while aspartic and glutamic acid are negatively charged above pH 3. In the physiological pH range, both the carboxylic acid and the amino groups of peptides and amino acids are completely ionised resulting in a zwitterionic molecule. Therefore, peptides and proteins will tend to be more hydrophilic than many other biologically active molecules. This feature would most likely preclude absorption of peptides and proteins by transcellular diffusion unless the charges were neutralised through ion pairing. Another factor is the negative charge at physiological pH associated with intracellular gaps which will tend to attract positively charged or neutral compounds and favour the paracellular absorption relative to anionically-charged compounds.

Size

The potential therapeutic agents in this class tend to be very large with molecular weights of 700 - 30,000 daltons. The extent of absorption tends to be very low above a molecular weight above 1,000 daltons, since aqueous channels will exclude high molecular weight compounds from entry.

Lipophilicity

The lipophilicity of peptides and proteins is generally very low, with most compounds having a partition coefficient between 0.02 and 0.08. The ability to traverse the transcellular pathway is therefore extremely limited.

2. Degradation

Perhaps even more important than the permeability barrier in limiting the absorption of intact peptides and proteins from the gastrointestinal tract is the enzymatic barrier. This barrier is well designed to digest proteins to a number of

amino acids and small quantities of peptides consisting of two to six amino acid residues prior to their appearance in the portal circulation. Hydrolysis of peptides and proteins occurs at three sites: in the lumen, at the brush border, and intracellularly.

Proteases in the brush border and the cytosol of the enterocyte are potentially the most important deterrent to the absorption of small, biologically active peptides across the intestinal mucosa. Typically, proteases are anchored in the apical membrane with the active site in an extracellular environment. In addition to the membrane-bound proteases, luminal proteases including trypsin, chymotrypsin, and other pancreatic proteases may be adsorbed onto the brush border of the enterocyte thereby assisting in proteolysis of oligopeptides and proteins. Brush border proteases as a group tend to cleave tripeptides and tetrapeptides although they are also capable of readily hydrolysing peptides in the range of two to ten amino acid units. Specifically, about 60% of the cellular proteolytic activity against tripeptides and 90% of the activity against tetrapeptides can be found in the brush border. In contrast, the general cytosolic proteases attack smaller di- and tripeptides with very little activity against tetrapeptides. The most active area proteolytically is the ileum, which is due to the high activity of brush border aminopeptidase N and diaminopeptidase

Circumventing the barriers

a). Modify the Backbone

Modifications to the amino acid backbone have been extensively used for example cyclization, alkylation of specific amino acid residues, and amide bond surrogates. For example, substitution of D-Ala for L-Gly in the N- , terminal amino acid in methionine enkephalin and the amidation of its C-terminal methionine produces a more stable analogue which has a half-life of hydrolysis that is 15 times that of methionine enkephalin. Modification of the amino acid in the primary structure of the peptide may also improve its lipophilicity. This may be useful in minimising contact of the peptide with the proteolytic enzymes at the site of absorption, since diffusion across the absorptive membrane will be facilitated. The compound 1-deamino-8-D-arginine vasopressin , DDAVP, permeates the intestinal wall of the rat 35 times better than the aminated vasopressin (2). Substitutions with unnatural amino acids such as Oic (3aS,7aS-

octahydroindol-2- yl-carbonyl), **Tic** (1,2,3,4-tetrahydroisoquinolin-3-yl-carbo- nyl), and **Thi** (3-2-thienylalanyl) have been used to improve the stability and receptor binding of peptides and proteins.enzymes at the site of absorption, since diffusion across the absorptive membrane will be facilitated (3).

(b) Administer Protease Inhibitor

Coadministration of protease inhibitors has been used as a method of increasing the absorption of peptides and proteins. As early as 1959, investigators were looking into the effects of proteolytic inhibitors on its intestinal absorption. Danforth and Moore (4), using ligated sections of normal and depancreatised rat jejunum, measured the extent of the absorption of insulin both with and without protease inhibitors. They found that when insulin was introduced into the intestine along with diisopropyl-fluorophosphate (a serine protease inhibitor), there was a marked and consistent blood glucose depression whereas insulin alone (up to 800 USP units/kg) had no effect when administered via the intestinal lumen.

Saffran et al. (5) looked at the effects of aprotinin, a potent inhibitor of trypsin, on the absorption of the peptide hormones lysine, vasopressin (LVP) and (1-deamino, 4-valine)-8-D-arginine-vasopressin (DDAVP) after intragastric administration. The results showed that inclusion of the protease inhibitor enhanced the oral activity of both vasopressin and the synthetic analogue, increasing both the intensity and duration of effect.

(c) The use of absorption enhancers

Certain chemicals and drugs can increase (and decrease) mucosal permeability. Drugs such as oxyphenisatin, dihydroxy bile salts, ricinoleic acid and long- chain fatty acids have all been shown to increase the permeability of the colon and the use of long-chain fatty acids to increase the absorption of cefmetazole has been studied recently (6). Penetration enhancers are of four major types: *chelators* such as EDTA, citric acid, salicylates, N-acyl derivatives of collagen, and enamines; *surfactants* such as sodium lauryl sulphate, polyoxyethylene-9-lauryl ether, and polyoxyethylene-20-cetyl ether; *bile salts* such as sodium deoxycholate; and *fatty acids* such as oleic acid and monoolein . Whether these agents act on the cell (transcellular route) or the junctions (paracellular route) is not clear. The incorporation of permeation enhancers into a dosage form may be

essential in the systemic delivery of macromolecules. These chemicals would need to be co-released at the site of drug absorption. The risk involved, however, is increased absorption of potentially toxic or immunogenic substances from the GIT. The ideal absorption enhancer should be highly permi-selective.

(d) Site specific delivery

The colonic route of drug delivery can be used for systemic administration of drugs. Protecting peptide and protein drugs from hydrolysis in the duodenum and jejunum and subsequently releasing these agents in the ileum or colon may result in greater systemic bioavailability. There is also evidence to suggest that the proteolytic activity of the colonic mucosa may be much less than that observed in the small intestine

It would be predicted that absorption of most drugs would be slower from the colon by comparison with that in the small intestine, since the colonic mucosa has a much reduced surface area being flat with fewer folds and the absence of villi. Furthermore, the lumen of the colon is wider, movement more sluggish and the volume of dissolution fluid available is low. However, transit through the colon is considerably slower than that through the small intestine and thus the mean residence time of a drug in the colon can compensate possibly leading to greater amounts of drug being absorbed than might be expected.

Factors affecting residence in the colon are dependent on gastric filling and emptying, on rates of passage in the small intestine and on colonic metabolism (7,8). The rate can vary greatly from individual to individual and even in the same person (9). Transit can be relatively short (8-10 h) or as long as 78 h. The residence time in the various regions of the colon is a further factor which is expected to be important in influencing drug delivery. In most individuals the ascending colon is sufficiently liquid to allow diffusion of dissolved drug across the lumen to the epithelium, whereas further along the colon as more water and electrolytes are absorbed, the milieu becomes dryer, hindering diffusion (Figure 2). Residence time in the colon has been calculated to be 31.9 ± 17.8 h. for American males and 38.8 ± 18.5 h. for females (10) with 18-19% of the time spent in the ascending colon giving a calculated transit time of between 5.8 and 7 hours.

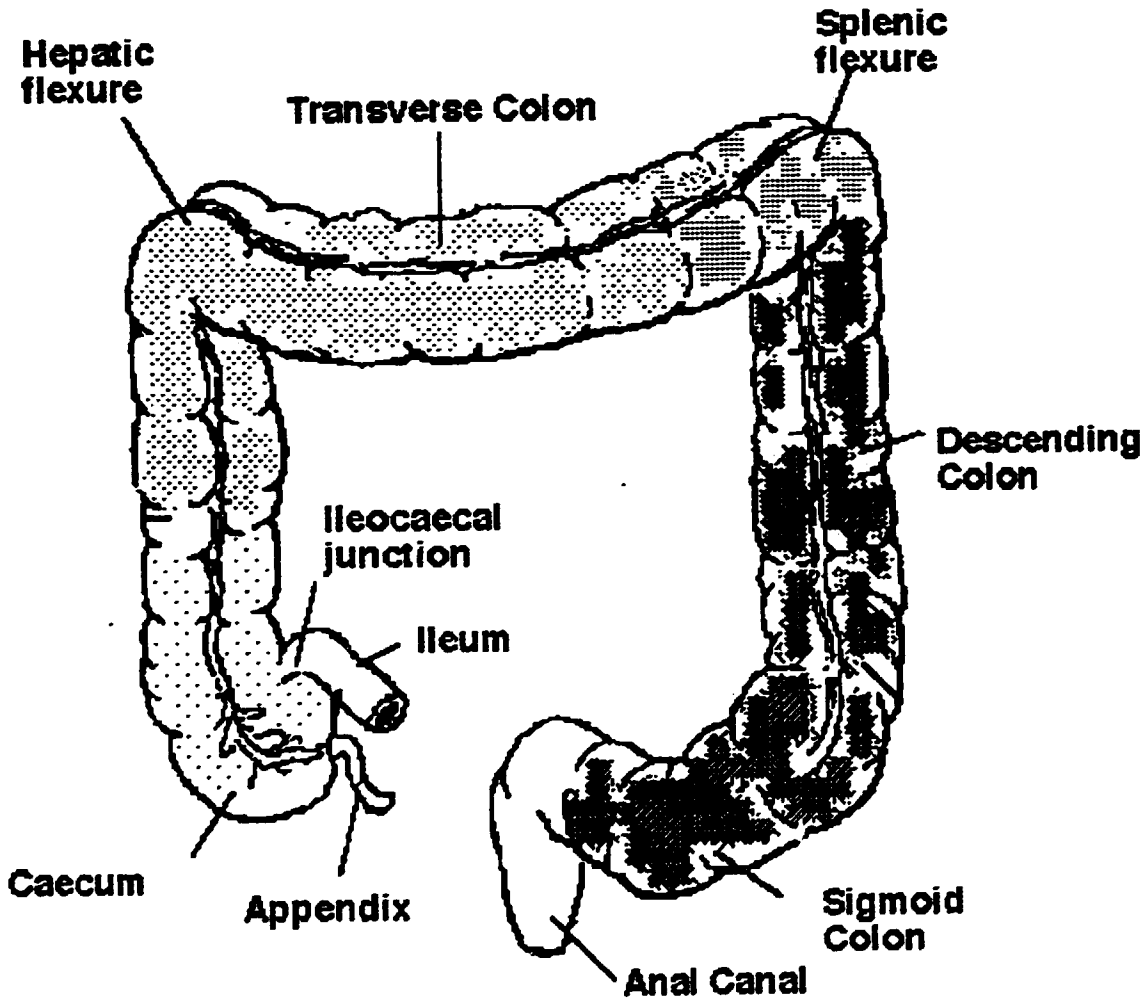

Figure 2. The Colon

Shading illustrates gradual consolidation of contents

Scintigraphic studies conducted within our laboratories on the behaviour of sustained release dosage forms have shown that the ascending and transverse limbs of the colon are important and under-rated sites of drug absorption (11,12, 13). Scintigraphic data also suggests that to optimise the delivery of drugs to the proximal colon a multiparticulate preparation should be considered which remains intact for approximately 5 hours after administration to the fasted patient. This would allow time for gastric emptying and transit through the small intestine. The drug preparation should then disperse allowing release of the material over the following 10 to 12 hours in the ascending and transverse colon. Residence times indicate a figure of 12.9±3.7 hours (range 9.7 - 23.2 hours) for the proximal colon and a further 12 hours for the descending colon (14). Additionally, the right colon exhibits an adaptation for capacitance (15) by comparison with the left colon where motility patterns are strongly propulsive (16). Extending the release profile over longer time periods would not give an added advantage, due to the progressively slower diffusion of drugs through consolidating luminal contents and to the variability of excretion patterns.

The use of enteric coating in formulations of drugs which are unstable in gastric acid or for preparations which cause nausea or irritation when they disintegrate in the stomach is well established. More recently, polymer coatings have found application in the construction of formulations whose delayed release presents the drug to the distal regions of the gut, rather than the upper small intestine. The application of such a principle, which minimises systemic absorption from the small intestine, is obvious in the treatment of diseases affecting the colon. The success of such therapy is dependent on two physiological principles; namely that the pH of the small intestine will be sufficiently high to ionise the carboxylic acid moieties of the polymer (17) and secondly that small intestinal transit is relatively unaffected by eating, nature of the diet and normal everyday activity to ensure that sufficient reproducibility in man. Numerous studies employing gamma scintigraphy have demonstrated that small intestine transit is generally a remarkably robust parameter in gastroenterology and influences of diet, daily habits, posture and even pharmacologically active materials are confined to the stomach and the distal ileum and colon. Davis and others (18) have summarised the data on many investigations completed by the Nottingham group which have shown small intestinal transit to be around four hours with little variation due to

size of meal. Thus the use of a gastroresistant formulation with an appropriate thickness of polymer to deliver drug to the terminal ileum and colon appears to be a workable premise.

Dew and co-workers had shown using X-contrast agents that positioned release could be achieved for capsules and tablets using Eudragit S polymer at a coating thickness of 120 μ. (19). Using an alternative polymer system, our own group has demonstrated caecal release of capsule contents in normals treated to simulate a diarrhoeal state similar to that noted in diarrhoea predominant irritable bowel syndrome (20).

Research on site specific delivery- new methodology

A recent development involving neutron activation techniques, allowing controlled-release dosage forms to be radiolabelled under conditions where conventional methods with short-lived gamma-emitting radioisotopes are impractical, has significantly increased the potential for in vivo analysis of delivery systems under physiological conditions. Such techniques allow the incorporation of a stable isotope, such as barium-138, samarium-152 or erbium-170, into the formulation prior to manufacture in the same manner as any other excipient. Subsequent neutron activation of the intact dosage form thus permits the manufacture under industrial scale conditions at a time not determined by proposed study dates and without the risk of contamination to personnel and equipment. This technique also has the advantage that it is applicable for radiolabelling enteric coated capsules without compromising the integrity of the dosage form (21).

A major problem with the utilisation of neutron activation is the high cost of the enriched erbium needed for tablet preparation. Recent studies in our laboratories (22,23) have investigated the possibility of using non-enriched substrates. Samarium contains a natural abundance of 26.7 % ^{152}Sm, and when irradiated produces ^{153}Sm, a gamma emitter with a half-life of 46.7 hours. Although peptide delivery systems has not yet been investigated, we have utilised a preparation consisting of samarium oxide -loaded drug polymer microspheres (Eudragit RS/ sulphasalazine) . Samples were irradiated for 90 minutes at a neutron flux of 1012/n/cm^2/s. Drug release profiles indicate a satisfactory labelling efficiency with

minimum radiolysis of drug. This system was used to investigate models of bowel disease in man (24).

Pulsatile systems based on a swelling hydrogel have been discussed earlier in the course by Professor Graham. This system has been commercialised (Scherer DDS Limited, Clydebank, Scotland) and was recently used by Davis and colleagues to investigate the absorption of an angiotensin-converting enzyme inhibitor, captopril [(1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline (S,S-isomer)] in 8 volunteers (25). The unit was loaded with 25 mg captopril and 5 mg ^{111}In-labelled diethylenetriaminepentaacetic acid (1 MBq) and sealed by means of a hydrogel plug. In six subjects, the drug was delivered to the colon and in two subjects, the terminal ileum. Absorption was extremely variable and it appears that the drug is poorly absorbed in the distal gut, perhaps by metabolism by the gut flora.

This study may reveal a final problem in the delivery of drugs to the colon; the colonic microflora. The human alimentary canal is highly populated with bacteria and other microflora. The density is greatest in the colon/rectum but there is a surprising diversity of organisms in the buccal cavity. In the gastrointestinal tract between these two sites, the GIT is very sparsely populated with microorganisms. The gut bacteria are capable of catalysing a wide range of metabolic events. Many colon- specific drug delivery systems for conventional drugs rely on enzymes unique to gut microflora to release active agents in the colon. However, only two or three enzyme systems have been exploited in this area: azoreductases and glycosidases (including glucuronidase). A large number of polysaccharides are actively hydrolysed by gut microflora leading to the possibility of using naturally occurring biopolymers as drug carriers. In addition, etheral sulphate prodrugs or carboxylated prodrugs may be metabolised in the colon to the parent drug leading to local delivery in the colon (26). Perhaps the utilisation of a delivery system based on a fermented prodrug approach will be the method of choice of the future, provided that the released peptide survives the bacterial endopeptidases.

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Mr. Robert F. Weir

THE DEVELOPMENT OF ADVANCED PHARMACEUTICAL FORMULATIONS

REGULATORY CONSIDERATIONS

by Robert F Weir

CONTENTS

THE DEVELOPMENT OF ADVANCED PHARMACEUTICAL FORMULATIONS

REGULATORY CONSIDERATIONS

by Robert F Weir

1. Introduction and History

In the development of pharmaceutical production and marketing, government intrusion is a relatively modern phenomenon.

The exception is the United States. In 1937 Samuel Massengil distributed an elixir of sulfanilanide. Included in the formulation as a solvent was diethylene glycol. 107 people died as a consequence, including many children. This prompted the passing of the 1938 Food Drug and Cosmetic Act which necessitated the approval of any new medicinal product by the FDA before marketing.

Elsewhere in the world the trigger for government controls was the thalidomide tragedy. In 1957 the German company Chemie Grunenthal marketed thalidomide as Contergan in Germany. The product was licensed to various overseas corporations, such as Distillers in the UK and Horner and Merrel in Canada. One of the attractions of Contergan was its claim to be free from toxic effects. However, in 1959 the first reports of CNS interactions appeared and in 1960 phocolmenia (sealed limbs) was reported in Germany. Further reports of teratogenicity (that is, causing congenital malformations in children) led to the withdrawal of the product from the German and British markets in 1961 and to a similar fate in Canada in 1962.

Thalidomide was still awaiting approval in the United States at this time. The appointed assessor had had concerns about reports of peripheral neuritis and for that reason had delayed approval of the product.

The thalidomide tragedy generated world-wide effects. Thus, in 1962 the Food Drug & Cosmetic Act in the United States was strengthened, demanding (among other things) that any proposed clinical trial be approved through the submission of an Investigational New Drug Application (IND), that adverse events be notified to the FDA and that standards of Good Manufacturing Practice (GMP) be implemented.

In the United Kingdom in 1963 the Committee on Safety of Drugs (The Dunlop Committee) was formed. Submission of pre-marketing data to this body was voluntary, but the industry acted in a responsible way. Only in 1971, following the passing of the Medicines Act, did statutory controls come into force, necessitating a major review of the safety implications of a proposed new compound prior to marketing.

Holland, Germany and other European countries introduced similar regulations for pre-marketing approval. Today every country exerts some measure of control. As this paper will illustrate, there are significant moves towards a harmonisation of standards on a world-wide basis.

For completeness it should be stated that the United Kingdom had introduced controls for a limited range of products as early as 1925. The Therapeutic Substances Act was inspired by the problems of quality control connected with biological products, or products requiring biological testing. The Act, which

applied to such products as vaccines and sera, antigens and insulin, included controls on the release of each batch by an independent state laboratory and the right to inspect manufacturing facilities.

2. Intra-Company Government Initiatives

2.1 Operating Standards

Increasingly on an international basis governments are setting standards for research development and production. These standards have been embodied in codes or guidelines (depending on the particular country) known as:

Good Manufacturing Practice (GMP)
Good Laboratory Practice (GLP)
Good Clinical Practice (GCP)

We will discuss later the actions which governments may take in order to ensure compliance with the standards. All scientific and technical aspects of company operations are embraced and may be summarised as follows:

a) **People** A company must employ individuals of adequate academic experience according to the tasks in question. These individuals must be exposed to appropriate industrial experience and trained accordingly. Records of their training must be maintained.

b) **Facilities** Buildings must be adequate in terms of quality and space. They must provide the environment appropriate to the operation; this means control of the microbiological surroundings and of the particulate content of the air.

The housing and use of animals in experimental programmes is similarly standardised in terms of environment, hygiene, segregation and humane systems.

Production facilities must be designed to avoid cross-contamination. In particular cases, such as the manufacture of penicillin containing products, significant physical separation is demanded by certain government authorities.

Equipment and instrumentation must be adequate for the stated tasks. Documented procedures for cleaning, calibration and routine maintenance must be in place, and there must be evidence that staff have been trained to a sufficient level of competence to handle the equipment and instruments.

c) **Documentation** The adequacy of operating systems is proved by the quality of documentation. Similarly, records must be generated and maintained for all relevant activities.

d) **Operations** Unit operations in manufacturing and in laboratories must be validated in order to prove their adequacy and correctness. The actual manufacturing processes must comply with the information submitted to the government as part of the original pre-marketing approval.

Clinical operations, whether within the company or in hospitals, must similarly be controlled in order to confirm compliance with the original approved protocol.

2.2 The Qualified Person

For the purpose of maintaining the quality of pharmaceutical products throughout the European community, an EC directive was established creating the role of Qualified Person. This demands that within each pharmaceutical company one individual is responsible for the decision to release a supply of sales goods to the marketplace.

This role was first conceived in France in the form of the "Pharmacien Responsible". With the creation of the Single European Market on 31 December 1992, and consequent steps towards the free movement of goods, the Qualified Person becomes an extremely critical function for the healthcare industry in Europe.

National governments are charged with approving individuals as Qualified Persons. For this purpose, academic attainment, industrial experience and targeted training are defined. In the United Kingdom an individual meeting these standards and wishing to become a Qualified Person must undergo assessment by one of the professional bodies charged with the maintenance of standards.

3. **Government Involvement in Manufacturing Authorisation**

Prior to the approval for marketing of a new pharmaceutical product, systems exist for the authorisation of the actual manufacturing facility. However, there is great country to country variation in the associated procedures, and harmonisation is far from complete.

Just as government demands that standards of Good Practice (GMP, GLP and GCP) be maintained, so many governments demand the right to inspect facilities. This generally takes place prior to marketing authorisation and may be repeated from time to time. Compliance may lead to the provision of a specific licence. Failure of compliance leads to government actions in order to generate improvements and will normally lead to a delay in the grant of marketing authorisation. Failures of compliance have necessitated the withdrawal of products from the market from time to time.

In the United Kingdom a specific Manufacturer's Licence is issued, granting permission for the manufacture of certain products. This is linked with the marketing authorisation for the products themselves. Inspection systems similarly exist in continental Europe. There is a measure of cross-acceptance of National Inspection Reports within and beyond Europe, frequently through the medium of the Pharmaceutical Inspection Convention (PIC). The US Inspection systems are considered to be the most intensive and far reaching, frequently being undertaken prior to the grant of clinical trial permission (that is, pre IND inspection) as well as before marketing authorisation.

4. **Government Involvement in the Authorisation of Human Studies**

Studies on human beings are generally categorised into four groups:

Phase 1 studies, involving kinetic examinations on healthy human volunteers.
Phase 2 studies, for dose ranging investigations.
Phase 3 studies, involving major clinical investigations.
Phase 4 studies, post-marketing clinical investigations.

Phase 1 studies are universally free from government intrusion, but generally require some form of local hospital approval, such as by an Ethical Review Committee. Government controls of clinical studies are far from universal, and even in Europe where relatively sophisticated systems of market authorisation exist, it may not be necessary to seek approval to commence studies on patients.

The original (but not commonly used) system for approval in the United Kingdom involves an application for a Clinical Trial Certificate (CTC). In order to accelerate the commencement of clinical studies, the UK government introduced a system rather similar to that of the US Investigational New Drug (IND) arrangements. The UK system is known as the Clinical Trial Exemption Procedure (CTX).

Both the CTX and IND systems involve negative vetting. This means that clinical studies may commence assuming no objections are raised by the authority within a certain number of days (35 in the case of the UK).

An application for an IND or CTX approval must be made according to the stated government requirements. The degree of novelty of the active constituent

influences the extent and nature of data required. Also critical are the intended route of administration, the dosage, frequency of administration and the duration of the proposed clinical study. The use of a novel excipient or drug delivery system would also require documentation.

The UK government authority (the Medicines Control Agency) may determine that a CTX application is inappropriate. In such an example there is no right of appeal and the corporation must either submit a modified CTX or revert to a full CTC application.

Where the proposed product is based on biotechnology or involves a novel drug delivery system, it is preferable not to submit an application without pre-discussion at the government agency. Failure to do so will almost certainly lead to delays. Informal discussions or a pre-planned seminar for government agency reviewers should serve to develop a mutual understanding of the critical issues involved. This should also serve to accelerate the review and approval process.

5. **Government Involvement in Marketing Authorisations**

The three major marketing targets for a multinational corporation are undoubtedly United States, Japan and Europe. Although the European community does not have a single marketing authorisation system yet it moves relentlessly, albeit rather slowly, towards that goal.

The data requirements for the three blocks are rather similar but not identical. The US may demand more toxicity and clinical data, with an emphasis on bio statistics. The Japanese authorities generally demand toxicity and clinical information oriented to their own population.

The standard format for EC applications is as follows:

There are five parts listed I - V.

Part I is a summary of the dossier comprising:

I(a) **Administrative Data.** This includes information on the name of the product, its active ingredient, the dosage form, the name and address of the company and the official signatures.

I(b) **Summary of Product Characteristics.** This includes qualitative and quantitative particulars of the composition, covering both active ingredient and excipients. The dosage form and route of administration are stated together with information on the pharmacology and clinical indications, contra indications,

precautions and special warnings. The dosage should be defined in this section, together with relevant information about storage limitations.

I(c) **Contains the Expert Reports.** Under EC legislation it is necessary to file 3 Expert Reports covering chemical and pharmaceutical information, toxicological and pharmacological documentation and clinical documentation. These reports, prepared by an internal or an external Expert, are expected to review the relevant sections of the main dossier in a critical way. Guidance notes exist for those embarking on the writing of Expert Reports.

Part II comprises chemical, pharmaceutical and biological documentation.

II(a) Covers the composition of the product as well as the containers and closures.

Important within this section is the development pharmaceutics information. This would describe the problems encountered during development and the means by which they were overcome. Information on incompatibilities and interactions should be included as well as data on variations between clinical trial formulations and final compositions.

The incorporation of a unique delivery system would be discussed in detail in this section.

II(b) Describes the method of manufacture and the dosage form. This would include information on batch size and unique equipment required for successful manufacture. The individual stages in processing would be described and the validation of these would be discussed in detail.

II(c) Concerns the control of starting materials. For new active ingredients much detail would be expected here about the structure, description, development chemistry, controls during synthesis, impurities, batch analysis and specifications.

II(d) Describes the controls on intermediate products. This may be important where the manufacturing technique generates a controlled release formulation and in-process controls become critical.

II(e) Describes the controls on the finished product. These would cover physical tests such as, dissolution, microbiological procedures and chemical control tests. The results of batch analyses on preferably five consecutive full-scale production batches should be incorporated.

II(f) Describes the stability of the product. Justification for the design and protocol of the studies may be required, particularly where these vary from normal standards. The objective is to prove that the product remains adequately pure and potent during its shelf-life. Stability data may be required on a new active ingredient as well as the finished product.

Part III provides pharmaco toxicological documentation divided as follows:

III(a) - Single Dose Toxicity.

III(b) - Repeated Dose Toxicity.

III(c) - Reproduction Studies.

III(d) - Mutagenic Potential

III(e) - Carcinogenic Potential

III(f) - Pharmacodynamics

III(g) - Pharmacokinetics

III(h) - Local Tolerance/Toxicity

Part IV provides the clinical documentation divided as follows:

IV(a) - Clinical Pharmacology

IV(b) - Clinical Experience

IV(q) - Other Clinical Information

Part V provides for special particulars.

V(a) Relates to the dosage form and would provide information on packaging together with a draft of the label and the package insert.

V(b) Refers to samples which are required for applications in some, but not all, EC territories. The size of the sample, when required, also varies.

V(c) Relates to manufacturing authorisation. A copy of the manufacturing

authorisation should be provided where it already exists for the product in any EC territory.

V(d) Concerns marketing authorisation. Copies of any existing marketing authorisations from countries within or outside the EC should be provided.

Single State or Multi-State Authorisation in the EC?

Corporations operating within the EC now have a choice. They may elect to file individual market authorisation requests in each member state. Alternatively, they may choose (and are increasingly choosing) to follow the so-called CPMP or multi-state route. They may actually elect to initiate both procedures and cancel one or the other at a later time period.

The single state procedure clearly involves the submission of an appropriate dossier to each regulatory agency. Since there is limited standardisation between the member countries this is a cumbersome and costly process for the corporation. The CPMP multi-state procedure is almost certainly the precursor of a single EC market authorisation process and is becoming the route of choice for most of the pharmaceutical companies. The procedure operates as follows:

The first step is achieved when market authorisation takes place in one member state. Thereafter, the multi-state system would begin. The rapporteur, that is the acting agent in the outgoing member state (that is the country which has already granted marketing authorisation) is a key individual and should be consulted prior

to the initiation of the relevant documentation. The rapporteur obtains an assessment report about the product within his own state and sends this to the authorities in the other concerned member states.

The corporation files its application in all of the concerned member states, with a copy to the Secretariat of The Committee for Proprietary Medicinal Products (CPMP). When all of the concerned member states have received the valid application, the 120 day approving period begins. During this time each concerned member state must notify their reasoned objections to the granting of an authorisation. If no objections are raised the state is obliged to grant marketing authorisation. All reasoned objections are sent to the rapporteur and the company involved. Following consultations with the rapporteur, the applicant may appeal against the objections. After submitting the appeal documentation a minimum of 30 working days are required before the CPMP Meeting can review the information. Prior to the meeting, and through the mediation of the rapporteur, the company may elect for a personal hearing, including the presence of Experts to address the CPMP Meeting.

Certain categories of products may not be processed according to the multi-state system. However, this procedure is gaining in popularity because the approval times are generally much less than those recorded for single-state applications.

