# Percutaneous delivery of thalidomide and its N-alkyl analogues for treatment of rheumatoid arthritis

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"Great works are performed not by strength

but by perseverance"

SAMUEL JOHNSON

To my parents

# Abstract


**Title: *Percutaneous delivery of thalidomide and its N-alkyl analogues for treatment of rheumatoid arthritis***

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease associated with high levels of tumour necrosis factor-alpha (TNF-α) in synovial fluid and synovial tissue (Saxne ***et al.,*** 1989). Thalidomide is a proven inhibitor of the biological synthesis of TNF-α (Sampaio ***et al.,*** 1991) and is believed to rely on this action for its suppression of the wasting of tissue which accompanies RA. Oral administration of thalidomide has proven to be effective in RA, but unacceptable side effects are easily provoked (Gutiérrez-Rodríguez, 1984). Administration of thalidomide *via* the dermal route can down-regulate TNF-α production in and around the affected joint, and this without raising the systemic blood level to a problematical level.

Based on thalidomide's physicochemical properties, it is unlikely that it can be delivered percutaneously at a dose required for RA. Therefore, we have embraced the idea of using N-alkyl analogues of thalidomide. The most important feature that an analogue of this compound might contribute is decreased crystallinity and increased lipophilicity. Ordinarily both these parameters should favour percutaneous delivery. The current study was primarily aimed at exploring the feasibility of percutaneous delivery of thalidomide and subsequently, three of its odd chain N-alkyl analogues (methyl, propyl and pentyl) *via* physicochemical characterization and assessment of their innate abilities to diffuse through skin as an initial step towards developing a topical dosage form for the best compound. The biological activities, more specifically their potential to inhibit the production of TNF-α was determined for thalidomide and its N-alkyl analogues.

In order to achieve the objectives, the study was undertaken by synthesizing and determining the physicochemical parameters of thalidomide and its N-alkyl analogues. A high level of crystallinity is expressed in the form of a high melting point and heat of fusion.

This limits solubility itself, and thus also sets a limit on mass transfer across the skin. Generally, the greater a drug's innate tendency to dissolve, the more likely it is that the drug can be delivered at an appropriate rate across the skin (Ostrenga ***et al.,*** 1971). Therefore, the melting points and heats of fusion were determined by differential scanning calorimetry. Aqueous solubility and the partition coefficient (relative solubility) are major determinants of a drug's dissolution, distribution and availability. N-octanol/water partition coefficients were determined at pH 6.4. Solubilities in water, a series of n-alcohols and mixed solvents were obtained, as well as the solubility parameters of the compounds in study. Secondly, *in vitro* permeation studies were performed from these solvents and vehicles using vertical Franz diffusion cells with human epidermal membranes. Thirdly, tumour necrosis factor-alpha (TNF-$\alpha$) inhibition activities were assessed for thalidomide and its N-alkyl analogues.

By adding a methyl group to the thalidomide structure, the melting point drops by over 100°C and, in this particular instance upon increasing the alkyl chain length to five $-CH_2-$ units the melting points decrease linearly. Heats of fusion decreased dramatically upon thalidomide's alkylation as well. Methylation of the thalidomide molecule enhanced the aqueous solubility 6-fold, but as the alkyl chain length is further extended from methyl to pentyl, the aqueous solubility decreased exponentially. The destabilization of the crystalline structure with increasing alkyl chain length led to an increase in lipophilicity and consequently an increase in solubility in nonpolar media. Log partition coefficients increased linearly with increasing alkyl chain length. Solubilities in a series of n-alcohols, methanol through dodecanol, were found to be in the order of pentyl > propyl > methyl > thalidomide. The N-alkyl analogues have more favourable physicochemical properties than thalidomide to be delivered percutaneously. The *in vitro* skin permeation data proved that the analogues can be delivered far easier than thalidomide itself. N-methyl thalidomide showed the highest steady-state flux through human skin from water, n-alcohols and combination vehicles. Thalidomide and its N-alkyl analogues were all active as TNF-$\alpha$ inhibitors.

Finally, active as a TNF-$\alpha$ inhibitor, N-methyl thalidomide is the most promising candidate to be delivered percutaneously for treatment of rheumatoid arthritis, of those studied.

Key Words: Percutaneous delivery; thalidomide; N-alkyl analogues; physicochemical properties; solubility; solubility parameter; tumour necrosis factor-alpha; lipophilicity; partition coefficient; rheumatoid arthritis.

# Opsomming


**Titel:** ***Perkutane aflewering van talidomied en N-alkiel analoë vir die behandeling van rumatoïede artritis.***

Rumatoïede artritis (RA) is 'n chroniese inflammatoriese gewrigsiekte wat geassosieer word met verhoogde vlakke van tumor nekrose faktor alfa (TNF-α) in sinoviale vloeistof en weefsel (Saxne ***et al.,*** 1989). Talidomied inhibeer die biologiese sintese van TNF-α (Sampaio ***et al.,*** 1991) en dit hou waarskynlik verband met die afname in weefselkwyning wat voorkom in RA. Daar is bewys dat orale toediening van talidomied effektief is in die behandeling van RA, maar veroorsaak egter ongewensde newe-effekte (Gutiérrez-Rodríguez, 1984). Deur talidomied *via* die dermale roete toe te dien, kan TNF-α produksie in en om die geaffekteerde gewrig verlaag word, sonder om sistemiese bloedvlakke problematies te verhoog.

Die fisies-chemiese eienskappe van talidomied veroorsaak dat dit nie in genoegsame hoeveelhede om RA te behandel perkutaan afgelewer kan word nie. Daarom is die N-alkiel analoë van talidomied oorweeg. Die belangrikste eienskap wat 'n analoog van talidomied kan bydra is verlaagde kristalliniteit en verhoogde lipofiliteit. Gewoonlik sal beide hierdie eienskappe perkutane aflewering verhoog. Die primêre doelwit van hierdie studie was om perkutane aflewering van talidomied te ondersoek. Hierna is die onewe ketting N-alkiel analoë (metiel, propiel en pentiel) gesintetiseer en gekarakteriseer en hul vermoë om perkutaan afgelewer te word, bepaal. Dit was 'n aanvanklike stap om 'n topikale doseervorm van die beste verbinding te ontwikkel. Die biologiese aktiwiteite van talidomied en die N-alkiel analoë is bepaal deur hul potensiaal om TNF-α produksie te onderdruk.

Om hierdie doelwitte te bereik, is die studie aangepak deur eerstens die fisies-chemiese eienskappe van talidomied en die N-alkiel analoë te karakteriseer. 'n Hoë vlak van kristalliniteit is aanwesig by verbindings met 'n hoë smeltpunt en smeltingshitte.

Dit beperk oplosbaarheid en stel dus 'n limiet aan massa-oordrag deur die vel. Hoe groter 'n geneesmiddel se aangebore neiging om op te los, hoe groter is die kans dat die middel teen 'n geskikte snelheid perkutaan afgelewer kan word (Ostrenga ***et al.,*** 1971). Gevolglik is die smeltpunte en smeltingshitte bepaal deur differensiële skanderingskalorimetrie. Wateroplosbaarheid en die verdelingskoëffisiënt (relatiewe oplosbaarheid) is beslissende faktore van 'n verbinding se dissolusie, distribusie en beskikbaarheid. N-oktanol/water verdelingskoëffisiënte is bepaal by 'n pH van 6.4. Oplosbaarhede in water, 'n reeks n-alkohole en oplosmiddel mengsels is bepaal, asook die oplosbaarheidsparameters van die verbindings. Tweedens is *in vitro* permeasie studies vanaf hierdie oplossings en draerstowwe uitgevoer deur gebruik te maak van vertikale Franz-diffusieselle en menslike epidermis. Derdens is talidomied en die N-alkiel analoë se TNF-$\alpha$ inhiberende aktiwiteite bepaal.

Deur 'n metielgroep aan die talidomiedstruktuur te heg het die smeltpunt gedaal met meer as 100°C. In hierdie bepaalde geval het die smeltpunte lineêr verlaag deur die alkielketting tot vyf $-CH_2-$ eenhede te verleng. Die smeltingshitte het ook drasties gedaal deur talidomied te alkileer. Deur die talidomied molekule te metileer, het die wateroplosbaarheid ses keer verhoog, maar deur die alkielketting te verleng van metiel tot propiel, het die wateroplosbaarheid eksponensieel verlaag. Die destabilisering van die kristallyne struktuur met toenemende alkielkettinglengte, het gelei tot 'n toename in lipofiliteit en gevolglik 'n toename in die oplosbaarheid in nie-polêre media. Die log verdelingskoëffisiënt neem lineêr toe met 'n toename in alkielkettinglengte. Die oplosbaarhede in 'n reeks n-alkohole, metanol tot dodekanol, is in die orde van pentiel > propiel > metiel > talidomied. Die N-alkiel analoë het beter fisies-chemiese eienskappe as talidomied om perkutaan afgelewer te word. Die *in vitro* vel permeasie het bewys dat die analoë beter afgelewer kan word as talidomied. N-metiel talidomied het die hoogste gelykvlak fluks deur mensvel vanaf water, n-alkohole en kombinasies van drastowwe getoon. Talidomied en die N-alkiel analoë is almal as TNF-$\alpha$ inhibeerders aktief.

Uit hierdie studie volg dat N-metiel talidomied die mees belowende kandidaat is om perkutaan afgelewer te word vir die behandeling van rumatoïede artritis.

Sleutelwoorde: Perkutane aflewering; talidomied; N-alkyl analoë; fisies-chemiese eienskappe; oplosbaarheid; oplosbaarheidsparameter; tumor nekrose faktor alfa; lipofiliteit; verdelingskoëffisiënt; rumatoïede artritis.

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# Chapter 1

# *Introduction and statement of the problem*

Thalidomide is a piperidinedione hypnotic and is derived from a natural endogenous α-amino acid (glutamic acid). It is described as a *N*-phthaloyl-glutamic acid imide, and the chemical name is α-(*N*-phthalimido) glutarimide. It was developed in the 1950's as an exciting example of a new class of non-barbiturate sedatives and was thought to be the safest sedative ever discovered. Unfortunately, it caused strange birth defects in infants born to women who had taken thalidomide during pregnancy. It was withdrawn from the market in 1961, but still remained available in certain countries for defined research purposes under strict control. Despite its history, thalidomide is currently being used clinically for more than 20 different indications in North America (it is still banned in Europe and Japan) (Stirling ***et al.,*** 1997). Most of the conditions being treated have existing anecdotal data to support the use of the drug. The main areas of current clinical application are listed in Table 1-1. There is understandably a definite scare factor with thalidomide, but this should not impede its use. The real tragedy of thalidomide, however, is not that it can cause birth defects but that this property of the drug was not known in the 1950's, when thalidomide was prescribed in good faith for pregnant women. Many drugs in common use today can cause birth defects, and many of these are used by women in their childbearing years. However, there is no great outcry over the use of these drugs because prescribing physicians and their patients are aware of the dangers they pose and therefore special precautions can be made.

The discovery of thalidomide's activity, other than sedation was entirely serendipitous. In 1964, Sheskin, an Israeli physician, noted that thalidomide produced not only the desired sedating effects but also improved one of the more debilitating aspects of leprosy, namely ENL (erythema nodosum leprosum), a severe inflammatory condition (Sheskin, 1965). Sheskin's discovery has been largely confirmed by leprologists worldwide and today, thalidomide is recognized as the single most effective agent against ENL, and the drug is used world-wide for this indication.

It was only relatively recently that the drug's mechanism of action in ENL was uncovered, and the therapeutic potential of thalidomide in other chronic inflammatory disorders appreciated. Dr. Gilla Kaplan, a scientist at the Rockefeller University (Sampaio ***et al.,*** 1991), established that thalidomide's therapeutic effects in ENL are due to the drug's ability to reduce levels of tumour necrosis factor-alpha (TNF-α). Through the efforts of Dr. Kaplan, we now know that thalidomide is an effective and substantially selective suppressor of the production of TNF-α. It doesn't totally inhibit, but significantly down-regulates production of this cytokine, mainly by peripheral blood mononuclear cells (PBMC's), when stimulated with an appropriate agonist, e.g., microbial lipopolysaccharide (LPS). It accomplishes this seemingly without affecting the production of other essential cytokines. TNF-α is one of a number of cytokines that is essential to immunological responses in which inflammation is observed (Old, 1987). It is produced by a variety of cell types, notably mononuclear cells (macrophages and monocytes), immune system cells which are principal effectors of inflammation. Though inflammation is the normal immune system response to infection or injury, serving to rid the body of foreign agents and to clear wounds of dead and dying tissue, chronic or high levels of TNF-α lead to either a persistent or an overly robust inflammatory response. For example, elevated levels of this cytokine are known to be responsible for the wasting of tissue that occurs in ENL. For the same reason it is effective in treating ENL, it appears thalidomide might also be useful in treating other diseases where tissue wasting is part of the disease expression. Consequently, TNF-α's protracted presence has been tied directly to the debilitating symptoms of infectious diseases, and to certain immune-related disorders, such as rheumatoid arthritis (RA). This fact has led to a growing feeling in the scientific community that therapeutic intervention aimed at suppressing the wasteful, abiding production of TNF-α would be of benefit in controlling some of the most troublesome symptoms associated with autoimmune diseases. TNF-α was detected in RA synovial tissue and fluid and the generation of TNF-α locally within the RA joint has also been confirmed histologically, using *in situ* hybridization techniques and immunostaining. These studies have indicated that cells of the monocyte/macrophage lineage appears to be the principal source of TNF-α within the synovium, although other cells e.g. T-cells and endothelial cells for TNF-α, also contribute (Chu ***et al.,*** 1991). The initial relief of systemic symptoms by thalidomide in RA is coupled with a concomitant drop in circulating TNF-α in the serum of rheumatoid arthritis patients (Stirling, 1996). The identification of TNF-α as one of the key mediators of inflammation in rheumatoid arthritis has led to randomised trials using anti-TNF-α monoclonal antibody which were reported to have beneficial results (Elliott ***et al.,*** 1994 and Rankin ***et al.,*** 1995). These results support the hypothesis that reducing TNF-α concentrations is an attractive goal in the treatment of rheumatoid arthritis.

**TABLE 1-1:** Main areas of clinical application of thalidomide.

| **Current investigational uses of thalidomide** | |
|---|---|
| ❖ Mycobacterial Diseases<br>• Leprosy<br>• Tuberculosis | ❖ Cancer and Associated Disorder<br>• Cachexia<br>• Chronic graft-versus-host disease |
| ❖ HIV / AIDS and Related Disorders<br>• Wasting syndrome<br>• Aphthous ulcerations<br>• Mycobacterial infections<br>• Chronic diarrhea | ❖ Autoimmune Diseases<br>• Rheumatoid arthritis<br>• Multiple sclerosis<br>• Inflammatory bowel disease<br>• Systemic lupus erythematosus |
| ❖ Miscellaneous Uses<br>• Behcet's syndrome<br>• Prurigo nodularis<br>• Pyoderma gangrenosum | • Macular degeneration<br>• Diabetic retinopathy |

(Stirling ***et al.,*** 1997)

Although the etiology of rheumatoid arthritis is unknown, the synovial membrane lining the joints are infiltrated by large numbers of immunological active cells, including lymphocytes. During this process, multiple inflammatory cytokines are elaborated into the synovial fluid, which exert destructive effects on articular cartilage and ultimately compromise joint function. Because of the mass of infiltrated inflammatory cells, the joint appears swollen and feels puffy and boggy to the touch. The increased blood flow that is a feature of the inflammation makes the joint warm. Usually, both sides of the body are affected similarly and the arthritis is said to be "symmetrical". Any joint can be affected, but the wrists and knuckles are almost always involved and often the knees and the joints of the ball of the foot. Patients with RA describe feeling much like they have a virus, with fatigue and aching in the muscles, except that, unlike a usual viral illness, the condition may persist for months or even years (Fries, 1986). The condition usually appears in midlife, in the forties or fifties, although it can begin at any age. Since RA is so common and because it can sometimes be severe, it is a major global health problem. Affecting about one

percent of the population worldwide, rheumatoid arthritis accounts for more disability expenditures by the U.S. federal government than any other. It can result in difficulties with employment, problems with daily activities, and can severely strain family relationships. In its most severe forms, and without good treatment, it can result in deformities of the joints.

Figure 1-1 represents a coronal view through a normal (left) and rheumatoid (right) synovial joint (Harris, 1997). In corticoid bones, (A) and (B) are pointing to the normal synovium that is continuous with the sub-synovium and joint capsule (E). C is the apparent joint space. In the normal joint, this is a "virtual" space containing only a small amount of synovial fluid. F is the subchondral bone plate, impervious to fluid and without penetrating blood vessels in adults. CA is articular cartilage.

In the rheumatoid joint, the joint (1) becomes enormously thickened, similar to the synovium (4), which can increase over 100-fold in weight. The synovium invades under subchondral bone (left) as well as cartilage. The joint space (2) contains an excess amount of fluid. Cortical bone can be degraded (3) resulting in subluxation.

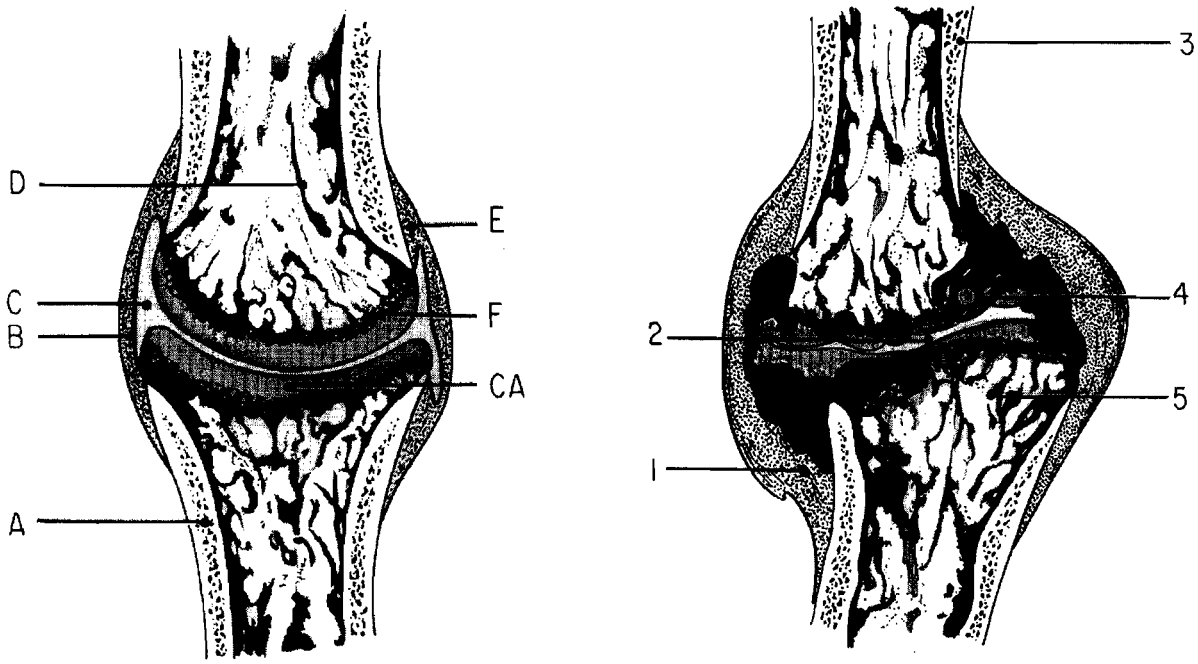


**FIGURE 1-1:** A coronal view through a normal (left) and rheumatoid (right) synovial joint.

Oral therapy of thalidomide has proven to be effective in RA, but unacceptable side effects such as drowsiness, constipation, eosinophilia, swelling of the lower limbs and the most significant, peripheral neuropathy, are easily provoked (Gutiérrez-Rodríguez, 1984). Administration of thalidomide *via* the dermal route can bypass liver metabolism and provide high local tissue drug

levels without systemic complications. There is every reason to believe thalidomide's action is on TNF-α's expression at the local tissue level. Therefore, if the drug's delivery can be targeted, it should be feasible to treat localized inflammation selectively. A localized delivery system should enable rheumatoid arthritis to be treated since it is the joints in the extremities that are most effected. Local applications of the drug over the affected joints will allow us to down-regulate TNF-α production in and around the joint, and this without raising the systemic blood level to a problematical level.

Before a drug can be considered seriously as a candidate for percutaneous delivery, it is necessary to have a thorough understanding of a drug's physicochemical properties, particularly its absolute and relative solubilities and related partitioning tendencies (Sloan ***et al.,*** 1986 and Flynn & Yalkowsky, 1972). Since membrane permeation is a function of skin/permeant and solvent/permeant interactions, an effort has been made to model absorption through skin by quantitating these interactions using solubility parameters. The philosophy and some results were described by Sloan (1990). A drug's solution behaviour relative to its dose may dictate the type of physical system most appropriate for administration of the drug. Two reference behaviours will be employed in the solubility analysis, primarily, ideal solution behaviour and secondarily, regular solution behaviour (Hildebrand ***et al.,*** 1970). An ideal drug being delivered percutaneously should have a low molecular weight (e.g. < 350 g/mole). A high level of crystallinity is expressed in the form of a high melting point and high heat of fusion. This limits solubility itself, and thus also sets a limit on mass transfer across the skin. Generally, the greater a drug's innate tendencies to dissolve, the more likely it is that the drug can be delivered at an appropriate rate across the skin (Ostrenga ***et al.,*** 1971). Therefore, the melting point should be low.