6. The EC Concertation Procedure for High Technology and Biotechnology Products

A significant advance took place in July 1987 with the establishment of the EC Directive for certain high technology medicinal products. This has become known as the "High-Tech Directive". This concertation procedure has certain objectives, including the following:

1. To allow an early exchange of views among assessors in European Regulatory Agencies about the content of complex submissions.

2. To accelerate the EC approval process.

3. To present a 10 year protection against a second applicant from the date of granting the first marketing authorisation.

The High-Tech Directive divides the relevant products into List A and List B products:

List A products are developed by means of the following:

> Biotechnological processes; recombinant DNA technology; controlled expression of genes coding for biologically active proteins in prokaryotes and eukaryotes, including transformed mammalian cells; and hybridoma and monoclonal antibody methods. Companies wishing to obtain EC approval for products in these categories **must** follow the High-Tech Directive route.

List B products are:

> Other high technology medicinal products that, in the opinion of the competent authority concerned, constitute a significant innovation or area of significant therapeutic interest. These include other biotechnological processes; medicinal products administered by means of a new delivery system; medicinal products containing a new substance or for an entirely new indication; new medicinal products based on radio-isotopes and medicinal products manufactured using processes that demonstrate a significant technical advance. The use of the High-Tech Directive is optional for products based on List B.

The obligation falls to the company to prove that the High-Tech route is appropriate for the product in question. The timetable for the consultation procedure is 180 days. However, the clock may be stopped when the obligation turns to the applicant to provide supplemental information. A period of 4 - 6 weeks is normally required to enable the rapporteur Member State to produce an initial assessment report. As for the general CPMP Multi-State Application (see Chapter 5), a rapporteur is selected and becomes a key individual.

An Appeals procedure exists, similar to that for products in the multi-state CPMP system.

Experience with the High-Tech concertation procedure is limited so far. A large proportion of applications have been from smaller companies aiming for

specialised markets. The benefits from the processing of difficult biotech applications by this route remain to be determined. However, the system appears to be an important step in the evolution of the EC market approval arrangements.

7. Inter - Government Initiatives

The previous chapters have indicated some of the steps which are taking place towards a common EC registration system. More details of these may be gained by studying the EC publications (see Chapter 9).

Outside of the EC, the Product Evaluation Report (PER) scheme was founded in 1979 within the European Free Trade Association (EFTA). This scheme aims to simplify the registration process, and thereby facilitate intercompany trading in pharmaceutical products. The initial member countries were Austria, Finland, Norway, Sweden and Switzerland. Germany, Italy, the Netherlands and United Kingdom have joined. After a lengthy period of collaboration with Sweden, Australia joined the scheme in 1990, as did Canada. It is likely that Hungary has now been accepted into the PER.

The scheme which is controlled from EFTA headquarters in Geneva operates through the exchange of Evaluation Reports on pharmaceutical products with the objective of speeding the individual state registration process.

Various consultation documents describe further steps towards simplification of the government role in marketing authorisation throughout the EC. These include the establishment of the European Medicines Evaluation Agency (EMEA). This body, which is intended to be responsible for both human and veterinary pharmaceutical products, would not replace national authorities but function as a co-ordination point for scientific and technical assessment of quality, safety and efficacy. It would also co-ordinate the reporting of adverse events

(pharmacovigilence), a subject of increasing concern to regulatory authorities. In the long term the EMEA could be expected to grow and become the registration authority for the EC.

8. Other Regulatory Considerations

8.1. Medical Devices

In most countries a distinction is drawn between a pharmaceutical product and a medical device. In the United States a simplified form of processing exists for recognised devices which are considered to be 'like and similar' to those previously marketed. Marketing authorisation is achieved through the use of the 510K documentation system.

Within the EC attempts are being made to standardise the approach to the authorisation of devices. It is possible that certain novel drug delivery systems may be categorised as devices.

The Active Implantable Medical Device Directive was published in 1990. A medical device is defined as an instrument, apparatus, appliance, material or any other article whether used alone or in combination, together with any accessories or software for its proper functioning intended by the manufacturer to be used for human beings in the diagnosis prevention monitoring treatment or alleviation of disease or injury, investigation, replacement or modification of the anatomy or of a physiological process and the control of conception, and which does not achieve its principal intended action by pharmacological, chemical, immunological or metabolic means, but which may be assisted in its function by such means.

An active medical device is defined as any medical device relying for its functioning on a source of electrical energy, or any source of power other than that directly generated by the human body or gravity.

An active implantable medical device is defined as any active medical device which is intended to be totally or partially introduced surgically or medically, into the human body or by medical intervention into a natural orifice and which is intended to remain part of the procedure.

In total, a series of four directives should cover the complete field of medical devices and will lead to a standardisation in EC authorisation.

8.2 Drug Master Files

It is important to bear in mind that, in making a request for market authorisation, a company is obligated to submit "all relevant information". On occasions this may be difficult because part of the "relevant information" has not been made available to the company. For example, the basic details of a drug delivery system which the company has licensed-in may be covered by strict confidentiality arrangements, preventing the company submitting sufficient details to the government agency.

In these circumstances, the difficulty is overcome by the use of the Drug Master File system (DMF), which enables the out-licensing company to provide, in confidence, an information package to the government agency with a cross-reference to any marketing authorisation request.

The EC system was revised in 1992 and the concept of a European Drug Master File (EDMF) was initiated. Confidential information covering a delivery system, a unique active constituent, or an excipient is provided in EDMF format in parallel with the submission of the marketing authorisation request.

Under US regulations two types of Drug Master Files exist. The so-called Type 2 DMF fulfils the same role as the EDMF and operates in essentially the same pattern. The close association between the EDMF and the marketing authorisation request within the EC is not necessarily so strict under US rules. Additionally, the FDA recognises a Type 1 DMF which describes the facility prepared and proposed for pharmaceutical manufacturing. This latter submission should stimulate a visit by the FDA Inspector, leading to a decision about the acceptability of the facility. Deficiencies will be listed on document 483 and classified according to severity. Correction of these deficiencies may be necessary before the approval of a New Drug Application (NDA) can take place.

8.3. Abridged Applications

In most western countries the opportunity exists to recognise the case for approval of certain products on the basis of a limited supply of information. This is known as an Abridged or Abbreviated Application (an ANDA according to the US procedures).

In order to achieve classification as an Abridged Application, the proposed new product must comply with one of several classifications. Thus, the active constituents should have been approved already in comparable dosages, the

proposed therapeutic indication may be different or the combination of known and approved active constituents is different. As justification for such applications already published data may be submitted. The abridged application system recognises the ethical considerations of avoiding unnecessary repeat animal and human studies. It is conceivable that, in certain circumstances, it may be justified to seek marketing authorisation under these rules for a product based on a unique drug delivery system. However, the definitions, as outlined in the previous paragraph, must be taken into account.

9. Further Reading

9.1 Mann, R.D. (1989). The historical development of medicines regulations. International medicines regulations: Proceedings of Centre for Medicines Research Workshop. Ciba Foundation, London 20/21 September 1988. Edited by Walker, S.R. and Griffin, J.P. Kluwer Academic, London.

9.2 Morris, J.M. (1990) Development pharmaceutics and process validation, Drug Development and Industrial Pharmacy, 16(11) 1749-1759.

9.3 Matthews, B. R. (1990) The drug master file, Pharmaceutical Manufacturing International, 115-118, ISSN 0951-9696, Sterling Publications International Ltd, London.

9.4 Poggiolini, D & Donawa, M. E. (1990), EEC pharmaceutical regulations: the multistate procedure and the CPMP. Pharm. Technol. 2, 104, 106, 108 and 110.

9.5 Cartwright, A. C and Matthews, R. B. (1991) Pharmaceutical Product Licensing - requirements for Europe. Ellis Horwood Ltd, Chichester, England.

9.6 **Rules governing medicinal products in the European community** - These have been published in the form of a 5 volume set.

Volume I "The Rules Governing Medicinal Products for Human

Use in the EC". Published in English, Spanish, Danish, German, French, Italian, Dutch and Portugese.

Volume II "Notice to Applicants for Marketing Authorisation for Medicinal Products for Human Use in the Member States of the EC". Published in English, Spanish, German, French and Italian.

Volume III "Guidelines on the Quality, Safety and Efficacy for Medicinal Products for Human Use". Published in English, Spanish, German, French and Italian.

Volume IV "Guide to Good Manufacturing Practice for Medicinal Products". Published in English, Spanish, Danish, German, Greek, French, Italian, Dutch and Portugese.

Volume V "The Rules Governing Medicinal Products for Veterinary Use in the EC". Published in English, Spanish, Danish, German, Greek, French, Italian, Dutch, Portugese.

An addendum to Volume IV has been published containing guidelines on such topics as Analytical Validation, European Drug Master Files, Production and Quality Control of certain products using biotechnological processes and human monoclonal antibodies, Good Clinical Practice and Clinical Testing.

These publications may be obtained from:

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4611 F Assembly Drive

Lanham

MD 20706-4391 USA

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Guidelines on the production and quality control of medicinal products derived by recombinant DNA technology

Commission of the European Communities notes to applicants for marketing authorizations*

Committee for Proprietary Medicinal Products *Ad Hoc* Working Party on Biotechnology/Pharmacy

1 Introduction

Developments in molecular genetics and nucleic acid chemistry now enable the genes coding for natural, biologically active proteins to be identified, analysed in fine detail, transferred between organisms, and expressed under controlled conditions so as to obtain synthesis of the polypeptide for which they code.

A common strategy in the development of recombinant DNA (rDNA) derived products is the insertion of naturally occurring or synthetic nucleotide sequences into a vector which is introduced into a suitable host organism so as to ensure the efficient expression of the desired gene product. Many vectors in use at present are bacterial plasmids and much gene cloning has been carried out in *Escherichia coli* and other prokaryotes. However, other vector–host cell systems involving eukaryotes, including yeasts and cell lines of mammalian or insect origin, have been developed and are, in some cases, in use for production. The factors affecting the expression of foreign genes introduced into a new host using a suitable vector are complex and the efficient, controlled expression of stable, cloned DNA sequences is an important aspect of current research.

The ability to synthesize and manipulate nucleic acids allows the construction of genes coding for modified products possessing enhanced biological activity and/or diminished undesirable characteristics, or entirely novel products. In addition, large quantities of useful medicinal products which were previously difficult to prepare from natural sources can now be manufactured using such rDNA technology.

A flexible approach to the control of these products should be adopted so that requirements can be modified in the light of experience of production and use, and with the further development of new technologies. Implementation of these requirements for an individual product must reflect its intended clinical use.

This document is not intended to apply to the control of genetically modified live organisms designed to be used directly in man, for example as live vaccines.

2 Points to consider in manufacture

This document is intended to facilitate the collection and submission of data to support applications for marketing authorization within the EEC for medicinal products derived by rDNA technology and intended for use in man.

Extensive 'scale-up' may be required as laboratory developments progress to full scale commercial production, and this may have considerable consequences for the quality of the product and thus implications for control testing. Although comprehensive characterization of the final product is essential, considerable emphasis must also be placed on 'in-process' control, a concept which has been highly effective in the quality control of bacterial and viral vaccines prepared by conventional methods.

Requirements relating to establishments in which biological products are manufactured (e.g. Revised Requirements for Biological Substances No. 1; WHO TRS 323) will apply to products derived by recombinant DNA methodology as will several of the general requirements for the quality control of biological products. Thus, appropriate attention needs to be given to the quality of all reagents used in production, including components of fermentation media; specifications for these are to be included in documentation and they must comply with any relevant EEC requirements. Tests for potency, abnormal toxicity, pyrogenicity and sterility etc., which apply to products made by conventional methods, will also apply to products made by recombinant DNA technology. It is undesirable to use in production agents which are known to provoke sensitivity reactions in certain individuals, such as, for example, penicillin or other β-lactam antibiotics.

Certain factors may compromise the safety and efficacy of rDNA-derived products: these should be given special attention and are outlined below:

(i) Products from naturally occurring genes expressed in foreign hosts may deviate structurally, biologically or immunologically from their natural counterparts. Such alterations can arise either at the genetic or post-translational level or during production or purification. Other products may be entirely novel in structure, produced by manipulation of naturally occurring genes or by chemical synthesis of new ones. These products may have enhanced biological features and/or diminished undesirable effects compared with their naturally-occurring counterparts. It should be recognized, however, that they may also have unexpected and undesirable biological properties.

(ii) The choice of manufacturing procedure may influence the nature and range of potential contaminants. Thus rDNA-derived products may contain potentially hazardous contaminants not normally present in their equivalents prepared by conventional methods and which the purification processes must be shown capable of removing. Examples of these are endotoxins in products expressed in bacterial cells and DNA of oncogenic potential in products expressed in transformed mammalian cells.

(iii) Unintended variability in the culture during production may lead to changes which favour the expression of other genes in the host/vector system or which cause alterations in the polypeptide product. Such variation might result in decreased yield of the product and/or quantitative and qualitative differences in the impurities present in the product. Consequently, procedures to ensure consistency of production conditions as well as the final product are imperative.

Whilst the requirements set out below should be considered to be generally applicable, individual products may present particular quality control problems. Thus, the production and control of

This document is the final draft generated by the Ad Hoc Working Party on Biotechnology/Pharmacy and approved (in June 1987) by the European Committe for Proprietary Medicinal Products. Address for correspondence: Pharmaceutical Division, DGIII/B/6, Commission of the European Communities, 200 Rue de la Loi, B-1049 Brussels, Belgium.

each product must be given careful individual consideration taking fully into account any special features

3 Strategy for cloning and expression

3.1 Expression vector and host cell. A description of the host cell and expression vector used in production should be given. This should include details of the origin and identification of the gene which is being cloned and the construction, genetics and structure of the expression vector. The method by which the vector is introduced into the host cell and the state of the vector within the host should be described. The association of the vector and host cell may be permanent, allowing continuous expression of the product; or self-limiting, for example where the vector is an acceptable cytopathogenic virus.

3.2 Sequence of the cloned gene. Full details of the nucleotide sequence of the gene insert and of flanking control regions of the expression vector should be provided. All relevant expressed sequences should be clearly identified. The DNA sequence of the cloned gene should normally be confirmed at the seed lot stage and at least once after a full scale fermentation. In certain systems, for example where multiple copies of the gene are inserted into the genome of a continuous cell line, it may be inappropriate to sequence the cloned gene at the production level. Under these circumstances Southern blot analysis of total cellular DNA or sequence analysis of the mRNA may be helpful and particular attention should be paid to the characterization of the final product.

3.3 Expression. The strategy by which the expression of the relevant gene is promoted and controlled during production should be described in detail.

4 Control of seed lot

It is essential that production is based on a well defined seed lot system involving a master seed and manufacturer's working seed bank. During the establishment of the seed no other cell lines should be handled simultaneously in the same laboratory suite or by the same persons. The origin, form, storage, use and details of life expectancy at the anticipated rate of use must be described in full for all seed materials. Attention should be paid to the stability of the host–vector expression system in the seed stock under conditions of storage and recovery. Any known instability should be reported. New seed lots should be fully characterized.

A critical part of quality control will involve the full characterization of seed material. Where higher eukaryotic cells are used for production, distinguishing markers of the cell, such as specific isoenzyme and immunological features or karyology, will be useful in establishing the identity of the seed. Details of the tumourigenicity of continuous cell lines should also be obtained and reported. Likewise, where microbial cultures are used, specific phenotypic features which form a basis for identification should be described.

Evidence that the seed lot is free from potentially oncogenic, where appropriate, or infective adventitious agents (viral, bacterial, fungal or mycoplasmal) must be provided. Special attention should be given to viruses which can commonly contaminate the animal species from which the cell line has been derived. For instance, cell lines of murine origin should be checked for contamination according to Appendix I of the *Notes to Applicants for Marketing Authorizations on Requirements for the Production and Quality Control of Monoclonal Antibodies of Murine Origin Intended for Use in Man* (*Trends in Biotechnology*, in press). Certain cell lines contain endogenous viruses, e.g. retroviruses, which may not readily be eliminated. The expression of these organisms, under a variety of conditions known to cause their induction, should be tested for and reported. Furthermore, the purification process should be shown to be capable of removing and or inactivating any such virus which may inevitably be present in the seed as an endogenous agent or part of the expression vector.

5 Production

Ideally not more than one cell line should be cultivated simultaneously in the same production area. If other cell lines are cultivated in parallel, records must be kept of the cell lines handled and evidence presented for the absence of cross-contamination between them.

5.1 Production at finite passage. Details of the fermentation or culture used to manufacture the product should be provided. For each production run, the presence, extent and nature of any microbial contamination in the culture vessels immediately prior to all harvesting must be thoroughly examined. Detailed information to confirm the adequate sensitivity of the methods used to detect contamination should be provided and acceptable limits of contamination set.

Maximum permitted passage levels for production should be defined and should be based on information concerning the stability of the host cell vector system upon serial sub-cultivation up to and beyond the level of production. Data on consistency of growth of the culture and on the maintenance of yield of the product should be presented. Criteria for the rejection of culture lots should be established.

Monitoring of the host cell vector characteristics at the end of production cycles should also be undertaken. For example detailed information on plasmid copy number and degree of retention of the expression vector within the host cell, as well as restriction mapping of the vector containing the gene insert, may be of value.

5.2 Continuous culture production. This approach should only be undertaken when special consideration has been given to the control of production based on continuous culture. Where it is undertaken monitoring of the production system is necessary throughout the life of the culture. The required frequency and type of monitoring will depend upon several factors including the nature of the expression system and product. Information should be obtained on the molecular integrity of the gene being expressed and of the phenotypic and genotypic characteristics of the host cell after long term cultivation. Evidence should be provided that the yield does not vary beyond defined limits and that the nature and quality of the product does not change with respect to specified parameters. The acceptance of harvests for further processing should be clearly linked to the schedule of monitoring applied. The period of continuous cultivation should be specified and this should be based on information concerning the stability of the system and consistency of the product up to and beyond this limit. In cases of long term continuous cultivation, the cell line and product should be completely re-evalu-

Glossary

Master Seed Bank – A homogeneou suspension of the original cells alread transformed by the expression vect containing the desired gene, aliquoted int individual containers for storage (e.g. in liquid nitrogen refrigerator). In some case it may be necessary to establish separat master seed banks for the expressior vector and the host cells.

Manufacturer's Working Seed Bank – A homogeneous suspension of the see material derived from the master see bank(s) by a finite passage level, aliquotec into individual containers for storage (e.g in a liquid nitrogen refrigerator).

In both seed banks, all containers ar treated identically during storage, and onc removed from storage, the containers ar not returned to the seed stock.

Production at Finite Passage – Thi cultivation method is defined by a limitec number of passages or population doub lings which must not be exceeded d production.

Continuous Culture Production – The number of passages or population doublings are not restricted from the beginning of production. Criteria for the termination of production have to be defined by the manufacturer.

Bulk Harvest – This is a homogeneous pool of individual harvests or lysates which is processed in a single manufacturing run.

Bulk final processed product – This is the finished product, after completion of the manufacturing process, obtained from a bulk harvest. It is maintained in a single container and used in the preparation of the final dosage form. The generation of this final batch has to be clearly defined and unambiguously recorded by the manufacturer.

Final dosage form – The finished product is formulated and filled into final, sealed containers which hold the product in its final dosage form. The containers of a filling lot are processed together and are uniform in their contents and biological potency.

ated, as set out in sections 3.1, 3.2, 7.1 and 7.2, at intervals based on information concerning the stability of the system and the character of the product.

A clear definition of a 'batch' of product for further processing should be provided. Regular tests for microbial contamination should be performed in relation to the strategy for harvesting. Criteria for rejection of harvests and premature termination of the culture should be defined.

6 Purification of the product

6.1 Methods. Methods used to purify the product should be described in detail. Procedures which make use of affinity chromatography, for example employing monoclonal antibodies, should be accompanied by appropriate measures to ensure that these substances, or any additional potential contaminants arising from their use, do not compromise the quality and safety of the final product. Attention is drawn to the *Notes for Applicants for Marketing Authorizations on Requirements for the Production and Quality Control of Monoclonal antibodies of Murine Origin Intended for Use in Man* (*Trends in Biotechnology*, in press).

6.2 Validation of the Purification Procedure. The capacity of the purification procedure to remove unwanted host cell derived proteins, nucleic acids, carbohydrates, viruses and other impurities should be investigated thoroughly, as should the reproducibility of the purification process as regards its ability to remove specific contaminants and the consistent composition of the purified product with respect to any impurities which may be present. Pilot scale studies using, for example, a carefully selected group of viruses which exhibit a range of physico-chemical features relevant to their behaviour on purification, or radioactively labelled DNA intentionally mixed with the crude preparation (spiking) should be undertaken. A reduction factor for such contaminants at each stage of purification, and overall, should be established by using, if necessary, concentrations of DNA and viruses in excess of that expected during normal production.

7 Final processed product

7.1 Characterization of the Purified Active Substance. Rigorous characterization of the active substance by chemical and biological methods will be essential. Routine detailed characterization of the final product may be required if the nature of the expression system makes it impossible to characterize the gene at the production level. Particular attention should be given to using a wide range of analytical techniques exploiting different physico-chemical properties of the molecule; for instance, size, charge, isoelectric point, amino acid composition and hydrophobicity. It may be desirable to include suitable tests to establish that the product has the desired conformational structure and state of aggregation. Examples of techniques suitable for such purposes are: polyacrylamide gel electrophoresis; isoelectric focusing; size exclusion, reversed phase, ion exchange, hydrophobic interaction or affinity chromatography; peptide mapping; amino acid analysis; light scattering; UV spectroscopy; circular dichroism, and other spectroscopic techniques. Additional characterization of the product using for example, electron microscopy or relevant immunochemical techniques may provide valuable information. Biological and immunological characterization should include as wide a range of techniques as possible appropriate to the anticipated biological activity, use, system of administration of the product and duration of treatment. The determination of the specific activity of highly purified material will be of particular value (units of activity/weight of product).

Sufficient sequence information to characterize the gene product adequately should be obtained. The degree of sequence verification required will depend on the extent of other characterization tests. For some purposes partial sequence determination and peptide mapping may suffice, for others full sequence determination may be necessary. Attention should be paid to the possible presence of N-terminal methionine, signal or leader sequences and other possible N- and C-terminal modifications (for instance acetylation, amidation or partial degradation by exopeptidases). Other post-translational modifications, such as glycosylation should be indicated. Special consideration should be given to the possibility that such modifications are likely to differ from those found in a natural counterpart and may influence the biological and pharmacological properties of the product.

7.2 Purity. Data should be provided on contaminants whose presence is anticipated in the final processed product. The level of contamination considered as acceptable, and criteria for acceptance or rejection of a production batch should be given. It is important that the techniques used to demonstrate purity be assessed using as wide a range of methods as possible, including physico-chemical and immunological techniques. Particular emphasis should be placed on tests for viral and nucleic acid contamination and for other unwanted materials of host origin, as well as on materials which may have been added during the production or purification processes.

8 Routine batch control of bulk final processed product

A comprehensive analysis of the initial batches of a product should be undertaken to establish consistency with regard to identity, purity and potency. Thereafter, a more limited series of tests may be appropriate as outlined below.

8.1 Consistency. An acceptable number, for example five, of successive batches of the bulk processed product should be characterized as fully as possible to determine consistency of composition. The studies should include biological, chemical and immunological methods to characterize and assay the active substance and methods to detect and identify impurities. Any differences which occur between batches should be noted. The data obtained from these consistency studies should be used as the basis for product specification.

8.2 Identity. A selection of the tests used to characterize the purified active substance (see 6.1) should be used to confirm the product identity for each batch. The methods employed should include tests for the anticipated biological activity as well as physico-chemical and immunological methods. Depending on the extent of other identification tests, sequence verification of a number of amino acids at the N- and C-terminus or other methods such as peptide mapping should be performed.

8.3 Purity. The degree of purity and also the degree of consistency of the production process. In general, a very high degree of purity can be achieved for

desirable and attainable will depend on several factors; these include the nature and intended use of the product, the method of its production and purification most products by modern manufacturing procedures.

The purity of each batch should be established and be within specified limits. The analysis should include sensitive and reliable assays for DNA of host cell origin applied to each batch of product prepared from continuous lines of transformed mammalian cells. Strict upper limits should be set for DNA in the product. It is recommended that DNA analyses are also performed on each batch of product obtained from other eukaryotic cells, and limits set for DNA content, until further information on safety is obtained. DNA of prokaryotic expression systems (e.g. of vector or plasmid origin) should be tested for wherever appropriate to considerations of the quality and safety of the product. For products to be administered for an extended period of time, or in high doses, the residual cellular proteins should also be determined by an assay with appropriate sensitivity (e.g. ppm) and strict upper limits set.

8.4 Potency. The potency of each batch of the product should be established (e.g. units of biological activity per ml) using, wherever possible, an appropriate national or international reference preparation calibrated in units of biological activity (see Section 9).

In addition, information on specific activity (units of biological activity per unit weight of product) will be of considerable value and should be reported. A highly purified reference preparation is required to standardize measurements of specific activity (see Section 9).

It is recommended that correlations between potency measurements, involving biological tests, and the results of physico-chemical methods of assay are made and the information reported. If possible, batches should be calibrated using accurate physico-chemical tests, and the biological assays used to confirm – within stated limits – that the product is biologically potent.

9 Specification and reference materials

The studies described in Section 7 will contribute to a definitive specification for the product when considered together with the information obtained from the examination of successive batches, as indicated under Routine Batch Control (Section 8).

A suitable batch of the product, preferably one which has been clinically evaluated, should be fully characterized in terms of its chemical composition, purity, potency and biological activity, including where possible full amino acid sequencing, and retained for use as a chemical and biological reference material.

When appropriate the biological activity of the product and its physical characteristics, including the amino acid sequence, should be compared with that of a highly purified preparation of the naturally occurring molecule.

10 Finished product

The product in final containers should be shown to comply with the requirements of the EEC directives where appropriate. In circumstances where this is not possible the omission of tests should be justified by the manufacturer.

11 Pre-clinical safety tests

The requirements for a particular product need to be carefully evaluated in each case. In general, classical toxicological studies in animals may be of only limited relevance. However, some safety testing will usually be required for most products. Reference should be made to separate *Notes to Applicants on the Pre-clinical Biological Testing of Medicinal Products Derived by Biotechnology*.

Acknowledgements

The Ad Hoc Working Party thanks members of universities, the pharmaceutical industry and control institutions for helpful comments received during the preparation of these guidelines.

BIODEGRADABLE POLYMERS FOR DRUG DELIVERY

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CONTENTS

INTRODUCTION

Recent advances in genetic engineering recombinant DNA technologies has led to the development of novel therapeutic protein and peptide drugs. A partial list is shown in Table 1. However, these therapeutic agents are difficult to administer due to their molecular size, susceptibility to proteolytic breakdown, rapid plasma clearance and denaturation. Due to these properties protein and polypeptide drugs are currently generally administered by non-oral routes. Developments in the oral and nasal delivery of proteins and polypeptides will be discussed elsewhere in the Course.

Possible parenteral methods of administration include:

1) Solutions : i/v. s/c or i/m : frequent administration necessary
   : controlled infusion pump.
2) Oil based depot : i/m or s/c : possible, discussed in more detail under 'Multiple Emulsions'
3) Polymeric Implants or Depot of Microspheres : this approach has proved successful for the delivery of polypeptides and will be discussed in detail.

<u>Polymeric Implants for Delivery of Protein and Polypeptide Drugs</u>

Some non-biodegradable polymeric implants systems have been investigated for the sustained release of polypeptides e.g.

Cross-linked poly(vinyl alcohol) hydrogels (Langer & Folkman, 1976).

Ethylene/vinylacetate copolymer (Langer, 1984).

Silicone elastomers (Hsieh *et al.* 1985).

However, the major disadvantage of non-biodegradable systems is that the implant has to be surgically removed once their lifetime has expired. It is also difficult to achieve a constant rate of release (zero-order release).

A number of macromolecular, naturally occurring materials have also been considered, e.g. albumin, gelatin, collagen, etc. In general these can be immunogenic when

TABLE I: A PARTIAL LIST OF PROTEIN DRUGS APPROVED FOR USE OR UNDER INVESTIGATION

PROTEIN	USE/FUNCTION
Human insulin	Treatment of diabetes mellitus
Human growth hormone	To supplement inadequate levels of endogenous growth hormone secretion in children
Alpha interferon	Treatment of hairy cell leukemia and AIDS-related Kaposi's sarcoma
Hepatitis B vaccine	Vaccination against infection caused by hepatitis B virus
Tissue plasminogen activator	Lysis of thrombi obstructing coronary arteries
Erythropoietin	Stimulation of red blood cell production
Murine monoclonal antibody	As immunosuppressant to prevent kidney transplant rejection
Gamma interferon	Treatment of chronic granulomatous disease
Granulocyte colony–stimulating factor	Promotes production of granulocytes; As an adjunct to chemotherapy in correcting neutrophil deficiency
Granulocyte–macrophage colony–stimulating factor	Promotes production of granulocytes and macrophages; Treatment of non–Hodgkin's lymphoma, acute lymphoblastic leukemia and Hodgkin's disease associated with bone marrow transplantation
Interleukin–2	Modulates immune function; Renal call carcinoma
Factor VIII	Treatment of blood clotting factor deficiency in hemophiliacs
Macrophage colony–stimulating factor	Promotes production of macrophages; Treatment of fungal infections; Wound healing
Monoclonal antibodies	Bind to and inactivate endotoxins to prevent septic shock; As cancer-imaging agents

cross-linked.

Biodegradable polymers are clearly the materials of choice for implantation for sustained drug delivery. The polymeric drug carrier needs to be treated as a drug itself in terms of the safety, biocompatibility and lack of toxicity of the polymer and its degradation products. In general, there are two processes of biodgradation - surface erosion and bulk hydrolytic degradation. Polymers where degradation is hydrolytical ly rather than enzymically controlled are preferable in that there will be less patient-to-patient variation.

In general, polypeptide and protein drugs are too large to show significant diffusion through polymer matrices : thus the release of drug can be controlled by the degradation of the matrix. This can allow a more constant rate of release (i.e. closer to zero order release) than with a diffusion controlled release device, where the rate of release tends to decrease with time and fraction released (e.g. Higuchi diffusion control release). Unstable drugs can also be protected from the surrounding environment until released.

Biodegradable Polymers

A range of biodegradable polymers have been considered for the sustained release of protein/polypeptide drugs : e.g.

Poly(alkyl-α-cyanoacrylates)

Poly(ortho-esters)

Poly(amino acids) i.e. polypeptides

Poly(dihydropyrans)

Poly(anhydrides)

and the group of

Aliphatic polyesters :

Poly(lactic acid) or poly(lactide)

Poly(glycolic acid) or poly(glycolide)

Poly(lactic-co-glycolic acid) or poly(lactide-co-glycolide)

Poly(ϵ-caprolactone)

Poly(hydroxy butyrate)

Some structures are shown in Figure 1.

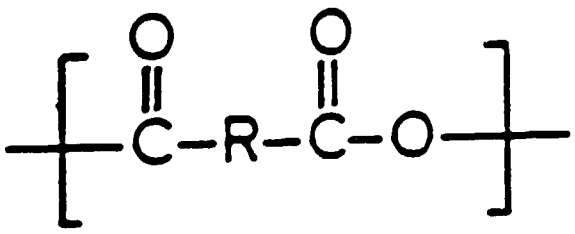
Biodegradable polymers for drug delivery have been reviewed by Leong (1991) and Smith *et al* (1990).

Attention will be focused on the aliphatic polyesters, in particular on the poly(lactic-co-glycolic acid) polymers which are used in current clinical practice for controlled drug delivery, having received regulatory acceptance for long-term parenteral use, in particular for once-a-month sub-cutaneous administration of LHRH agonists for the treatment of prostatic cancer (Zoladex and Prostap SR). They are inert and biocompatible and degrade to the naturally occurring lactic and glycolic acids.

The only other type of biodegradable polymer to be considered in detail is the polyanhydride group as controlled drug delivery systems using these polymers have been taken through to Phase 3 Clinical Trials.

Polyanhydrides

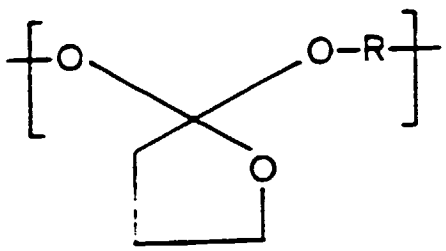
This class of polymer has the general structure shown here in Figure 2.



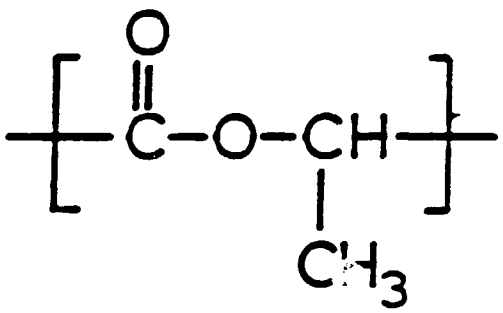
Polyanhydrides

FIGURE 2

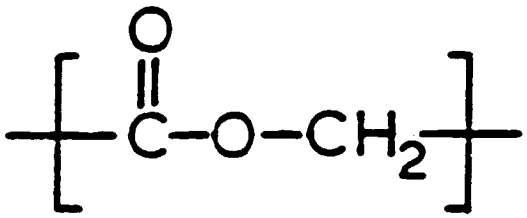
Poly(ortho-esters)



Poly(α-hydroxy acids)



poly(lactic acid)



poly(glycolic acid)

poly(ϵ-caprolactone)

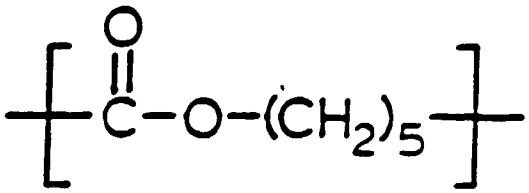


FIGURE 1

A particular polyanhydride has received much attention recently with its application in the controlled delivery of the cytotoxic nitrosourea, BCNU (or carmustine) to treat brain tumours. This work has been pioneered by Brem and his group at John Hopkins Medical Center, Baltimore, USA (Brem, 1990; 1990a) using the biodegradable polymer developed by Langer and co-workers at MIT. (Langer, 1988).

The particular polyandride used is poly(p-carboxyphenoxypropane-co-sebacic acid) PCPP-SA whose structure is shown in Figure 3.

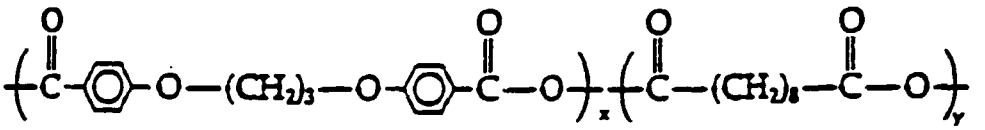
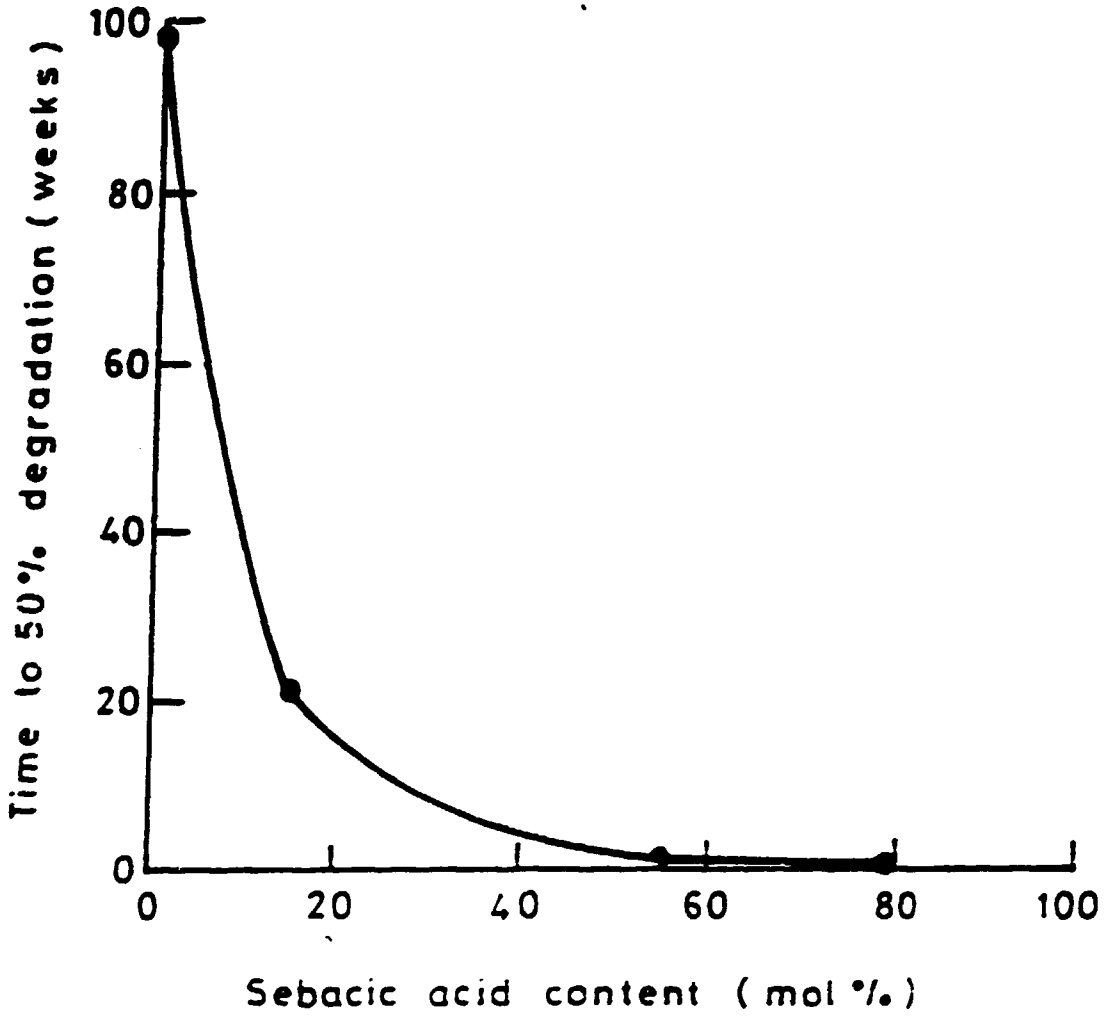


FIGURE 3

This is a hydrophobic, surface eroding polymer and hence can protect the BCNU (which has a half-life of only 12 min. in plasma and aqueous systems) until it is released in the proximity of the brain tumour. Implants are inserted at surgery into the cavity left by the removal of the tumour. Lack of toxicity of the polymer has been shown, as has the efficacy of the drug loaded implant : multi-centre Phase 3 Clinical Trials are being undertaken.

In the Department of Pharmaceutical Sciences at the University of Strathclyde we are following a similar approach but using the non-toxic, acceptable PLGA polymers loaded with carboplatin, which, being water soluble should not re-distribute from the brain, being unable to cross the blood-brain-barrier.

The degradation rate of PCPP-SA co-polymers can be controlled by the sebacic acid content, as shown in Figure 4. Thus, as the release rate is determined by surface



Effect of polymer composition on degradation rate of polyanhydrides.

FIGURE 4

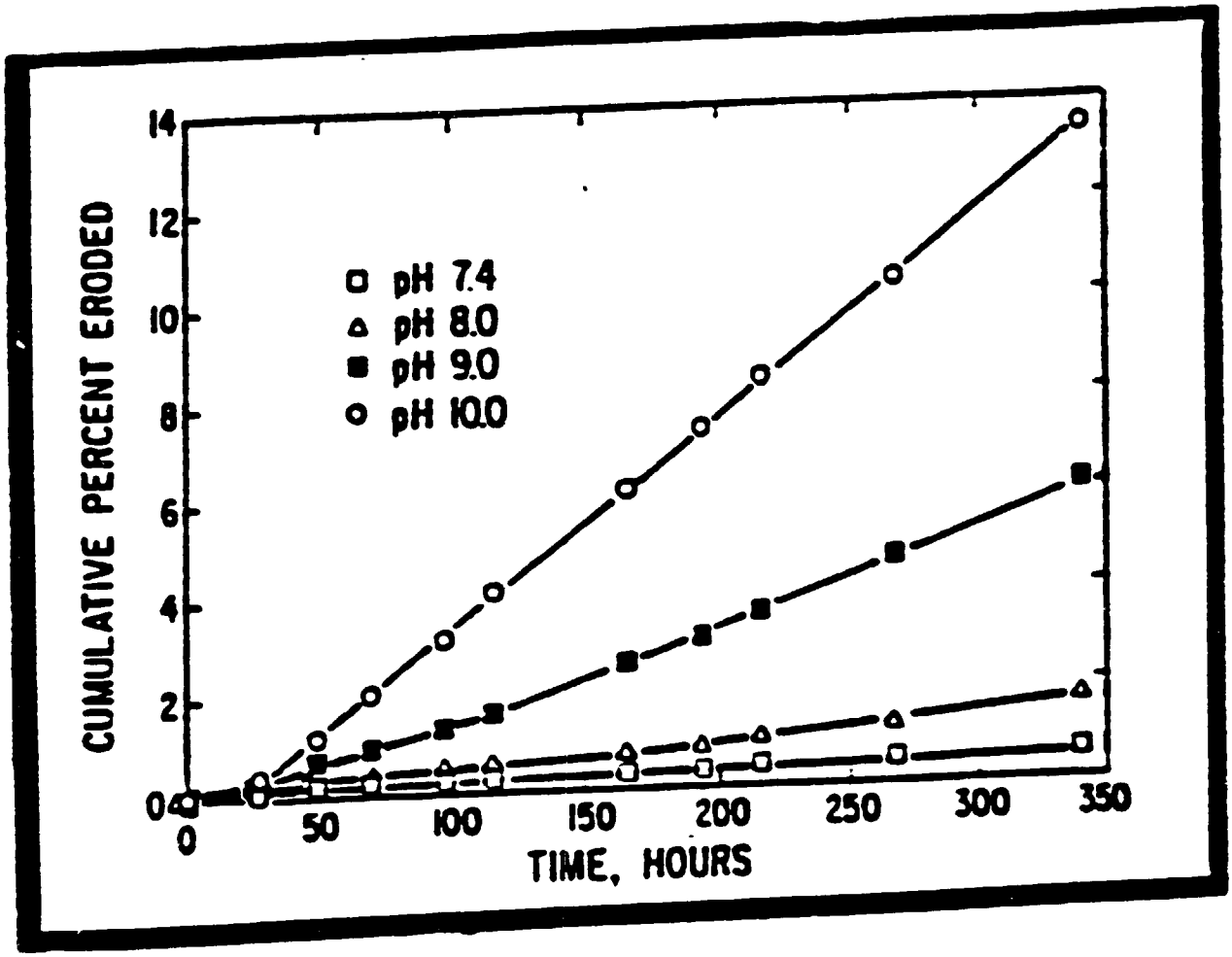
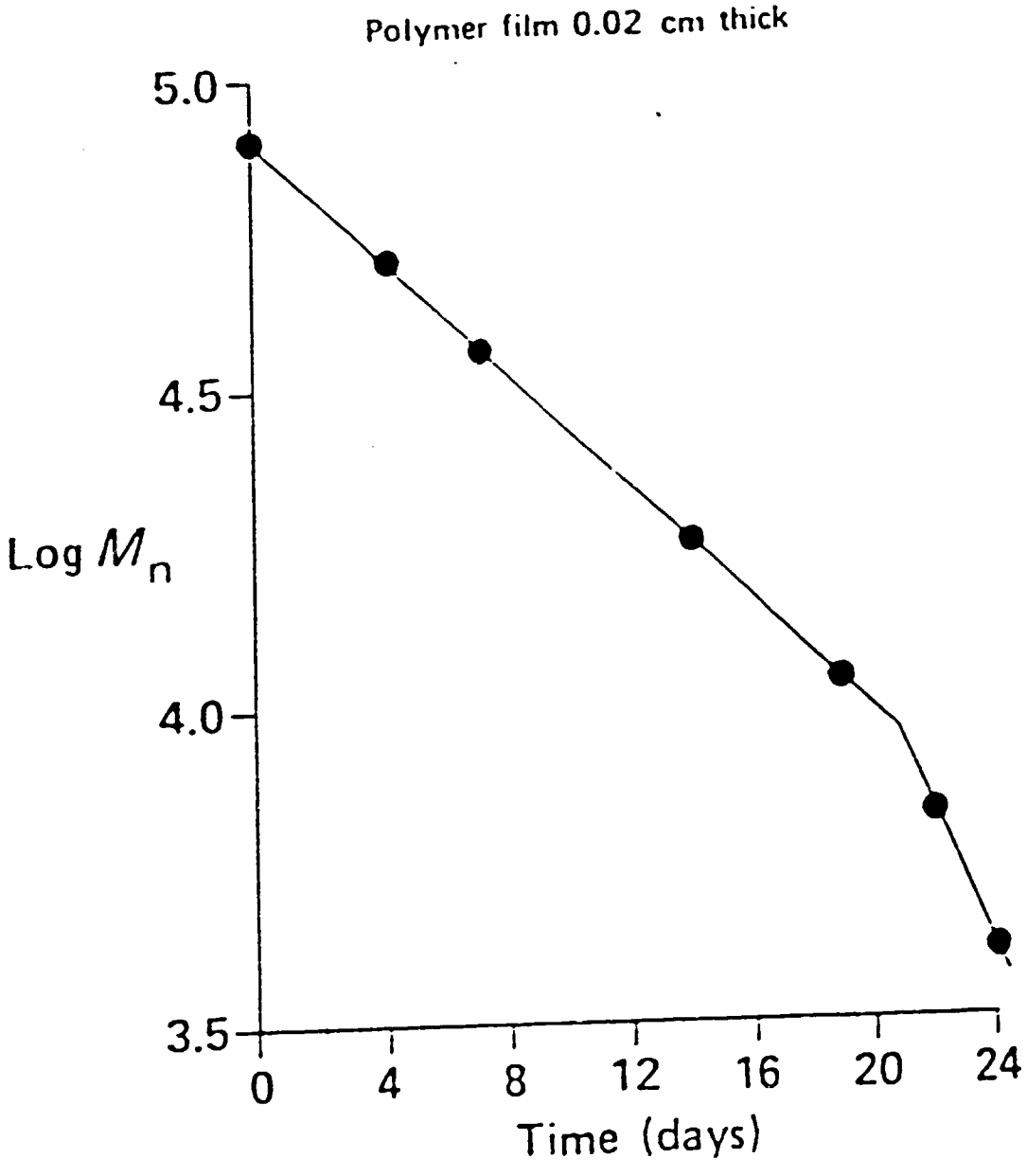

CUMULATIVE PERCENT ERODED
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
TIME, HOURS
pH 7.4
pH 8.0
pH 9.0
pH 10.0

FIGURE 5



In vitro degradation of poly (*d,l*-lactide-co-glycolide) at 37°C in buffer at pH 7.4. Number average molecular weight of degrading polymer as function of time.

FIGURE 9

erosion/degradation, a range of release rates can be readily achieved.

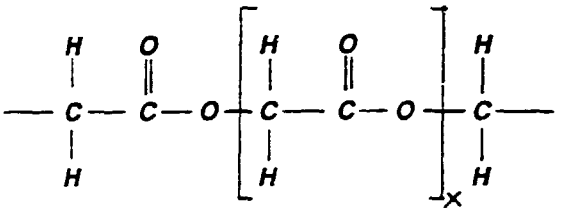
The rate of degradation of PCPP-SA polyanhydrides is sensitive to pH as shown in Figure 5. An increase in pH values causes a faster rate of degradation (Laurencin *et al*, 1990).

Poly(Lactic-co-Glycolic Acid) Polymers

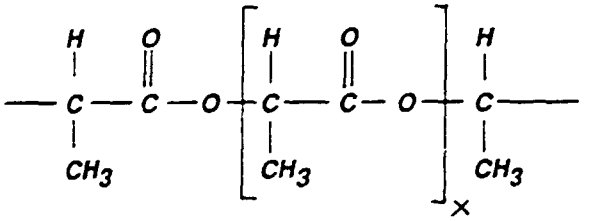
The structure of the monomeric lactic and glycolic acids is shown in Figure 6. It should be noted that lactic acid can exist in both D and L stereo isomeric forms; the L being the form occurring *in-vivo*. Both lactic and glycolic acids occur *in vivo* and are utilised in normal function.

The polymers and co-polymers are available commercially. They can be synthesised via the dimeric lactide and glycolide, whose structures are shown in Figure 7.

The structure of PLGA co-polymers is shown in Figure 8.



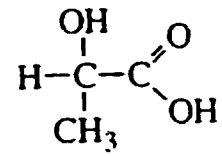
POLY (GLYCOLIC ACID)



POLY (LACTIC ACID)

FIGURE 8

The poly(glycolic acid) polymer is very crystalline and insoluble in solvents other than fluorinated ones. The poly(L-lactic acid) polymer is more crystalline than the poly(D,L-lactic acid) material where the chains cannot pack so well. Crystallinity decreases as glycolic acid is introduced into the poly(lactic acid) systems.

$CH_3 CH (OH) COOH$  Lactic acid

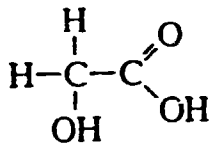
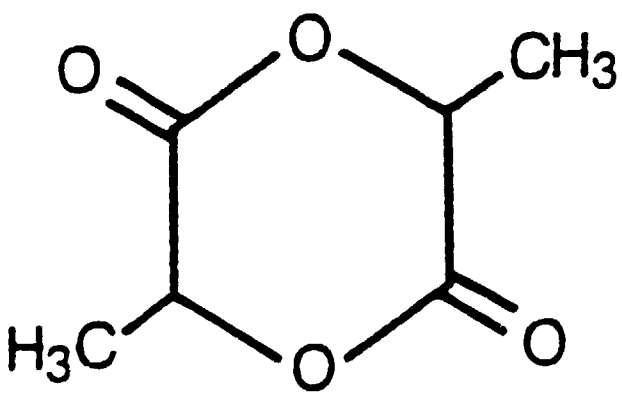
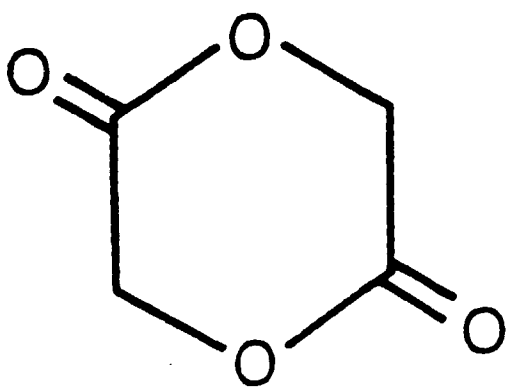
$CH_2 OH COOH$  Glycolic acid

FIGURE 6



Lactide



Glycolide

FIGURE 7

The PLGA co-polymers degrade by a hydrolytic mechanism, which occurs throughout the matrix i.e. it is not an enzymic or surface erosion mechanism. There is an increase in rate of hydrolysis at high and low values of pH typical of acid-base catalysed ester hydrolysis (Makino *et al*, 1985).

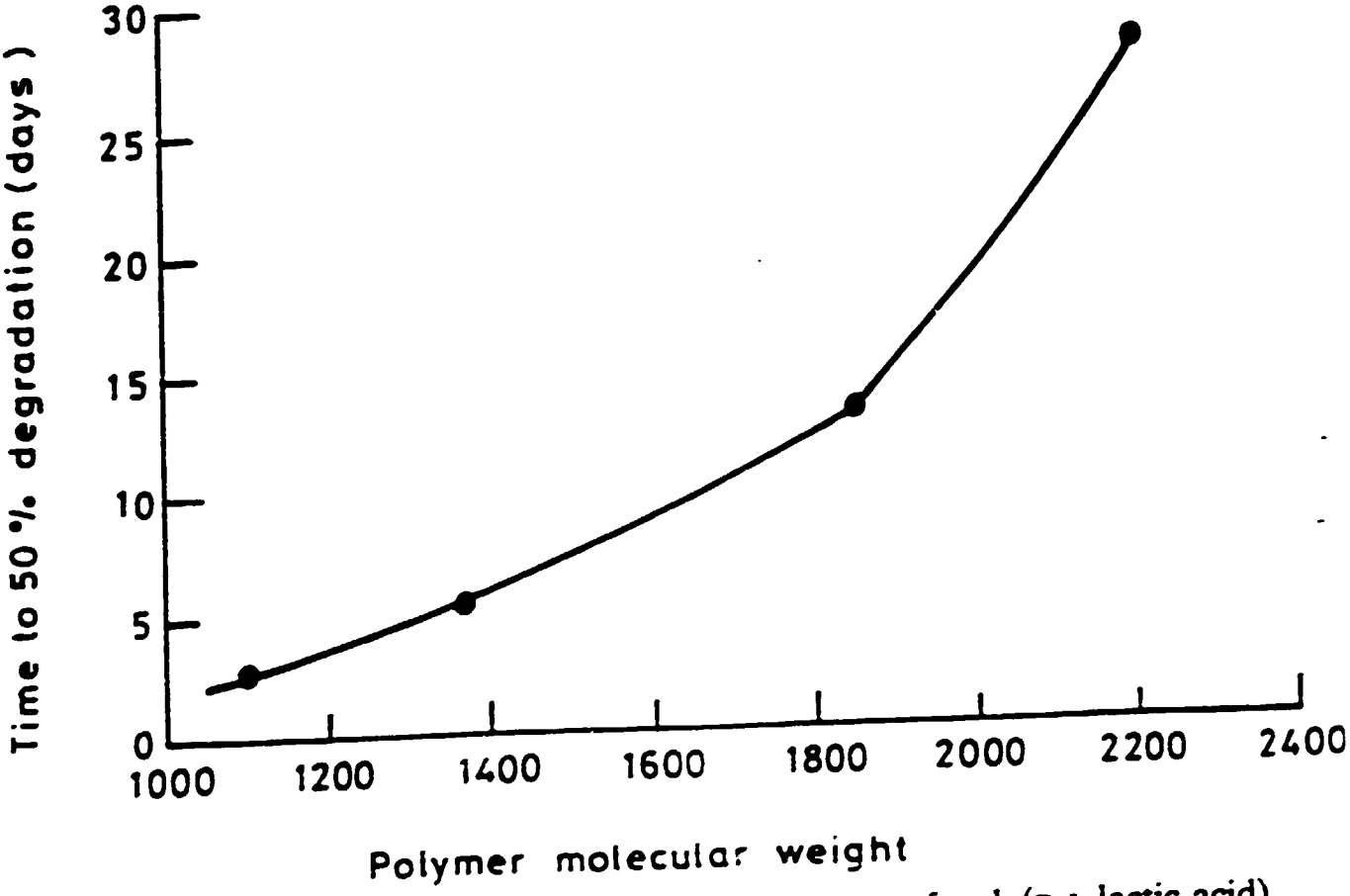
A typical *in vitro* degradation curve at 37°C and pH 7.4, as measured by molecular weight decrease, is shown in Figure 9 (Fildes *et al*, 1991).

There are three major variables which can affect the properties of the co-polymers i.e. molecular weight; ratio of lactic to glycolic acid and γ-irradiation. Each of these parameters has an effect on the degradation rate, which in many cases is the major factor controlling the rate of drug release

Figure 10 illustrates that an increase in molecular weight of PLA decreases the rate of degradation whilst Figure 11 shows that an increasing content of glycolic acid (up to 60-70%) causes an increase in the rate of degradation (Kaetsu *et al*, 1987). It should be noted that the rate of degradation decreases again as the percent of glycolic acid approaches 100%, due to the greater crystallinity in these materials.

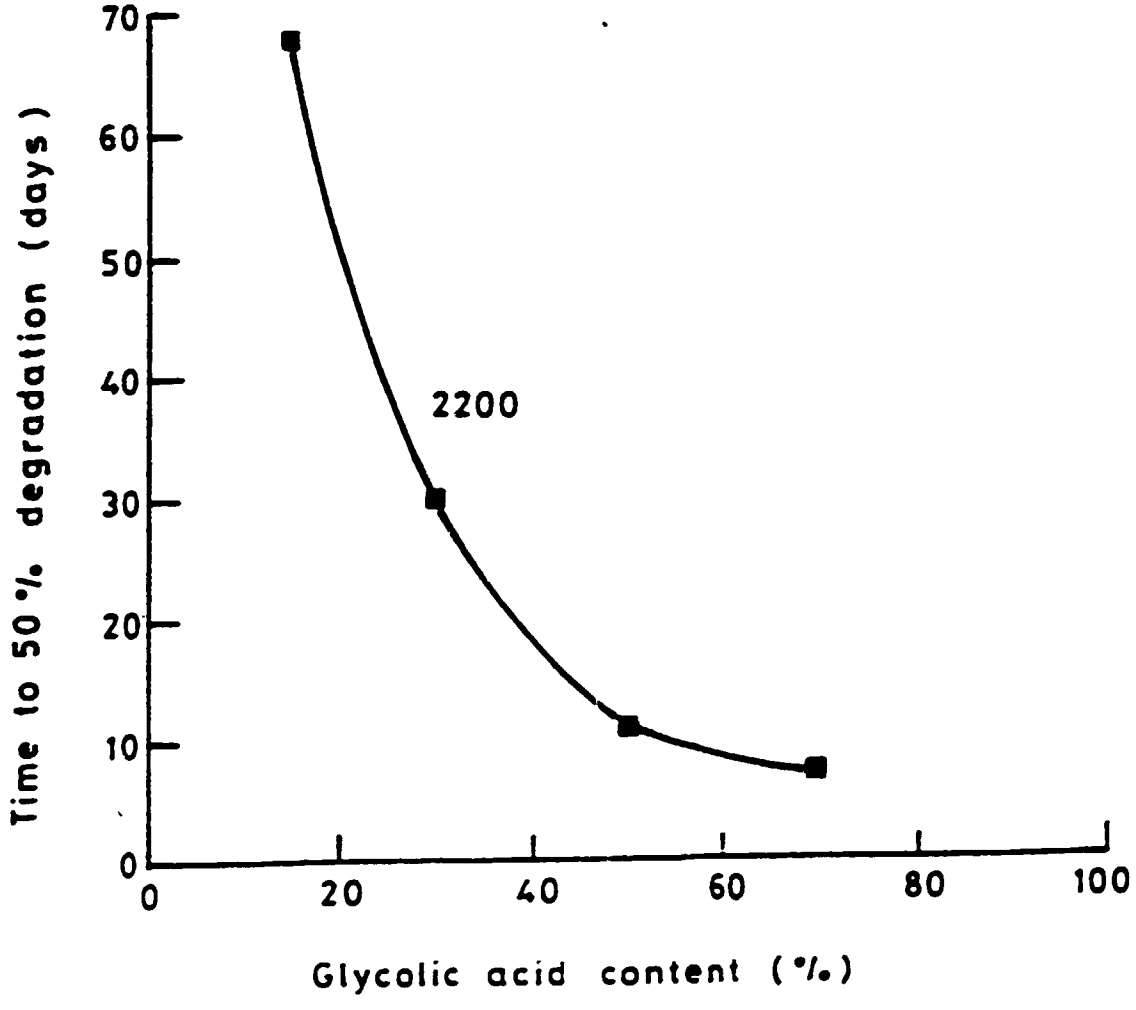
The effect of γ-irradiation (often used for terminal sterilisation of PLGA systems) on the polymer molecular weight (as indicated by the intrinsic viscosity), as illustrated in Figure 12, shows a near-linear decrease in molecular weight with increasing irradiation dose. Thus, γ-irradiation sterilisation has the effect of reducing the molecular weight of the polymer, with consequent effect on the rate of degradation, as previously discussed. These three factors allow control of the rate of degradation of PLGA polymers and consequently on the rate of drug delivery.