Aqueous solubility and the partition coefficient (relative solubility) are the major determinants of a drug's dissolution, distribution and availability. The hydrophobicity of a compound is a key determinant of its ease of skin transport. Hydrophobicity is well reflected in the relative abilities of drugs to partition between "oil" and water. The ***stratum corneum*** has for many years been identified as, to a first good approximation, a nonpolar membrane. Its "solvent" properties have therefore, been mimicked by various nonpolar liquids including hexane, ether and octanol. A drug having been released from a topical formulation, will partition into the ***stratum corneum*** and then into the underlying epidermis. When the drug reaches the viable tissue it encounters a phase change; it has to transfer from the predominantly lipophilic intercellular channels of the ***stratum corneum*** into the living cells of the epidermis, which will be largely aqueous in nature. Therefore, skin permeants must have reasonable solubilities in oil and water, but should favour the oil. A preferentially oil soluble drug may have difficulty leaving the ***stratum corneum*** and on the other hand, an extremely polar drug will have trouble partitioning into the ***stratum***

***corneum*** from its vehicle. Other investigators have proved that the log partition coefficient should be between 1 and 2.5, for percutaneous delivery (Yalkowsky ***et al.,*** 1983 and Yano ***et al.,*** 1986).

Thalidomide has some serious physicochemical limitations to overcome, in order to be delivered percutaneously. It has a melting point of 275°C and a melting point this high portends relatively low solubilities as evidenced in its water and n-octanol solubilities. Low solubilities and lipophilicity, in turn, suggest delivery difficulties and insufficiencies. Based on thalidomide's physicochemical properties, it is unlikely that it can be delivered percutaneously at a dose required for rheumatoid arthritis. Therefore, we have embraced the idea of using N-alkyl analogues of thalidomide. The most important feature that an analogue of this compound might contribute is decreased crystallinity and increased lipophilicity.

De & Pal (1975) found that the addition of alkyl chains to the thalidomide structure softens the crystallinity of thalidomide in such way that the melting points decreased dramatically. It is known that the N-methyl analogue of thalidomide is an active teratogen (Jönnson, 1972) and therefore we assume that the N-alkyl analogues will also suppress the synthesis of TNF-α. If active as TNF-α inhibitors, such analogues would be very interesting given their increased lipophilicities and lower levels of crystallinity, both important and positive physicochemical properties for percutaneous delivery. Even if the N-alkyl analogues prove TNF-α inactive, their study as penetrants of the skin will fortify basic principles within the field of structure-permeability relationships and would provide clear points for synthesizing compounds which are active and that could be successfully delivered across the skin barrier.

The objectives of this study were thus to:

- synthesize thalidomide and select N-alkyl analogues;
- characterize those physicochemical properties of test compounds that are relevant to their percutaneous delivery;
- determine how thalidomide and its N-alkyl analogues permeate in human skin from saturated aqueous solutions and from solvents which might eventually act as vehicle components;
- develop relationships which exist between the physicochemical properties of the selected compounds and their percutaneous delivery, and
- as a matter of choice of compounds for further study, determine the comparative biological activities (TNF-α inhibition) of selected compounds.

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# Chapter 2

# *Percutaneous absorption*

## 2.1 Introduction

Skin delivery systems are placed against the skin to deliver drugs to: (1) the local tissues immediately beneath the application site, (2) deep tissues to effectuate or accentuate the pharmacological actions of the drug within musculature, vasculature, joints, etc., beneath and around the application site, and (3) the systemic circulation to mediate pharmacological changes somewhere totally removed from the site of application (Flynn, 1993).

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease associated with high levels of tumour necrosis factor-alpha (TNF-α) in synovial fluid and synovial tissue (Saxne ***et al.,*** 1989). Oral therapy of thalidomide has proven to be effective in RA, but unacceptable side effects are easily provoked. TNF-α has been detected in rheumatoid synovium and synovial fluid samples, which suggests that this cytokine is produced locally in inflamed tissue (Chu ***et al.,*** 1991). There is every reason to believe thalidomide's action is on TNF-α's expression at the local tissue level. Local applications of thalidomide over the affected joints will allow us to down-regulate TNF-α production in and around the joints and this without raising the systemic blood level to a problematical level.

The aim of this study was to characterize the physicochemical properties of thalidomide and three of its odd chain N-alkyl analogues, relevant to percutaneous delivery and to assess their solubilities and percutaneous absorption through human skin in select solvents. In order to fulfil the above-mentioned aim, a literature study was done on:

- the process of percutaneous absorption;
- properties that influence percutaneous absorption and
- the mathematical model of skin absorption.

### 2.1.1 The process of percutaneous absorption

The phenomenon of diffusive penetration of the skin by drugs and chemicals is known as **percutaneous absorption**. The process of percutaneous absorption can be described as follows and a scheme of events can be seen in Figure 2-1. When a drug system is applied topically, the drug diffuses passively out of its carrier or vehicle and into the surface tissues of the skin, specifically and most importantly the ***stratum corneum*** and the sebum-filled pilosebaceous gland ducts. A net mass movement continues through the full thickness of the ***stratum corneum*** and ducts into the viable epidermal and dermal strata. A concentration gradient is thus established across the skin that essentially terminates at the outer reaches of the skin's microcirculation in the dermal layer. Each step in the diagram is potentially rate limiting, depending on the drug and how it interacts with the vehicle and the skin. Two principal absorption routes are identified: (1) the transepidermal route, corresponding to diffusion directly across the ***stratum corneum***; and (2) the transfollicular route, corresponding to diffusion down the follicular pore. Whether the transepidermal route or transfollicular route is followed depends on the relative affinities of the respective tissues for the drug, the fractional areas of the routes and the ease of diffusion through the respective phases. Regardless of which pathway is followed, the drug must partition into and diffuse through underlying viable tissues to be effective (Flynn, 1979).

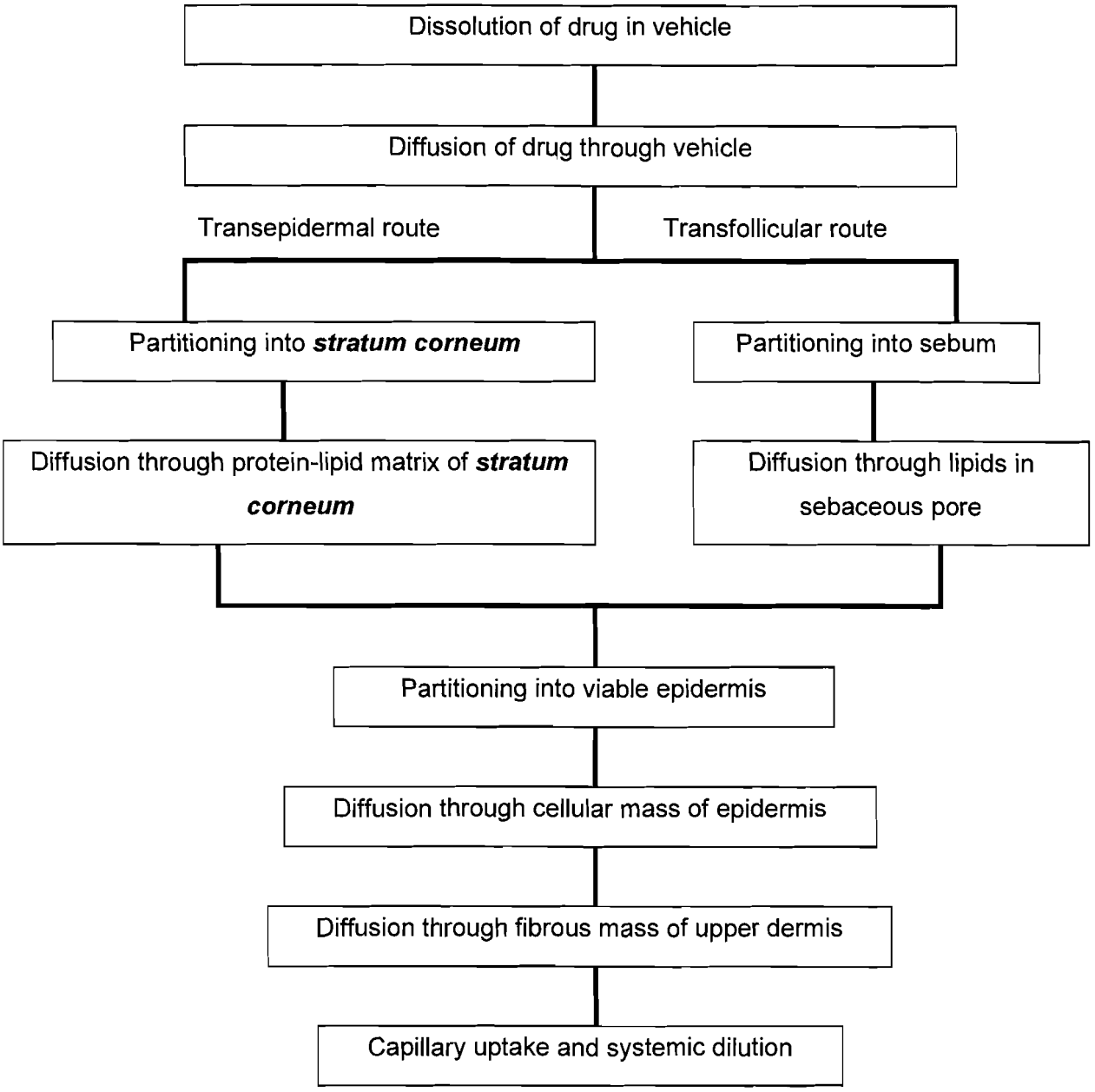


**FIGURE 2-1:** Schematic representation of events for percutaneous absorption (Flynn, 1979).

A concentration gradient is established through the skin via passive diffusion. This concentration gradient is very steep in the ***stratum corneum*** because of its barrier function and less steep in the viable layers of the epidermis. The further decrease in concentration in the dermis is often slight. As a consequence of the passive diffusion a decreasing concentration gradient from the ***stratum corneum*** to the subcutaneous tissue is found (Schalla & Schaefer, 1982).

The driving force for absorption or transport of any penetrant is proportional to the concentration gradient of that penetrant within the skin. It follows that any degradation or other modification of the penetrant to another species reduces the concentration of the penetrant species in the skin, and therefore reduces the flux (Smith, 1990).

### 2.1.2 Routes of penetration

When a molecule gets onto the intact skin, either from the external environment or from a vehicle, it first makes contact with the sebum, cellular debris, bacteria and other exogenous materials which coat the skin. The ***stratum corneum*** can offer two possible routes of penetration: one transcellular, the other *via* the tortuous but continuous intercellular lipid. The route through which permeation occurs is largely dependent on the penetrant's physicochemical characteristics, the most important being the relative ability to partition into each skin phase (Walters, 1989).

### 2.1.3 Advantages of percutaneous delivery

If one compares the percutanoues route of administration with the most popular method of taking drugs, oral administration, the percutanoues approach has several advantages. The gastrointestinal (GI) tract presents a fairly hostile environment to a drug molecule. The low gastric pH or enzymes may degrade a drug molecule, or the interaction with foods, drinks and/or other drugs in the stomach may prevent the drug from permeating through the GI wall. Even if a drug passes through the GI wall, it first must pass through the liver to be degraded (metabolised). This is referred to as the "first-pass" effect. Percutaneous delivery avoids the vagaries of the GI milieu and does not shunt the drug directly through the liver, thereby avoiding the "first-pass" effect. For oral and parenteral drugs with very short biological half-lives and body clearance rates, a percutanoues dosage form can provide a steady maintenance of blood level over a predictable period of time (Cleary, 1993).

### 2.1.4 Properties that influence percutaneous absorption

Absorption or transport of drugs, toxicants or other chemicals into or through the skin depends on a number of factors such as characteristics of the penetrant, condition and type of skin, other chemicals (e.g. vehicles or enhancers) present with the penetrant and external conditions such as temperature, humidity and occlusion. Under most conditions, the factor with perhaps the greatest influence on the rate or extent of skin absorption is the character of the penetrant. Various chemical characteristics, including solubility, lipophilicity, ionization and stability are important in influencing transport of penetrants across skin (Smith, 1990).

### 2.1.4.1 Biomedical factors

#### *2.1.4.1.1 Skin age*

Intact adult skin functions well as a barrier but pre-term infant skin is a less effective barrier. Differentiation of the epidermis to ***stratum corneum*** takes place in the third trimester of fetal life with the formation of completely keratinised cells, and by the time full-term gestation is reached the barrier property of the ***stratum corneum*** is essentially equivalent to that of adult skin (Holbrook, 1992). Thus, pre-term infants are susceptible to enhanced skin permeability and therefore, will be more susceptible to systemic toxicity from a topically applied agent. The full-term newborn infant, on the other hand, has a well-developed skin that possesses excellent barrier properties, similar to those of adult skin.

#### *2.1.4.1.2 Skin condition*

The skin is a tough barrier to penetration, but only if it is intact. Many agents can damage the tissue. Vesicants such as acids, alkalis and mustard gas injure barrier cells and thereby promote penetration. Probably the most widespread cause of an alteration in skin condition is a disease. Injury to the tissue, with resultant inflammation, occurs more often in skin than in any other organ of the body (Barry, 1983).

#### *2.1.4.1.3 Species differences*

Mammalian skin from different species display wide differences in anatomy in such characteristics as the thickness of the ***stratum corneum***, the body mass, the numbers of sweat glands and hair follicles per unit surface area and the condition of the pelt. The behaviour and distribution of the papillary blood supply and the sweating ability differ between humans and the common laboratory animals. Such factors will obviously affect both the routes of penetration and the resistance to penetration (Barry, 1983).

#### *2.1.4.1.4 Regional skin sites*

Few fundamental studies have been done in order to investigate the variation of drug absorption with body site. Tsai & Naito (1982) investigated the percutaneous absorption of indomethacin from ointment applied to the skin surface, and found that the indomethacin applied to the skin surface was influenced by the anatomical site of skin treated. Dorsal sites led to higher levels than abdominal sites, which in turn produced higher levels than in the case of application to the thigh areas. The hair and hair follicles at the dorsal site are thicker than those of the abdomen and thigh, and this may provide a partial explanation for the differences in absorption.

#### *2.1.4.1.5 Disease*

The skin is the part of the body, which comes into direct contact with the environment and hence it is usually the first part of the body to sustain damage or be exposed to irritant substances. Dermatitis is thus a fairly common complaint. Some dermatoses can reduce barrier action and lead to increased permeability of the skin to drugs, while some do not modify the permeation through the ***stratum corneum***. To the latter group belong diseases in which the pathological process is situated in the deeper skin layers without the superficial layers being involved (Washington & Washington, 1989).

#### *2.1.4.1.6 Drug/skin metabolism*

The body has several ways to protect itself from xenobiotics and one of the major ways is by using its drug-metabolising enzyme systems. Skin is the largest tissue, separating the body from the outside environment. It is, therefore, not surprising that it was also found to have enzymatic activity. Such local first-pass metabolism is at least a potential constraint on percutaneous delivery and should be looked into during development.

However, all current experience suggests that drug/skin metabolism is not going to be a frequently encountered problem. For one thing, the skin has far less capacity to metabolise drugs than have either the liver or the gastrointestinal mucosa, the former of which is a central detoxifying organ (Flynn & Weiner, 1993).

### 2.1.4.2 Physicochemical factors

As described previously, the major source of resistance to penetration and permeation of the skin is the ***stratum corneum***. This coherent membrane, which is 15-20 μm thick over much of the human body, primarily consists of proteinaceous cells embedded in a multilamellar three-dimensional lipid domain (Walters, 1989). Thus, the ***stratum corneum*** is a structural composite of unique mechanical and barrier properties, both important to the percutaneous delivery of drugs. Although only 10% to 15% of the total ***stratum corneum*** mass comprises lipids, these lipids largely dictate the overall skin permeability properties.

The relevant physicochemical parameters, which determine the rate and extent of drug penetration across human skin, can be identified by considering the mechanism by which drugs penetrate the skin. A schematic representation of the sequential physicochemical steps involved in percutaneous delivery is shown in Figure 2-2.

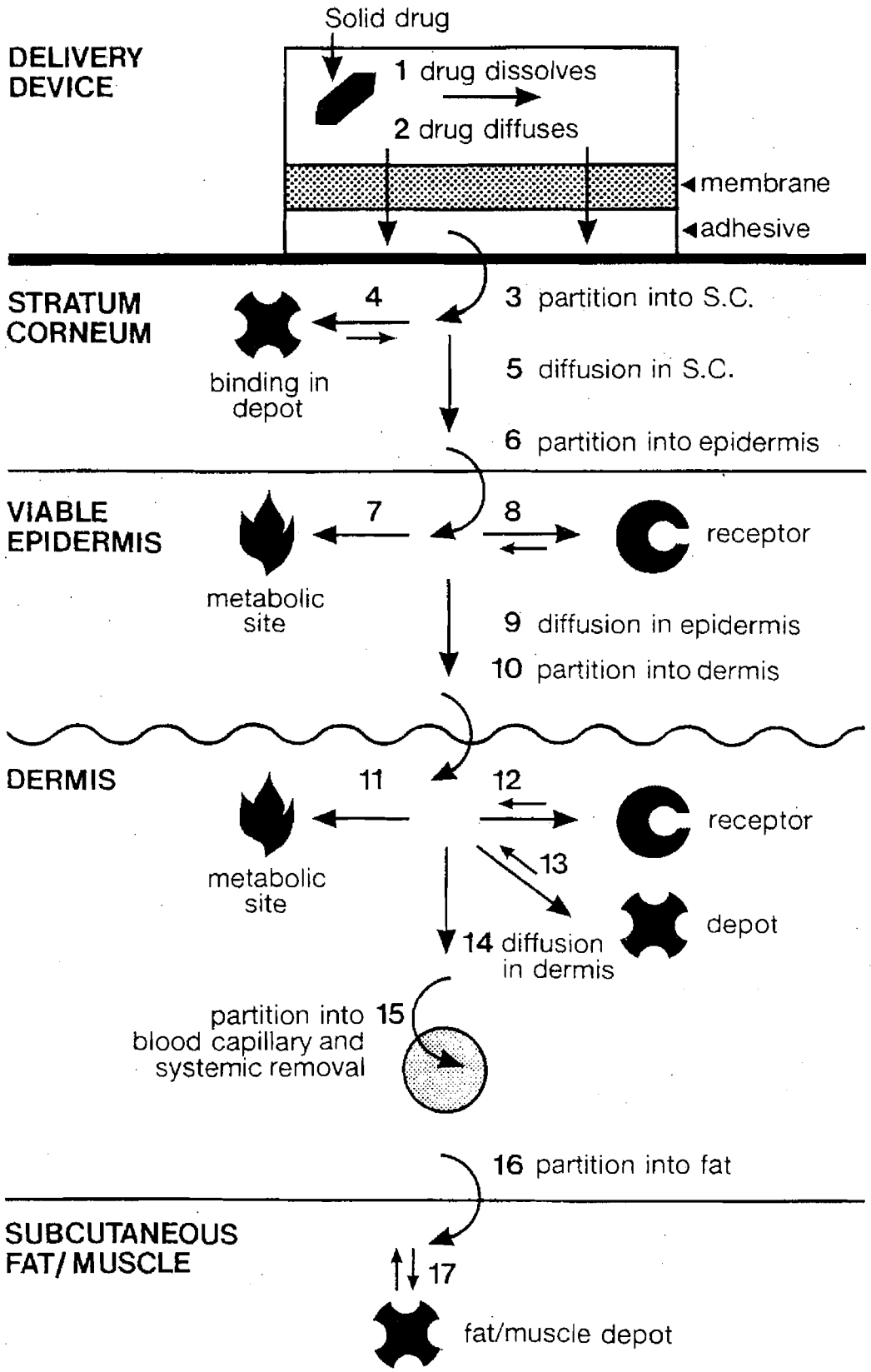


**FIGURE 2-2:** Sequential physicochemical steps involved in percutaneuos delivery (Barry, 1987).

The sequential steps in percutaneous delivery are:

- diffusion or transport of penetrant to the skin surface.
- partitioning of the chemical into the ***stratum corneum***.
- diffusion through (the intercellular lipids of) the ***stratum corneum***.
- partitioning of the chemical from the lipophilic ***stratum corneum*** into the aqueous viable epidermis.
- diffusion through the viable epidermis and upper dermis.
- uptake of penetrant into the cutaneous blood vessels and partitioning into subcutaneous fat, muscle, joints, etc.

From a physicochemical point of view the most important processes to consider are therefore the partitioning and diffusion steps that occur in the transport into, through and out of the ***stratum corneum***. These can be summarized as follows:

#### *2.1.4.2.1 Solubility in the stratum corneum*

The drug having been released from the formulation will partition into the outer layers of the ***stratum corneum***. The solubility of the penetrant in the ***stratum corneum*** plays a large part in determining the rate of penetration. While it is the concentration of penetrant within the skin that controls the rate of transport, that concentration is dependent on the concentration and solubility of the penetrant in the vehicle on the skin surface. A critical point in understanding skin transport is that penetrant concentration in the vehicle does not determine the rate of transport. Rather, the chemical potential of the penetrant in the vehicle determines the rate of transport (Smith, 1990).

The ***stratum corneum's*** lipid domain and sebum, are mostly comprised of lipids. Lipophilic compounds have fewer functional groups, which are responsible for strong hydrogen and dipolar bonding within the crystalline state. Therefore, they melt at lower temperatures and consume less energy per mole in doing so. Consequently, lipophilic compounds exhibit higher absolute solubilities in non-polar media, including the lipids of the ***stratum corneum.***

The solubility parameter of the skin has been estimated as $\approx$ 10 and therefore drugs, which possess similar values, would be expected to dissolve readily in the ***stratum corneum*** (Liron & Cohen, 1984). Thus, penetrants with high solubilities in the ***stratum corneum*** will tend to exhibit high fluxes, or at least will not be limited by solubility considerations.

The use of solubility parameters of drugs and vehicles to describe the transport of drugs through skin is based on the efforts of Hildebrand ***et al.*** (1970) and is presented in Equation 2-1. The solubility parameter of an organic solute ($\delta_2$) in the ***stratum corneum***, can be estimated from Equation 2-1, if the solubility of the solute in a non-polar organic solvent (like hexane) is known, as well as the solute's heat of fusion and melting point, and the solubility parameter of the solvent (hexane):

$$\ln X_2 = \frac{-\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) + \frac{\Delta C_p}{R}\left[\frac{T_f - T}{T} - \ln\frac{T_f}{T}\right] - \frac{V_2\phi_1^{\,2}}{RT}(\delta_1 - \delta_2)^2 \qquad \text{(Equation 2-1)}$$

Where:

- $X_2$ is the solute's mole fraction solubility in hexane
- $\Delta H_f$ is the heat of fusion of a solid
- R is the gas constant
- $T_f$ is the melting point of the solid in degrees Kelvin
- T is any experimental temperature less than $T_f$
- $\Delta C_p$ is the difference in heat capacity between the solid form and the hypothetical super-cooled liquid form of the compound, both at the same temperature
- $V_2$ is the molar volume of the liquid solute
- $\phi_1$ is the volume fraction of the solvent
- $\delta_1$ is the solubility parameter or square-root of the cohesive energy density of the solvent (hexane)
- $\delta_2$ is the solubility parameter or square-root of the cohesive energy density of the solute

#### *2.1.4.2.2 Diffusion through the stratum corneum*

Once the drug has dissolved in the outer skin lipids it will diffuse according to Fick's laws of diffusion down its concentration gradient. The rate constant $k_1$ ($h^{-1}$) is a first order approximation for diffusion and its magnitude is related to the molecular size through the molecular mass M by the equation:

$$k_1 = 0{,}9\ M^{-0.33} \qquad \text{(Equation 2-2)}$$

In order to increase the flux of drugs across the ***stratum corneum*** it is necessary to decrease the diffusional resistance in the structured lipids by making them more fluid. This can be achieved by the use of penetration enhancers such as isopropyl myristate ester and citric acid. The effect of these will be to increase the value of $k_1$ but the overall change in absorption degree or rate can only be gauged by a total assessment which takes account of the physicochemical properties of the penetrant (Hadgraft & Wolff, 1993).

#### *2.1.4.2.3 Partitioning*

Once the penetrant has crossed the ***stratum corneum***, it must partition into the underlying layers of epidermis, dermis and subcutaneous fat, muscle, joints, etc. When the drug reaches the viable tissue it encounters a phase change. It has to transfer from the predominantly lipophilic intercellular channels of the ***stratum corneum*** into the living cells of the epidermis, which will be largely aqueous in nature and essentially buffered to pH 7,4 (Hadgraft & Wolff, 1993 and Smith, 1990). Therefore, skin permeants must have reasonable solubilities in oil and water, but should favour the oil. A preferentially oil soluble drug may have difficulty leaving the ***stratum corneum*** and on the other hand, an extremely polar drug will have trouble partitioning into the ***stratum corneum*** from its vehicle.

The kinetic model for percutaneous penetration takes partitioning at this interface into account by the use of $k_3$ in Figure 2-3. The larger the lipophilic characteristic of the drug, the larger the value of $k_3$, i.e. it preferentially resides in the ***stratum corneum***. It has been demonstrated empirically that $k_3$ is linked to the octanol pH 7,4 partition coefficient (K) by

$$\frac{k_3}{k_2} = \frac{K}{5} \qquad \text{(Equation 2-3)}$$

$k_2$ describes diffusion through the viable tissue as discussed below (Hadgraft & Wolff, 1993).

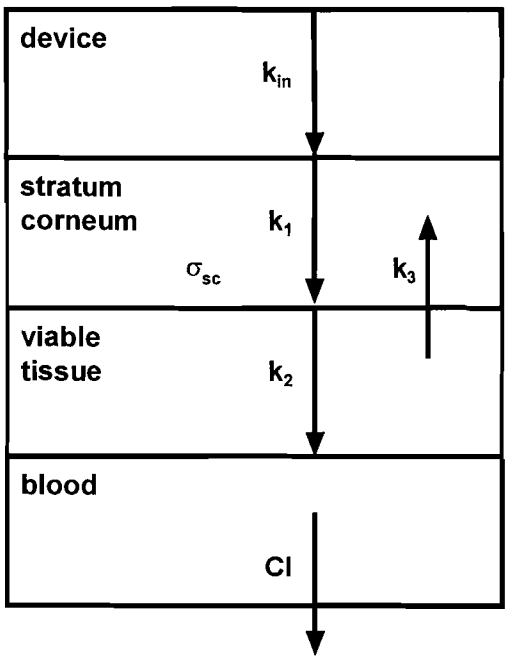


**FIGURE 2-3:** Kinetic model of skin (Hadgraft & Wolff, 1993).

#### *2.1.4.2.4 Diffusion through the viable tissue*

When the drug has dissolved in the upper regions of the viable tissue it will diffuse down a concentration gradient to the epidermal-dermal junction. It is unlikely that the presence of any formulation constituents in this area will significantly alter the diffusional characteristics. The diffusion process is probably not very dependent on molecular size and the first order rate constant assigned to it in the kinetic model is given by:

$$k_2\ (h^{-1}) = 14{,}4\ M^{-0.33} \qquad \text{(Equation 2-4)}$$

When the drug reaches the base of the viable tissue it is taken into the blood vessels and redistributed around the body (Hadgraft & Wolff, 1993), or it will partition into the deeper tissues to effectuate or accentuate the pharmacological actions of the drug within musculature, vasculature, joints, etc.

### 2.1.4.3 Other factors

#### *2.1.4.3.1 Skin hydration*

When water saturates the skin, the tissue softens, swells and wrinkles and its permeability dramatically increases. Skin hydration may be increased by some drugs, which can rapidly penetrate the skin to yield tissue concentrations that are high enough to exert an osmotic effect.

Hydration of the ***stratum corneum*** facilitates the penetration of most drugs through the skin (Barry, 1983).

#### *2.1.4.3.2 Drug-skin binding*

It is logical to assume that the high activation energies observed for the transport process of penetrants in general arise from the binding of the penetrant to the ***stratum corneum*** membrane. Any influence which decreases the heat of activation for desorption, thus aiding desorption, helps the transport process through the skin (Barry, 1983).

#### *2.1.4.3.3 Vehicle-skin interactions*

The skin interacts dynamically with the environment, and thus the epidermal microenvironment changes throughout a normal day's activity. When we apply to the skin pharmaceutical vehicles such as solutions, lotions, creams, ointments, gels, powders and aerosols, these materials may well superimpose further changes on the physical state of the integument and they may affect its permeability. If the applied vehicle does modify the skin permeability, its mechanism of action will probably be a solvent action on the ***stratum corneum***, a hydration effect, or an effect on the skin temperature (Barry, 1983).

#### *2.1.4.3.4 Effect of temperature*

Skin temperature increases under occlusive dressings or in diseased states. Under occlusion, sweat cannot evaporate nor can heat radiate as readily and the surface temperature may rise by a few degrees. However, any consequent increased permeability is small compared with the more dramatic effect that the resultant increased hydration causes. In diseased skin, other effects such as the disruption of the ***stratum corneum*** are much more important than an elevated temperature in promoting penetration. Cooling lotions are even less important for their temperature effect on skin penetration. A solvent with a low boiling point cools the skin as it evaporates, but the change is transitory (Barry, 1983).

#### *2.1.4.3.5 Penetration enhancers*

As mentioned before, the ***stratum corneum*** is a compact and highly keratinised tissue with its lipids and proteins contributing to a complex structure which is relatively impermeable to water and other substances (Kim ***et al.,*** 1993). The ***stratum corneum*** provides the principal barrier to the percutaneous penetration of topically applied substances (Ogiso ***et al.,*** 1992). Approaches to improve skin absorption have included prodrugs, iontophoresis and barrier perturbation with penetration enhancers. Penetration enhancers can be defined as pharmacologically inert, cosmetically acceptable substances which immediately, specifically and reversibly lower the barrier resistance of the ***stratum corneum*** (Dolezal ***et al.,*** 1993), and thus

allow the drug to penetrate to the viable tissues and enter the systemic circulation. Since the main role of the ***stratum corneum*** is to act as a barrier, enhancers must interact with and alter the proteins and/or the lipids to make the lipid moiety of the layer easier for molecules to diffuse through (Kim ***et al.,*** 1993). Drugs that are poorly absorbed through the skin cannot be used percutaneously, but the addition of an appropriate enhancer may increase the flux through the skin sufficiently that therapeutic levels can be achieved in the plasma or deeper tissues.

Stringent conditions have to be placed on appropriate enhancers since they will be in contact with the skin for extended periods of time. A number of criteria can be proposed for their properties:

- They should elicit no pharmacological action of their own.
- They should be specific in their action.
- They should act immediately with a predictable duration.
- Their action should be reversible.
- They should be chemically and physically stable.
- They should be compatible with the drug and other formulation components.
- They should be colourless, odourless and tasteless.
- They should be nontoxic, nonallergic and nonirritant.

It is unlikely that any enhancer will possess all of the above properties and compromises will have to be accepted (Guy & Hadgraft, 1989a).

Two of the first transdermal penetration enhancers were DMSO and Azone (Francoeur ***et al.,*** 1990), but the list has grown substantially, including isopropyl myristate ester, fatty acids (lauric acid and citric acid), *N*-methyl pyrrolidone and simple solvents like water and alcohols.

- *Dimethyl sulfoxide (DMSO)*

DMSO is a dipolar aprotic solvent, which is miscible with both water and organic solvents and is, therefore, easily incorporated into pharmaceutical formulations. DMSO is a powerful solvent and it increases drug penetration, but at the same time, it alters the biochemical and structural integrity of the skin and operates by direct insult to the ***stratum corneum*** (Gummer, 1985). Studies done on the penetration enhancement of DMSO strongly support the conclusion that DMSO causes a certain degree of irreversible biological damage to the ***stratum corneum*** as well as a more reversible physicochemical damage to the barrier that may be rescinded by removing the enhancer. Kurihara ***et al.*** (1986) have presented strong evidence that the mode

of action of DMSO involves a combination of elution of DMSO-soluble components from the ***stratum corneum***, delamination of the ***stratum corneum***, and denaturation of its proteins. This type of irreversible alteration of the stratum corneum is one reason curtailing the usefulness of DMSO in transdermal systems, but the major drawback is that significant permeability enhancement is only obtained when it is present at concentrations in excess of 70% (Kurihara ***et al.,*** 1986).

- *Fatty acids*

Long-chain fatty acids have been shown to be effective penetration enhancers. Aungst ***et el.*** (1986) examined the effects of a series of saturated fatty acids ranging from heptanoic (C7) through stearic (C18) and showed that maximum enhancement of flux was obtained for lauric acid (C12).

- *Esters of fatty acids – Isopropyl myristate ester (IPM)*

Sato ***et al.*** (1988) examined the effects of a series of binary mixtures of isopropyl myristate ester (IPM) and propylene glycol (PG). The flux was markedly increased by addition of 1% IPM, compared with that of the neat PG. When the skin was deprived of the ***stratum corneum*** by stripping, the difference between the IPM-PG vehicle and the neat PG vehicle, as seen with full-thickness skin, vanished. Therefore, it was suggested that IPM had a direct effect on the ***stratum corneum***.

- *Pyrrolidones – N-methyl pyrrolidone (NMP)*

*N*-methyl pyrrolidone (NMP) is a derivative of the naturally occurring pyrrolidone carboxylic acid and has been effective in enhancing the permeation of drugs (Barry & Bennett, 1986). Metronidazole permeation has been investigated by Møllgaard (1993) using full-thickness human skin *in vitro*. 5% NMP was incorporated into a hydrophilic vehicle, propylene glycol (PG), and into a lipophilic vehicle, isopropyl myristate ester (IPM). NMP alone and in a mixture with IPM promoted the drug permeation three- to fourfold, compared with neat PG.

- *Water*

Water reacts with and forms hydration shells around the polar groups of ceramides and sphingolipids in the lipid double layer (Walters, 1989). This disturbs the packing configuration of the lipids which creates a more liquid and permeable hydrophobic route. This also causes a thickening of the hydrophilic layer with a resulting increase in mobility. Penetration of polar penetrating substances is enhanced by the latter effect (Wiechers, 1989).

- *Alcohols*

Some of the lower alcohols have been shown to possess the ability to enhance permeation across skin. Analogues implicated in this respect include methanol and ethanol. The enhancing ability of these alcohols appears to be related to their ability to extract ***stratum corneum*** lipids and in most cases, the increase in permeation rate is slight because only the polar lipids are significantly affected (Walters, 1989). Ethanol is an ingredient in numerous transdermal products and is used to solubilize drugs as well as to enhance the skin permeation of drugs. Estraderm®, a controlled-release transdermal system contains ethanol, where it substantially increases the rate of estradiol delivery through skin (Good ***et al.,*** 1985). Ethanol has a relatively low incidence of topical reactions such as contact dermatitis.

The penetration enhancement of indomethacin by a series of long-chain primary alcohols was evaluated by Tsuzuki ***et al.*** (1988). They dicovered/stated that the alcohols may play two possible roles in the penetration enhancement of indomethacin. Firstly, the alcohols may increase the solubility of indomethacin in a vehicle so that the release of indomethacin may be increased and this may in turn increase the penetration rate of indomethacin. Secondly, if the alcohol did release from the vehicle it may interact with the lipid components of the skin by swelling the skin lipids or causing them to become a more fluid-like material. This may change the permeability characteristics of the skin, thus reducing its resistance to indomethacin.

#### *2.1.4.3.6 Drug-vehicle interactions*

When the drug-vehicle interactions are considered, the impermeability of the ***stratum corneum*** can be ignored and thus the rate of release of the drug from the topical vehicle provides the rate-limiting step in the overall diffusion process while the skin functions as a perfect sink. This could happen clinically if the ***stratum corneum*** severely disrupts or is absent because of disease or injury, or when diffusion of the drug in the vehicle is exceptionally slow (Barry, 1983).

#### *2.1.4.3.7 Drug-vehicle-skin interactions*

When the more usual situation is considered where the impermeability of the ***stratum corneum*** cannot be ignored, the ***stratum corneum*** provides the rate-limiting step in the overall percutaneous absorption process (Barry, 1983).

The release of a topical drug from its vehicle occurs at the interface between the skin surface and the applied layer of product. The physicochemical relationship between the drug and the vehicle determines the rate and amount of drug released. Considerations such as the solubility of the drug in the vehicle, its diffusion coefficient in the vehicle and its partition coefficient into sebum and the ***stratum corneum*** are significant to its efficacy. A drug with a strong affinity for

the vehicle is released less readily than one whose solubility in the vehicle is lower. Likewise, a drug with a proper balance of polar and hydrocarbon moieties (a partition coefficient approaching 1) penetrates the ***stratum corneum*** more readily than drugs that are either highly polar or highly lipoidal since that portion of the skin possesses both hydrated proteins and lipids (Zanowiak & Jacobs, 1982).

### 2.1.5 Mathematical model of skin absorption

It is important to be able to model the absorption of materials through the skin for both pharmaceutical and toxicological reasons. Ideally, it should be possible to predict the rate and extent of absorption from knowledge of the simple physicochemical properties of the penetrant.

Ignoring formulation effects, a drug administered onto the skin surface, will first partition into the outer layers of the ***stratum corneum***. The major route of penetration through the ***stratum corneum*** is *via* the lipid-rich intercellular channels. Thus, the first physicochemical constant that can be identified as being significant is the lipid/water partition coefficient of the drug. Another parameter that should be taken into account is the solubility of the drug in the skin lipids.

One of the major difficulties in generating a good model is the inherent biological variation of the skin; the disease or damage condition of the skin also presents obstacles. Regional variations in the permeability of the ***stratum corneum*** are important but are difficult to quantify (Guy & Hadgraft, 1989b). The structure of the skin relevant to the production of a mathematical model is given in Figure 2-4.

The various layers of the skin are considered to be heterogeneous. Thus, the layers themselves have different properties, but within a layer the barrier function is assumed constant. It is, therefore, possible to consider diffusion through the applied formulation, the ***stratum corneum***, the viable epidermis and transport away from the site by the cutaneous vasculature (Guy & Hadgraft, 1989b).

The simplest way of modelling the process of skin absorption is to assume that Fick's first law of diffusion is applicable (Guy & Hadgraft, 1989b). Fick's first law states that the quantity of a diffusing substance J which migrates in 1 second through 1 $cm^2$ in the direction x from the skin surface into the ***stratum corneum*** is equal to the diffusion coefficient D, multiplied by the gradient - dc/dx of the concentration, c.

$$J = -D \cdot \frac{dc}{dx} \qquad \text{(Equation 2-5)}$$

The assumption that Fick's first law is applicable may be a valid approximation in some *in vitro* experiments, but it is unlikely to be a true assumption *in vivo*, due to the fact that the ***stratum corneum*** is a very impermeable barrier and therefore a long time is required to establish steady-state conditions (Guy & Hadgraft, 1989b).

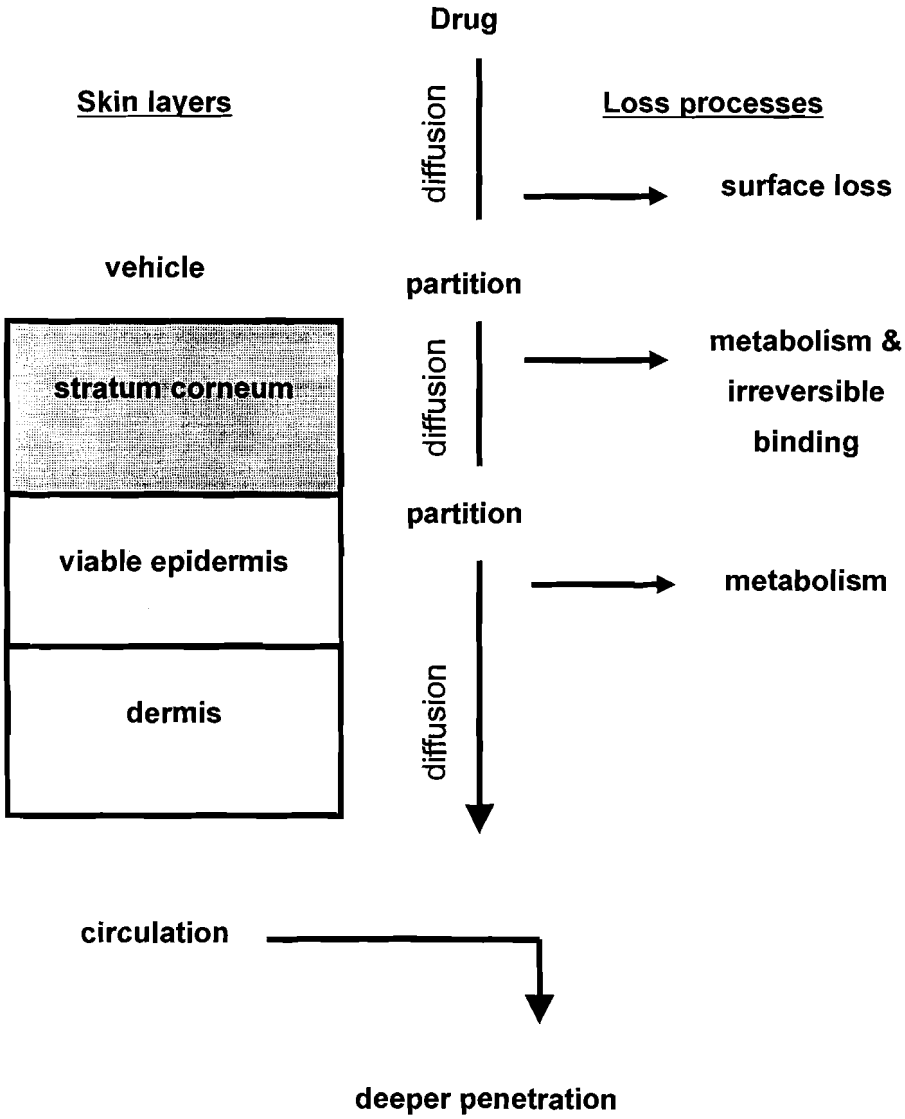


**FIGURE 2-4:** Schematic representation of the skin (Guy & Hadgraft, 1989b).

It has already been mentioned that in real situations steady-state conditions are unlikely to be established during the penetration of drugs across the skin (Guy & Hadgraft, 1989b). Under these conditions, the concentration gradient in the distribution space is reduced during the diffusion of a drug into the ***stratum corneum***. Fick's second law describes the change in concentration over time (dC/dt) at a specific surface. Concentration changes with time like flux (J) changes as a function of distance (x) (Martin ***et al.,*** 1983).

$$\frac{dC}{dt} = -\frac{dJ}{dx} \qquad \text{(Equation 2-6)}$$

Neither the ***stratum corneum*** nor the whole skin is a unique inert membrane. Therefore, the drug concentrations in the formulation are not the same as at the skin surface but are related by the vehicle membrane distribution coefficient $K_m$. When the difference between the concentration at the upper membrane surface and its lower surface is $\Delta C$ and the thickness of the skin is d, the equation can be stated as follows:

$$J = \frac{K_m \cdot D.\Delta C}{d} = K_p \cdot \Delta C \qquad \text{(Equation 2-7)}$$

Where:

J = flux of drug

$K_m$ = vehicle membrane distribution coefficient

D = diffusion coefficient

$\Delta C$ = concentration difference between the upper surface and lower surface of the skin

d = thickness of the skin

$K_p$ = permeability coefficient

The parameters in Equation 2-7 can be measured or calculated.