Figure 13 shows the process for the preparation of Zoladex type implants for the once-a-month administration of LHRH agonists (Asano *et al*, 1991). The mixture of high and low molecular weight poly(lactic acids), tailored to give the correct degradation and release rate, are melt mixed prior to a further melt mixing with the LHRH agonist. This



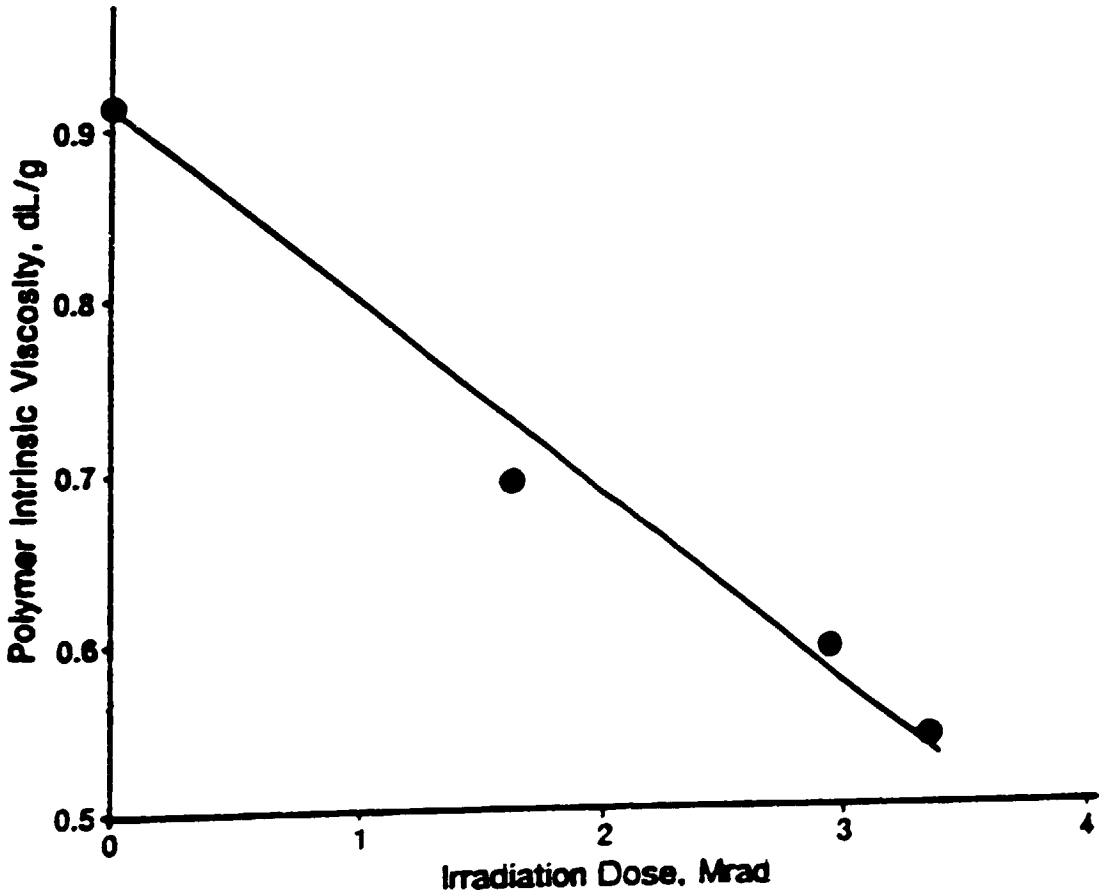
Effect of polymer molecular weight on degradation rate of poly(D,L-lactic acid)

FIGURE 10



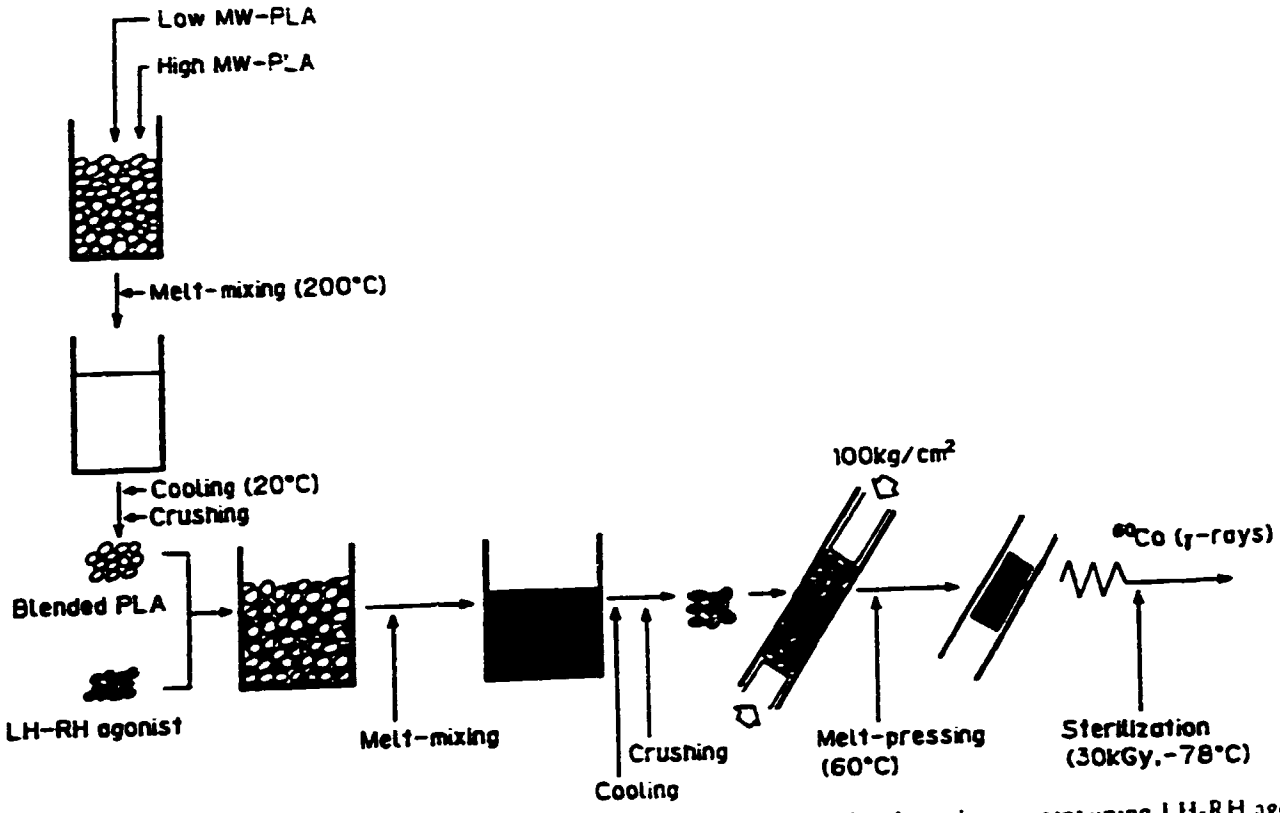
Effect of polymer composition on degradation rate of poly(L-lactic acid/glycolic acid)

FIGURE 11



Effect of gamma-irradiation on intrinsic viscosity of 69:31 co-polymer.

FIGURE 12



Schematic diagram illustrating the preparation of small cylinders of blended PLA formulations containing LH-RH agonist by the melt-pressing technique.

FIGURE 13

mixture is warm-pressed at 60°C and sterilised by γ-irradiation. A special syringe delivery system has been devised for the Zoladex product : however, a 16 gauge needle is needed and this can necessitate the use of a local anaesthetic for the sub-cutaneous injection. Microsphere suspension preparations have been developed, as discussed in the lecture on "Microencapsulation and Microspheres" which can be injected using smaller needles of 21 to 23 gauge. An excellent review of the development of Zoladex is given by Fildes *et al*, (1991).

The general properties of the release of drugs from PLGA systems is well-illustrated in the data in Figures 14a and 14b. These systems are cis-platin loaded microspheres of 75:25 PLGA of molecular weight 58,000 prepared by the oil-in-water emulsion solvent evaporation method with the external phase saturated with the water-soluble cis-platin (Spenlehaner *et al.*, 1988).

Figure 14a shows the release over a 48 hour period for three different drug loadings (18%, lowest curve; 32%, middle curve and 41%, top curve). This data illustrates the general phenomena that a faster rate of release is found with higher drug loadings, over the initial stages of release where degradation of the matrix is not rate-controlling but release is determined by a diffusion-dissolution mechanism. The explanation can be understood by consideration of Figure 15 which shows the distribution of drug crystals (cis-platin and mitomycin C will be insoluble in the polymer and dichloromethane solvent and will be present in the matrix as dispersions of crystals) within the polymer matrix at the 3 different drug loadings. At the highest drug loading, crystals in contact with the surface can dissolve and form channels for water to leach out crystals in the interior. Essentially all the crystals can be part of a network connected to the external aqueous phase.

In the case of medium loading ony a limited number of crystals are in contact with the aqueous phase. Isolated crystals in the interior can only release drug via the diffusion

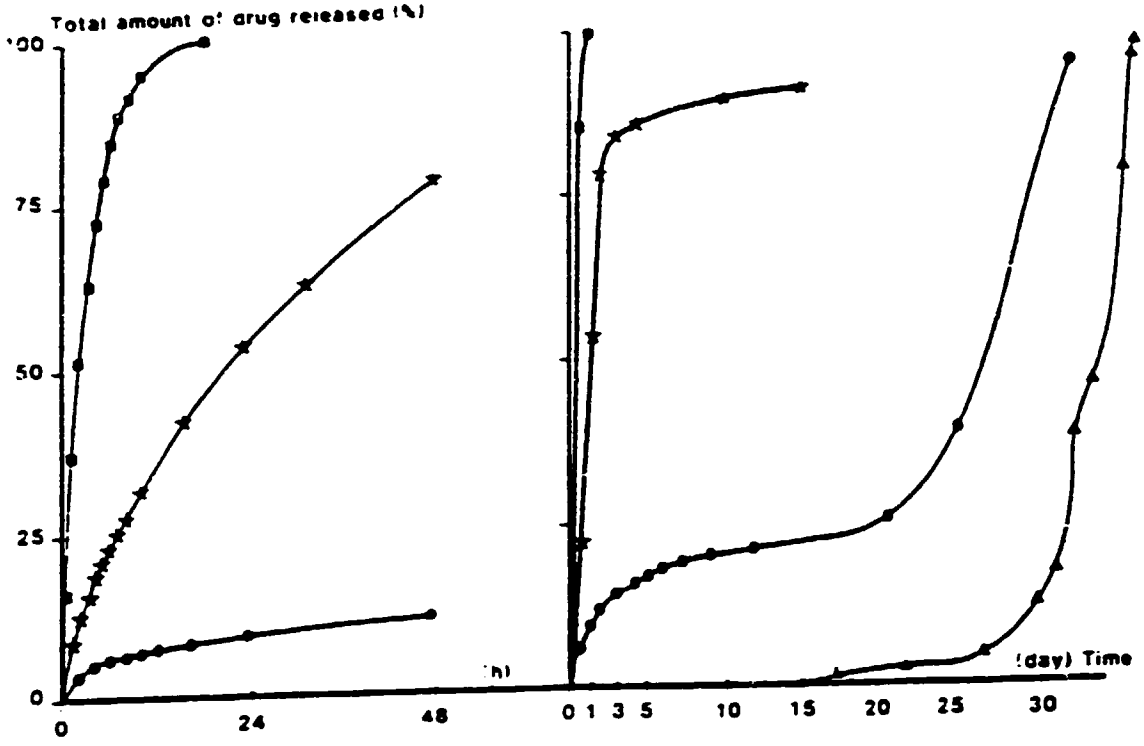


FIGURE 14a

FIGURE 14b

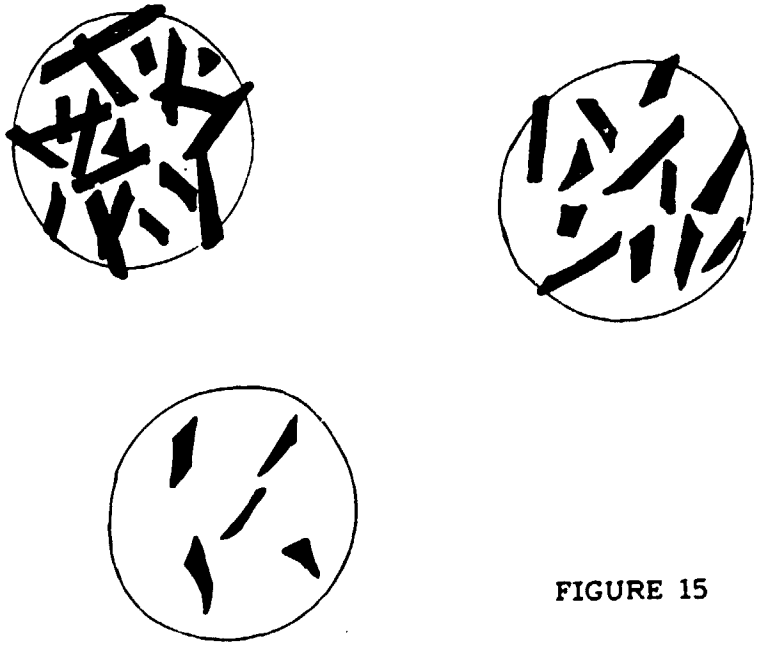


FIGURE 15

of water through the matrix and dissolution, clearly a slower process. At low loading of drug, essentially all of the drug particles in the interior are isolated and as shown in Figure 14a only 10% is released at 48 hour.

Figure 14b, shows data for the same systems of Figure 14a over a time period of more than 30 days (the rightmost curve is for a loading of 9.1%, which showed zero release over the initial 15 day and hence is not included in Figure 14a).

The rapid increase in the rate of release at 20-30 day for the two lower loadings of drug is due to the massive collapse of the polymer matrix due to its hydrolytic degradation. The curve for the drug loading of 18% shows a typical 3 phase release pattern i.e. (1) an initial burst due to material at or close to the surface, (2) a phase of very little release, followed by (3) a rapid release on collapse of the matrix following hydrolytic degradation. The question of the importance of diffusion on degradation controlled drug release has recently been discussed by Shah *et al*, (1992) and Bodmer *et al*, (1992).

The effect of gamma-irradiation on drug release from PLGA matrices is well-illustrated in Figure 16 for PLGA microspheres loaded with 29% cis-platin.

γ-irradiation does not affect the rate of release in the initial few days. It is the massive release on collapse of the matrix which is dramatically affected : γ-irradiation (37.7 kGy) causes the matrix collapse to occur at 10-15 days rather than at 60-70 days in the un-irradiated materials. These results correlate with the data previously presented (Figure ?) where the molecular weight (as indicated by intrinsic viscosity) of PLGA was shown to decrease essentially linearly with γ-irradiation dose.

An injectable 90 day delivery system for testosterone based on PLGA has been reported (Atkins, 1992).

The factors influencing the rate of release of thyrotropin releasing hormone (TRH) from poly(d,l-lactic/glycolic acid) has been investigated in detail recently (Heya *et al*, 1991, 1991a). In particular, the ionic interaction between the basic group of TRH and the

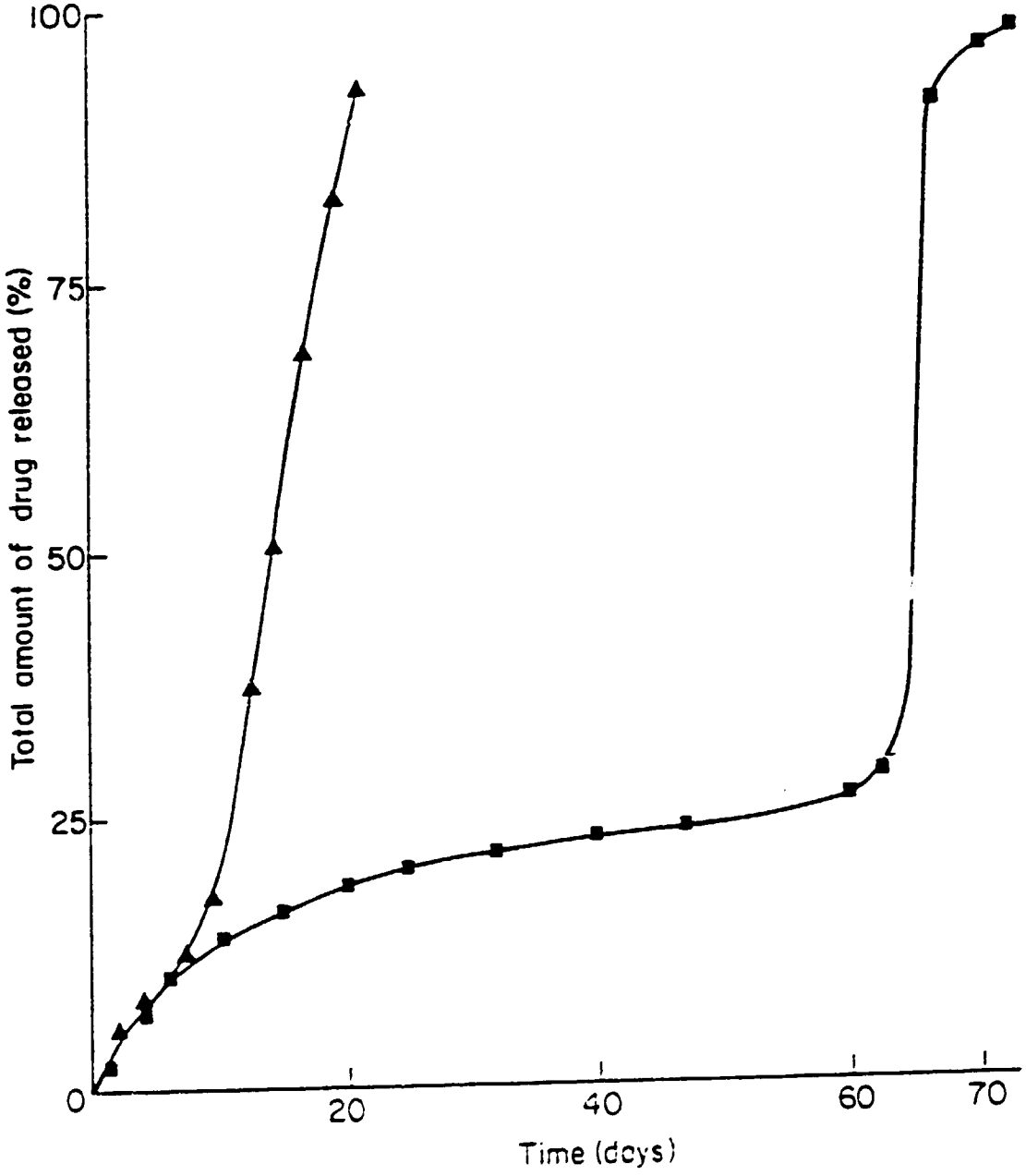

100
75
50
25
0
Total amount of drug released (%)
20
40
60
70
Time (days)

FIGURE 16

carboxylic end group of PLGA affected both the rigidity of the microsphere matrix and the rate of release.

The effect of various additives on the release rate of drugs from PLGA systems has been investigated by a number of groups. The effect of the additon of a low Mw.PLA (2,000) to thin films of a high Mw PLA (120,000) on the release rate of salicylic acid (6.7% loading) is shown in Table 2. Increasing amounts of the low Mw PLA increase the rate of release and the glass transition temperature is reduced (Bodmeier *et al*, 1989).

TABLE 2

High : Low Mw PLA	% Released at 24 hr
100 0	15
75 25	20
50 50	40
25 75	70
0 100	92

The addition of the Pluronic L101 to PLA films eliminated the burst effect for the release of BSA. With 30% L101 there was 15% hydration at 30 days and no loss of L101. The formation of a liquid crystalline phase of H_2O-L101 was postulated (Park *et al*, 1991).

The incorporation of basic amine drugs, can enhance the rate of polymer hydrolysis due to microenvironmental pH effects e.g. thioridazine reduces the polymer molecular weight to one half during the preparation of microspheres. The drug is released rapidly, i.e. 50% in 3 days at pH 7.4 : if the amine group is blocked, the release time is extended. The effect of drug loading on release rate is clearly seen for fluphenazine in poly(D,L-lactide) microspheres. For polymer of molecular weight, 2,000, the T_{50} values for loadings of 10, 20 and 30% were 5, 17 and 48 days respectively. A similar effect was

found for polymer of 16,000 molecular weight (Ramtoola *et al* 1992).

A number of interesting aspects with regard to the degradation of PLGA have been reported.

(a) glycolic acid is released faster than lactic acid.

(b) Degradation is faster in the interior (of implants) due to the lower microenvironmental pH following chain scission but before oligomers in the interior are able to diffuse out (Vert, 1992).

(c) two types of water have been shown by NMR to be present in melt-pressed implants: either dissolved or as water in pores.

(d) free radicals (OH) have been shown to increase the rate of degradation *in vitro* Ali *et al*, 1992).

In general the 50:50 lactide/glycolide co-polymer shows the lowest crystallinity, highest hydrophilicity and maximum rate of degradation. However, samples of 50:50 PLGA of similar molecular weights can show different solubility properties due to the presence of blocks of glycolide (as opposed to a random distribution of glycolide and lactide). Glycolide is highly crystalline and blocks of glycolide result in reduced solubility. Large differences in the 'blockiness' of a sample can be detected by ^{13}C-NMR (Bendix, 1990).

The question of the stability of the drug in the PLGA matrix has obviously to be addressed. For example the cytotoxic drug, etoposide, was found to degrade with a similar mechanism as in aqueous solution, over a period of days in a PLA matrix (Aso *et al*, 1992).

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Multiple w/o/w Emulsions as Drug Vehicles

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CONTENTS

I. INTRODUCTION

Multiple emulsions are emulsions in which globules of the dispersed phase encapsulate smaller droplets, which normally consist of a liquid miscible and, in most cases identical with, the continuous phase.[1] The two major types of multiple emulsions are water-in-oil-in water (w/o/w) in which the internal and external aqueous phases are separated by an oil layer and oil-in-water-in oil (o/w/o) in which water separates the two oil phases. Because of this structure and the presence of the liquid barrier, multiple emulsions are also known as liquid membrane systems. A typical photomicrograph of a w/o/w emulsion is shown in Figure 5-1. The internal droplets that usually contain the therapeutic agent are dispersed in the oil phase (the primary w/o emulsification stage) which in turn is dispersed in the external aqueous phase (the secondary emulsification stage). Drug is released either by internal droplet coalescence with the external aqueous phase or in stable systems by diffusion across the liquid membrane. Obviously if prolonged release is to be

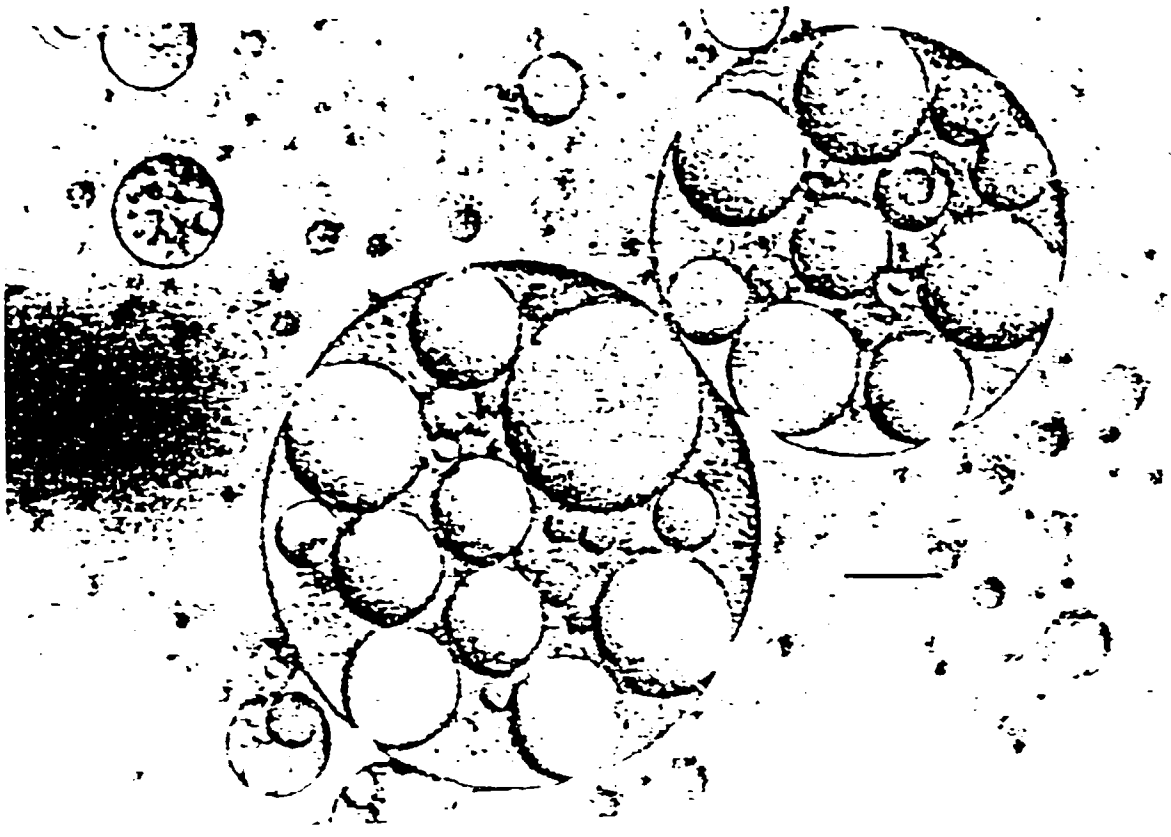


FIGURE 5-1
Photomicrograph of a [illegible] multiple emulsion indicating the polydispersity of both external and internal droplets but marked [illegible] 50 μm.

achieved stable systems may not yet be. Such emulsions have the additional advantage of being less viscous than comparable water in oil emulsions and therefore can be easier to inject.

Multiple emulsions have potential uses as adjuvant vaccines,[1] as prolonged parenteral drug delivery systems,[2] as an adjunct to errors in drug overdosage treatments,[3] and immobilization of enzymes.[4] The nonpharmaceutical uses of liquid membranes have included the separation of hydrocarbons,[5] hydrometallurgical recovery of metal ions,[6] and the removal of toxic materials from air to water,[7] all of which depend on the selective transport of solutes across the liquid membrane. This review is concerned with the potential for the use of these systems for sustaining the action of drugs in vivo by prolonging their release from a depot or for modifying the toxic effects of certain drugs.

The literature up to 1982 relating to the formulation, mechanisms of breakdown, and factors that control the stability of multiple emulsions was recently reviewed by one of us.[8] Although multiple emulsions, especially water-oil-water systems, have potential applications in controlled release systems for drug delivery and in separation procedures, [illegible] have been limited by their lack of stability.

The production [illegible] of both aqueous [illegible] without coalescence [illegible] tion is to be realized.

II. COMPOSITION OF THE MULTIPLE EMULSION AND STABILITY: INFLUENCE OF THE NATURE OF OIL PHASE

A number of factors have been identified as affecting the stability of w/o/w emulsions. These include their method of preparation, the nature of entrapped materials, particularly the effect of electrolytes, their phase volumes, and the concentration and type of emulsifiers.[12] The influence of the oil phase on the stability of the resulting emulsion has until recently received little attention.

Davis and Walker[13] used 6-carboxyfluorescein as an internal marker to measure the yield of multiple emulsion droplets prepared from mineral oils (liquid paraffin and squalene) and vegetable oils (sesame oil, maize oil, and arachis oil). The yield of multiple emulsion droplets was found to be dependent upon the nature of the oil and decreased in the order liquid paraffin > squalene > sesame oil > maize oil > arachis oil. Magdassi et al.[14] observed that optimal stabilities of multiple emulsions were obtained when there is a similarity between the hydrophobic part of the emulsifier and the oil phase. This suggests, not surprisingly, that the stability of multiple emulsions depends to a large extent like ordinary emulsions on the adsorption of the emulsifiers at the oil–water interface and on the properties of the adsorbed layer at that interface. The nature of the oil phase affects the nature of the interfacial film and together these are of crucial importance in determining globule size and stability.

The stability of multiple w/o/w emulsions prepared with isopropylmyristate and a range of pure hydrocarbons has been studied by the present authors.[15] Both the internal aqueous droplets and the multiple oil drops varied in size according to the nature of the oil used in preparing the emulsions and a correlation between the interfacial tension at the oil–water interface and the droplet size was reported. Figure 5-2 shows the change in number of multiple oil drops with time. There is no significant change in number of multiple oil drops prepared with hydrocar-

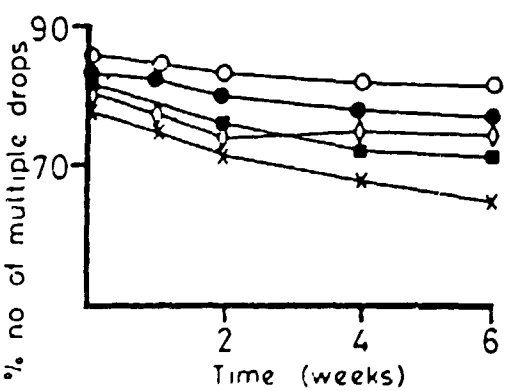


FIGURE 5-2
Stability of multiple w/o/w emulsions prepared with hydrocarbon oils: (○) octane, (●) dodecane, (■) cyclohexane, (◇) hexadecane, (×) toluene. Emulsions were prepared with 2.5% span 80 and 0.2% BSA as primary emulsifiers and 1% polysorbate 80 as secondary emulsifier. From Ref. 15.