Because of the great complexity of the skin barrier, an exact mathematical model for the diffusion of a drug into the ***stratum corneum*** is impractical. The main reasons for this difficulty are the numerous compartments and the slow kinetics. The prolonged epicutaneous absorption is impressively demonstrated by the fact that the ***stratum corneum*** reservoir is not completely empty even two weeks after a single topical application (Schalla & Schaefer, 1982).

One of the biggest limitations of percutaneous drug delivery is the limited skin permeability of most drugs. Therefore, one has to determine if the drug can permeate the skin in high enough quantity to show its therapeutic effect. This is usually done by conducting *in vitro* skin permeation studies. A wide variety of experimental procedures have been developed and used to assess skin absorption. However, at present there are no generally accepted techniques that are completely satisfactory for assessing skin absorption. The fundamental questions addressed in most skin absorption investigations are concerned with how much, how fast and what the modulating factors are which may influence the penetration and percutaneous rate of topically applied agents.

## 2.2 Summary

From the perspective of absorption, the skin forms a complex barrier to the external environment, maintaining body fluids within our system and excluding harmful substances. The important steps involved in percutaneous absorption have been identified as the partitioning of the drug from the delivery vehicle to the ***stratum corneum***, transport through the ***stratum corneum***, partitioning from the lipophilic ***stratum corneum*** into the more aqueous viable epidermis, transport across the epidermis and uptake by the cutaneous microvasculature with subsequent systemic distribution. It is clear that percutaneous absorption is a complex physicochemical and biological process. The skin is not just a protective envelope surrounding the body; it is a dynamic, living tissue and as such its permeability characteristics are susceptible to constant changes. The major advantage claimed for percutaneous absorption is that transdermal administration avoids the vagaries of the GI milieu and does not shunt the drug directly through the liver, thereby avoiding the "first-pass" effect. The major disadvantages for percutaneous absorption are related to the barrier properties of the skin.

The major route of penetration through the ***stratum corneum*** is *via* the lipid-rich intercellular channels. Thus, the first physicochemical constant that can be indentified as being significant is the lipid/water partition coefficient of the drug. Another parameter, which should be taken into account, is the solubility of the drug in the skin lipids. Ideally, it should be possible to predict the rate and extent of percutaneous absorption from the knowledge of the simple physicochemical properties of the penetrant.

The aim of the present research was to determine the physicochemical properties of thalidomide and its N-alkyl analogues relevant to skin transport and their percutaneous delivery through human skin *in vitro*, as well as their biological activities.

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# Physicochemical Properties and Solubility Analysis of Thalidomide and its N-Alkyl Analogues

Chapter 3

## 3.1 Introduction

Synthesized in Germany in 1954, thalidomide was introduced into the pharmaceutical market in 1956 as a sedative and hypnotic drug. Soon after, it was also used as a tranquilizer and anti-emetic for pregnant women. Some years later, it was found that thalidomide had a teratogenic effect and was withdrawn from the market in 1961, but still remained available in certain countries for defined research purposes. In 1964 Sheskin discovered that thalidomide was beneficial in the treatment of erythema nodosum leprosum (ENL) (Sheskin, 1965). Clinical and immunologic similarities between the leprotic reaction of ENL and diffuse connective tissue diseases, particularly rheumatoid arthritis (RA) have been observed (Maini, 1977). Gutiérrez-Rodríguez (1984) proved that systemic administration of thalidomide to patients with RA results in rapid clinical improvement, but unacceptable side effects was observed and was severe in some cases. Alternative means of delivering thalidomide, to minimize some of the adverse effects, is desirable. By the very nature of the route of administration the toxicity problems would be avoided by a percutaneous thalidomide-containing preparation and could result in a significant therapeutic advantage with respect to treating RA.

Before a drug can be considered seriously as a candidate for percutaneous delivery, it is necessary to have a thorough understanding of a drug's physicochemical properties, particularly its absolute and relative solubilities and related partitioning tendencies (Flynn & Yalkowsky, 1972 and Sloan ***et al.,*** 1986). Generally, the skin can be considered as a trilaminate structure, consisting of the outer lipophilic ***stratum corneum***, the underlying viable hydrophilic epidermis, and dermis.

The amphiphilic nature of the skin dictates, that its permeability will be highly dependent on the lipophilicity of a penetrant. Also, the greater a drug's innate tendencies to dissolve, the more likely it is that the drug can be delivered at an adequate rate across the skin (Ostrenga ***et al.,***

1971 and Spruance ***et al.,*** 1984). Based on thalidomide's physicochemical properties (melting point and lipophilicity), it is unlikely that it can be delivered percutaneously at a dose required for RA. It is for this reason that we have embraced the idea of using N-alkyl analogues of thalidomide. The most important feature that an analogue of this compound might contribute is decreased crystallinity and increased lipophilicity. Therefore, the lipophilicities of thalidomide and its N-alkyl analogues and their solubilities in select solvents have been assessed and such important physicochemical properties of solubility as melting points, fusion energies and cohesiveness have also been investigated in this chapter.

Given the clinical efficacy of thalidomide in a wide range of immunologic disorders (Hawkins, 1992 and Vogelsang ***et al.,*** 1992 and Hamza, 1986 and Sheskin, 1965), especially rheumatoid arthritis (Gutiérrez-Rodríguez ***et al.,*** 1989), it seems worthwhile to investigate thalidomide and some of its N-alkyl analogues. Thus, the aim of this chapter is to characterize thalidomide's and its N-alkyl analogues's physicochemical properties relevant to skin transport.

## 3.1.1 Theoretical background

A reaction between a liquid solvent and an excess of a crystalline solute is at a state of equilibrium when the thermodynamic activity of the solute in solution ($a_2$) equals the thermodynamic activity of solid solute ($a_2^s$). This is of course only true as long as the solvent does not penetrate the crystalline mass and alter in an appreciable way the solid state form. There are fundamentally two concerted but at the same time thermodynamically separable processes at work as the equilibrium is approached and reached, fusion of the solute to form liquid solute and equilibrium of the liquid solute with the solvent. If the solute's activity also equals its mole fraction composition over the entire concentration range up to and including saturation, the solution is, by definition, an ideal solution. In an ideal solution, the solute's and solvent's self-interactions are identical and indistinguishable between like and unlike species, and there is no heat of mixing ($\Delta H = 0$) nor change in volume upon mixing. For solutes that are crystalline at ambient temperature, the selected standard state is an ordinarily hypothetical state referred to as the super-cooled liquid state. To thermodynamically achieve this state, a solid is heated to its melting temperature, melted and the resulting melt is cooled back to ambient temperature (without re-crystallization). By summing enthalpy and entropy contributions from each step, the total free energy change is quantified. The activity of the solid and thus of the saturated solution can be expressed as:

$$\ln a_2^s = \frac{-\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) + \frac{\Delta C_p}{R}\left(\frac{T_f - T}{T}\right) - \frac{\Delta C_p}{R}\left(\ln\frac{T_f}{T}\right) \qquad \text{(Equation 3-1)}$$

Where: $\Delta H_f$ is the heat of fusion of the solid at melting point, $T_f$. $\Delta H_f$ is the quantity of heat absorbed or evolved by the system during the process to maintain its isothermal condition. T is the ambient temperature, R is the gas constant and $\Delta C_p$ is the difference in heat capacity at constant pressure between the solid form and the hypothetical super-cooled liquid form of the compound, both at the same temperature. The heat capacity of the solid form of a compound, in general, increases with respect to temperature at a rate equal to or greater than the heat capacity of the corresponding liquid (Neau & Flynn, 1990). Since the heat capacity of the super-cooled liquid form normally cannot be determined, two assumptions for the estimate of $\Delta C_p$, can be considered. First, the heat capacity of the super-cooled liquid form is generally not extraordinarily different from the heat capacity of the solid and, $\Delta C_p$, is often assumed to be negligible, especially if the temperature range $T_f$ - T is small (Yalkowsky ***et al.,*** 1972 and Hagen & Flynn, 1983). Thus, Equation 3-1 reduces to the familiar expression:

$$\ln a_2^s = -\frac{\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) \qquad \text{(Equation 3-2)}$$

The alternate assumption is that the super-cooled liquid form would consistently have a heat capacity higher than the solid form heat capacity and, $\Delta C_p$ is assumed to be equal to the entropy of fusion, $\Delta S_f$, where: $\Delta S_f = \Delta H_f/T_f$ (Hildebrand ***et al.,*** 1970).

The thermodynamic activity of a crystalline solute is therefore dependent on the intrinsic properties of the crystal lattice and can be estimated from experimentally obtainable values of $\Delta H_f$ and $T_f$. The activity of a compound can always be expressed as the product of a concentration and an activity coefficient, e.g., $a_i = \gamma_i c_i$., and it follows that the activity of a solute in solution is defined as directly proportional to the mole fractional concentration of the solute ($X_2$):

$$a_2^s = \gamma_2 X_2 \qquad \text{(Equation 3-3)}$$

where $\gamma_2$ is the solute's mole fractional activity coefficient. Because the activity coefficient of an ideal solution is unity (by definition), $a_2^s$ also represents the mole fraction ideal solubility.

### 3.1.1.1 Regular solution analysis

Solutes rarely exhibit ideal behaviour in real solvents since seemingly slight differences in molecular functionality between solvent and solute can result in large differences in their intermolecular interactions. Real solutions thus have non-ideal aspects to their behaviours, and these can be quite complex. Perhaps the simplest source of non-ideality and the most

ubiquitous occurs when the solute and solvent have different abilities to react within themselves than between themselves but otherwise form a random mix, the regular solution. In the Regular Solution Theory, the limiting situation is described as one in which the dominant molecular interactions between solute-solute, solvent-solvent and solute-solvent are London forces (Scathard, 1931 and Hildebrand ***et al.,*** 1970). This force is only dependent on the distance between atoms and the instantaneous orientation of their electrons. The molecular model for regular solution behaviour, therefore, allows for only an excess enthalpy of mixing arising from differences in cohesive energies between solute and solvent. Completely random mixing is assumed; thus there is no excess entropy of mixing. The condition $\Delta H_{mixing} = 0$ is met when the cohesive energies of solute and solvent are the same so that molecules 'A' and 'B' do not experience a change in their molecular environments when taken from the neat state to the mixed state. When the non-ideality arises strictly from differential cohesiveness, which occurs when dispersion forces alone are involved, and a solid solute is in equilibrium with its saturated solution, the mole fractional regular solution solubility is given by,

$$\ln X_2 = \frac{-\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) + \frac{\Delta C_p}{R}\left[\frac{T_f - T}{T} - \ln\frac{T_f}{T}\right] - \frac{V_2\phi_1^{\,2}}{RT}(\delta_1 - \delta_2)^2 \qquad \text{(Equation 3-4)}$$

where:

- $X_2$ is the solute's mole fraction solubility
- $\Delta H_f$ is the heat of fusion of a solid
- R is the gas constant
- $T_f$ is the melting point of the solid in degrees Kelvin
- T is any experimental temperature less than $T_f$
- $\Delta C_p$ is the difference in heat capacity between the solid form and the hypothetical super-cooled liquid form of the compound, both at the same temperature
- $V_2$ is the molar volume of the liquid solute
- $\phi_1$ is the volume fraction of the solvent
- $\delta_1$ is the solubility parameter or square-root of the cohesive energy density of the solvent (hexane)

- $\delta_2$ is the solubility parameter or square-root of the cohesive energy density of the solute

The above equation indicates that, if one knows the solubility of an organic solute in an apolar organic solvent, the solute's heat of fusion, the solute's melting point, and the solubility parameter of the solvent, it is possible to calculate the solubility parameter of the solute (Yalkowsky ***et al.,*** 1972 and Hagen & Flynn, 1983). It is assumed that a regular solution has no excess entropy of mixing, with the entropy of mixing being equal to the statistical mixing found in an ideal solution. This has proved to be a valid assumption whenever there is no excess volume of mixing. It should be noted in passing that the right-side-most term in the equation is equal to $-\ln\gamma_2$ where $\gamma_2$ is the solute's activity coefficient. Regular solution theory predicts a parabolic relationship between the mole fractional solubility of a given solute and the solubility parameters for solvents, which span the solute's value. Solvents such as hexane, cyclohexane, carbon tetrachloride, benzene and toluene, which self-interact exclusively through London forces, were selected to test the hypothesis that the solubility of a solute like thalidomide and its N-alkyl analogues would conform to regular solution behaviour in nonpolar solvents as these. The theoretical maximum solubility is the ideal solubility. This is so because, at the peak of the regular solution curve, the cohesive energy differential between solute and solvent, $\delta_1$-$\delta_2$, is zero. In other words, a positive excess enthalpy of mixing and a solubility less than ideal is always associated with regular solution behaviour (Yalkowsky ***et al.,*** 1972 and Hagen & Flynn, 1983). Most solute-solvent mixtures do not behave ideally, and solute mole fractional concentrations often differ greatly from their activities.

# 3.2 Experimental

## 3.2.1 Materials and methods

For solubility studies reagent grade organic solvents (Aldrich, Milwaukee, WI, USA) were used, and included hexane, cyclohexane, carbon tetrachloride, toluene, benzene, n-octanol and ethanol (95%). Each solvent was used as received. HPLC-grade acetonitrile (Fisher Scientific, Pittsburg, PA, USA) and double-distilled deionized water were used for the chromatography procedure and HPLC-grade methanol (Fisher Scientific, Pittsburg, PA, USA) was used to dilute samples in preparation for HPLC analysis. Thalidomide, N-methyl thalidomide, N-propyl thalidomide and N-pentyl thalidomide were synthesized according to literature methods (Budavari, 1989 and De & Pal, 1975) with some modifications in the purification procedures. Identification and levels of purity (>96%) were assured through Element Analysis (EA), Electron Impact Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR) spectroscopy, High-pressure Liquid Chromatography (HPLC) and by the sharpness of melting points.

### 3.2.1.1 Synthesis Method

*Thalidomide*: One mole (39 g) of N-phthaloyl-DL-glutamic anhydride (Aldrich, Milwaukee, WI, USA) was melted with 2 moles (18 g) of urea (Aldrich, Milwaukee, WI, USA) in an oil bath at 170-180°C for 45 min. After cooling, the crude thalidomide was dissolved in ± 60-ml ethanol (95%), heated to 60°C and recrystallized 4 times at room temperature. Thalidomide was obtained in an almost white powder.

*N-Methyl Thalidomide*: One mole (20 g) of N-phthaloyl-DL-glutamic anhydride (Aldrich, Milwaukee, WI, USA) and 1.2 moles (46.32 ml) of a 2 M methylamine solution in methyl alcohol (Sigma Chemical Co., St. Louis, MO, USA) were heated in an oil bath at 200°C for 8 hr. After cooling, the crude imide was purified by recrystallization from 95% EtOH at 4°C. The recrystallization process was repeated 5 times. De & Pal (1975) purified N-methyl thalidomide by sublimation and a melting point of 131-133°C was reported. In this study N-methyl thalidomide was purified by recrystallization from 95% EtOH and a melting point of 159°C was obtained. According to HPLC, element analysis and the sharpness of the melting point, a high purity (≥ 98%) was obtained for N-methyl thalidomide in the present study.

*N-Propyl Thalidomide*: One mole of N-phthaloyl-DL-glutamic anhydride (20 g) (Aldrich, Milwaukee, WI, USA) and 1.2 moles of propylamine (7.62 ml of 99% propylamine) (Sigma Chemical Co., St. Louis, MO, USA) were heated in an oil bath at 200°C for 8 hr. After cooling, the crude imide was purified by recrystallization from EtOH (95%) at -20°C. The recrystallization process was repeated 5 times and a melting point of 136°C was determined in contrast to the 66-68°C melting point reported by De and Pal.

*N-Pentyl Thalidomide*: One mole of N-phthaloyl-DL-glutamic anhydride (20 g) (Aldrich, Milwaukee, WI, USA) was heated with 1.2 moles of N-amylamine (10.74 ml) (Sigma Chemical Co., St. Louis, MO, USA) in an oil bath at 200°C for 8 h. 99% Amylamine was used for this reaction. According to the literature method (De & Pal, 1975), N-pentyl thalidomide was purified by distillation *in vacuo* and a 19-21°C melting point was reported. Unfortunately, the distillation process was unsuccessful in this study and the crude N-pentyl thalidomide was purified by silica gel column chromatography (diethyl ether : hexane) using a stepwise gradient (10:90; 20:80; 30:70; 40:60; and 45:55). A melting point of 105°C was determined for N-pentyl thalidomide. The volume of each mobile phase used was 2 times the column volume and N-pentyl thalidomide was collected during the 45% diethyl ether mobile phase. It was dried *in vacuo* and recrystallized once from diethyl ether, to give white crystals of N-pentyl thalidomide in an overall yield of 40 – 60%.

### 3.2.1.2 High-Pressure Liquid Chromatographic (HPLC) Procedure

The HPLC system consisted of a Beckman 114M solvent delivery system and a Spectraflow 783 variable wavelength ultraviolet detector, operated at 220 nm with a sensitivity setting of 0.05 AUFS. A Perkin-Elmer ISS-100 autoinjector was used to inject samples (20 µl) onto a Spheri-5 (RP-8, 5 µm, 220 x 4.6 mm) column (Alltech Associates, Inc., Deerfield, IL, USA). A NewGuard precolumn (RP-18, 7 µ, 15 x 3 mm) insert (Alltech Associates, Inc., Deerfield, IL, USA) was used and changed frequently to prolong column life. The mobile phase comprised of acetonitrile/water (1:4) for thalidomide and the pH (2.0) was adjusted with ortho-phosphoric acid (Fluka Chemical Co., Ronkonkoma, NY, USA). Separation was achieved at a flow rate of 1.2 ml/min. Data handling was performed using a Hewlett Packard, HP 3395 integrator. The concentrations of the N-methyl, N-propyl and N-pentyl analogues were determined by the same HPLC method described, with only slight modifications in the mobile phase (25, 35, and 45% Acetonitrile, respectively). Under the chromatographic conditions described above, the retention times of thalidomide and its N-methyl, N-propyl and N-pentyl analogues were approximately 7, 6, 7, and 8 min, respectively and showed a single peak at 220 nm. Calibration curves showed excellent linearity over the entire concentration range.

### 3.2.1.3 Solubility Determination

The solubility of thalidomide and its N-alkyl analogues in several organic solvents (hexane, cyclohexane, carbon tetrachloride, toluene and benzene) (Fisher Scientific, Pittsburg, PA, USA) were obtained by equilibrating large excesses of the solute with each solvent. Temperature was maintained at 25°C by a constant–temperature water bath and vigorous stirring was supplied by magnetic bars to hasten the attainment of equilibrium. An excess of solute was always present in the slurries. Preliminary work indicated that equilibrium was obtained well within 30 hr. Therefore the equilibration times for all the studies were $\leq$ 30 hr. Samples were taken, filtered (PTFE filter media with Polypropylene housing, 0.45 µm pore size, Whatman Inc., Haverhill, MA, USA), measured with respect to volume and the solvent from each individual sample was then evaporated. The initial 25% of each filtrate were discarded to eliminate the possibility that adsorption of the compounds on the filter and/or filtering apparatus might influence the solubility determination. The sample's solute residue was reconstituted in an appropriate amount of methanol and assayed by HPLC. All samples were done in triplicate. No decay products or impurities were detected by the HPLC assay.

### 3.2.1.4 Differential Thermal Analysis

The heat of fusion ($\Delta H_f$) and the entropy of fusion ($\Delta S_f$) of thalidomide and its N-alkyl analogues were determined with a Perkin-Elmer DSC7 Differential Scanning Calorimeter (DSC), which was

calibrated with an indium standard. Accurately weighed samples (4-5 mg) were layered evenly over the bottom of a 40-μl aluminum crucible on which an aluminum lid was crimped. An empty aluminum crucible and lid served as the reference. Samples were heated at 10°C/min for melting point and enthalpy of fusion determinations. The molar heat of fusion was calculated from the area of the melting endotherm, moles of sample used and the calibration coefficient. The entropy of fusion was obtained by dividing the heat of fusion by the absolute temperature of melting, $T_f$. All tracings were repeated four times. There were no appreciable differences in the thermograms for any compound from run to run.

### 3.2.1.5 Melting Point

The melting points of thalidomide and its N-alkyl analogues were determined by two methods: (1) differential thermal analysis (as described above) and (2) controlled-heating thermal microscopy (Mettler Hot Stage with FPS Temperature Regulator and a Zeiss Standard Microscope). The heating rate for both methods was 10°C/min.

### 3.2.1.6 Determination of Partition Coefficient

Equal volumes of n-octanol and phosphate buffer (pH 6.4) were saturated with each other for at least 24 hr before the experiment. Solutions of each compound in this study (30 μg/ml) were prepared with the pre-saturated n-octanol phase as a solvent. 5 ml of these solutions were transferred to 10-ml assay tubes containing equal volumes (5 ml) of phosphate buffer. Three tubes of each compound were stoppered and agitated for 1 hr and another set of three was agitated for 2 hr. After centrifugation at 2000 g for 10 min, the n-octanol and buffer phases were analyzed by HPLC for drug concentrations. The aqueous phases were diluted with the respective mobile phase for each compound and the n-octanol phases were appropriately diluted with methanol before injecting onto the HPLC column. The pH of the buffer was measured before and after each drug was added, to ensure that the compounds had no influence on the pH. Partition coefficients ($K_{oct}$) were calculated as the ratio of drug concentration in the n-octanol phase to that in the buffer phase. There was no difference in the value of $K_{oct}$ when the tubes were agitated for 1 or 2 hr.

## 3.3 Results

Table 3-1 summarizes the physicochemical properties of thalidomide and three of its N-alkyl analogues. The molecular weight of each compound was determined theoretically as well as experimentally with electron impact mass spectroscopy (EI-MS). There were no differences in the theoretical and experimental molecular weight of the compounds in study. Enthalpies of fusion, $\Delta H_f$, and entropies of fusion, $\Delta S_f$, calculated from these data are also shown in Table 3-1. These values are the mean of four determinations, all with standard deviations < 4%. Thalidomide, N-propyl thalidomide and N-pentyl thalidomide exhibited only one thermal transition. The endotherms at 275, 136 and 105°C, correspond to the melting of these crystals, respectively. N-methyl thalidomide showed an endotherm at 159°C and a small endotherm at 165°C. The endotherm at 159°C corresponds to the melting point of N-methyl thalidomide. Melted samples of all the compounds, assayed by HPLC showed only trace amounts of decomposition with essentially 100% retention of the compounds and it was assumed that the endotherms represent primarily energy consumed on melting. Figure 3-1, represents the trend in melting points as a function of alkyl chain length.

A physical parameter that is necessary for solubility analysis is the molar volume of liquid solute. The molar volume, $V_2$, of thalidomide was determined from its molecular weight divided by its crystalline density (Reepmeyer ***et al.,*** 1994). Since the crystalline densities of the N-alkyl analogues are unknown, their molar volumes were estimated, starting with thalidomide's true molar volume and then by summation and subtraction of functional-group molar volumes which distinguish the individual compounds from thalidomide (Yalkowsky & Zografi, 1972). The estimation of the molar volumes for the N-alkyl analogues can be seen in Table 3-2.

The aqueous solubilities ± standard deviation (SD) of thalidomide and its N-alkyl analogues are listed in Table 3-3, along with their octanol/water ($K_{oct}$) partition coefficients ± standard deviations (SD). N-alkylation of the glutarimide ring in the thalidomide molecule results in compounds (N-methyl, N-propyl and N-pentyl analogues) that are more lipophilic. Figure 3-2 illustrates that the log [partition coefficients] (log $K_{oct}$) increases linearly with increasing alkyl chain length. Figure 3-3, on the other hand, illustrates the relationship between partitioning and the intrinsic cohesiveness (both parameters that reflect the level of polarity) of the compounds in study. The inverse partition coefficient was chosen for the x-axis so that polarity increases from left to right as the water/octanol partition coefficient increase.

**TABLE 3-1:** Physicochemical properties of thalidomide and its N-alkyl analogues.

| Physical Parameter | Thalidomide | N-Methyl Thalidomide | N-Propyl Thalidomide | N-Pentyl Thalidomide |
|---|---|---|---|---|
| Molecular weight (g/mole) | 258 | 272 | 300 | 328 |
| Crystalline density (g/ml) | 1.48 | 1.43 | 1.35 | 1.28 |
| Molar volume, $V_2$ (ml/mole) | 174 | 191 | 223 | 255 |
| Melting Temperature, $T_f$ (°C) ± SD | 275 ± 0.11 | 159 ± 0.11 | 136 ± 0.90 | 105 ± 0.26 |
| Heat of fusion, $\Delta H_f$ (kcal/mole) ± SD | 8.61 ± 0.27 | 4.33 ± 0.06 | 6.52 ± 0.25 | 5.73 ± 0.08 |
| Entropy of fusion, $\Delta S_f$ (cal/mole/K) | 15.71 | 10.02 | 15.94 | 15.16 |
| Activity of solid phase $a_2^s$, $\Delta C_p = 0$ * | $1.29\times10^{-3}$ | $1.03\times10^{-1}$ | $5.18\times10^{-2}$ | $1.29\times10^{-1}$ |
| Activity of solid phase $a_2^s$, $\Delta C_p = \Delta S_p$ * | $8.07\times10^{-3}$ | $1.54\times10^{-1}$ | $7.89\times10^{-2}$ | $1.64\times10^{-1}$ |

* Ideal activity of solid phase, $a_2^s$ estimated from:

$$\ln a_2^s = \frac{-\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) + \frac{\Delta C_p}{R}\left(\frac{T_f - T}{T}\right) - \frac{\Delta C_p}{R}\left(\ln\frac{T_f}{T}\right)$$ with one or the other assumption.

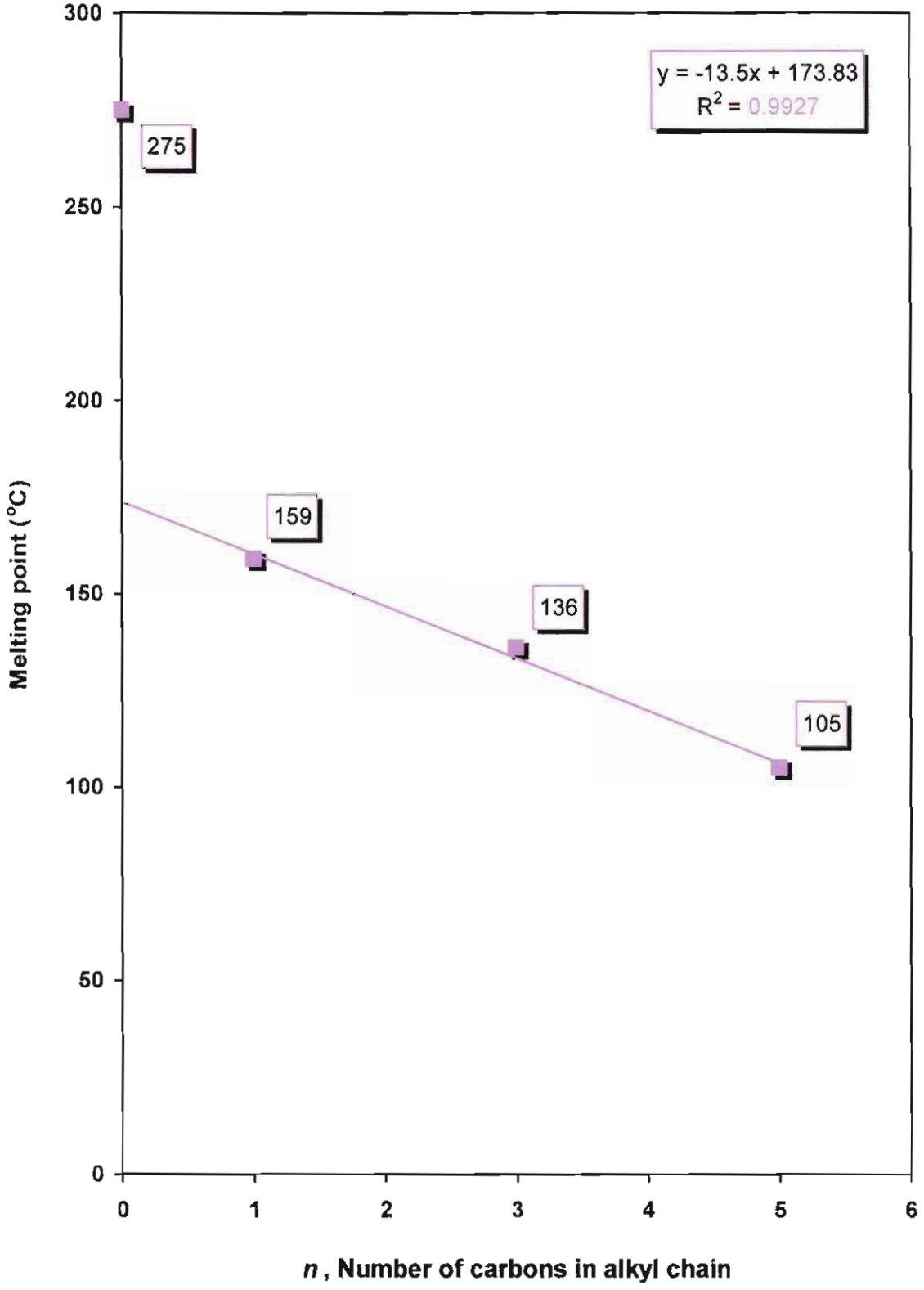


**FIGURE 3-1:** Melting points of thalidomide (data plotted on y-axis) and its N-alkyl analogues *versus* alkyl chain length.

**TABLE 3-2:** Estimation of molar volumes for the N-alkyl analogues.

| Functional group or atom | Partial molar volume contribution per group (ml/mole) | N-Methyl Thalidomide | N-Propyl Thalidomide | N-Pentyl Thalidomide |
|---|---|---|---|---|
| Thalidomide | 174.33 | 174.33 | 174.33 | 174.33 |
| H | 3.1 | -3.1 | -3.1 | -3.1 |
| CH2 | 16.2 | | +32.4 | +64.8 |
| CH3 | 19.3 | +19.3 | +19.3 | +19.3 |
| Estimated Molar Volume | | **191** | **223** | **255** |

**TABLE 3-3:** Solubility and partition coefficients of thalidomide and its N-alkyl analogues.

| Compound | SOLUBILITY (25°C) | | $K_{oct}$ ± SD |
|---|---|---|---|
| | Water (pH 6.4) μg/ml ± SD | Hexane μg/ml ± SD | |
| Thalidomide | 52.1 ± 1.49 | 0.1 ± 0 | 3.09 ± 1.03 |
| N-Methyl Thalidomide | 275.9 ± 6.39 | 90 ± 0 | 14.1 ± 1.05 |
| N-Pentyl Thalidomide | 57.3 ± 1.46 | 220 ± 10 | 129 ± 1.05 |
| N-Propyl Thalidomide | 6.54 ± 0.52 | 530 ± 10 | 1023 ± 1.06 |

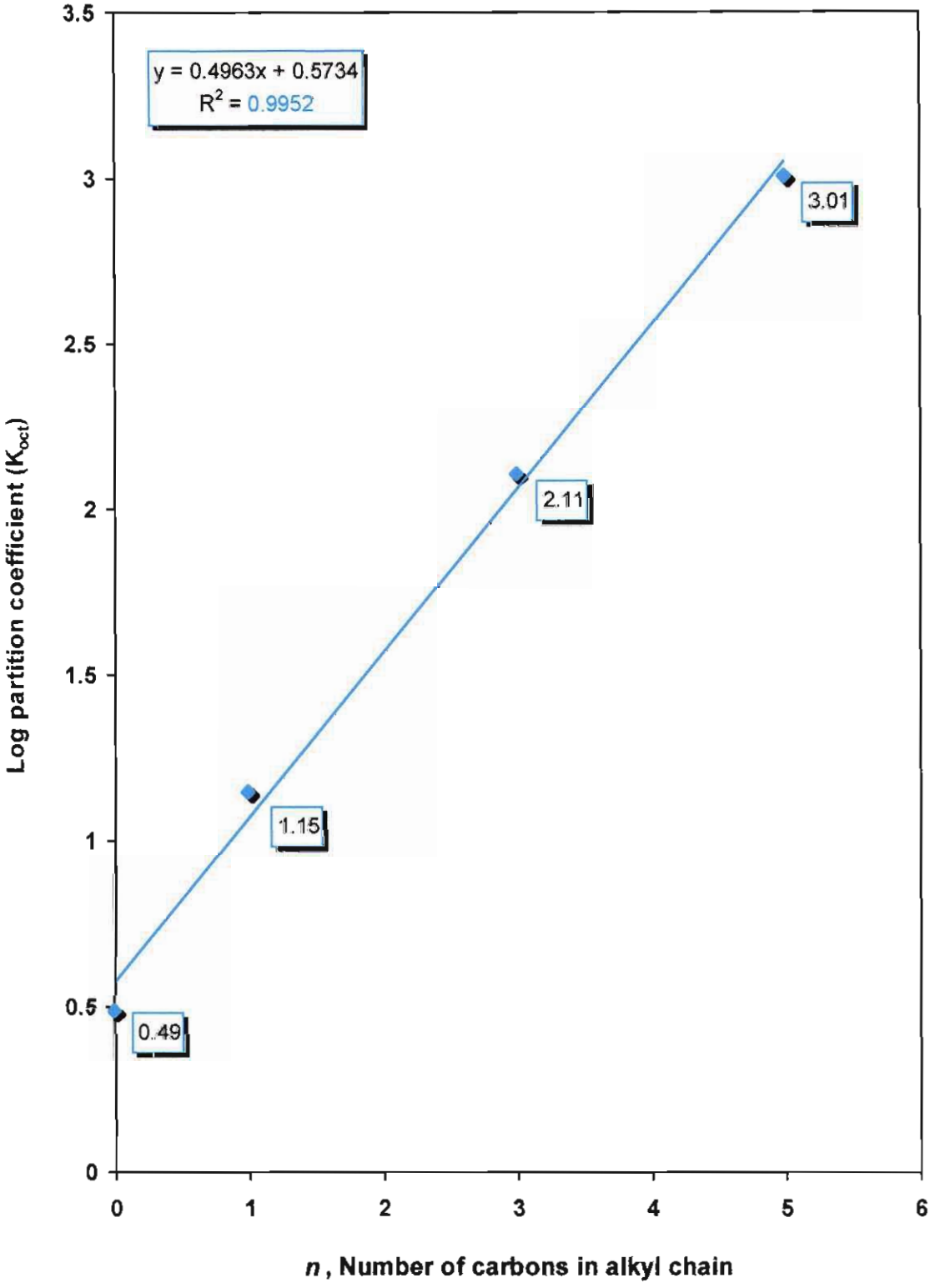


FIGURE 3-2: Linear plot showing the log [octanol/water] partition coefficients of thalidomide (arbitrarily plotted on y-axis at $n$ = 0) and its N-alkyl analogues as a function of alkyl chain length.

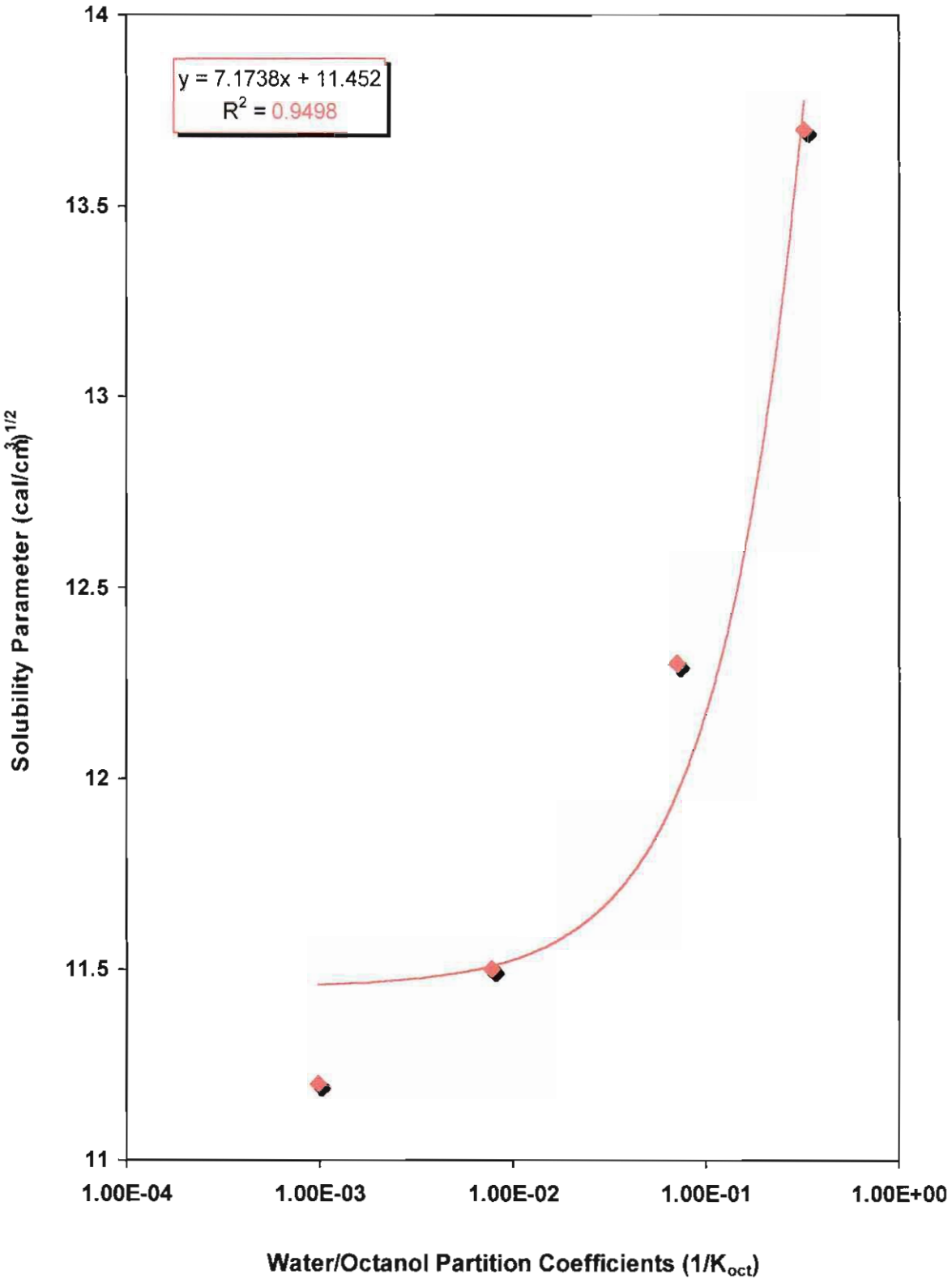


**FIGURE 3-3:** Plot of solubility parameters *versus* the water/octanol partition coefficients of thalidomide and its N-alkyl analogues.

The hexane solubilities of the compounds can be seen in Table 3-3. The assumption that the volume fraction of hexane in the saturated solutions ($\phi_1$), is unity, was made (Hagen & Flynn, 1983). Using this surmise, the solubility parameters for all the solutes were calculated according to Equation 3-3 and the assumption that $\Delta C_p = 0$ or $\Delta C_p = \Delta S_f$. The differences in solubility parameters and in the ideal solubility (based on the values of the thermodynamic activities, $a_2^s$, in Table 3-1), created by the alternate assumptions for $\Delta C_p$, are given in Table 3-4. Regular solution theory predicts a parabolic relationship between the mole fraction solubility of a solute and the solubility parameters of "regular" (essentially nonpolar) solvents. The mole fraction solubilities (ln X) of thalidomide and its N-alkyl analogues in several organic solvents at 25°C are listed in Table 3-5, as well as the values of the solubility parameters of the solvents. To show the extent to which thalidomide's, N-methyl thalidomide's, N-propyl thalidomide's and N-pentyl thalidomide's solubility behaviour might conform to regular solution behaviour, the regular solution solubility parabolas for all four compounds were calculated (using Equation 3-3 with the assumption, $\Delta C_p = \Delta S_f$) about the midpoints of 13.7, 12.3, 11.5 and 11.2 $(cal/cm^3)^{1/2}$, respectively, where in each case the solutions are considered ideal (Figures 3-4 through 3-7). The regular solution parabolas for all four compounds were also calculated when the assumption, $\Delta C_p$ equals zero, was used (Figures 3-4 through 3-7).

TABLE **3-4:** Comparison of solubility parameters and ideal solubility from experimental results.

| Compound | Melting Point (°C) | $\delta_2$ (Hexane) $(cal/cm^3)^{1/2}$ | | Ln $X_{2,ideal}$ | |
|---|---|---|---|---|---|
| | | $\Delta C_p = 0$ | $\Delta C_p = \Delta S_f$ | $\Delta C_p = 0$ | $\Delta C_p = \Delta S_f$ |
| Thalidomide | 275 | 13.2 | 13.7 | -6.65 | -4.82 |
| N-Methyl | 159 | 12.2 | 12.3 | -2.27 | -1.87 |
| N-Propyl | 136 | 11.4 | 11.5 | -2.96 | -2.54 |
| N-Pentyl | 105 | 11.2 | 11.2 | -2.05 | -1.81 |

**TABLE 3-5:** Mole fraction solubility of thalidomide and its N-alkyl analogues.

| **Solvent** | $\delta_1$* **(cal/cm$^3$)$^{1/2}$** | **Mole fraction solubility (ln X) at 25°C** | | | |
|---|---|---|---|---|---|
| | | Thalidomide | N-Methyl Thalid. | N-Propyl Thalid. | N-Pentyl Thalid. |
| n-Hexane | 7.27 | -16.80 | -10.05 | -9.25 | -8.46 |
| Cyclohexane | 8.19 | -16.25 | -9.44 | -8.31 | -7.25 |
| Carbon Tetrachloride | 8.55 | -12.60 | -6.34 | -4.75 | -2.98 |
| Toluene | 8.93 | -10.02 | -4.58 | -3.72 | -2.47 |
| Benzene | 9.16 | -9.68 | -4.40 | -3.17 | -2.13 |
| Water (pH 6.4) | 23.0 | -12.53 | -10.91 | -12.58 | -14.84 |

* (Hoy, 1970).