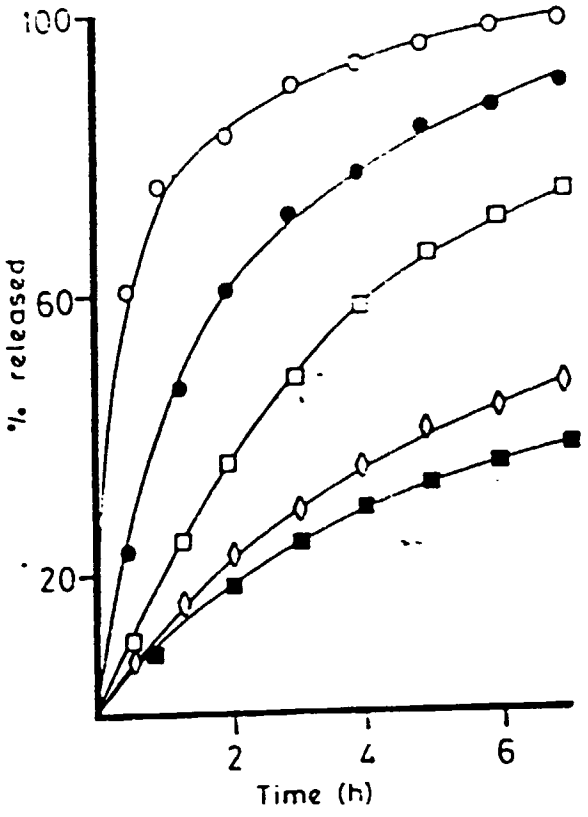


FIGURE 5-3
Release of 5-FU from multiple w/o/w emulsions. The emulsions contained 2.5% Span 80 and 0.2% BSA as primary emulsifiers, with 5-FU (1 mg ml^{-1}) in the internal phase and the following oil phases: (■) octane, (◇) cyclohexane, (□) dodecane, (●) hexadecane, (○) toluene, 1% polysorbate 80 served as the secondary emulsifier. From Ref. 15.

bons indicating stability in these systems. *In vitro* release studies of 5-fluorouracil (Figure 5-3) from w/o/w emulsions showed that release was faster for those systems with smaller internal droplets due to increased interfacial area.[15]

III. METHODS AVAILABLE FOR STABILIZING w/o/w MULTIPLE EMULSIONS

In spite of the potential applications of multiple emulsions, there have been very few reports in the literature of attempts to improve their stability. The principal modes of breakdown involve coalescence of internal or external droplets, expulsion of the internal droplets, or osmotic swelling or shrinkage.[16] The methods in the literature can be classified into three: gelation of either internal or external phases, formation of interfacial complex stabilizing films, and the determination of the optimal HLB of surfactant mixtures for the primary and secondary emulsification steps, each of these being combined sensibly with attempts to minimize the osmotic gradient across the liquid membrane.

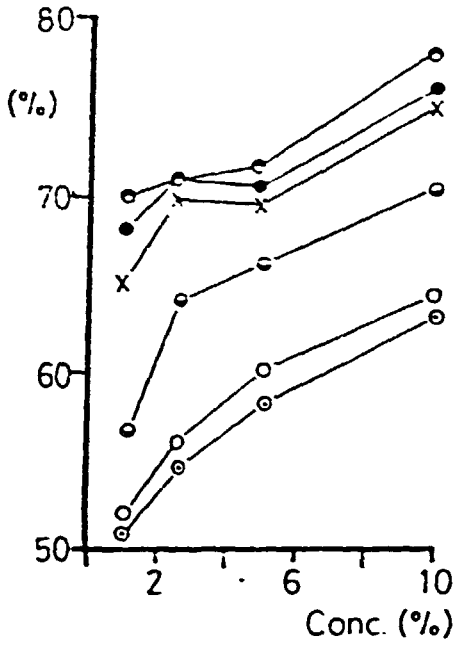
A. Gelation of Either Internal or External Water Phase

Following an earlier attempt to improve the stability of a w/o/w emulsion by use of an "oil" that solidified at room temperature (e.g., octadecane), Florence and Whitehill[17] investigated the possibility of stabilizing multiple systems by forming a polymeric gel in either the internal or external aqueous phase[15] using a nonionic surfactant—a poloxamer that could be polymerized *in situ*.[18] Irradiation of the system resulted in the bulk polymerization of the stabilizer (the poloxamer), with the consequence that the multiple globules became enmeshed in a network of cross-linked surfactant molecules. Polyacrylamide has also been used to demonstrate this technique, but less toxic systems are required for pharmaceutical use. The main disadvantage of the use of γ irradiation is that the drug has to be incorporated at the primary emulsification step and is therefore exposed to γ irradiation. To overcome this difficulty a series of acryolyl derivatives of poloxamer (Pluronic) surfactants (including Pluronic L44) was found to increase the stability of w/o/w emulsions following cross-linking by irradiation at the interfacial polymerization oil–water interface.[19] The primary w/o emulsion in a typical formulation contained 5% Span 80 in isopropylmyristate and 0.9% of the diacryloyl derivative of Pluronic L44 in the aqueous phase with 4% $(NH_4)_2S_2O_8$ as initiator. Polymerization in the external aqueous phase increases its viscosity, increasing stability by reducing the close approach of particles. However, a polymerized interfacial membrane has been found to be more effective in providing stability.

B. Formation of Interfacial Complex Films

During recent years, work in our laboratory has focused on the formation of stable interfacial films. Surfactants from a series of chemically modified polymerizable, nonionic surfactants can form *in situ* cross-linked membranes after adsorption at the o/w interface.[20] The resulting interfacial membrane was found useful for maintaining the stability of the liquid membrane. This approach, like the previous one, suffers from the disadvantages of possible drug degradation during the polymerization process and/or the presence of residual polymerization initiator in the final product.

Another approach to the formulation of stable interfacial membranes for improving the stability of w/o/w emulsions is by interfacial interaction between a macromolecule such as albumin, or a polyanion such as polyacrylic acid in the internal aqueous phase and a lipophilic nonionic surfactant in the oil phase.[21,22] The formation of an interfacial complex at the primary water–oil interface was found to enhance the stability of the w/o/w emulsion and to cause a delayed release of solutes entrapped in the internal phase of the emulsion. Here, increased stability allows the film to withstand thinning, but unfortunately this stable film acts as an inadequate barrier to solute transfer when it has thinned. A range of PEO–PPO–PEO block copolymers (poloxamers) was found to form a stable interfacial membrane with either polyacrylic acid or albumin.[21,22]



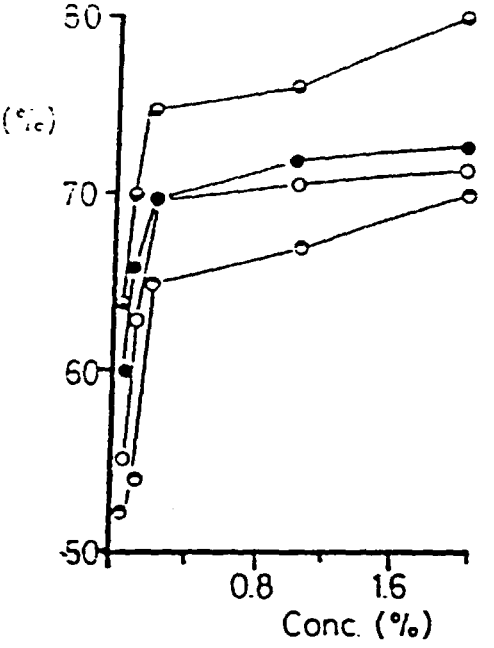


FIGURE 5-4 (left)
The effect of Span 80 concentration on the entrapment of NaCl in w/o/w emulsions of constant BSA concentration in the internal aqueous phase: (⊙) no BSA; (○) 0.05% BSA; (◓) 0.1% BSA; (×) 0.2% BSA; (●) 1% BSA; (◓) 2% BSA. From Ref. 23.

FIGURE 5-5 (right)
The effect of BSA concentration on the NaCl entrapment in w/o/w emulsions: (◓) 1% Span 80; (○) 2.5% Span 80; (●) 5% Span 80; (◒) 10% Span. From Ref. 23.

In a multiple w/o/w emulsion stabilized by interfacial interaction between Span 80 and albumin, the entrapment efficiency of a w/o/w emulsion was studied by incorporating NaCl in the internal phase of the emulsion.[23] Increasing the concentration of Span 80 from 1 to 10% (Figure 5-4) over all the bovine serum albumin (BSA) concentrations increased the amount of NaCl entrapped, which appears to parallel the observed increase in the number of internal aqueous droplets in the emulsions. The effect of BSA on the quantity of NaCl entrapped is not linear (Figure 5-5); there is a region of rapid increase in yield (at all Span 80 concentrations) within the range of 0.05–0.2% BSA. This is followed by only a gradual increase in yield at BSA concentrations above 0.2%. The quantity of NaCl initially entrapped (Figure 5-6) increased with increasing concentration of NaCl, probably due to the formation of a rigid interfacial layer as a result of the salting out effect of NaCl at the inner water–oil interface.

Two competitive effects of NaCl in the internal phase of w/o/w emulsion can be distinguished: because of the "salting out" effect, NaCl has a stabilizing influence on w/o/w emulsions. However, the presence of NaCl in the internal phase of the emulsion could create an osmolarity difference between the two aqueous phases of the emulsion that would induce osmotic flux of water from the external phase to the internal phase of the emulsion. The influx of water into the internal droplets results in the continuous thinning of the oil layer. Successive growth of

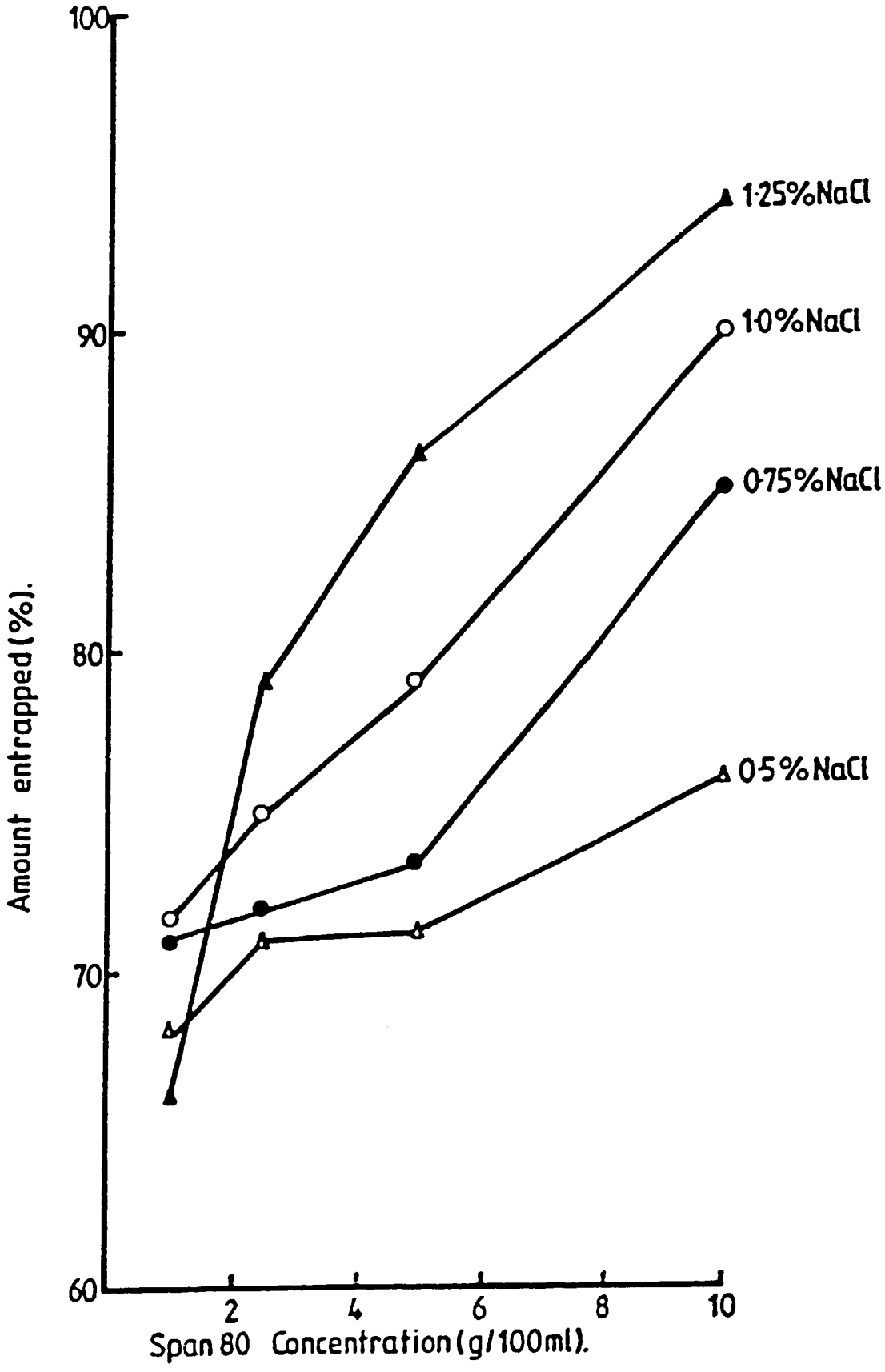


FIGURE 5-6
The effect of NaCls concentration in the inner aqueous phase on the entrapment efficiency of w/o/w emulsions prepared with 0.2% BSA in the internal aqueous phase and varying concentrations of the Span 80 in the isopropyl myristate phase.

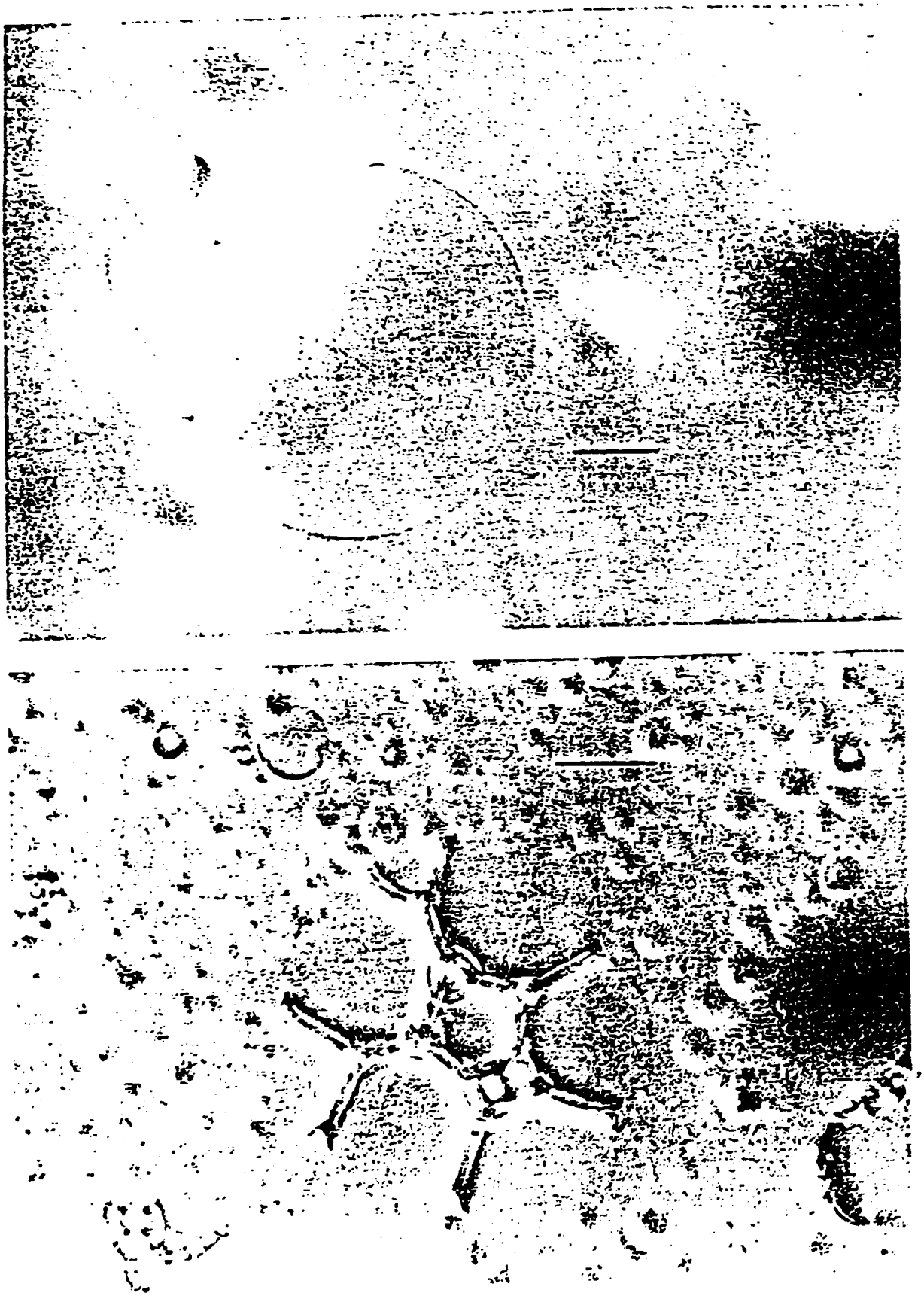


FIGURE 5-7
Upper: phase contrast photomicrograph of a swollen w/o/w emulsion containing 1.25% NaCl in internal phase after 4 weeks storage. 0.2% BSA was included in the internal aqueous phase of 1% Tween 80 in the octane (oil) phase. Lower: a similar system after aging for 4 weeks. Bar marker: 10 μm. The very thin external lamellae can be seen.

the internal droplets will depend on the elasticity of the interfacial film and the extent to which it could maintain a low interfacial tension as the interfacial area increases. Unstable systems will break down, thereby releasing the encapsulated salt to the external phase. Multiple oil droplets carrying such thin oil membranes were prepared by interfacial interaction between Span 80 and BSA and were found to be stable for several weeks (Figure 5-7).

C. Hydrophilic–Lipophilic Balance (HLB) Approaches

Multiple emulsions consist of w/o and o/w emulsions and require at least two stabilizing surfactants, a low HLB one to form the primary emulsion and a second higher HLB to achieve the secondary emulsification.[24] Migration of surfactant from one interface to the other on formation of the completed multiple system is one cause of instability. Solubilization of molecules of the primary (lipophilic) surfactant in the external aqueous phase when the concentration of secondary (hydrophilic) surfactant exceeds its critical micelle concentration can also contribute to instability. As the concentration of secondary hydrophilic surfactant increases, more of the primary surfactant may be incorporated into the secondary surfactant micelles, causing the concentration of primary surfactant at the interface to fall, and leading to rupture of the oil layer, which results in the loss of the internal aqueous drops. This has led to studies into the optimal HLB required for both primary and secondary emulsification steps in the formulation of multiple emulsions.

Inversion of multiple w/o/w emulsion to o/w emulsion was studied as a function of the HLB of the external emulsifier, its concentration, and droplet sizes.[24] Inversion was found to occur only when droplet size is reduced below a critical size or if the weighted HLB of the total emulsifiers present in the system approaches the required HLB of the oil phase. Magdassi and co-workers have studied the HLB shift caused by emulsifier migration to the external phase.[25] They observed the optimal HLB for multiple emulsions to be dependent on the concentration of both primary and secondary emulsifiers. A linear correlation was reported between the optimal HLB, the concentration of hydrophilic emulsifier, and the reciprocal concentrations of the lipophilic emulsifier.

When multiple emulsions were prepared with 0.7–1.5 wt% of secondary emulsifier, the yield of preparation did not change significantly (70–80%) at any HLB of the secondary emulsifier. However, at higher concentration of secondary emulsifier (>1.5 wt%), there was only one optimal HLB the yield of the multiple emulsion, decreasing with increase in secondary emulsifier.[20] The difference in behavior of the two multiple emulsions formed from the two concentrations was attributed to the significant difference in the multiple droplet size. Low concentrations of secondary emulsifier produced multiple droplets of high droplet size (low surface area) whereas high secondary emulsifier concentrations produced smaller multiple droplets (large surface area), which have a greater tendency of being expelled from the multiple oil drops.

In a later report, Magdassi et al. investigated the effect of emulsifier type on

the preparation and stability of multiple emulsions.[26] The influence the HLB of secondary emulsifier on the yield of preparation and stability was found to be different for each emulsifier. It is obvious from these studies that in optimizing a multiple emulsion formulation, both the HLB values and surface activities of the surfactant have to be considered.

IV MECHANISMS OF TRANSPORT OF SOLUTES

Mechanisms of transport of solutes from the inner aqueous phase to the external aqueous phase are not well understood. In emulsions where breakdown and coalescence of the internal droplets are minimized, diffusion of unionized materials represents the most important route of release and will be affected by the nature of the solute (its size, lipophilicity or affinity for the particular oil phase, and its dissociation constant). Other factors that can affect the release rate include the pH of the internal phase and the nature of the oil.

To deal with the kinetics of solute release from w/o/w emulsions, the emulsion is usually simplified such that the internal drops are assumed to form one large coalesced subdrop with the oil phase forming a spherical shell of thickness *(L)* around the internal phase. As both the internal drops and the multiple drop are in constant motion, the internal aqueous phase is assumed to be well mixed, transport of the solute from the bulk of internal drops to the w/o interface is considered to be very fast with the middle liquid membrane serving as a barrier against transport. Furthermore, the oil membrane thickness is assumed to be negligible compared to the drop radius, so that it may be considered that the mass transfer area is constant, i.e., uniform flat membrane thickness (Figure 5-8). The equation governing the release from a slab with a nonconstant source has the form[27]

$$M_r = \frac{M_\infty}{V_1 V_2}\left\{V_2 \exp\left[\frac{-ADK(V_1 + V_2)t}{LV_1V_2}\right] + V_1\right\} \qquad (5\text{-}1)$$

where M_r is the amount of drug retained in the internal drops, M_∞ is the initial amount of drug, A is the surface area of the multiple oil drops, K is the partition coefficient of the solute in the oil phase, L is the thickness of the liquid membrane, V_1 is the internal volume of the w/o/w emulsion, and V_2 is the sink solution volume. Since $V_2 \gg V_1$ the equation simplifies to

$$M_r = M_\infty \exp\left(\frac{-ADKt}{LV_1}\right) \qquad (5\text{-}2)$$

or

$$\log M_r = \log M_\infty - \left(\frac{ADKt}{2.303LV_1}\right) \qquad (5\text{-}3)$$

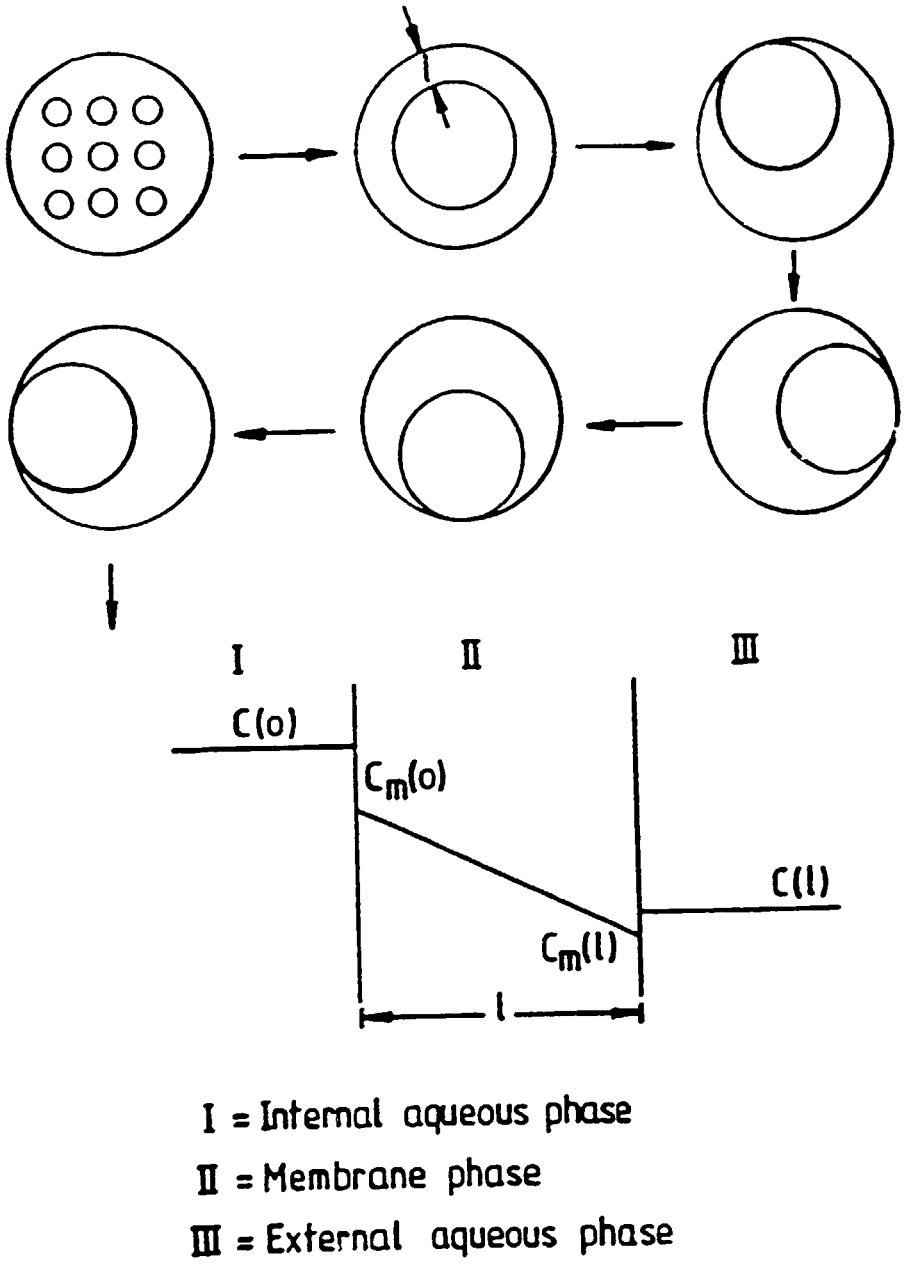


FIGURE 5-8
Uniform planar-sheet model of a w/o/w emulsions, in which internal droplets are assumed to have coalesced into one subdrop with the oil phase forming a spherical shell of thickness, l.

This equation is in the form of a first-order kinetic expression and permits evaluation of release rate constants. It can be seen that the release rate of entrapped solute from w/o/w emulsion will be affected by the diffusion coefficient, the partition coefficient of the solute in the oil phase, and the emulsion droplet size.

A. Micellar Transport

Simple diffusion accounts for the transport of substances between the two aqueous phases of w/o/w emulsions but is of itself insufficient to account for the observed facts particularly with transport of water and electrolytes. Transport in water-loaded inverse micelles and/or swollen micelles of both hydrophilic and lipophilic

surfactants has been suggested,[28] evidence of which was found by Florence and Whitehill.[1] The flux of water through the oil phase will affect the release of ionized species. The permeability of the oil layer of multiple emulsions to water has been studied by using osmotic flux produced by a solute to which the oil membrane is impermeable: this osmotic flux method yields an osmotic permeability coefficient.[29] Water flux through layers of isopropylmyristate and hexadecane has been measured using the microgravimetric method of Schatzberg[30] and Petersen.[31] The total flux (J_t) is the sum of the flux due to micellar transport (J_m) and that contributed by the molecular diffusion (J_s):

$$J_t = J_s + J_m. \tag{5-4}$$

If Fick's law is assumed Equation (5-4) can be written as

$$D_t \frac{dC_t}{dx} = D_s \frac{dC_s}{dx} + D_m \frac{dc_m}{d_x} \tag{5-5}$$

where D_m is the diffusion coefficient of the micelle, C_m is the increase in solubility due to the presence of surfactant, C_s is the solubility of water in the oil phase, and D_s is the molecular diffusion of water in the oil phase. It is assumed that the diffusion layer thickness (dx) is identical for each species. It can be seen that the steady mass loss of water through the oil layer depends on the amount of water carried by the micelles, on their saturation, and the rate of diffusion.

Higuchi[32] has discussed the concept of an effective diffusion coefficient (D_{eff}) that is particularly useful in a system with more than one diffusing species.

$$D_{eff} = \frac{D_s C_s + D_m C_m}{C_t} \tag{5-6}$$

where $C_t = C_s + C_m$. The water permeability P is given by

$$P = \frac{D_{eff} C_t}{L} \tag{5-7}$$

where L is the thickness of the oil layer. In the absence of surfactant the permeability coefficient of 8.29×10^{-9} cm sec^{-1} yields a value of diffusion coefficient for water in hexadecane of 4.10×10^{-5} cm^2 sec^{-1}. In the presence of Span 80, the permeability coefficient increases to 12.7×10^{-9} cm sec^{-1}, illustrating the additional contribution to transport of water of swollen inverse micelles. Addition of hydrophilic surfactants into the oil layer containing Span 80 significantly increased the flux of water (Figure 5-9). The presence of hydrophilic surfactant in combination with lipophilic surfactant in the oil phase would facilitate the formation of swollen mixed micelles,[33] which could solubilize water-soluble compo-

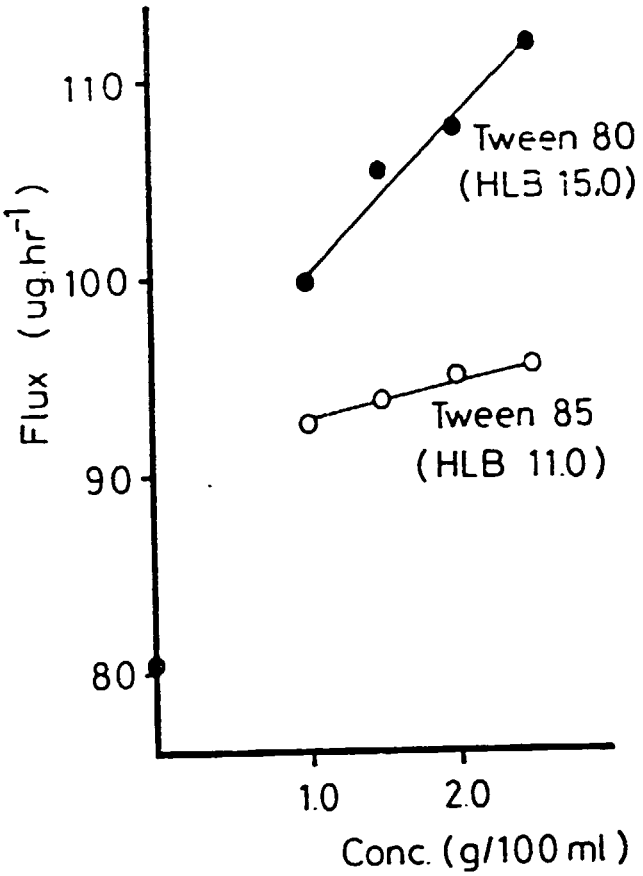


FIGURE 5-9
The influence of Span 80/Tween 80 and Span 80/Tween 85 combinations on the flux of water at 25° C through a thin layer of isopropylmyristate concentration of Span 80 kept constant.

nents. The large micellar units of both lipophilic and hydrophilic surfactants could act as carrier of water and ionized species.