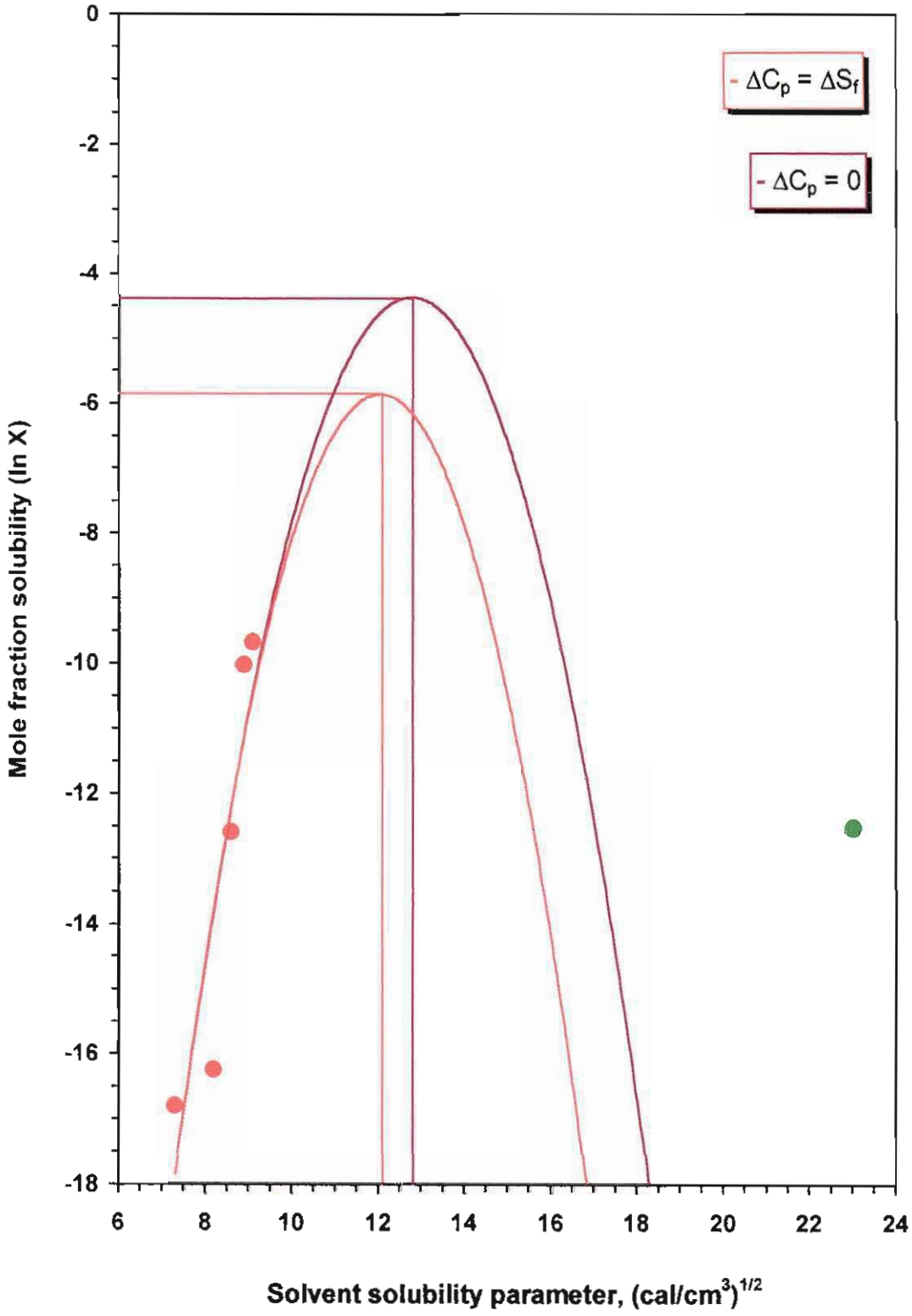


FIGURE 3-4: Regular solution parabola for thalidomide.

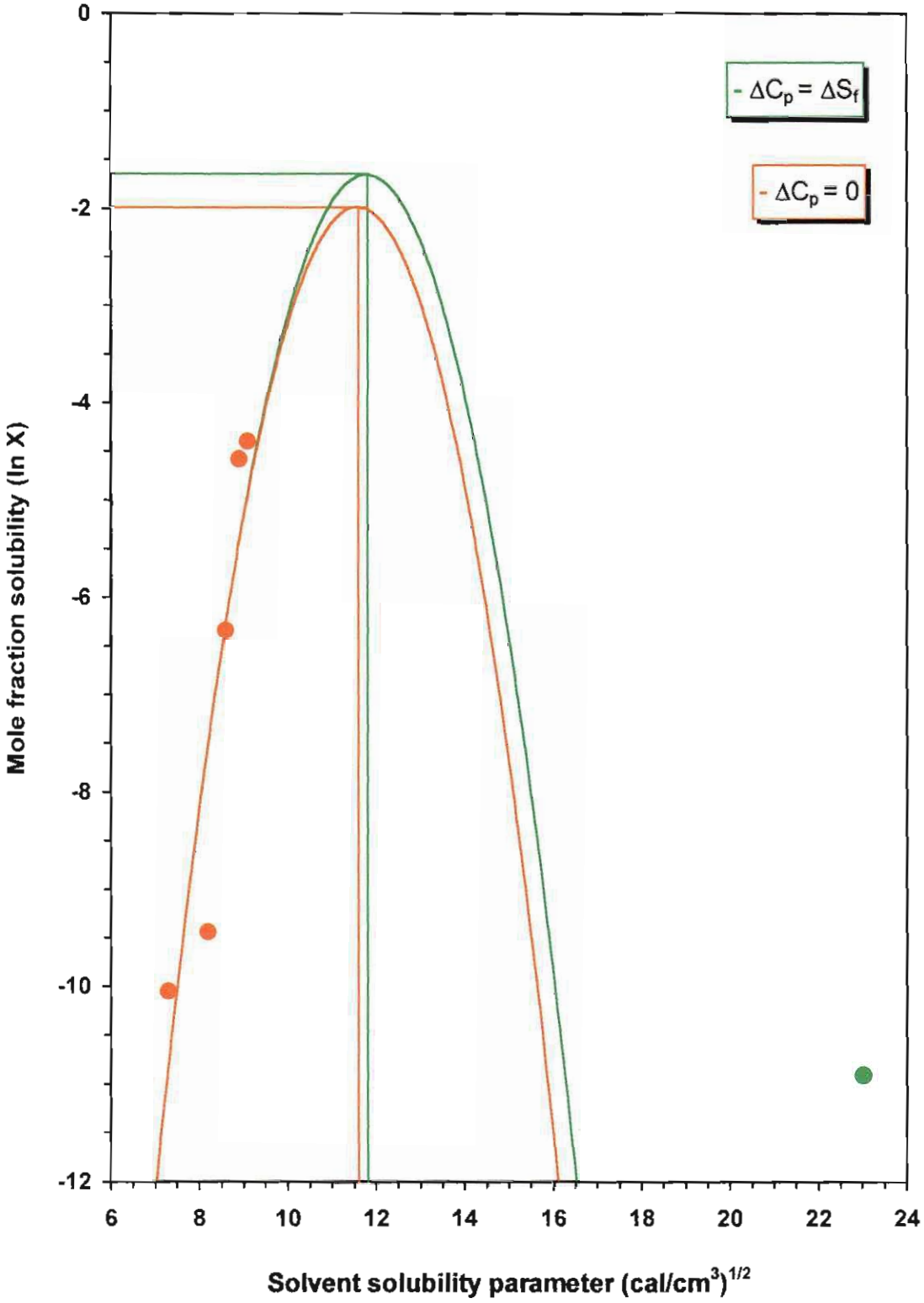


**FIGURE 3-5:** Regular solution parabola for N-methyl thalidomide.

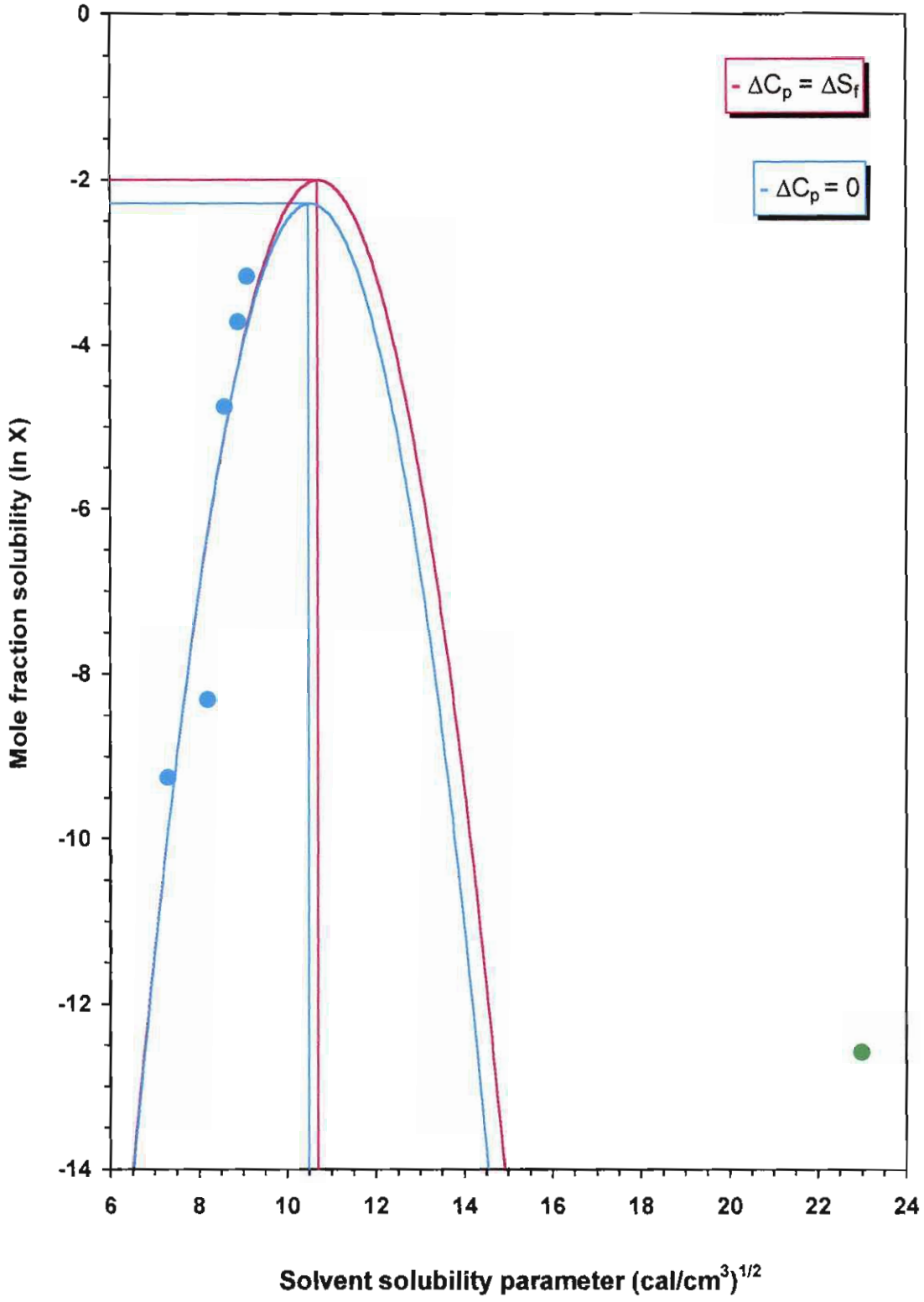


**FIGURE 3-6:** Regular solution parabola for N-propyl thalidomide.

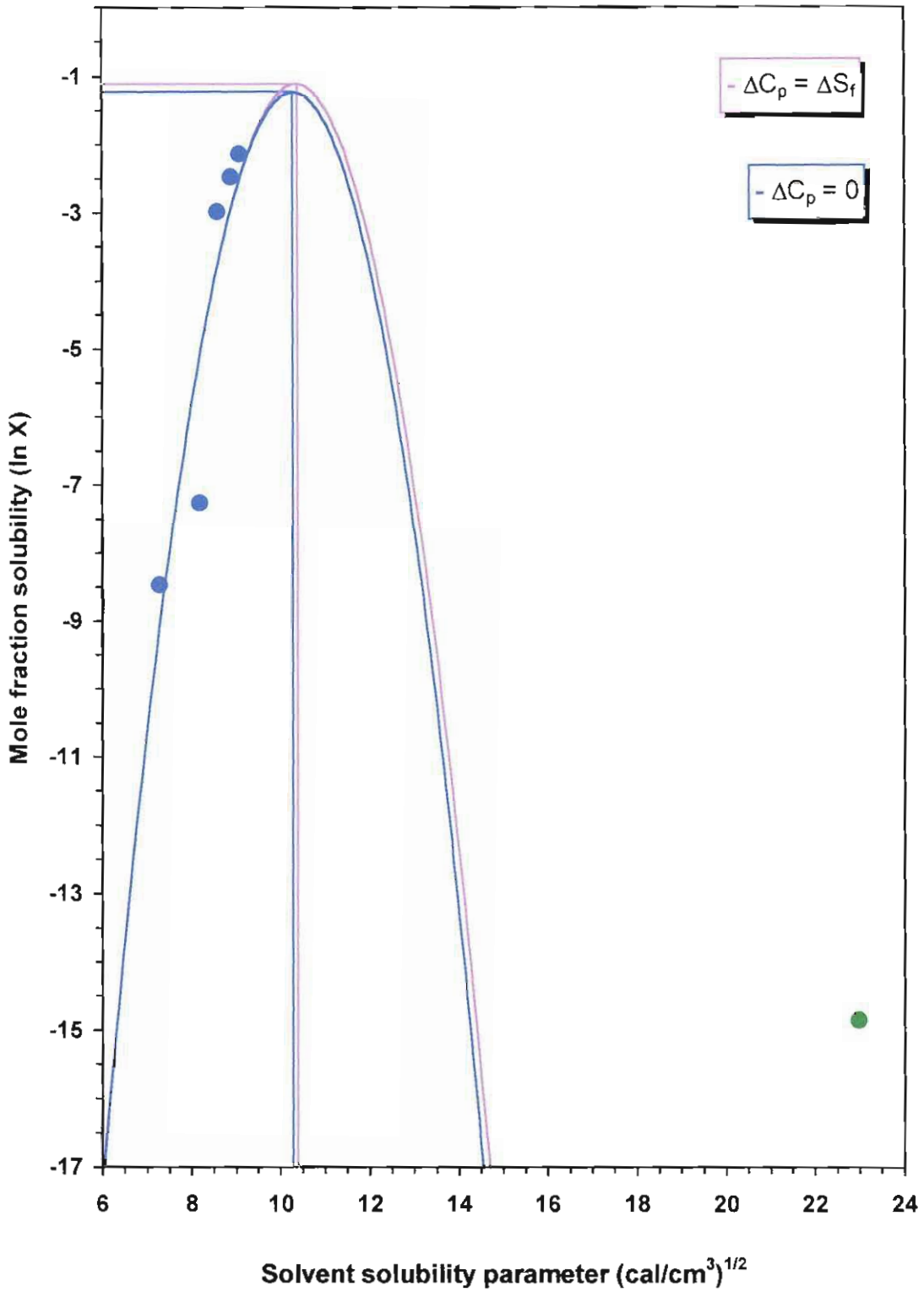


FIGURE 3-7: Regular solution parabola for N-pentyl thalidomide.

## 3.4 Discussion

The physicochemical properties of thalidomide and its N-alkyl analogues are summarized in Table 3-1. N-alkylation of the glutarimide ring in the thalidomide molecule replaces the imido hydrogen atom, which is responsible for strong hydrogen and dipolar bonding within the crystalline state. Therefore, the N-alkyl analogues melt at lower temperatures and consume less energy per mole in doing so. Figure 3-1, represents the trend in melting points as a function of alkyl chain length. By adding a methyl group to the thalidomide structure, the melting point drops more than 100°C and, in this particular instance upon increasing the alkyl chain length to five $-CH_2-$ units, the melting points decrease linearly. Other investigators who have studied the influence of extending alkyl chain length also report that melting points decrease overall, but usually not linearly (Stinchcomb ***et al.,*** 1995 and Yalkowsky ***et al.,*** 1972). Because only unequal carbon numbers N-alkyl analogues were studied at present, it cannot be concluded that all the melting points to carbon length five will decrease linearly. The melting points for the N-alkyl analogues in this series are all at least 100°C lower than thalidomide's melting point, illustrating the remarkable impact of eliminating the acidic imido hydrogen atom of the thalidomide molecule.

N-alkylation of the glutarimide ring in the thalidomide molecule results in compounds (N-methyl, N-propyl and N-pentyl analogues) that are more lipophilic. This is evident from the systematically declining solubility parameters through the series but is even better demonstrated in the octanol/water partition coefficients (Table 3-3). Figure 3-2 illustrates that the log [partition coefficient] (log $K_{oct}$) increases linearly with increasing alkyl chain length. Here one see a linear free energy relationship which mostly has developed around the incremental excess free energy expected to dissolve $-CH_2-$ groups in water. Figure 3-3, on the other hand, illustrates the relationship between partitioning and the intrinsic cohesiveness (both parameters that reflect the level of polarity) of the compounds in study. The inverse partition coefficient was chosen for the x-axis so that polarity increases from left to right as the water/octanol partition coefficient increases. As expected from past experience, even as the polarity increases from bottom to top, the solubility parameter increases. As the solubility parameter increases (reflecting increasing polarity), the water/octanol partition coefficient ($1/K_{oct}$) also increases, in other words the octanol/water partition coefficient ($K_{oct}$) decreases. There is little direct proportionality in these two effects, however. This is because the dominant effect in the partitioning case is that of the interaction of methylene units in water, as just stated, while cohesive energy densities and this square roots, the solubility parameters reflect the interactivity of the whole molecule. By either measure, adding alkyl groups to the thalidomide structure results in compounds with increased lipophilicity. Ordinarilly this favours the N-alkyl analogues with respect to transport across biological membranes, particularly with respect to them being

percutaneously delivered. A similar relationship exists between the water/octanol partition coefficients and the solute solubility parameters of selected narcotic analgesics (Roy & Flynn, 1988).

Table 3-1 contains estimates of the thermodynamic activities of thalidomide and its N-alkyl analogues at 25°C. These were obtained using Equation 3-1 in conjunction with the experimental values for $\Delta H_f$ and $T_f$. These values also represent the mole fractional ideal solubilities of thalidomide and its N-alkyl analogues. It can be seen that the inherent thermodynamic activity increases dramatically when the thalidomide structure is alkylated. However, there is no simple pattern to the thermodynamic activities of the analogues as a result of extending the alkyl chain. While it is inappropriate to directly relate the thermodynamic activity of one compound to that of another, as there is no provision in classical thermodynamics for doing so, it is still clear from these data that a high level of crystallinity is associated with low activity and *vice versa*. It must always be kept in mind that it is the thermodynamic activity of a solid drug in a formulation, which establishes its maximum practical driving force for permeation.

At 52 µg/ml (Table 3-3), the 25°C aqueous solubility of thalidomide is exceptionally low. Its low solubility in water is undoubtedly due in part to its exceptionally high level of crystallinity as reflected in its high melting point and enthalpy of fusion. There may be other factors here, which also have bearing on the value. By way of contrast, the aqueous solubility of N-methyl thalidomide, 276 µg/ml, is quite high. The loss of the H-binding imido hydrogen is more than compensated for by the reduced crystallinity of the compound. Based on this general behaviour of amines, one can also speculate that the change in structure also slightly increases the strength of the nitrogen atom as an H-bind receptor. In the instances of the N-propyl and N-pentyl analogues, further reductions in compound crystallinity are not sufficient to overcome the impact of making the compounds incrementally more hydrophobic.

As suggested in the introduction, there are two simplifications of Equation 3-1, which have been offend by previous investigators. In particularly, Neau & Flynn (1990) evaluated the assumptions regarding $\Delta C_p$ in ideal solubility estimations and compared the change in heat capacity, between the solid form and the hypothetical super-cooled liquid form of the solid with the entropy of fusion. They found that, for compounds that are rigid such as benzene and polycyclic aromatic hydrocarbons, $\Delta C_p$ is indeed closer to zero than to the entropy of fusion. However, for compounds that are not rigid, such as n-alkyl para-aminobenzoates, the value of $\Delta C_p$ is better approximated by the entropy of fusion. The thalidomide molecule contains a flat phthalimide moiety and a glutarimide moiety, which, though held within a plane, can rotate freely about its connection to the phthaloyl portion of the molecule. Therefore, thalidomide and its N-alkyl analogues are to believe molecules, which are not as rigid as the polycyclic aromatic

hydrocarbons, but somewhat more constrained than the alkyl-para-aminobenzoates. It is therefore hard to place them into the best category represented by these two choices. The influence of heat capacity assumptions on the estimation of solubility parameters and ideal solubility, were specifically studied by Neau ***et al.*** (1989). In this study the estimates were found to be essentially insensitive to the $\Delta C_p$ assumption ($\Delta C_p = 0$ or $\Delta C_p = \Delta S_f$) with 87°C as the largest difference between the melting point and the solution temperature. However, from physicochemical properties reported by Hagen & Flynn (1983), they noted that the heat capacity assumption might play a significant role in the evaluation of the ideal solubility, if the difference between the melting point and the solution temperature is large. With a 212°C melting point for hydrocortisone an appreciable difference in ideal solubility was observed between the two approximations even though the solubility parameters differed by only 0.3 hildebrand units. The influence of these two assumptions on the values of the ideal solubility and solubility parameters of thalidomide and its N-alkyl analogues, as determined by analyzing solubility data using regular solution theory and Equations 3-1 and 3-3 respectively, can be seen in Table 3-4. Thalidomide has an extremely high melting point (275°C) and its solubility parameter is clearly sensitive to the heat capacity assumption. However as anticipated based on from previous work reported (Neau & Flynn, 1990), the heat capacity assumption did not have an appreciable effect on the values of the solubility parameters of the three N-alkyl analogues in study. They all exhibit melting points $\leq$ 159°C. As can be seen in Table 3-4, the assumption, $\Delta C_p$ equals zero, leads to a value of ln $X_{2,ideal}$ for thalidomide of –6.65, while using the alternate assumption ($\Delta C_p = \Delta S_f$), the value of ln $X_{2,ideal}$ is –4.82. Thus, on a mole fraction basis, the ideal solubility of thalidomide is over 6 times higher, based on the latter assumption, than using the former assumption. However, the mole fraction ideal solubility of the N-methyl and N-propyl analogues was only 1.5 times higher and 1.3 times regarding the N-pentyl analogue. Therefore, the ideal solubility will be influenced by the $\Delta C_p$ assumption, especially if the difference in melting and solution temperature is large, as in the case of thalidomide. Taking the latter discussion into account and the fact that thalidomide and its N-alkyl analogues are not fully rigid molecules, as discussed earlier, $\Delta C_p$ in this series of compounds would seem to be better approximated by the entropy of fusion than by a value of zero. This is the approach, which will be the most emphasized in further discussions.