B. Facilitated Transport

This mechanism of solute release involves the incorporation of some material into either the internal aqueous phase or membrane phase, which reacts with the permeating compound to render it soluble in the middle liquid phase. The carrier compounds facilitate the transport of the permeating compound across the membrane. This approach has found widespread use in hydrometallurgical recovery of metal ions.

Much of the published work in the literature deals with liquid membrane formulations for Cu^{2+} recovery. Such formulations usually contain an aqueous solution of sulfuric acid in the internal droplets that is emulsified in hydrocarbon solvent containing a common hydrometallurgical complexing agent such as β-

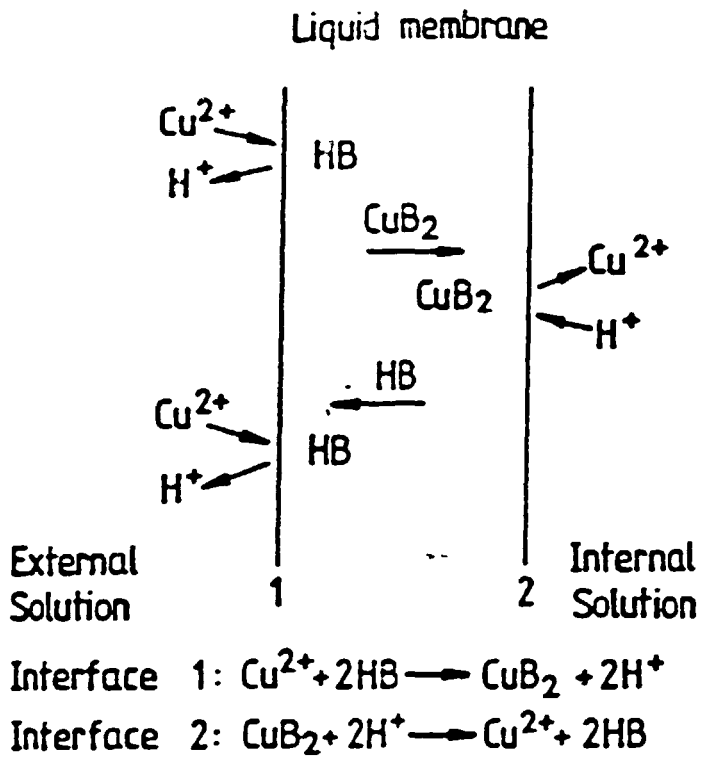


FIGURE 5-10
Simplified diagram of liquid membrane for copper extraction. From Ref. 35.

hydroxyoxime.[34] Other chelating agents for copper extraction have been reported: Kondo and co-workers[35] formulated liquid membranes for extraction of copper using liquid surfactant membranes containing benzoylacetone as a mobile carrier (Figure 5-10). Copper ions in the external aqueous phase diffuse toward interface 1 where the complex between copper ion and benzoylacetone was formed. Diffusion of the complex takes place in the membrane and on reaching the interface 2, a stripping reaction takes place to liberate copper ion into the internal aqueous phase. The rate-controlling step in the extraction procedure was considered to be the chelating complex formation and diffusion process in the aqueous phase close to the interface.[35]

V. *IN VIVO* STUDIES

Oil-in-water emulsion systems have been popular as dosage forms normally for oral administration of oils, as parenteral drug delivery systems, or in the form of cosmetics. Whereas w/o emulsions have high potential as vehicles for lipid-soluble materials and to provide a sustained release dosage system, their high viscosity, which makes them difficult to inject, has limited their use. Discrete depots may be formed and occasionally lesions appear after subcutaneous or intramuscular injection of the emulsions.[36] Multiple (w/o/w) emulsions have low viscosity and initially after injection will form diffuse depots as multiple oil drops; the external aqueous phase would be miscible with the body fluids and so dissipate leaving

globules of water in oil emulsion dispersed in the body fluids. Such systems might be expected to behave like w/o emulsions except that the drug released to the external phase during the secondary emulsification step would provide free drug for rapid release. Because of these apparent advantages w/o/w emulsions have potential to provide controlled release of antigenic material whose immunogenic response is enhanced by the presence of oil adjuvants and of anticancer drugs in cancer chemotherapy.

A. Formulation of Antigenic Material in w/o/w Emulsions

A sustained adjuvant effect due to delayed diffusion through the oil layer was reported when a w/o/w emulsion containing ovalbumin in physiological saline in the internal phase was inoculated subcutaneously into mice.[36] Mice treated with antigen in the multiple emulsion exhibited a slightly better response than those treated with w/o emulsion. Taylor and coworkers[2] studied in humans a comparison of the antibody response and reactions to aqueous influenza vaccine, influenza vaccine containing a mineral oil in simple emulsion, and influenza vaccine containing mineral oil as on multiple emulsion. The antibody response induced by the multiple emulsion preparation was found to be very satisfactory, greater than the response to the simple emulsion vaccine and substantially greater than the aqueous vaccine.

The stimulation by endotoxin encapsulated in a w/o/w emulsion of the nonspecific resistance of mice to bacterial infections was investigated by Hill and coworkers.[37] These workers attempted to encapsulate a bacterial endotoxin in a multiple emulsion in the hope that the administration of the endotoxin to mice would lead to reduced toxicity and a prolonged activity while maintaining the resistance of mice to bacterial infections. They found, however, that the multiple emulsion was as toxic as endotoxin dissolved in saline. This was attributed to the presence of nonencapsulated endotoxin in the external phase. While the protective action of the multiple emulsion was not observed, the multiple emulsion considerably enhanced the nonspecific resistance of mice to bacterial infections.

B. Cancer Chemotherapy

Emulsion systems have been used experimentally in cancer chemotherapy as a means of enhancing the uptake of anticancer agents into the lymphatic system.[38-42] Takahashi and co-workers investigated the possibility of selectively targeting 5-fluorouracil (5-FU) contained in o/w, w/o, and w/o/w emulsion systems to the lymph nodes.[43] The w/o/w and w/o emulsions were eight and three times more effective, respectively, than an aqueous solution of the drug. The same group of workers also found the concentration of bleomycin in the tumor tissue of rats was two to seven times as high after intratumoral injection of w/o and w/o/w emulsion than those produced by local administration of aqueous solution of the drug.[44] In rats bearing subcutaneous tumors AH-66, only trace amounts of bleomycin could be demonstrated in the tumor tissue 30 min after intratumoral

injection of an aqueous solution, whereas considerably higher concentrations of the antitumor agent were found in the tumor up to 3 hr following local injection in the form of an emulsion. In the clinical trials six of eight patients with either squamous cell carcinoma of skin or local recurrence of adenocarcinoma of the breast responded favorably to this treatment.

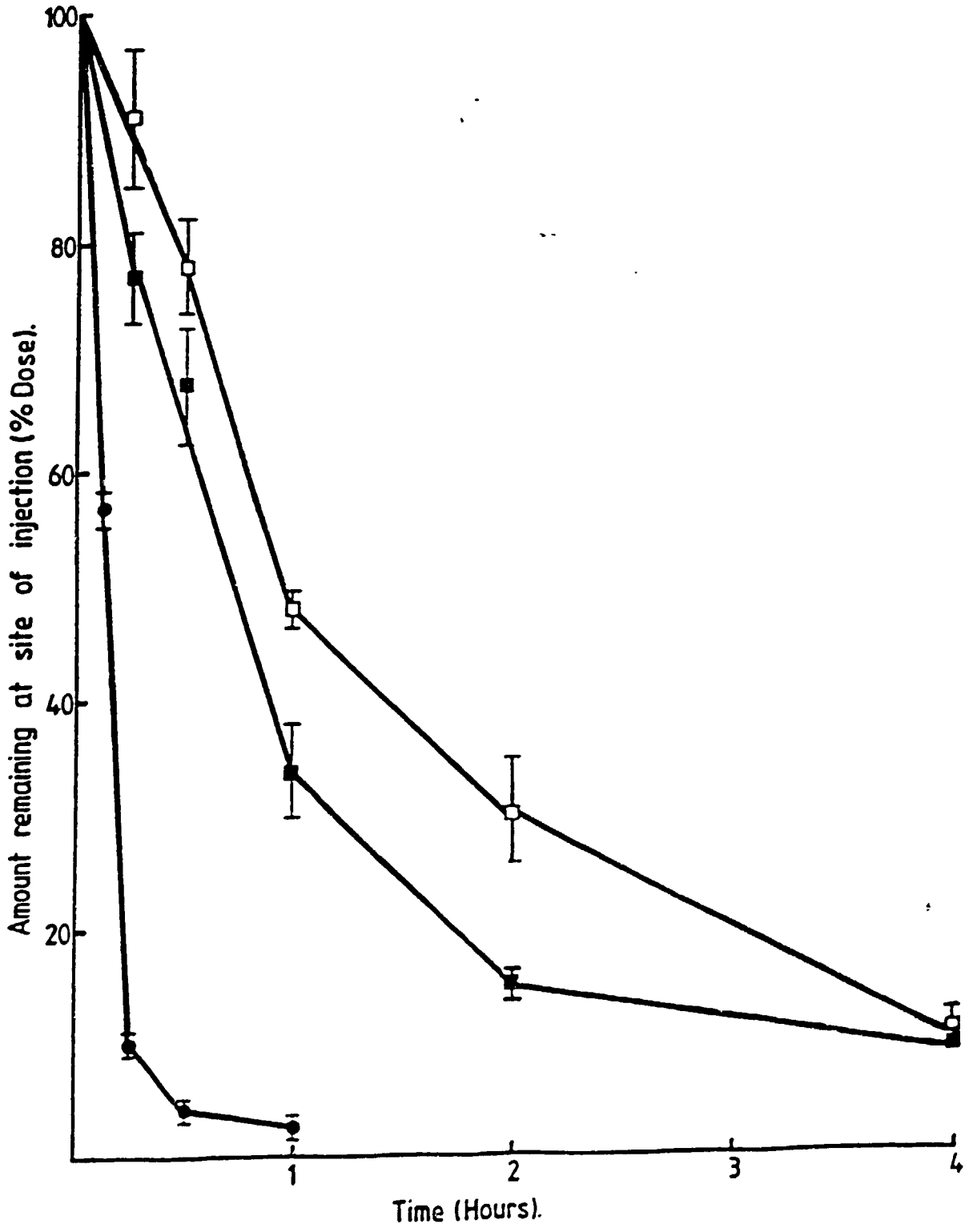


FIGURE 5-11
Disappearance of [5-[^{3}H]FU] radioactivity from the injection site following intramuscular injection of various formulations: (□) w/o emulsion prepared with hexadecane. (■) w/o/w prepared with hexadecane and (●) an aqueous solution of the drug.

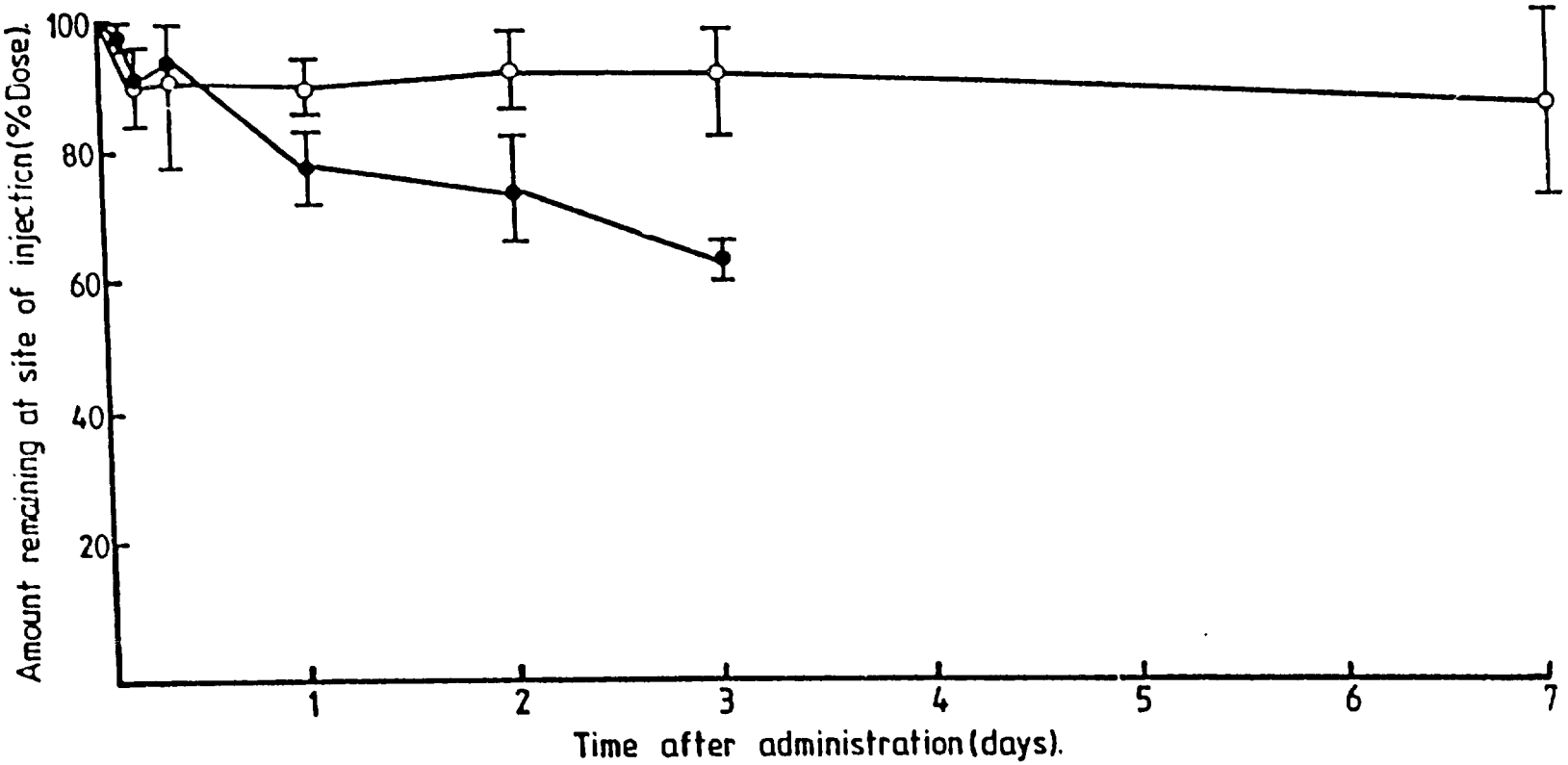


FIGURE 5-12
Effect of increasing Span 80 concentration in the oil phase on the clearance of [^{14}C]hexadecane w/o/w emulsions from injection sites following intramuscular injection; (O) 2.5% and (●) 10%.

Table 5-1
Studies with Drug Incorporation in Multiple Emulsions

Oil	Surfactant	Type	Drug	Test System	Reference
Mineral oil	Arlacel A, Tween 80	w/o/w	Ovalbumin	*In vivo*	36
Palmitic acid	Trioctanoin/sodium lauryl sulfate	w/o/w	Insulin	*In vivo*	46
Palmitic acid	Octyl-decyl triglyceride, sodium lauryl sulfate	w/o/w	Insulin	*In vivo*	47
Palmitic acid	Octyl-decyl triglyceride, sodium lauryl sulfate	w/o/w	Insulin	*In vivo*	48
Drakeol 6 VR	Arlacel A, Tween 80	w/o/w	Influenza vaccine	*In vivo*	2
Drakeol	Arlacel A, Tween 80	w/o/w	Endotoxin	*In vivo*	31
Peanut oil	Span 80, Tween 80	o/w/o	Naltrexone Tylmol	*In vitro*	49
Peanut oil	Span 65, Span 80/Tween 80	w/o/w	Naltrexone hydrochloride	*In vitro*	50
Light mineral oil	Span 80, Tween 20	w/o/w	Ephedrine hydrochloride	*In vitro*	51
Sesame oil	Span 80, Pluronic F68	w/o/w	5-[^{3}H]Fluorouracil	*In vivo*	43
Isopropylmyristate	Poloxamers/Triton X series	w/o/w	Sulfane Blue	*In vitro*	21
Isopropylmyristate	Poloxamers	w/o/w	Sulfonamides	*In vitro*	52
Isopropylmyristate	Span 80, Tween 80, Triton X-165, Pluronic F87, 88	w/o/w	Methotrexate	*In vitro*	53

Table 5-1 (continued)
Studies with Drug Incorporation in Multiple Emulsions

Oil	Surfactant	Type	Drug	Test System	Reference
Sesame oil Peanut oil	Sorbitan monooleate, Sorbitan sesquioleate	w/o/w	Cytarabine 5-fluorouracil	*In vitro*	54
Octane hexadecane dodecane, cyclohexane toluene Isopropylmyristate	Span 80, Tween 80	w/o/w	5-Fluorouracil	*In vitro*	15

Studies on methotrexate sodium formulated in a multiple w/o/w emulsion showed an enhanced therapeutic activity against L1210 leukemia compared with treatment with the drug in aqueous solution.[3] A single dose of methotrexate sodium formulated as a multiple emulsion tested in mice implanted with the R1 lymphoma was found to be more effective in preventing the death of mice, not only than a single aqueous injection of methotrexate sodium, but also of five daily aqueous injections of the drug at the same dose level.[5]

The present authors have studied the *in vivo* release of 5-fluoro[6-^{3}H]uracil from w/o/w emulsions stabilized by interfacial complexation. Comparative *in vivo* studies of aqueous solution, multiple w/o/w and w/o emulsions showed that formulating the drug in emulsion systems significantly sustained the release from intramuscular injection sites in the rat (Figure 5-11). Two other factors may contribute to the different release profiles observed between w/o/w and w/o emulsions: the higher viscosity of w/o emulsion and the presence of secondary hydrophilic surfactant that could accelerate the dispersion of w/o/w emulsions in the muscles.

Whitehill showed that the intestinal uptake of methotrexate was significantly increased when administered to mice in multiple w/o/w emulsions.[45] The presence of polysorbate 80 in the external phase of the emulsion was thought to account for the enhancement of absorption of methotrexate from the gastrointestinal tract.

C. Insulin Therapy

Another potential area of interest is in insulin and other protein/polypeptide drug therapy. It is known that insulin does not exhibit hypoglycemic activity when administered orally. The possible use of w/o/w emulsions to facilitate gastrointestinal absorption of normally nonabsorbed water-soluble biopolymers has been investigated in rats. Intraduodenal injection of a w/o/w emulsion containing insulin resulted in a significant hypoglycemic activity.[46]

The enteral absorption of w/o/w insulin emulsions in rabbits has been investigated. Multiple w/o/w emulsions containing 100 U/ml of insulin when administered to the jejunum at doses of 100 U/kg produced a significant and consistent increase in plasma insulin followed by a fall in blood glucose.[47] Oral administration of w/o/w emulsion containing insulin produced definite responses in three out of seven rabbits which indicated a possible means of protecting the insulin molecule from proteolytic destruction and for facilitating intestinal absorption of insulin. The same group of workers investigated the potential effectiveness of w/o/w emulsions as oral insulin preparations for short-term treatment of alloxan-diabetic rats.[48] A clear reduction in urinary glucose levels was observed in alloxan-diabetic rats that received insulin formulated in a w/o/w emulsion intrajejunally at a dose of 25–50 U/100 g body weight. Further studies are necessary to elucidate the possible interaction of surfactants, oil, and therapeutic agent. Table 5-1 summarizes some of the multiple emulsion systems that have been studied.

While problems of the long-term stability of multiple emulsions have nearly been solved, it is still unlikely that these systems will be able to produce prolonged release over more than about 6 hr although theoretically, because of the

slow dispersal of some oils from the musculature, (Figure 5-12) a system stabilized by polymers rather than surfactants might prolong the action of water soluble drugs. The traditional surfactant stabilizers, by forming micelles in the oil phase act as carriers that speed up drug loss from the depot.

However the protection these systems afford labile drugs from the environment and the protection afforded the tissues to irritant drugs, might be useful attributes. In addition the lymphotropic properties of these emulsions given orally might also be of value in the formulation of drugs that should be targeted to the lymphatics[55], regardless of any sustained-release potential the emulsions might possess.

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LABORATORY SESSIONS

Christopher R. Moran

UNIDO WORKSHOP

THE DEVELOPMENT OF ADVANCED PHARMACEUTICAL FORMULATIONS

22nd - 27th NOVEMBER 1992

PRACTICAL LABORATORY SESSION

Automatic Multi-Cell, Multi-Lambda Membrane Permeability System

Membrane Diffusion

When a system is at equilibrium, the chemical potential, u_i, of the system components is the same in all phases. If equilibrium does not exist then there will be flow from the phases of higher chemical potential to those where it is lower until the equilibrium has been re-established.

Diffusion is the process by which concentration differences are reduced by the flow of matter. Although the driving force for diffusion is the difference in the chemical potential of the components it is more usual to consider diffusion in terms of concentration gradients. Fick's first law states that the flow of matter is directly proportional to the concentration gradient, Equation (1).

$$J = \frac{-D.dc}{dx} \qquad (1)$$

where J = The Flux of a Component Across a Plane of Unit Area

D = The Diffusion Coefficient

$\frac{dc}{dx}$ = The Concentration Gradient

Fick's second law states that the rate of change of concentration in a volume element within the diffusional field is proportional to the rate of change in

the concentration gradient at that point in the field, Equation (2).

$$\frac{dc}{dt} = \frac{D.d^2c}{dx^2} \qquad (2)$$

When a permeable membrane is introduced between two phases which have differences in chemical potential, flow between the two phases must occur through the membrane phase. Simple diffusion through a membrane is governed by Fick's laws. When a substance enters a homogeneous membrane from an external phase it dissolves in the membrane material to form a thermodynamically stable mixture. The concentration of the substance within the membrane is well defined and determined by the activity of the substance in the external phase. This equilibrium solubility of a substance within a membrane can be expressed as the Partition Coefficient, K.

$$K = \frac{\text{Concentration of solute in membrane, } C_m}{\text{Concentration of solute in external solution, } C_0} \qquad (3)$$

The dissolved molecules are able to share in the molecular motion within the membrane phase and so undergo translation within it. When a particular solute has an affinity with the membrane phase, the concentration of that solute can increase over that in the external phase giving a K value of greater than 1. A K value of unity would indicate that the solute has an equal affinity to either phase and a K value of less than 1 would indicate a preference of the solute for the external phase or solution.

The diffusion of a solute through a membrane from one solution to another can be shown schematically by Figure 1.

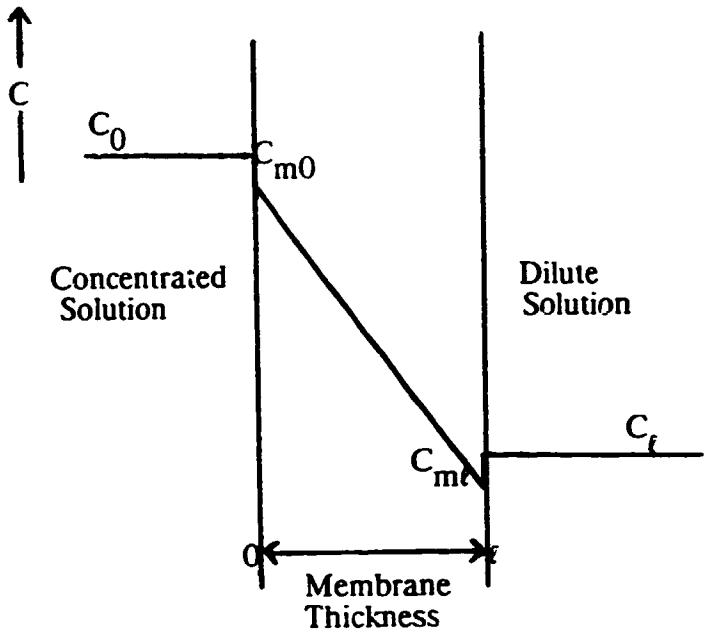


Figure 1. The diffusion of a solute through a membrane.

The flux through the membrane from Fick's first law is

$$J = \frac{D.(C_{m0} - C_{m\ell})}{\ell} \tag{4}$$

where C_{m0} = The Concentration at the Upstream Membrane Surface.

$C_{m\ell}$ = The Concentration at the Downstream Membrane Surface.

ℓ = The Membrane Thickness.

By using Equation (3), for the partition coefficient, K, of the solute between the two phases, the following relationships can be obtained

$$C_{m0} = K.C_0 \quad \text{upstream surface} \tag{5}$$

$$C_{m\ell} = K.C_\ell \quad \text{downstream surface} \tag{6}$$

The flux at steady state can be expressed as

$$J = \frac{D.K.(C_0 - C_\ell)}{\ell} \tag{7}$$

$$= \frac{D.K.\Delta C}{\ell} \tag{8}$$

where C_0 = The Upstream Bulk Concentration.

C_ℓ = The Downstream Bulk Concentration.

ΔC = The Concentration Difference between the two solutions.

The values of D and K are not often easily measured and, commonly, values of the Permeability Coefficient, P, are used where

$$P = D.K \tag{9}$$

Thus, the mass transfer of a solute across a membrane at steady state conditions can be expressed by Equation (10).

$$\frac{dMt}{dt} = \frac{A.D.K.\Delta C}{\ell} \tag{10}$$

where Mt = The Mass of Solute Transferred.

A = The Membrane Cross-Sectional Area.

From Equation (9)

$$\frac{dMt}{dt} = \frac{A.P.\Delta C}{\ell} \tag{11}$$

and by rearranging

$$P = \frac{(dMt/dt).\ell}{A.\Delta C} \tag{12}$$

Thus, we have an expression for the permeability of a membrane made up of readily measurable experimental parameters.

Figure 2. shows a typical mass transfer profile for the diffusion of a solute through a membrane. The time taken for the establishment of a constant rate of diffusion, or steady-state, is known as the Time Lag, τ. The time lag, τ, the membrane thickness, ℓ, and the diffusion coefficient, D, can be related by the following equation

$$\tau = \frac{\ell^2}{6.D} \qquad (13)$$

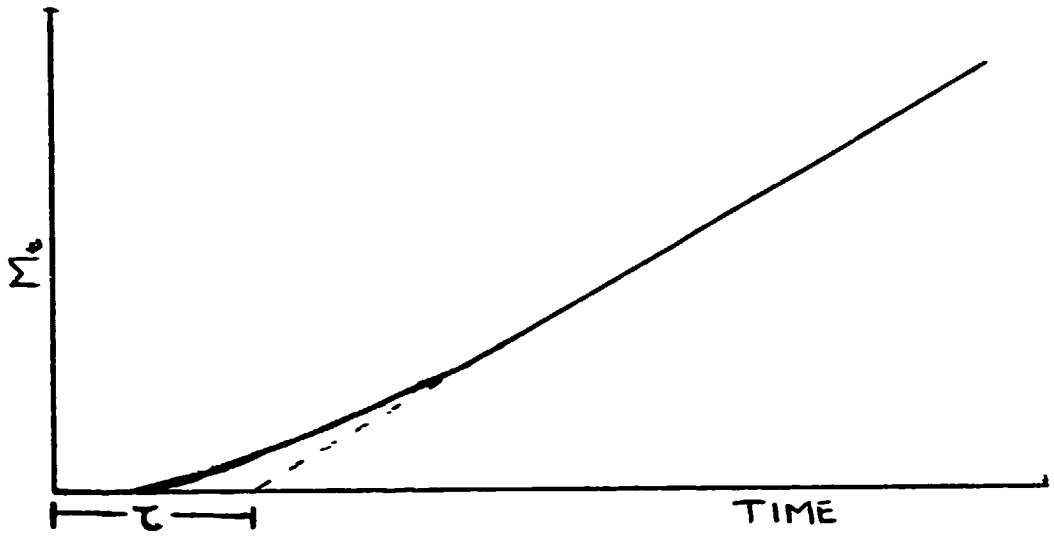


Figure 2. Typical mass transfer profile for the diffusion of a solute through a membrane.

By extrapolating the steady-state gradient back onto the time axis, a value for τ can be obtained. Using Equation (13), a value for the diffusion coefficient, D, can be calculated. The substitution of the values obtained for P and D into Equation (9) will yield a value for the partition coefficient, K.

The Automatic Multi-Cell, Multi-Lambda Membrane Permeability System

An automated 6 cell, multiwavelength diffusion system has been established in conjunction with Dr. R. Paterson's group at The University of Glasgow. The system consists of a series of up to 6 membrane diffusion cells coupled to a UV/Visible spectrophotometer and a computer which permits automatic logging of the data and calculation of the membrane permeabilities. The system provides a powerful and flexible tool for the analyses of up to 6 different sets of data at up to 6 different wavelengths (i.e. 6 different species can be analysed at one time).

The diffusion cell is of the Stagnant Point Flow design. The solution is introduced down a central flow pipe to impinge on the membrane surface, breaking up any stagnant layers before mixing back down the cell and leaving at the rear. The cell has a thermostatic jacket for temperature control. The cell is assembled with the membrane clamped between the two cell halves using a bull-clip. The exposed membrane area is 2.27cm^2 and the half-cell volume, 8.0ml.

Permeability experiments are carried out by firstly circulating distilled water or aqueous buffer solution around the downstream or sink side of the membranes. The downstream loops are passed through flow-cells in the UV spectrophotometer (Cecil, CE 5501 Double Beam UV/Visible Spectrophotometer). The spectrophotometer is fitted with a six-cell unit and an RS 232 interface which provides the link to an IBM PC. Solutions of the solutes under examination are introduced on the upstream or reservoir side of the membranes and the diffusion of solute down the concentration gradient for each cell is monitored by the UV spectrophotometer. The data is automatically collected by the computer, processed and displayed. The mass transfer profiles obtained for each diffusion experiment can be used to calculate Permeability Coefficients, Diffusion Coefficients, Partition Coefficients and Lag Times.

The rate of mass transfer of a solute is detemined from the gradient of the steady-state portion of the mass transfer profile and the permeability can then be calculated using Equation (12).

$$P = \frac{(dMt/dt).\ell}{A.\Delta C} \qquad (12)$$

For experimental purposes, the concentration gradient or difference, ΔC, is assumed to be equivalent to the concentration of the reservoir solution as the concentration of the sink is always negligible relative to the reservoir

concentration i.e. infinite sink conditions were assumed.

The gradient of the steady-state portion of the mass transfer profile can be used to calculate the time lag, τ, and consequently, values for the diffusion coefficient, D, and the partition coefficient, K, can be obtained. The sophisticated computer program enables this information to be calculated quickly and easily for up to six cells and six different solutes.

The automatic diffusion cell system is not only suitable for the investigation of our own and similar novel membrane materials but may be used for the evaluation of existing commercial membranes. The system is capable of carrying out continuous analyses over periods lasting from only a few minutes, up to several weeks.

Chris Moran
University of Strathclyde
November 1992

EXPERIMENTAL

The following experimental data can be entered into the computer and processed for the calculation of Permeability Coefficient, Diffusion Coefficient, Lag Time and Partition Coefficient. The operation of the automatic diffusion cell system will also be demonstrated.

DATA SHEET

Name of Operator	
Date	
Experimental Reference	UNIDO WORKSHOP
Solute	Guaiacol Glyceryl Ether
Molecular Weight of Solute	198.20
Solute Reservoir Concentration (mg/ml)	10.00
Solute Sink Volume (ml)	50.00
System Temperature ($^{\circ}$C)	37
System pH	5.0
System Flow Rate (ml/min)	20.00
Analysis Wavelength (nm)	273.4
Membrane Reference	PUUII/10%AMG/0.45μmHV
Swelling of Membrane (pph)	20.00
Membrane Thickness (cm)	0.015
Membrane Area (cm^2)	2.27

Experimental Data

POINT	TIME(s)	ABSORBANCE
1.	0.0	0.000
2.	313.0	0.003
3.	701.0	0.006
4.	1090.0	0.008
5.	1477.0	0.014
6.	1864.0	0.023
7.	2251.0	0.034
8.	2637.0	0.046
9.	3023.0	0.061
10.	3409.0	0.075
11.	3794.0	0.089
12.	4181.0	0.104
13.	4567.0	0.118
14.	4952.0	0.133
15.	5339.0	0.148
16.	5724.0	0.163
17.	6112.0	0.179
18.	6497.0	0.196
19.	6885.0	0.212
20.	7271.0	0.227
21.	7657.0	0.243
22.	8042.0	0.258
23.	8429.0	0.275

Dr. Marion E. McNeill

1. Measurement of the diffusion coefficient of proxyphylline in a fully hydrated poly(ethylene oxide) hydrogel.

Theory

The diffusion coefficient, D, of a water-soluble compound in hydrated hydrogels is a measure of permeability through water-swollen matrices based on Fick's Laws of Diffusion. Crank related the fractional release M_t/M_∞ of a solute from an infinite slab of thickness, ℓ, to the square root of time, $t^{0.5}$.

$$\frac{M_t}{M_\infty} = 4\left(\frac{Dt}{\pi \ell^2}\right)^{0.5} \quad \text{for} \quad \frac{M_t}{M_\infty} \leq 0.6$$

When $M_t/M_\infty = 0.5$, i.e. the half life time, $t_{1/2}$, this equation simplifies to

$$D = 0.0492\ell^2/t_{1/2} \ cm^2s^{-1}$$

These equations assume release of solute from the slab into an aqueous sink is uni-dimensional from the top and bottom surfaces and release from the edge is negligible.

The value of D depends on the molecular weight of the compound, any interaction between the solute and the polymer, the degree of hydration of the polymer, and the temperature.

Method

Hydrogel : PEO3800/1HT Composition, PEG $\bar{M}_n$ 3800, 1 mol 1,2,6 hexane triol/mol PEG and the stoichiometric equivalence of dicyclohexyl methane-4,4'diisocyanate.

Proxyphylline (Sigma), hydroxypropyl theophylline, MW238

Xerogel slabs a) thin 1.02 ± 0.02mm x 30mm x 42mm
b) thick 2.85 ± 0.05mm x 30mm x 41 ± 1mm

Samples a) were swollen in 6 mg/ml proxyphylline in water at 37°C to equilibrium.
Samples b) were swollen in 2 mg/ml proxyphylline in water at 37°C to equilibrium.

Caleva Tablet Dissolution Apparatus Model 88T conforming to US Pharmacopoeia XXI linked to Cecil UV Spectrophotometer 550 containing flow cells. Solution pumped by a Watson Marlow Peristaltic Pump 503U.

λ max for proxyphylline : 273nm
Volume of sink : 800 ml double distilled water
Temperature of sink : 37°C

a) Circulate distilled water through flow cell.
Set paddle speed to 60 revs/min.
UV spectrophotometer : Autozero at 273nm
Read out scale 1A, Chart speed 60s/cm.
Scan Mode : Time course, Enter time constant, e.g. 2.

Remove swollen slab from swelling solution. Blot surface dry with tissue. Drop into cell of Caleva. Press scan. When absorbance is changing slowly, stop scan. Remove outlet from Caleva cell. Once the tubing has emptied into the cell remove inlet. The following morning, measure the absorbance to determine M_∞. Calculate M_t from the A_t measurements for a range of times, t. Plot M_t/M_∞ vs $t^{0.5}$.

For proxyphylline 1A = 25.9 μg/ml at 273nm

Results

t (mins)	$t^{0.5}$ $(mins)^{0.5}$	A_t	M_t (mg)	M_t/M_∞ (%)

When M_t/M_∞ = 50% $t_{1/2}$ =
Xerogel thickness ℓ_d = 1.02mm

Swelling and expansion of the xerogel is isotropic and depends on the polymer composition and the temperature of the water.

for PEO3800/1HT at 37°C the linear swelling factor is 1.54.

Therefore swollen hydrogel thickness, ℓ = 1.02 x 1.54
= 1.57 mm
= 0.157 cm

$D = 0.0492\ell^2/t^1_{/2}$

= $x10^{-6}cm^2s^{-1}$

b) The effect of thickness on the half life time could be observed by repeating experiment a) with a slab 3 times thicker. The half life should then be 9 times longer than $t_{1/2}$ for a). In order to fit the second experiment which illustrates the effect of an initially dry polymer on the release profile of proxyphylline into the 2 hour laboratory session, experiment 1b) will be run on a sequential TIME PLOT program along with 2a) and 2b).

t (mins)	$t^{0.5}$ $(mins)^{0.5}$	A_t	M_t (mg)	M_t/M_∞ (%)

When M_t/M_∞ = 50% $t^1/_2$ =
Xerogel thickness ℓ_d = 2.85 ± 0.05
Swollen thickness ℓ = 2.85 x 1.54
= 4.39mm
= 0.439cm

$D = 0.0492\ \ell_2/t_{1/2}$

= x10^{-6}cm^{2}s^{-1}

2. Release of proxyphylline from a dispersion in a poly(ethylene oxide) xerogel in the form of a) a tablet and b) a sphere.

Theory

Drugs can be incorporated into hydrogels by swelling the polymer with a solution of the drug then evaporating the solvent leaving the drug substance trapped in the matrix as a uniform dispersion. When the xerogel containing the drug is reswollen in an aqueous fluid the drug dissolves in the penetrating water and diffuses along the aqueous pathways and across the device boundary to the surrounding fluid. The main effects compared to the fully swollen systems investigated in the first pair of experiments are prolongation of the period of release which can be seen by an increase in the half life time, and also a flattening of the release profile. The release kinetics can be described by the following equation.

$$\frac{M_t}{M_\infty} = kt^n$$

When n = 0.5 the diffusion is Fickian. This was illustrated in Experiments 1a) and 1b). When n = 1 the release is zero order i.e. constant. The release profile for Experiments 2a) and 2b) will lie between n = 0.5 and n = 1 and is termed anomalous. It should be possible to calculate the value of n from the data generated by the following experiments.

Method

a) Tablet shaped xerogel : 10mm diameter 3.05 ± 0.5mm thick.
Polymer composition : PEO4050/1.2HT

This composition has a higher $\overline{M}_n$ than the PEG3800 used in the swollen releases but this is compensated by a small increase in the proportion of crosslinker so that the 2 compositions have practically the same equilibrium water uptake of 186pph (parts per hundred parts dry polymer) at 37°C.

Weight of tablets : 280 ± 4mg
Swelling solution concentration : 30mg/ml
Swollen weight of tablets : 814 ± 11mg
Dried weight of tablets : 300 ± 3 mg

b) Spheres : 6mm diameter
Polymer composition : PEO4050/1.2HT
Weight of spheres : 141mg
Swelling solution concentration : 60mg/ml
Swollen weight of spheres : 433 mg
Dried weight of spheres : 143 mg

For both 2a) and 2b) and also 1b).

Volume of sink : 800ml
Temperature : 37°C
Paddle speed : 60 revs/min.

Circulate distilled water from 3 Caleva cells through 3 flow cells in the Cecil UV. Go to 273nm. Accessories program, Accessories, Autozero, Delay 10 minutes, Enter, Delay, Number of Cycles 25, Start (blue button). Collect data and final absorbance values from all 4 experiments, next day in order to calculate M_∞.

Results

Tablet design

t (mins)	$t^{0.5}$ $(mins)^{0.5}$	A_t	M_t (mg)	$\frac{100M_t}{M_\infty}$	t-t′ (mins)	ln(t-t′)	$\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}$	$\ln\left[\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}\right]$

When M_t/M_∞ = 50% $t'_{1/2}$ =

Estimated end of burst effect t′ =

Sphere

t (mins)	$t^{.5}$ $(mins)^{0.5}$	A_t	M_t (mg)	$\frac{100M_t}{M_\infty}$	t-t′ (mins)	ln(t-t′)	$\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}$	$\ln\left[\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}\right]$

When $M_t/M_\infty = 50\%$ $t_{1/2}$ =

Plot M_t/M_∞ vs time t for both the tablet and the sphere

Is there an initial burst from surface proxyphylline? If yes, decide on the transition time, t′, (possibly 10 minutes) when the release profile becomes anomalous.

Estimated end of burst effect t′ =

In order to calculate the exponent, n, which fits the release profile from t′ until t for 60% complete the tables for the tablet and the sphere, then plot

$$\ln\left[\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}\right] \quad \text{vs} \quad \ln(t-t')$$

$$\ln\left[\frac{100M_t}{M_\infty} - \frac{100M_{t'}}{M_\infty}\right] = k + n \ln (t-t')$$

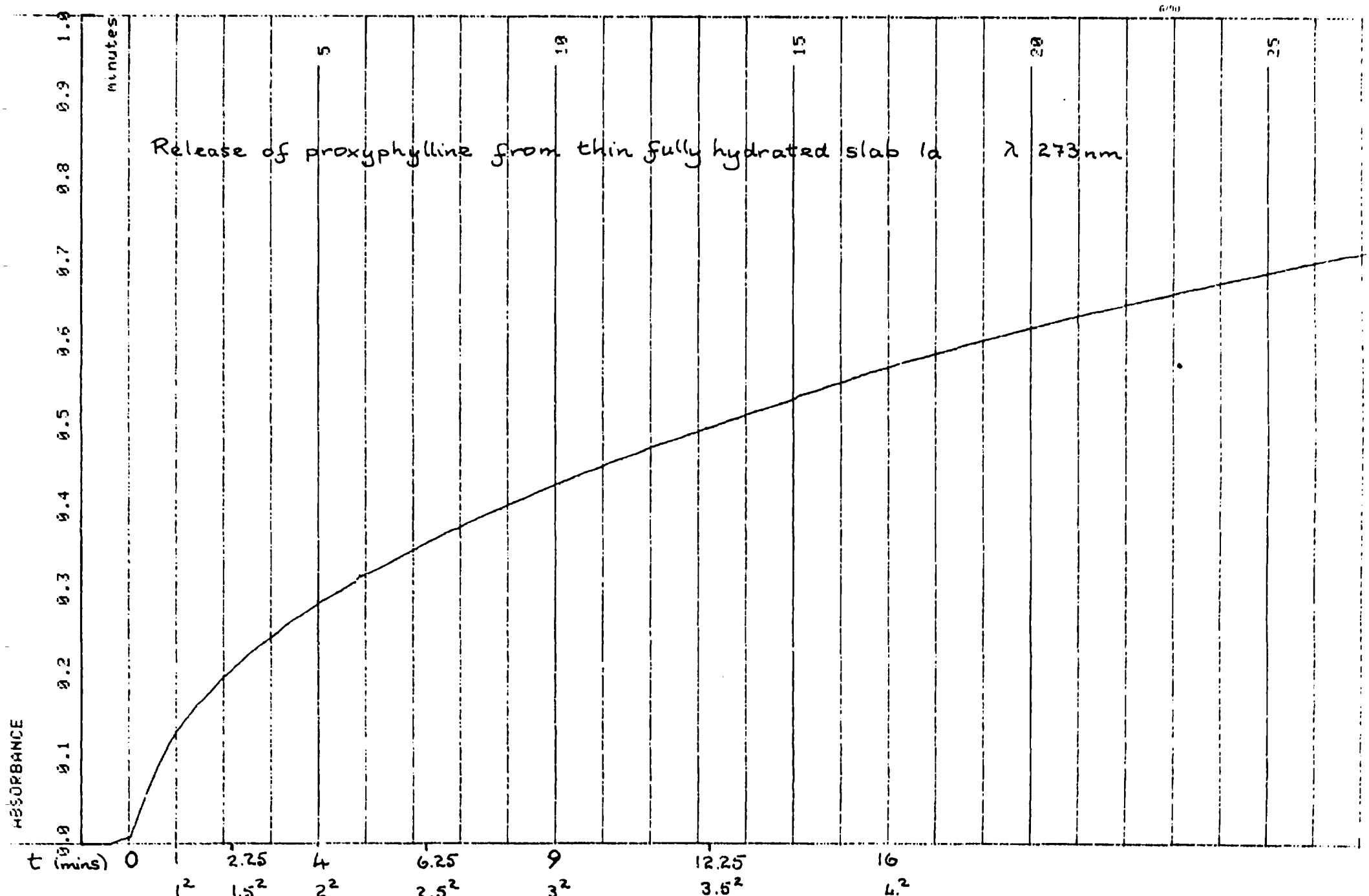
Determine the values of k and n from a regression program, or from the plot. n is the gradient and k the intercept.

Then $\frac{100M_t}{M_\infty} = \frac{100M_{t'}}{M_\infty} + k\,(t-t')^n$

For the tablet $\frac{100M_t}{M_\infty}$ = + (t-)

For the sphere $\frac{100M_t}{M_\infty}$ = + (t-)

CHART No. 5505 01 16



CECIL INSTRUMENTS CE5501 W DOUBLE BEAM SPECTROPHOTOMETER

CELL PROGRAM

Wavelength = 273.0 nm Bandwidth = 2.0 nm Cycle Delay = 10 minutes

Abs values

Sphere Tablet Thick slab

	CELL 1	CELL 2	CELL 3	TIME
CYCLE 1	0.000A	0.000A	0.000A	0h 0.0m
CYCLE 2	0.065A	0.064A	0.175A	0h10.0m
CYCLE 3	0.107A	0.109A	0.259A	0h20.0m
CYCLE 4	0.143A	0.147A	0.320A	0h30.0m
CYCLE 5	0.174A	0.180A	0.370A	0h40.0m
CYCLE 6	0.201A	0.209A	0.413A	0h50.0m
CYCLE 7	0.227A	0.239A	0.452A	1h 0.0m
CYCLE 8	0.250A	0.266A	0.488A	1h10.0m
CYCLE 9	0.272A	0.290A	0.520A	1h20.0m
CYCLE 10	0.292A	0.312A	0.550A	1h30.0m
CYCLE 11	0.312A	0.334A	0.579A	1h40.0m
CYCLE 12	0.331A	0.355A	0.603A	1h50.0m
CYCLE 13	0.349A	0.374A	0.627A	2h 0.0m
CYCLE 14	0.366A	0.393A	0.650A	2h10.0m
CYCLE 15	0.383A	0.411A	0.671A	2h20.0m
CYCLE 16	0.399A	0.428A	0.692A	2h30.0m
CYCLE 17	0.413A	0.446A	0.710A	2h40.0m
CYCLE 18	0.430A	0.462A	0.728A	2h50.0m
CYCLE 19	0.444A	0.477A	0.746A	3h 0.0m
CYCLE 20	0.458A	0.493A	0.761A	3h10.0m
CYCLE 21	0.471A	0.507A	0.776A	3h20.0m
CYCLE 22	0.483A	0.522A	0.789A	3h30.0m
CYCLE 23	0.497A	0.535A	0.805A	3h40.0m
CYCLE 24	0.508A	0.548A	0.817A	3h50.0m
CYCLE 25	0.520A	0.562A	0.829A	4h 0.0m

CECIL INSTRUMENTS CE5501 W DOUBLE BEAM SPECTROPHOTOMETER

CELL PROGRAM

Wavelength = 273.0 nm Bandwidth = 2.0 nm Cycle Delay = 0 minutes

Abs values

Sample 1 : readout: 1.041A λ: 273.0nm Thin slab t 17h $\sim A_\infty$

	CELL 1	CELL 2	CELL 3		TIME
CYCLE 1	0.831A	0.357A	1.054A	$\sim A_\infty$	t 16h0h 0.0m

Sam McFadzean

Theory of the Diaphragm Cell

In this example the permeant molecule is an ion. Two electrodes sensitive to this ion (iodide) are placed, one in the Top and the other in the Bottom half cell. The difference in electrical potential between these two electrodes is measured as a function of time during the diffusion experiment.

Theory requires that (in the steady state) the permeability of the membrane may be estimated by the equation:

$$\ln\left(\frac{\theta + r}{\theta - 1}\right) = \mathrm{P}\beta t$$

the symbols are defined in the theoretical development given below:

Proof Consider the Diaphragm Cell - in which the diaphragm is a test membrane

Nomenclature : C_B. C_T Concentrations of diffusant (bottom & top) (mol cm^{-3})

$\Delta C = C_B - C_A$

$\theta = C_B/C_A$

V_B. V_T Half cell volumes (cm^3)

A Area of diaphragm membrane (cm^2)

l Thickness " " (cm)

J Flux density (mol $cm^{-2}s^{-1}$) defined by Fick's Law

f Flux ($mols^{-1}$) = JA

D Diffusion coefficient of permeant (cm^2s^{-1})

Experimental Conditions :

1. The half-cell solutions are stirred continuously and are regarded as homogeneous.
2. We consider only the (pseudo) steady state in which there is a linear gradient across the

diaphragm.

3. Fick's Law applies in the membrane so that

$$J = D\frac{\Delta\overline{C}}{l} = \frac{D\alpha}{l}(C_B - C_T) \qquad (1)$$

where $\alpha = \Delta\overline{C}/\Delta C$ is the distribution coefficent for permeant between the membrane and solution phases.

In the steady state the loss of diffusant from the bottom half cell equals the gain in the top, so that defining the flux, f, from bottom to top

$$f = -\frac{dC_B}{dt}.V_B = +\frac{dC_T}{dt}.V_T \qquad (2)$$

$$\Delta C = C_B - C_T \qquad (3)$$

$$d\Delta C = dC_B - dC_T \qquad (4)$$

$$\frac{d\Delta C}{dt} = \frac{dC_B}{dt} - \frac{dC_T}{dt} = -f\left(\frac{1}{V_B} + \frac{1}{V_T}\right) \qquad (5)$$

from equation (1)

$$f = \left(\frac{DA\alpha}{l}\right)\Delta C = \mathbf{P}\Delta C \qquad (6)$$

from (5) & (6)

$$\frac{d\Delta C}{dt} = -\mathbf{P}\left[\left(\frac{1}{V_B} + \frac{1}{V_T}\right)\right]\Delta C$$

$(1/V_B + 1/V_T)$ is a constant - defined as β, the cell constant.

$$\int_{\Delta C_i}^{\Delta C_{t=0}} \frac{d\Delta C}{\Delta C} = -\mathbf{P}\beta \int_0^t dt \qquad (7)$$

since dx/x = dlnx, we obtain, on integration,

$$\ln \Delta C_{t=0} - \ln \Delta C = \mathbf{P}\beta t \tag{8}$$

at time zero $\Delta C_{t=0} = C_B^o$ in this experiment

$$\ln C_B^o - \ln \Delta C = \mathbf{P}\beta t \tag{9}$$

From the emf measured in this experiment we obtain the concentration ratio $C_B/C_T = \theta$

$$E = \frac{k}{n} \ln \theta \quad : \qquad \theta = \exp\left(\frac{nE}{k}\right) \tag{10}$$

where $k = RT/\mathbf{F}$

equation (9) deals with concentration differences $\Delta C = C_B - C_T$

We can obtain the final equation - if we remember that the system is sealed, so by conservation of diffusant (we neglect the very small amount of diffusant in the membrane).

$$C_B V_B + C_T V_T = C_B^o V_B \tag{11}$$

Let the volume ratio of the half cells be $V_T/V_B = r$. Rewriting eqn(11) in terms of Θ and r and rearranging gives:

$$C_T = \frac{C_B^o}{\theta + r} \tag{12}$$

and

$$C_B = \theta C_T = \frac{C_B^o \theta}{\theta + r} \tag{13}$$

From equations (3)(9)(12)(13), the working equation is obtained:

$$\ln\left(\frac{\theta + r}{\theta - 1}\right) = \mathbf{P}\beta t$$

PREPARATION OF LIPOSOMES

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PREPARATION OF LIPOSOMES

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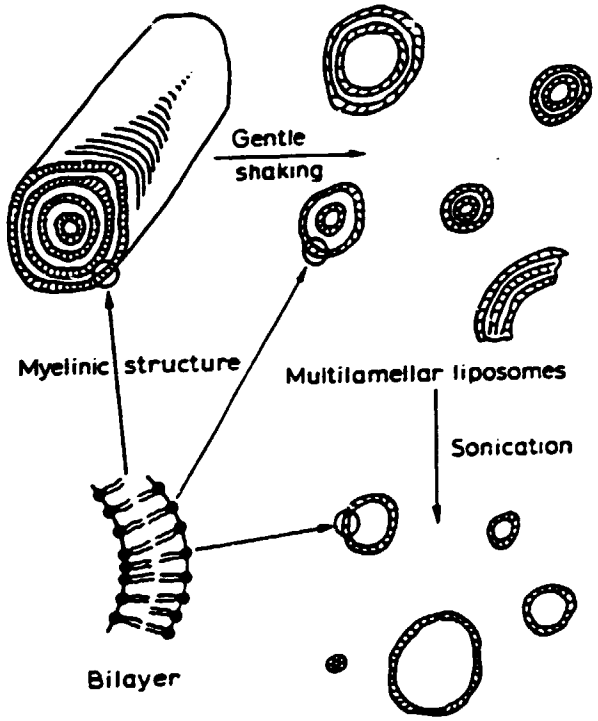
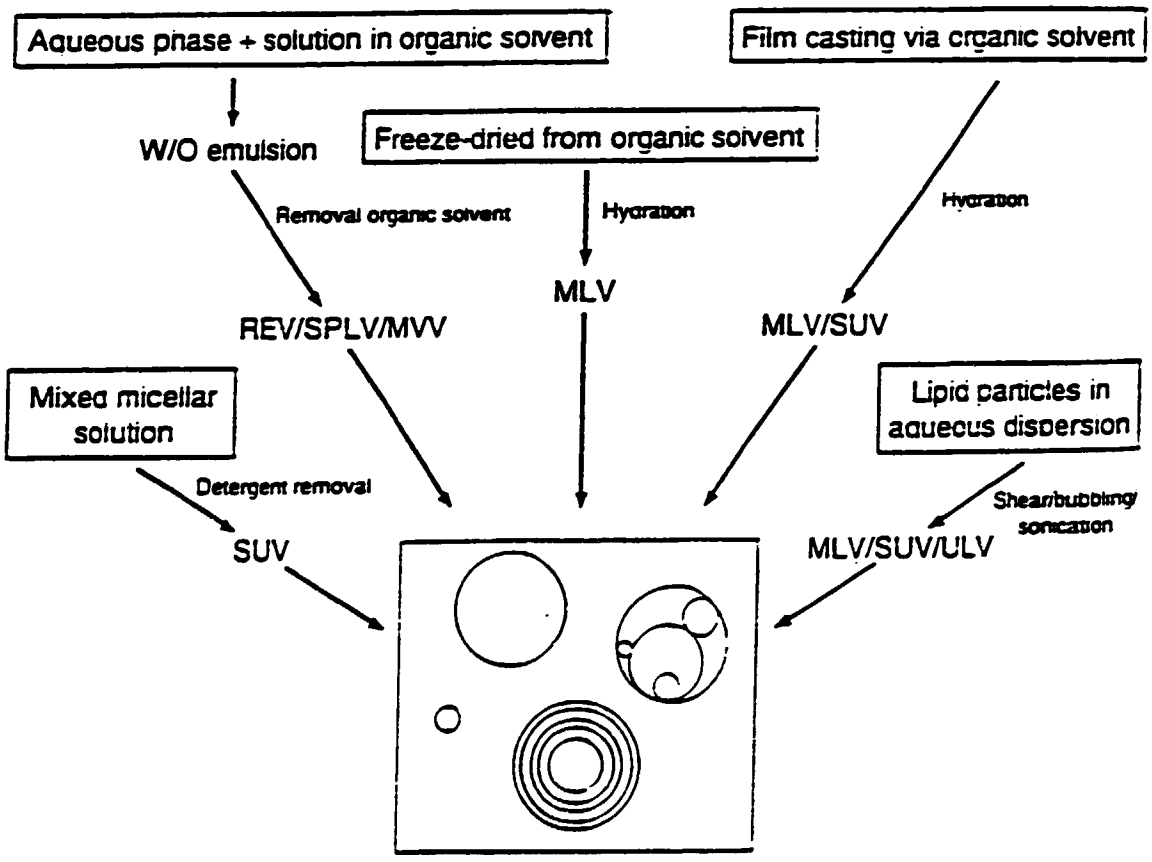
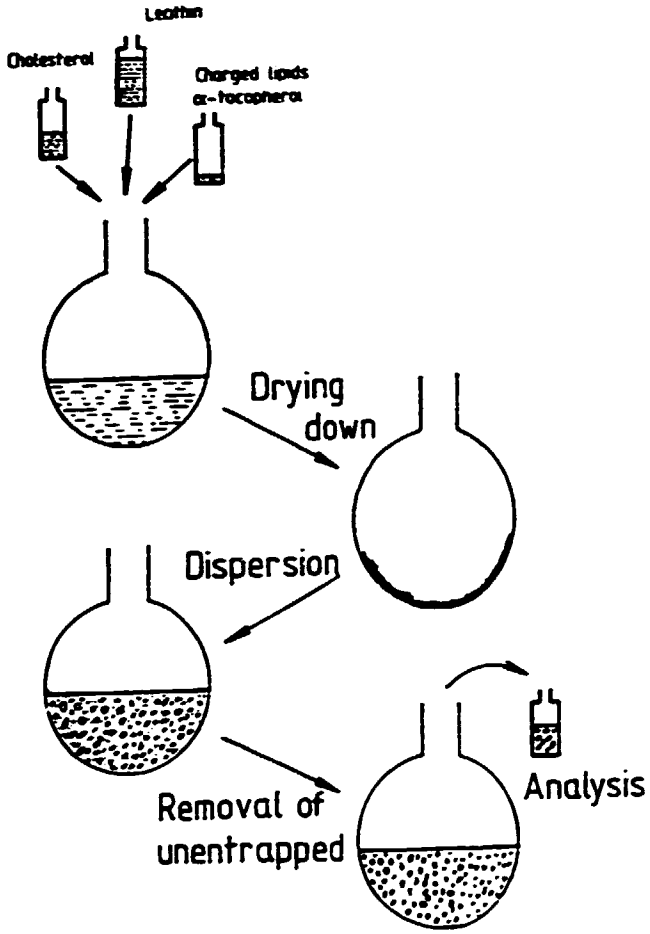
The general features involved at the molecular level in the preparation of liposome are shown in the following **Figure**



A summary of methods which are generally used for liposome preparation is given in this **Figure** . Some of these will be considered in more detail.