Table 3-5 summarizes the experimentally determined mole fraction solubilities of the compounds in various nonpolar solvents that self-interact exclusively through London forces at 25°C along with the known solubility parameters for the solvents. The mole fraction solubilities of the compounds in water at 25°C are also listed in Table 3-5. Regular solution theory predicts a parabolic relationship between the mole fraction solubility of a solute and the solubility parameters of "regular" (essentially nonpolar) solvents. The regular solution solubility parabolas

for all four compounds were calculated (using Equation. 3-4 with the assumption, $\Delta C_p = \Delta S_f$) about the midpoints of 13.7, 12.3, 11.5 and 11.2 $(cal/cm^3)^{1/2}$, respectively, where in each case the solutions are considered ideal (Figures. 3-4 through 3-7). The regular solution parabolas for all four compounds were also calculated when the assumption, $\Delta C_p$ equals zero, was used. As anticipated, the solubilities of the compounds in hexane, cyclohexane, carbon tetrachloride, toluene and benzene, closely fit to the respective curves. Figures 3-4 through 3-7 also demonstrate that regular solution theory is totally inappropriate for solubility estimation in solvents like water capable of extensive hydrogen bonding and other strong orienting bonding with the solute. Specifically the mole fraction water solubilities of thalidomide and its N-alkyl analogues, is far off scale. Figures 3-4 through 3-7 also reflect the differences in solubility parameters, as well as the ideal solubility of the compounds, when using each one of the assumptions involving $\Delta C_p$. Again, it is clear that the heat capacity assumption does have an appreciable effect on the estimation of solubility parameters and ideal solubilities. When the difference in melting and solution temperatures is large, as it is for thalidomide, the assumption impact is at its greatest, consistent with the expectations of Neau ***et al.*** (1989).

In conclusion, one can clearly see that alkylation of the thalidomide molecule result in compounds with physicochemical properties that appear to be better suited for percutaneous delivery. The N-alkyl analogues of thalidomide have lower melting points and consume less energy per mole in doing so. They are more lipophilic as evident in their higher octanol/water partition coefficients. Their absolute solubilities in nonpolar media, including the lipids of the skin barrier, are demonstrably higher, a factor which should favour their percutaneous delivery too. Thus, it is expected that the N-alkyl analogues of thalidomide will be more easily delivered through the skin than thalidomide itself. Moreover, it is expected that the rank order in skin permeability will be N-pentyl > N-propyl > N-methyl > thalidomide as the result of the fact that the flux determining physicochemical properties, partitioning and oil solubility, both more in the same direction. In continuing investigations we will determine the extent to which these basic expectations are actually met. Specifically in chapters 4 and 5 relative permeabilities of these compounds through human skin and their potentials as TNF-$\alpha$ inhibitors for treatment of rheumatoid arthritis are presented and discussed.

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# Chapter 4

# Percutaneous Delivery of Thalidomide and its N-Alkyl Analogues

## 4.1 Introduction

Thalidomide is a proven inhibitor of the biological synthesis of tumour necrosis factor alpha (TNF-α) and is believed to rely on this action for its suppression of the wasting of tissue which otherwise accompanies leprosy and other diseases. There is good reason to believe that the tissue wasting and damage done by arthritis can also be lessened or stopped upon use of the drug. However, the systemic levels which have to be attained to gain this benefit from thalidomide, in the instance of rheumatoid arthritis, are projected to have serious untoward effects. The question we would like to answer, therefore, is whether or not thalidomide or a similarly acting compound can be focused to act only in local areas by applying it topically. The goal here is thus to assess the possibility of delivering a drug of the kind through skin as the first step in getting an effective agent into the synovial fluid of an infected rheumatoid arthritis joint. The ultimate intent would be to inhibit TNF-α production within the joints beneath and around the site of application without raising circulating levels of a drug to the point of concern. Among other benefits, such percutaneous delivery levels out the peaks and valleys in blood levels seen with discrete oral dosages. Drug concentrations which can be achieved in deep tissues beneath the application (i.e. joints, masculature, etc.) are expected to be higher than can be achieved by oral administration of the same total body exposure to the drug.

Generally, molecules containing multiple hydrogen bonding centers and/or strong dipoles are high melting due to strong intracrystalline self-association. Such molecules have little tendency to dissolve in organic phases, and consequently, their partitioning into the lipoidal conduit phases of the skin is expected to be minimal. Permeation rates will therefore be low even from their saturated solutions. Clearly, a low capacity to dissolve in the transport phases is a major obstacle to percutaneous delivery.

In order to know whether a drug can be delivered percutaneously, one must assess the drug's permeability through skin. Thus *in vitro* diffusion cell methods were brought to bear on the percutaneous absorption assessment problem.

# 4.2 Experimental

## 4.2.1 Materials and methods

### 4.2.1.1 Materials

Thalidomide and three of its odd chain N-alkyl analogues (methyl, propyl and pentyl) were synthesized and purified according to previously described methodology (Chapter 3). Their identity and purity (≥ 96%) were confirmed by element analysis (EA), electron impact mass spectroscopy (EI-MS), differential scanning calorimeter (DSC), high-pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. Carbon, hydrogen, and nitrogen analysis were in excellent agreement with theory in all cases. The overall yields ranged from 40-60%. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburg, PA, USA) and all other reagents were of analytical grade.

### 4.2.1.2 Chromatography

Amounts of thalidomide and its N-alkyl analogues, which penetrated through skin mounted in diffusion cells had to be quantitatively determined and an HPLC assay, developed by Eriksson ***et al.*** (1992) was modified to do this. The HPLC consisted of a Beckman 114M solvent delivery system and a Spectraflow 783 variable wavelength ultraviolet detector. The latter was operated at a 220 nm wave length and a sensitivity setting of 0.05 AUFS. A Perkin-Elmer ISS-100 auto-injector was used to inject samples (20 µl) onto a Spheri-5 (RP-8, 5 µm, 220 x 4.6 mm) column (Alltech Associates, Inc., Deerfield, IL, USA). A NewGuard pre-column (RP-18, 7 µ, 15 x 3 mm) insert (Alltech Associates, Inc., Deerfield, IL, USA) was used to remove soluble biopolymers eluted from skin. This was changed frequently to minimize on column injection of such residues and to prolong column life. The mobile phase was comprised of acetonitrile/water (1:4) in the case of thalidomide. The pH of the mobile phase was adjusted to 2.0 with ortho-phosphoric acid (Fluka Chemical Co., Ronkonkoma, NY, USA). Baseline separation of thalidomide from other substances in the samples was achieved at a flow rate of 1.2 ml/min. Quantitation of the individual amounts in the samples was performed using a Hewlett Packard HP 3395 integrator. The concentrations of the N-methyl, N-propyl and N-pentyl analogues were determined by essentially the same HPLC method. However, slight modifications of the mobile phase had to be made to effect comparable separation. The mobile phases for these series contained 25, 35, and 45% acetonitrile, respectively. Under the chromatographic conditions described above, the retention times of thalidomide and its N-methyl, N-propyl and N-pentyl analogues were

approximately 7, 6, 7, and 8 min, respectively. A single peak was seen at the 220 nm wavelength for each compound, attesting to this high levels of purity. Standard curves showed excellent linearity over the concentration range experienced in analyzing the samples.

#### 4.2.1.3 Skin Preparation

The human cadaver skin used in the permeation studies was obtained from the Anatomical Donation Program at the University of Michigan. Samples of split-thickness skin were removed from the thigh and abdomen of cadavers within 24 hr postmortem, with the aid of a dermatome set at 250 µm. Epidermal layers were separated from split-thickness skin by immersing the skin in 60°C water for 1 min. The epidermal layer was gently teased away from the remaining tissue with forceps. The skin sections were cut into squares, wrapped in plastic film and stored in a freezer at -20°C until utilized. The frozen skin pieces were thawed and examined for defects before mounting them within the diffusion apparatus. The frozen tissue was either used within 2 months of its receipt or was discarded.

#### 4.2.1.4 Skin Permeation Method

Vertical Franz diffusion cells with a 4 ml capacity receptor compartment and a 0.8 $cm^2$ diffusion area were used in the permeation studies. The epidermal layer of the skin was mounted carefully onto the lower half of the cells of the diffusion apparatus with the ***stratum corneum*** facing up in the direction of the donor compartment. The donor "chimneys" were fastened to the receptor compartments with a clamp, with the skin acting as a seal between the half cells. The receptor compartments were filled with isotonic phosphate buffer (pH 6.4). Care was taken to see that there were no consequential air bubbles left in the compartments. The temperature of the cell system was maintained at 32°C by circulating water from a constant temperature water bath (Lauda K-2/RD, Beckman Instruments, Inc., Fullerton, CA, USA) through the jacket of the lower compartment. The receptor cell compartments were filled and equilibrated with buffer 1 hr before adding the drug-containing solution to the donor compartment. Stirring was maintained during the entire experiment. A small magnetic stirring bar was placed at the bottom of the receptor compartment to accomplish this. To begin an experiment, the donor compartment was charged with 300 µl of fresh prepared saturated solution of the drug and covered immediately with Parafilm to prevent any significant evaporation of volatile components of the applied medium during the absorption experiment. At predetermined times, the entire receptor volume was withdrawn and replaced with 32°C fresh buffer. This was done to insure that sink conditions existed throughout the experiment. 20 µl of these samples were directly assayed by HPLC to determine drug concentrations in the receiver fluid. The duration of the typical skin permeation experiment was $\leq$ 30 hr.

#### 4.2.1.5 Solubility Determination

The solubilities of thalidomide and its N-alkyl analogues in the vehicles used for delivery were obtained by equilibrating excess amounts of each of the compounds with phosphate buffer (pH 6.0), each of an extended series of n-alcohols and the specific combinations of solvents and penetration enhancers used as vehicles after placing them individually in stoppered, water-jacketed, glass containers. The temperature was maintained at 32°C by circulating water through the jackets from a constant temperature water bath. The slurries were vigorously and continuously mixed for 24 hr using magnetic stirring bars. On each and every occasion, an excess of solute was present in the slurries. Preliminary work indicated that under the conditions used, a saturated state was achieved well within one day. Therefore, samples were taken after 24 hr of vigorous mixing. These samples were filtered through filters (PTFE filter media with Polypropylene housing, 0.45 µm pore size, Whatman Inc., Haverhill, MA, USA) preconditioned to the experimental temperature. Syringes used for filtration were also brought to 32°C prior to their use. Each filtrate was appropriately diluted with methanol prior to its assay. Drug concentrations were determined by HPLC.

#### 4.2.1.6 Stability Determination

The stabilities of the compounds under the conditions of the flux experiments were checked. A stock solution of each compound (160 µg/ml) was prepared in methanol. A series of dilutions into three phosphate buffer solutions (pH 6.0, 6.4 and 7.4) was made to give final drug concentrations of 2 µg/ml containing less than 1% methanol. To obtain buffers at the two lowest pH's, the 0.1 M phosphate buffer (pH 7.4) was adjusted to pH 6.0 and 6.4 with ortho-phosphoric acid. The various solutions were incubated at 25°C and 32°C for 24 hr, with samples being taken at various times (1, 2, 4, 6, 8 and 24 hr). The hydrolysis reaction was immediately quenched by diluting the samples with the appropriate mobile phase (pH 2) of each compound. These were then stored at -20°C until analyzed by HPLC. Normal log [concentration] -time profiles were plotted. The slopes of these curves were taken as the rate constants. Half-lives were calculated by dividing 0.693 by the individual rate constants.

#### 4.2.1.7 Partition Coefficient Determination

Equal volumes of n-octanol and phosphate buffer (pH 6.4) were equilibrated with each other for at least 24 hr before measuring the partition coefficients so that the phases would be co-saturated with one another. Solutions of each compound in this study (30 µg/ml) were prepared with the pre-saturated n-octanol phase as the solvent. Five milliliters of these solutions were transferred to 10-ml assay tubes containing equal volumes (5 ml) of n-octanol equilibrated phosphate buffer. Three tubes of each compound were stoppered and agitated (Electronic IKA-

VIBRAX-VXR, Janke & Kunkel Typ VX2) for 1 hr and another set of three was agitated for 2 hr. After centrifugation at 2000 g for 10 min, the octanol and buffer phases were carefully pipetted to separate tubes and analyzed by HPLC for drug concentrations. The aqueous phase samples were diluted with the respective mobile phase for each compound. The octanol phases were appropriately diluted with methanol before injecting them onto the HPLC column. The pH of the buffer was measured before and after each drug was added to ensure that the compounds had no influence on the pH. Partition coefficients ($K_{oct}$) were calculated as the ratio of drug concentration in the octanol phase to that in the buffer phase. There was no difference in the value of $K_{oct}$ when the tubes were agitated for 1 or 2 hr, indicating 1 hr was sufficient for partitioning equilibration.

#### 4.2.1.8 Data Analysis

The permeability coefficient for a given run was calculated from Fick's law of diffusion:

$$P = \frac{V_R(dC/dt)}{A(\Delta C)}$$

where:

- dC/dt is the steady-state slope of a plot of the amount of substance which had penetrated the skin against time in terms of μg/h. It was determined by taking the ratio of the total amount permeated in an interval of time to the length of the time interval.
- P is the effective permeability coefficient (cm/h) which is calculated.
- A is the diffusional area, which was 0.8 $cm^2$ in this study.
- ΔC is the concentration differential existing across the membrane. This was effectively equal to the saturation concentration in the donor phase (μg/ml) as, through total exchange sampling, a near zero receiver concentration (sink condition) was closely approximated. ΔC is, in effect, the thermodynamic force driving mass transfer. The maximum driving force is seen at the saturation solubility (excluding supersaturation).
- $V_R$ is the volume of the receiver compartment (4 ml).

## 4.3 Results

The vast majority of published permeability coefficients for the skin have been determined using aqueous media in both the donor and receptor compartments. There is no other reasonable solvent to use for this purpose, as water is the least damaging solvent relative to the skin's barrier elements, particularly the ***stratum corneum***. The solubility of a drug in aqueous media used in the donor phase has to be known if one is to assess the permeability coefficient, using saturated solutions. Therefore, the aqueous solubilities (µg/ml) of thalidomide and its N-alkyl analogues were determined and are given in Table 4-1. The melting points and octanol/water partition coefficients ($K_{oct}$) of these compounds are also listed in Table 4-1.

The aqueous solubility data in Table 4-1 are plotted as the logarithm of solubility *versus* alkyl chain length in Figure 4-1. The slope of the statistically best line through the solubility data for the N-alkyl analogues up to pentyl is 0.404; thus, each methylene unit decreases the aqueous (buffer, pH 6.0) solubility by a factor of 2.54. Of course this line is drawn for these odd chain length analogues and therefore doesn't tell the whole story. Figure 4-2 is a logarithmic plot of experimentally derived permeability coefficients measured from water against the alkyl chain length. Octanol/water partition coefficients of each of the compounds are superimposed on the permeability coefficient plot. It is clear that both parameters increase exponentially with increasing alkyl chain length. While the slopes of the plots are clearly not the same, when the experimental permeability coefficients from water are plotted against the partition coefficients (Figure 4-3) a strong correlation is found between them. This correlation reflects the fact that skin partitioning is an element of the mass transport process, which share a common dependency with octanol/water partitioning. This alone indicates that the skin is acting to a first good approximation as a lipophilic barrier.

**TABLE 4-1:** Aqueous solubility and physicochemical parameters of thalidomide and its N-alkyl analogues.

| Compound | Melting Point (°C) | Aqueous Solubility at 32°C (µg/ml) | Log $K_{oct}$ |
|---|---|---|---|
| Thalidomide | 275 | 61.4 | 0.49 |
| N-Methyl Thalidomide | 159 | 370.4 | 1.15 |
| N-Propyl Thalidomide | 136 | 59.4 | 2.11 |
| N-Pentyl Thalidomide | 105 | 9.0 | 3.01 |

The permeation parameters (flux, J; lag time, $T_L$ and permeability coefficient, P) of thalidomide and its N-alkyl analogues from their saturated aqueous solutions (pH 6.0) and an ethanol/water/octanol vehicle are summarized in Table 4-2. The permeation data were plotted as the cumulative amount of drug penetrated through skin as a function of time. The steady-state flux was determined from the slope of the linear portion of the cumulative amount-time plot. The lag time ($T_L$) was determined by extrapolating the linear portion of the curve to its intersection with the x-axis. None of the compounds evidenced detectable lag times within the extended time frames of the permeation experiments from water. More specifically, the lag times were short and could not be distinguished from zero in experiments with 5 hr sampling periods. A bar plot of the mean steady-state flux and standard deviations (SD) for thalidomide and its N-alkyl analogues from water can be seen in Figure 4-4. Since thalidomide was actually not detected, its greatest possible mean steady-state flux from water was calculated according to the limit of detection of the HPLC method, which was 0.01 μg/ml. Thus, the flux of thalidomide was less than 0.01 $\mu g/cm^2/h$. The fluxes of thalidomide and its N-alkyl analogues were all statistically different from one another at $p<0.01$ with one exception. The statistical difference between N-methyl thalidomide's flux and that for N-propyl thalidomide was statistically different at a 90% confidence level ($p<0.1$). Typical cumulative amount permeated-time profiles for thalidomide and its N-alkyl analogues from the ethanol/water/octanol (Formulation C) vehicle are shown in Figure 4-5. In all cases, steady-state fluxes were attained within 3 hr after application of the drug solutions. These were maintained throughout the entire duration of the diffusion experiment.

**TABLE 4-2:** Permeation parameters of thalidomide and its N-alkyl analogues through human skin.

| Vehicle | Compound | J ± SD ($\mu g/cm^2/h$) | $T_L$ ± SD (h) | P ± SD × $10^{-3}$ (cm/h) at 32°C |
|---|---|---|---|---|
| Aqueous (pH 6.0) [a] | Thalidomide | 0.01 ± 0.00 [b] | [c] | 0.16 ± 0.00 [b] |
| | N-Methyl | 0.43 ± 0.08 | [c] | 1.17 ± 0.22 |
| | N-Propyl | 0.34 ± 0.13 | [c] | 5.73 ± 2.22 |
| | N-Pentyl | 0.18 ± 0.06 | [c] | 19.68 ± 6.31 |
| ($EtOH/H_2O$/ octanol) [d] | Thalidomide | 0.713 ± 0.218 | 2.2 ± 1.4 | |
| | N-Methyl | 6.450 ± 0.448 | 2.8 ± 0.5 | |
| | N-Propyl | 2.087 ± 0.292 | 1.8 ± 1.1 | |
| | N-Pentyl | 2.002 ± 0.178 | 3 ± 1.6 | |

[a] Each value from the aqueous donor is the mean ± standard deviation (SD) of 6 diffusion experiments. [b] Since thalidomide could not be detected, these values are calculated according to the limit of detection of the HPLC method (0.01 µg/ml). [c] $T_L$ could not be determined accurately because of relatively short lag times. [d] Ethanol/water(pH 6.0)/n-octanol: (57.5 : 40 : 2.5). Each value is the mean ± standard deviation (SD) of 3 diffusion experiments.