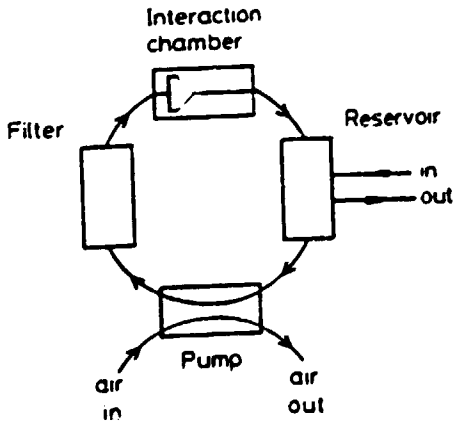


Perhaps the original method and a simple and staightforward one is the Dried-Film method illustrated in the diagram here.This is the method that will be demonstrated in the practical session. Several of the stages in this method are common to all methods e.g. removal of unentrapped drug and analysis and quality control.



Stages common to all methods of preparation of liposomes.

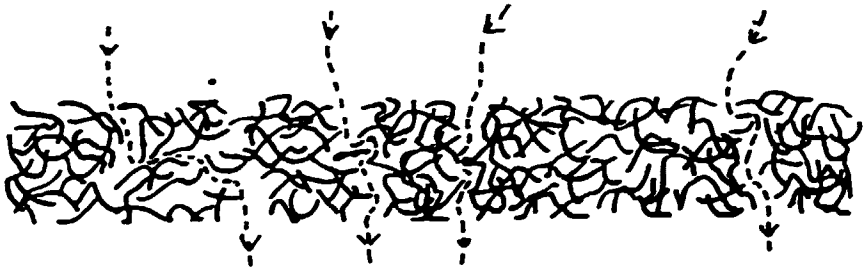
One method of producing small liposomes is shown in this **Figure** where a Micro-fluidiser is used.



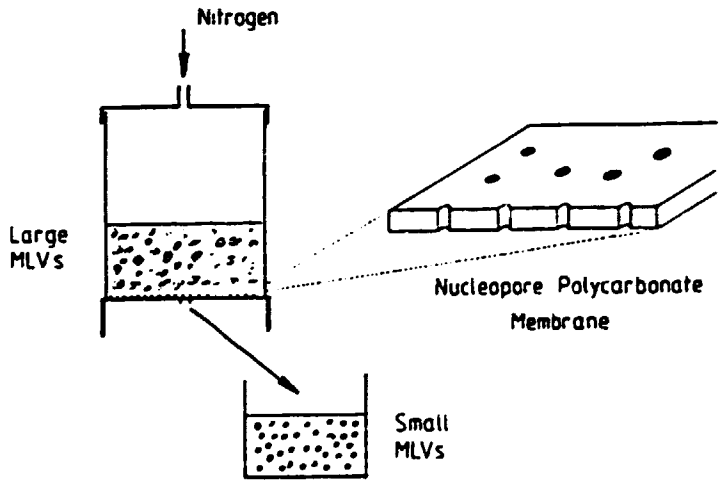
The suspension of large liposomes ,(either LUV's or MLV's) is pumped at high pressure into an interaction chamber:here,the flow is separated into two streams which collide with each other at high velocity.Large liposomes are broken down into smaller vesicles:these may be re-cycled throuh the system until the required size is obtained.

Nucleopore filter membranes have a pores of uniform diameter wheras normal Millipore type filters are of the tortuous path type as illustrated in the **Figure**

By extruding large liposomes through Nucleopore filters (perhaps several passes),liposomes of a similar size to the membrane pore size and with reasonable monodispersity can be obtained.



Tortuous path membrane. Compare the difference in size and shape of the pores of this membrane with that of the nucleation track membrane



Liposome sizing by extrusion through Nuclepore membranes.

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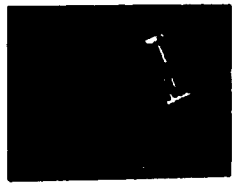

1- Clean the device with ethanol prior to use.

2- Insert the first half of the filter supporting system into the housing, leaving a few mm from the top edge.

3- Lay one or two filter(s) on the 'O' ring embedded in the filter support system.

4- Put the second half of the filter support system into the housing so that the "O" rings can be seen through the inspection hole.

5- Tighten the end caps by hand firmly to avoid twisting of the support system parts and to keep the dead volume as low as possible. The filter is pressed firmly between the "O" rings.

6- Hydrate and disperse the dry lipid in buffer to obtain multilamellar vesicles. Repeated freeze-thawing (e.g. 10 times) will improve the encapsulation efficiency.

7- Screw and press the syringe simultaneously to attach the syringe containing the prepared multilamellar liposomes to the luer in the filter support system. Usually 0.25-0.5 ml are being used. Repeat the test for higher quantities. By proper handling the total dead volume is only a few microlitres and reproducible

8- Pass the liposome emulsion through the filter(s), back and forth (usually 15-29 passes are sufficient).

9- Remove the liposomes from the opposite syringe to ensure that there are no unfiltered vesicles left.

References

LIFOCODE: [illegible]
AUTHORS: [illegible] macdonald rc/mecozzi s/subbarao nk/lee l
TITLE: small volume extrusion apparatus for preparation of large unilamellar vesicles
SOURCE: [illegible]

LIFOCODE: 91x8
AUTHORS: subbarao nk/macdonald rc/takeshita k/macdonald rc
TITLE: characteristics of spectrin-induced leakage of extruded phosphatidylserine vesicles
SOURCE: biochimica et biophysica acta/1063/91/147-154

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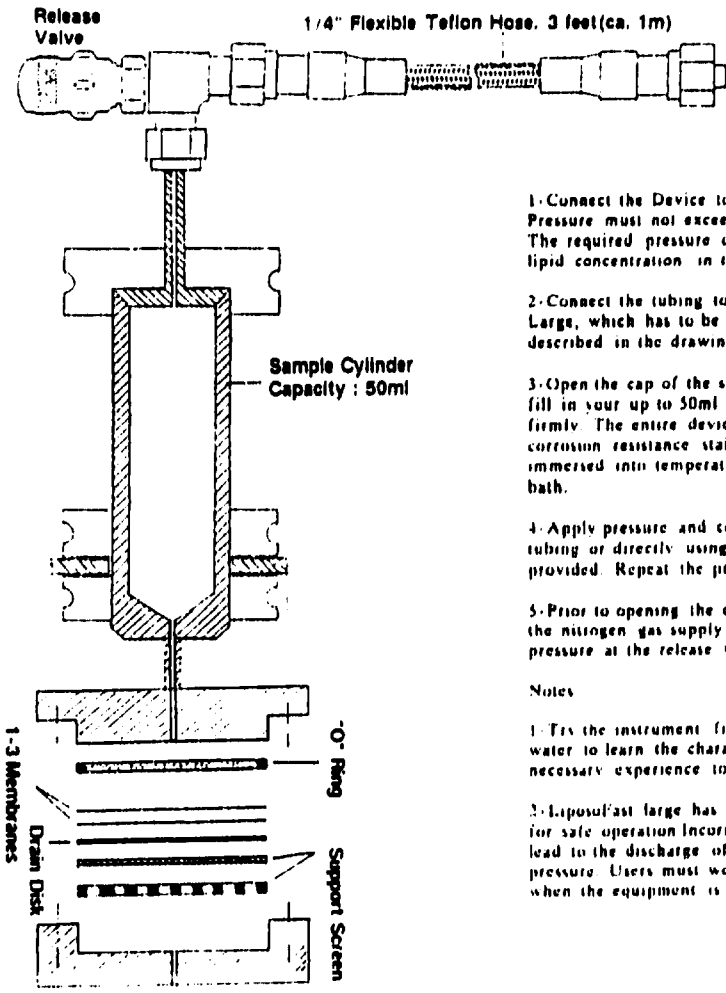

1- Connect the Device to the Nitrogen gas. Pressure must not exceed 800PSI (5.5 MPa) The required pressure depends mainly on the lipid concentration in the MLV emulsion.

2- Connect the tubing to your LiposoFast-Large, which has to be assembled as described in the drawing.

3- Open the cap of the sample cylinders and fill in your up to 50ml sample. Close the cap firmly. The entire device is made of corrosion resistance stainless steel and can be immersed into temperature controlled water bath.

4- Apply pressure and collect the sample via tubing or directly using the erlenmayer flask provided. Repeat the process 10-20 times

5- Prior to opening the cap, close the valve of the nitrogen gas supply and release the pressure at the release valve using wrench

Notes

1- Try the instrument first with distilled water to learn the characteristics and get the necessary experience to handle the steps

2- LiposoFast large has been manufactured for safe operation Incorrect operation may lead to the discharge of fluids under pressure. Users must wear eye protection when the equipment is being used

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Max-Planck-Institut fuer Biophysik, Frankfurt, Germany
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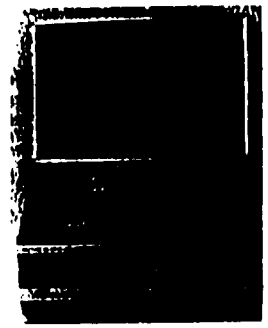

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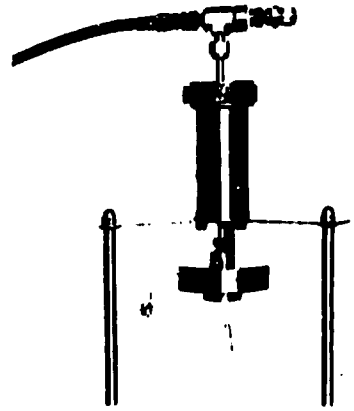
According to the following reference:

- MacDonald RC/MacDonald RI/Menco B Taleshita K/Subbarao NK/Hu L.
- Small-volume extrusion apparatus for preparation of large, unilamellar vesicles
- Biochimica et Biophysica Acta/1061/1991/297-303



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- Stainless Steel and corrosion resistence design Can be immersed in a temperature controlled water bath.
- Capacity : 50ml
- Simple and easy to use design

SIZING OF MICROSPHERES AND AEROSOLS

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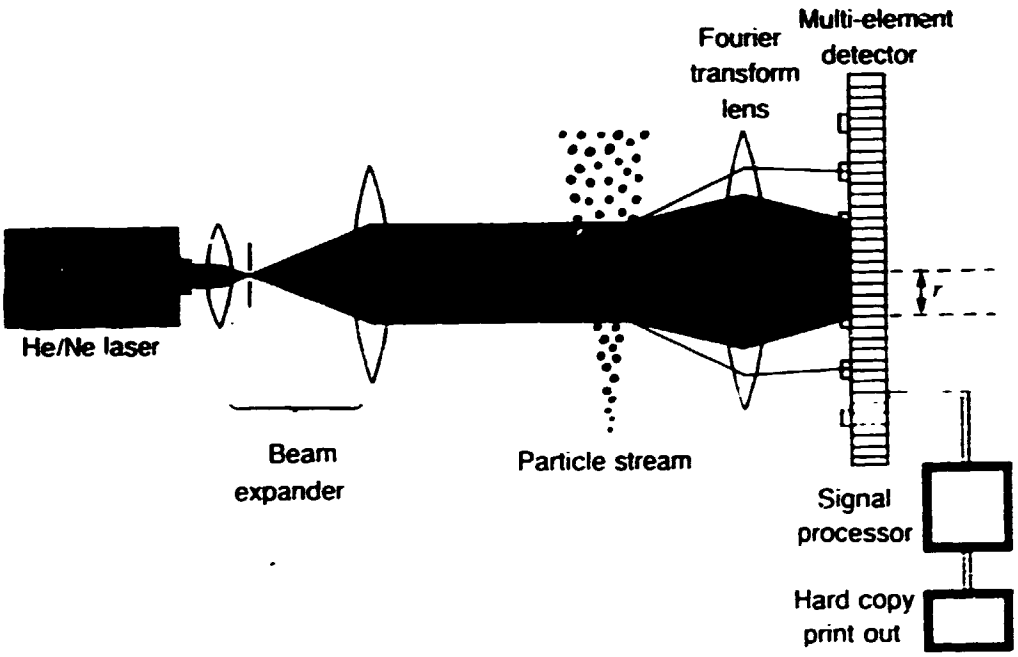
PARTICLE SIZE DETERMINATION OF AEROSOLS AND MICROSPHERES

For particles and droplets in the size range 2-1000 um the same method can be used:

MALVERN MODEL 2600 LASER DIFFRACTION PARTICLE SIZER

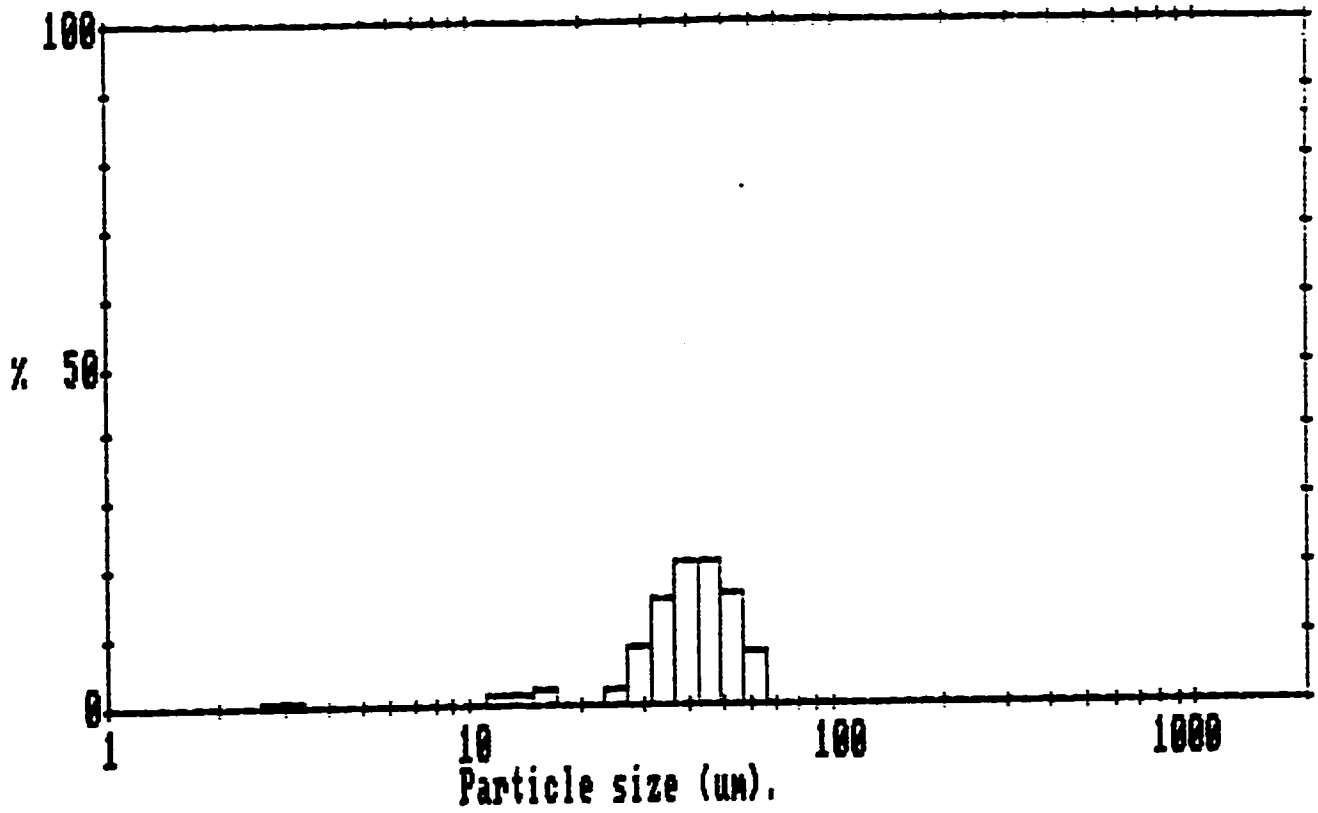
A diagram of the insrument is shown below.



Schematic diagram of the Malvern particle and droplet sizer

Once the sizer has been set up the following sequence of commands can be used to size both aerosols and suspensions of microspheres.

ALI	Align
MEA BAC	Measure background
INS SAM	Inspect Sample to adjust conc. of a suspension
MEA SAM	Measure sample (inject aerosol into beam)
CAL RES	Calculate results
SAVE DAT,1	
DIS RES	Display results
PLO HIS	Plot histogram
PRI	Print



Malvern Instruments MASTER Particle Sizer M3.1 Date 01-01-80 Time 02-55

Size microns	% under	Size band microns		%	Result source=Sample
					Record No. = 1
					Focal length = 63 mm.
118.4	100.0				Experiment type oil
54.9	89.9	118.4	54.9	10.1	Volume distribution
33.7	26.5	54.9	33.7	63.4	Beam length = 14.3 mm.
23.7	8.7	33.7	23.7	17.8	Obscuration =0.1400
17.7	8.3	23.7	17.7	0.4	Volume Conc. = 0.0103 %
13.6	4.2	17.7	13.6	4.1	Log. Diff. =4.58
10.5	2.5	13.6	10.5	1.8	Model indp
8.2	2.5	10.5	8.2	0.0	
6.4	2.5	8.2	6.4	0.0	D(v,0.5) = 40.4 µm
5.0	2.5	6.4	5.0	0.0	D(v,0.9) = 54.9 µm
3.9	2.4	5.0	3.9	0.0	D(v,0.1) = 26.0 µm
3.0	1.5	3.9	3.0	0.9	D(4,3) = 36.4 µm
2.4	0.0	3.0	2.4	1.5	D(3,2) = 23.6 µm
1.9	-0.0	2.4	1.9	0.0	Span = 0.7
1.5	-0.0	1.9	1.5	0.0	Spec. surf. area
1.2	-0.0	1.5	1.2	0.0	0.11 sq.m./cc.

Sample details:-ned ca2498v micronised ipa tween 80 probe sonicated 22/5/91

PREPARATION OF MICROSPHERES

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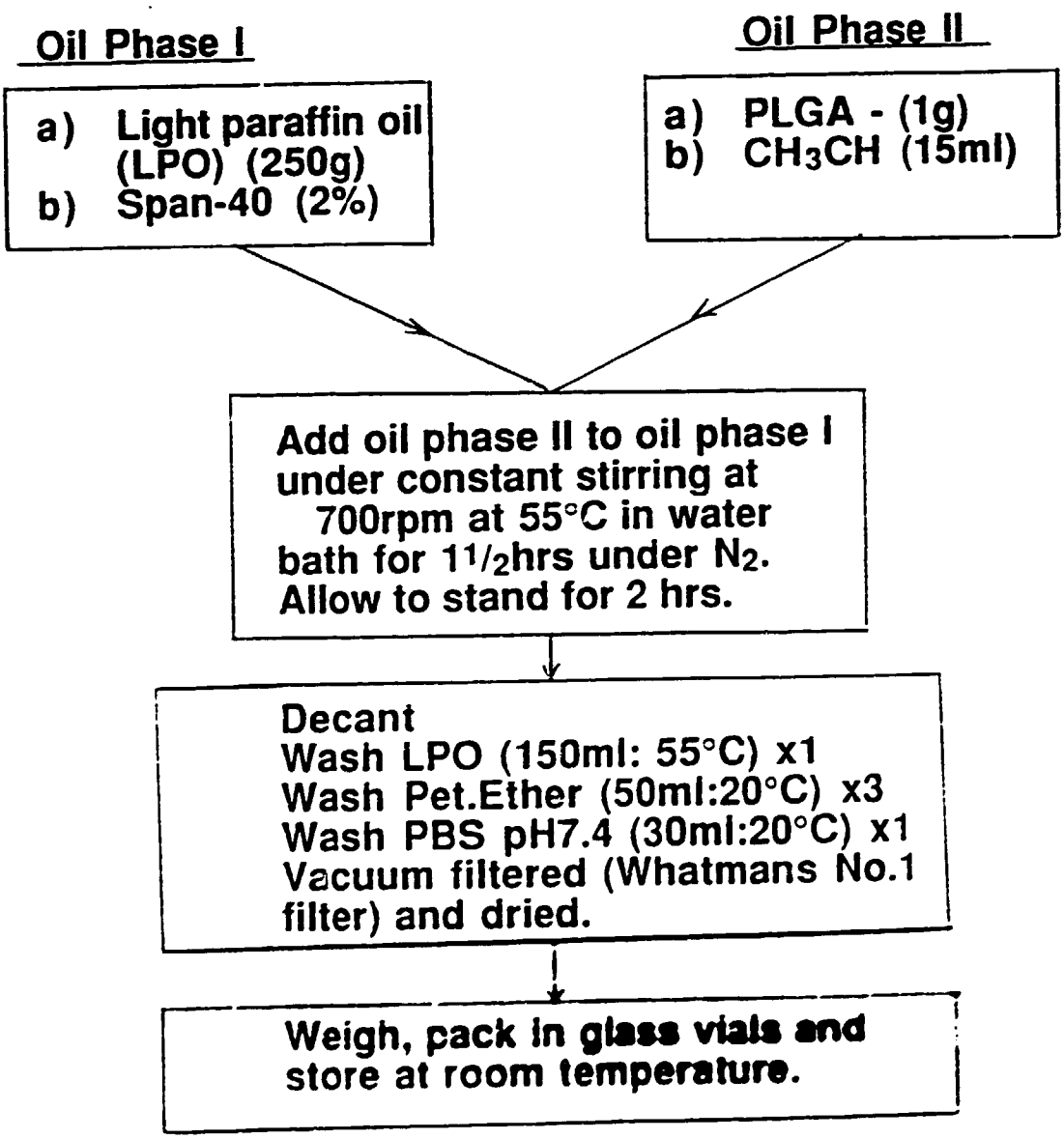
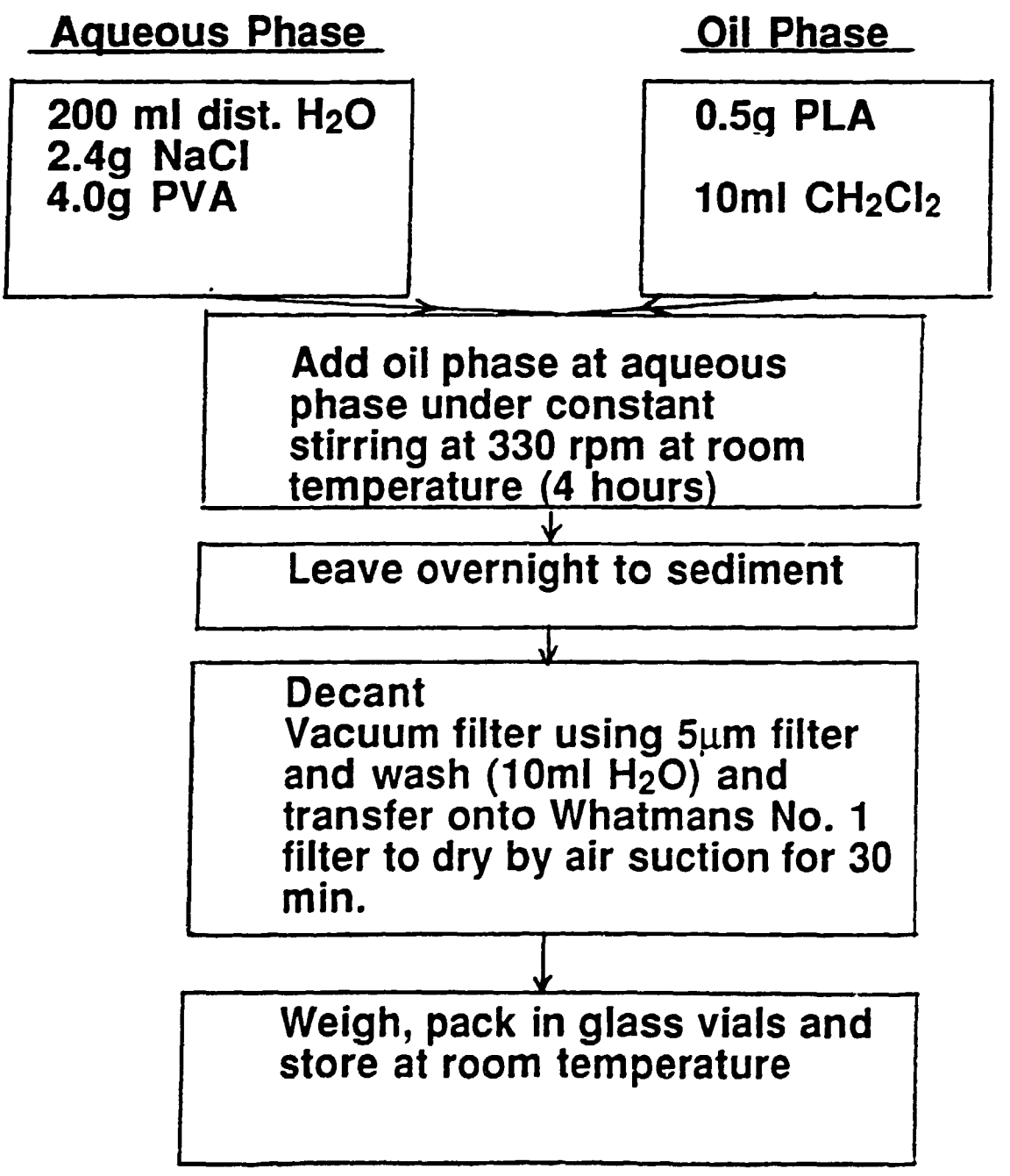
METHOD I. Oil-in-Oil

The oil-in-oil emulsion method (using acetonitrile + SPAN-40 in light liquid paraffin, stirring with Silverson at 690 rpm for 2 hours at 55°C under N2) yielded microspheres of adequate loading for the water soluble MMC but was not suitable for the preparation of small microspheres (<50μm).

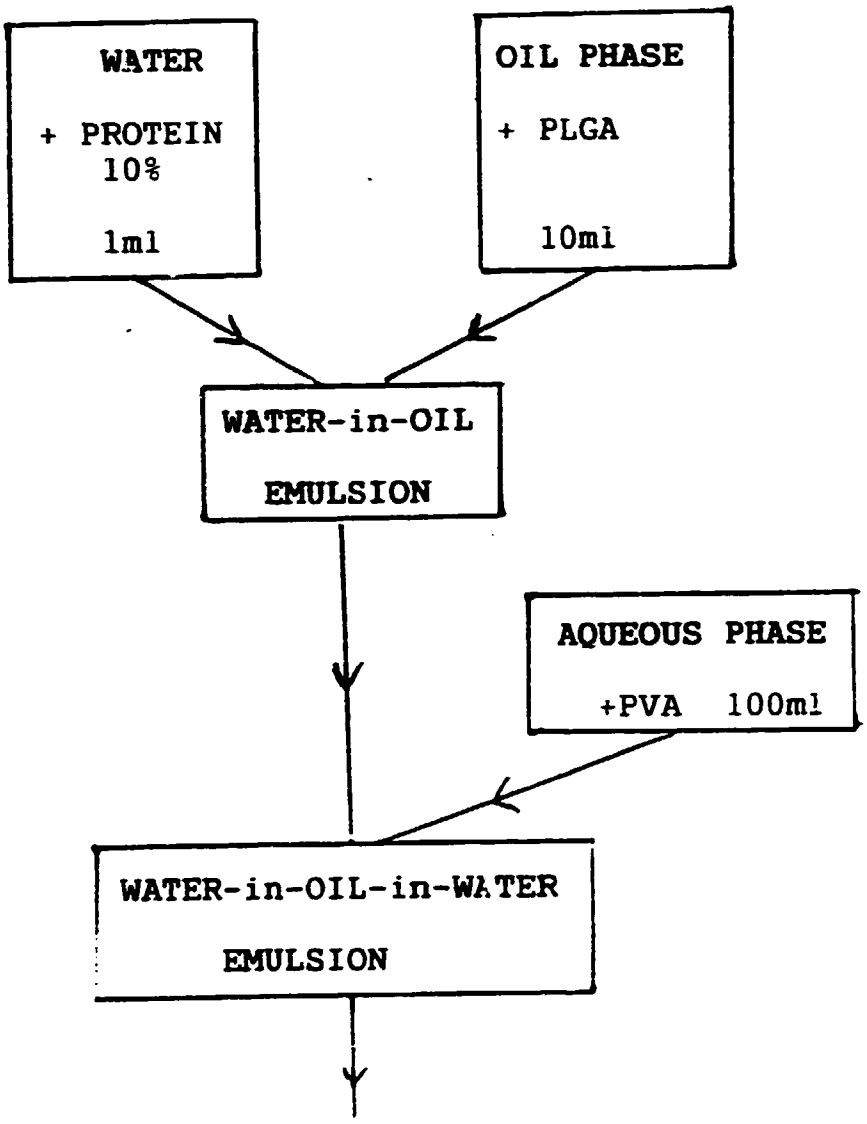


METHOD II. Oil in Water

The oil-in-water emulsion method not previously investigated for MMC (using dichloromethane, polyvinyl alcohol, and sodium chloride in water saturated with MMC stirred at room temperature with an overhead paddle at 330 rpm for 4 hrs) yielded microspheres of suitable size (40-50μm) and surface properties (*i.e.* hydrophilic) and of adequate loading of MMC. Solutions were decanted, filtered and dried under vacuum. Release rate studies were performed using U.S.P. Method II and HPLC assay.



METHOD III WATER-IN-OIL-IN-WATER



OIL IN OIL METHOD

ADVANTAGES	:	*good for water soluble drugs*
PROBLEMS	:	*difficult to prepare small microspheres*
	:	*aggregate in aqueous solutions due to hydrophobic surface*

OIL IN WATER METHOD

ADVANTAGES	:	*good for lipophilic drugs* *wide range of sizes readily prepared* *easily re-suspended (hydrophilic surface)*
PROBLEMS	:	*poor drug loading for water soluble drugs*

MICROENCAPSULATION WITH ETHYLCELLULOSE

SUSPENSION OF DRUG IN CYCLOHEXANE

AT 80^{O} PLUS

ETHYLCELLULOSE

cool slowly

↓

DRUG PARTICLES COATED WITH ETHYLCELLULOSE

↓

isolate, wash

DEMONSTRATION OF GAMMA-CAMERA

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