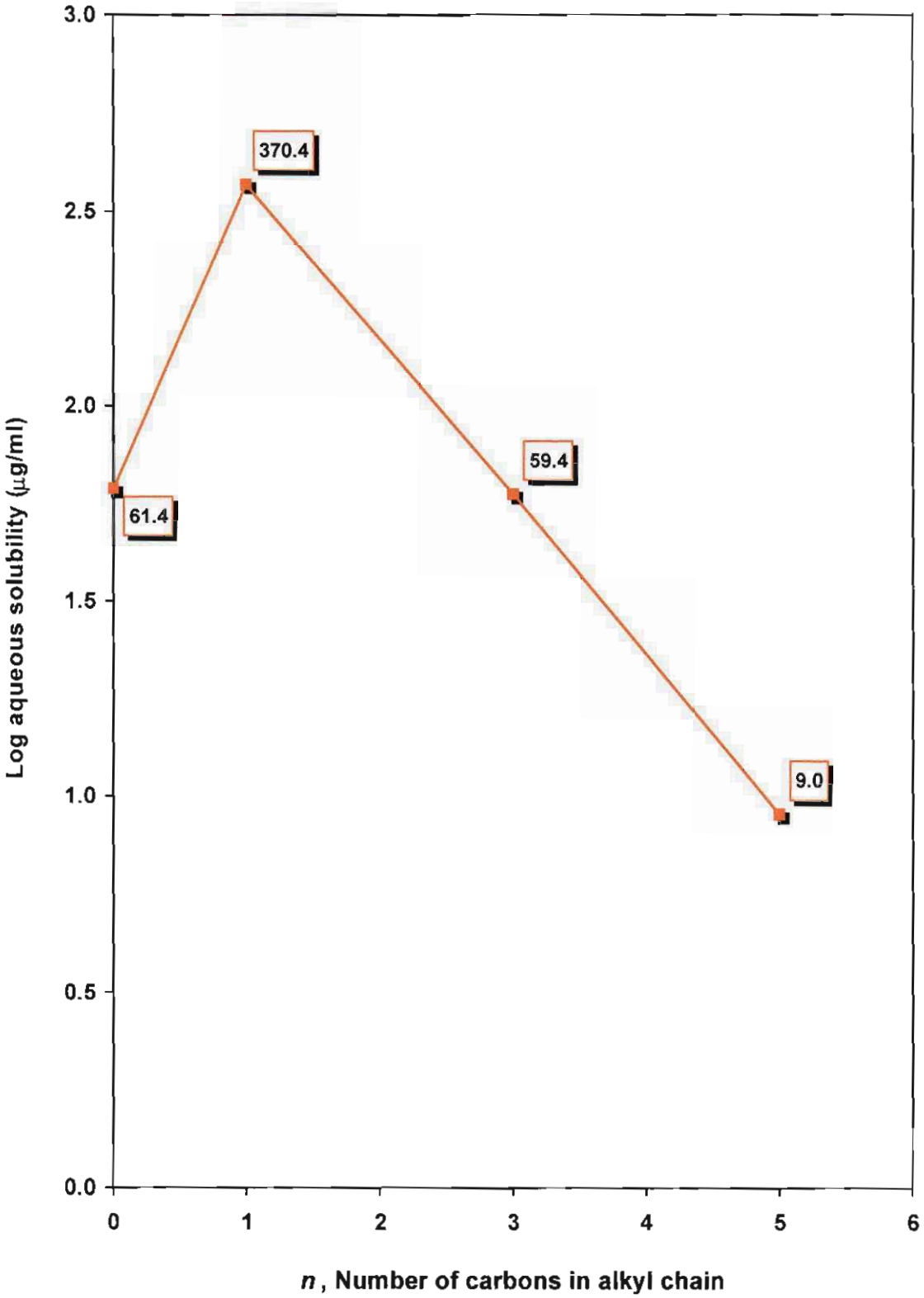



FIGURE 4-1: Aqueous solubility at 32°C of thalidomide (plotted on y-axis at $n = 0$) and its N-alkyl analogues *versus* alkyl chain length.

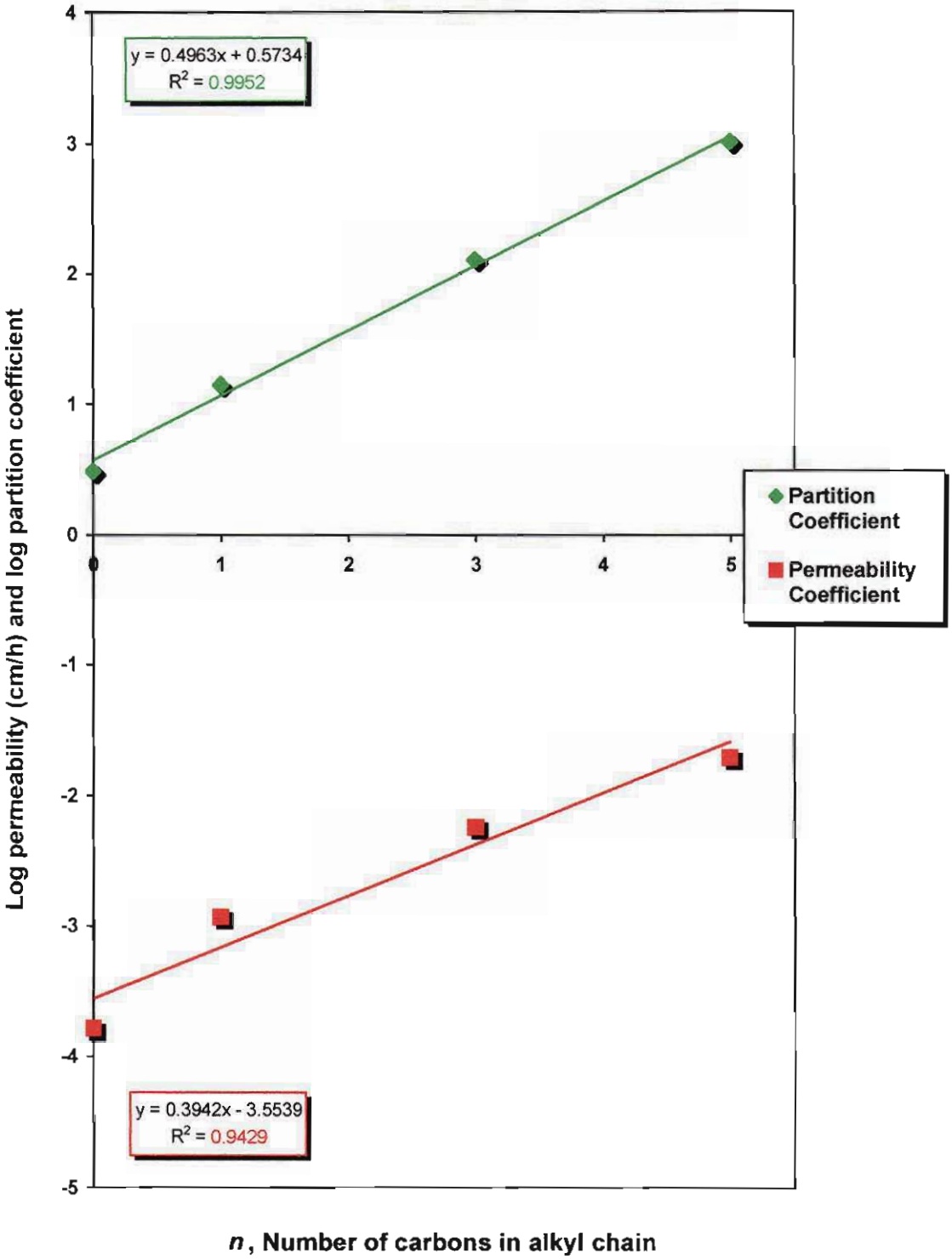



FIGURE 4-2: Logarithmic plot of experimentally derived permeability and partition coefficients of thalidomide and its N-alkyl analogues *versus* alkyl chain length. Thalidomide's values are plotted on the y-axis at $n = 0$.

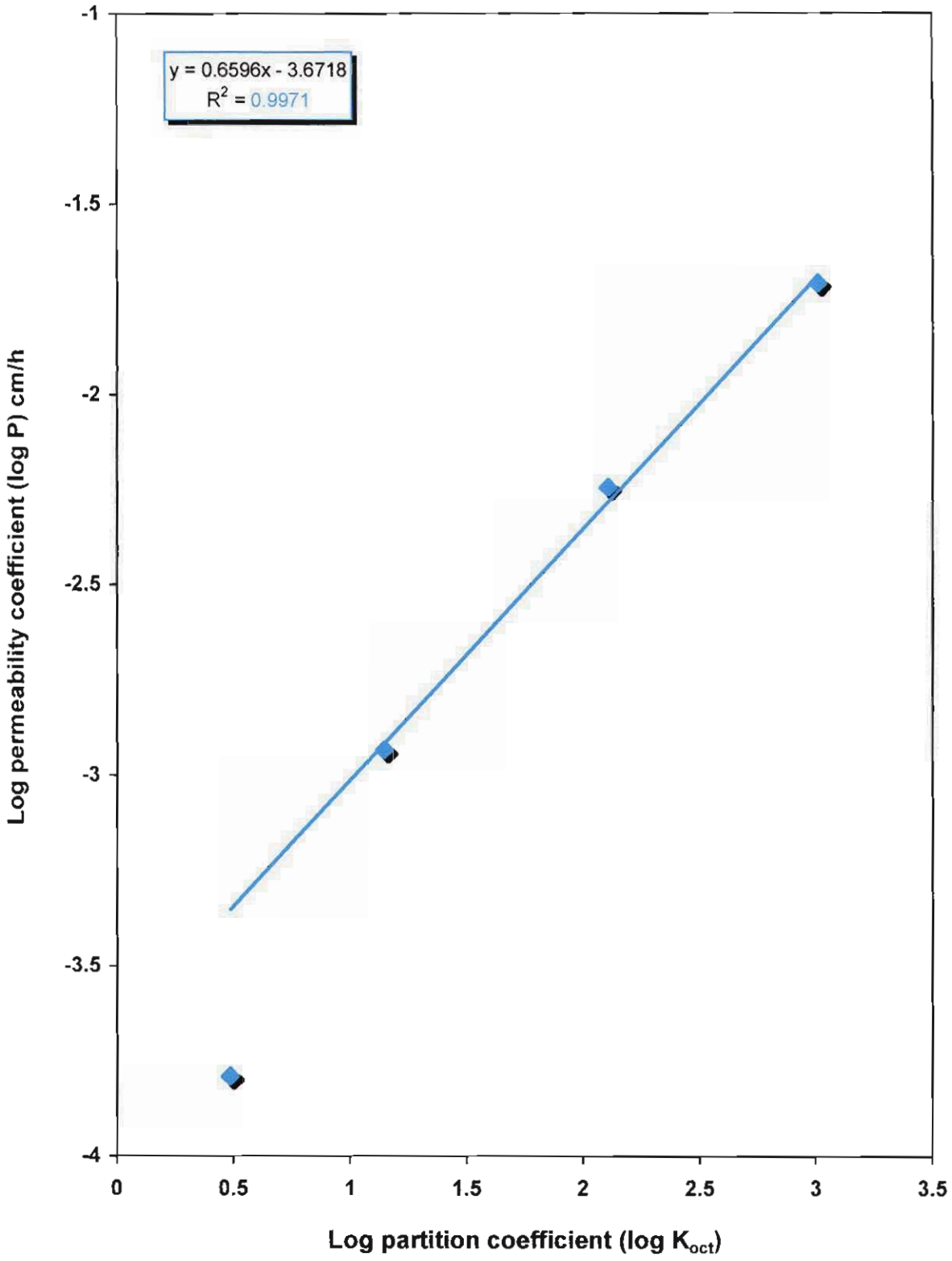



FIGURE 4-3: Permeability coefficient *versus* partition coefficient.

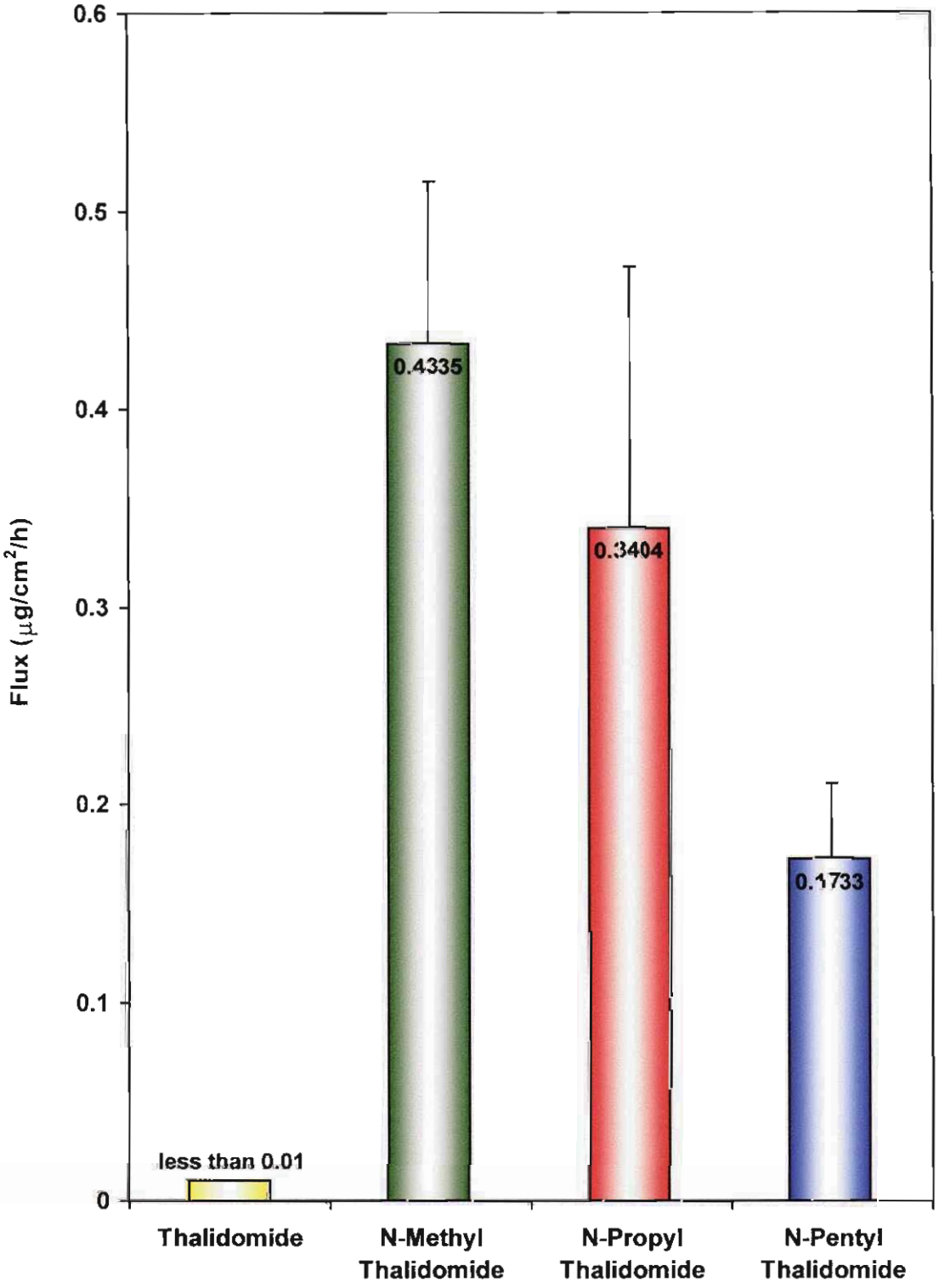



**FIGURE 4-4:** Mean (n = 6) ± SD steady-state flux of thalidomide and N-alkyl analogues from water at 32°C.

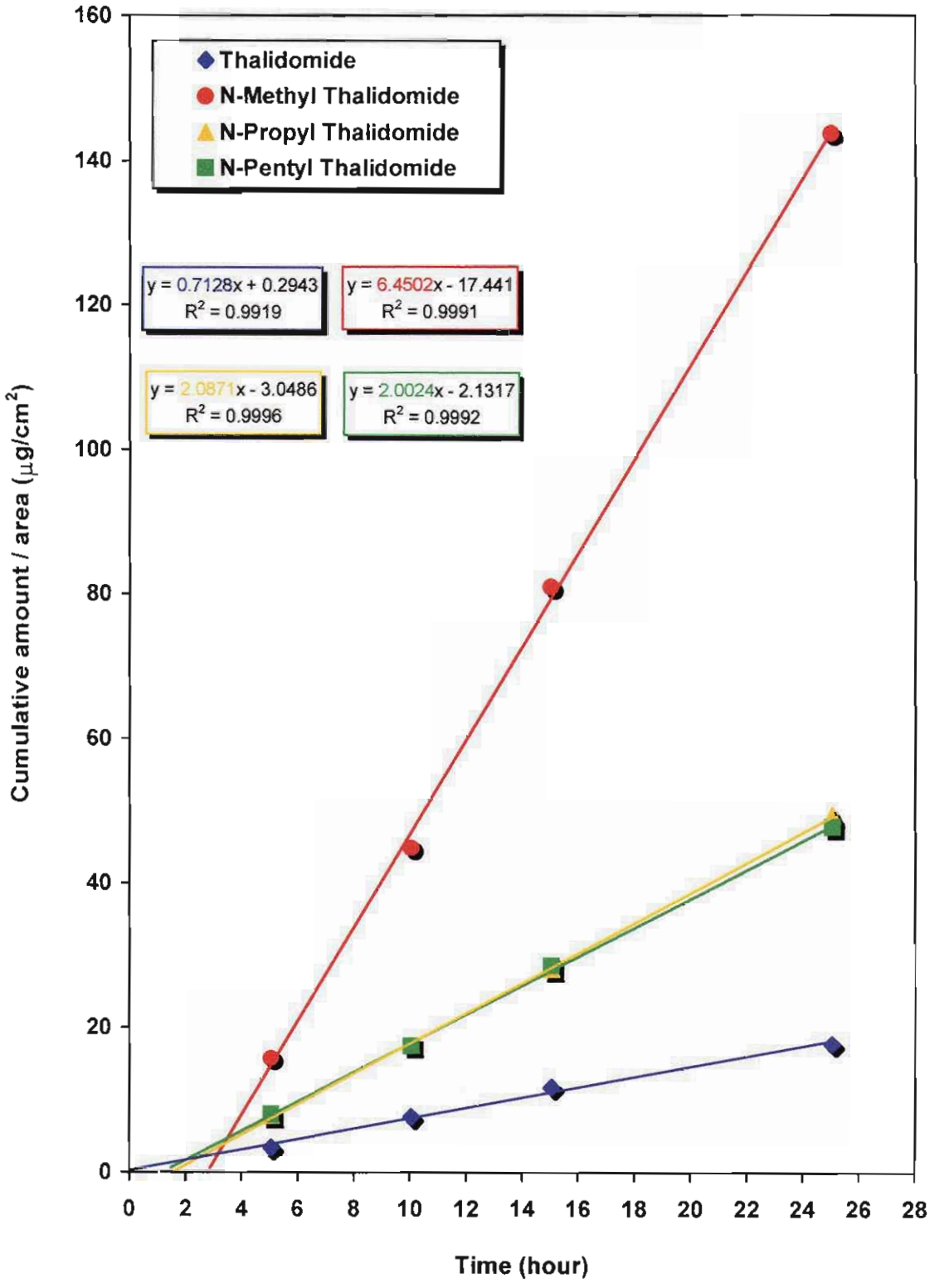



**FIGURE 4-5:** Representative permeation profiles of thalidomide and its N-alkyl analogues from formulation C at 32°C.

Several compounds are commercially delivered from transdermal systems having alcohol-containing reservoirs. Estradiol and fentanyl are two transdermal drugs which appears in formulations containing ethanol as a penetration enhancer. It was hypothesized that this solvent or a closely related compound might prove to be of benefit with respect to delivering thalidomide. Systematic studies were begun using a range of homologous alkanols to explore the hypothesis. The solubilities of thalidomide and its N-alkyl analogues in a series of n-alcohols, methanol through dodecanol, are given in Table 4-3. These values, determined at 32°C are plotted in Figure 4-6. It can be seen that for every solute the solubilities across the homologous series of solvents decrease systematically with increasing alkanol chain length. Figure 4-6 also establishes a relationship that exists between solubility and alkylation of the thalidomide molecule. The permeabilities of thalidomide and its N-alkyl analogues through human skin at 32°C were determined using the n-alcohols as solvents as used in the solubility studies. These data are presented in Figure 4-7 as the steady-state flux ($\mu g/cm^2/h$) against the number of carbons in the n-alcohols. As was seen from the n-alcohol solubility profiles the permeability profiles within each compound also decreased, albeit far more irregularly.

In order to enhance the skin flux and simultaneously determine which analogue in the study penetrates the skin best, various combinations of solvents and penetration enhancers were combined and used as vehicles. The compositions of these formulations, A-D, are given in Table 4-4. The formulations were chosen based on the results of early experiments aimed at formulating thalidomide into a percutaneous application. The solubilities of thalidomide and its N-alkyl analogues in these formulations are provided in Figure 4-8. The steady-state fluxes of thalidomide and its N-alkyl analogues, from formulations A-D can be seen in Figure 4-9. In order to make comparisons of these values, the same skin specimen was used for the compounds applied within each individual formulation. The flux of N-methyl thalidomide is statistically higher ($p<0.05$) than that of thalidomide and the other analogues in formulations A, B and C. Although the flux of N-methyl thalidomide is not statistically separable ($p<0.05$) from fluxes obtained for the N-propyl and N-pentyl analogues when using formulation D, all the N-alkyl analogues penetrated the skin more readily than does thalidomide ($p<0.05$). In formulation B, only N-methyl thalidomide is statistically higher and separable ($p<0.05$) from the other compounds and, in formulation A, only N-pentyl thalidomide is not statistically different ($p<0.05$) from thalidomide. There is no statistical difference ($p<0.05$) between the fluxes of the N-propyl and N-pentyl analogues in formulation C, but both are statistically higher ($p<0.05$) than the flux found for thalidomide.

**TABLE 4-3:** Solubility of thalidomide and its N-alkyl analogues in a series of n-alcohols.

| n-Alcohol | SOLUBILITY (mg/ml) at 32°C | | | |
|---|---|---|---|---|
| | **Thalidomide** | **N-Methyl Thalidomide** | **N-Propyl Thalidomide** | **N-Pentyl Thalidomide** |
| Methanol | 1.13 | 13.78 | 18.78 | 42.60 |
| Ethanol | 0.40 | 6.91 | 13.20 | 29.63 |
| Propanol | 0.26 | 5.78 | 10.55 | 26.65 |
| Butanol | 0.19 | 4.47 | 10.35 | 26.11 |
| Pentanol | 0.16 | 3.98 | 8.56 | 24.08 |
| Hexanol | 0.12 | 3.26 | 7.26 | 21.78 |
| Heptanol | 0.09 | 2.93 | 6.45 | 20.34 |
| Octanol | 0.07 | 2.64 | 6.21 | **20.19** |
| Nonanol | 0.06 | 2.41 | 5.83 | 15.87 |
| Decanol | 0.05 | 2.38 | 3.87 | 12.53 |
| Undecanol | 0.04 | 2.29 | 3.73 | 11.67 |
| Dodecanol | 0.04 | 1.91 | 3.18 | 11.56 |

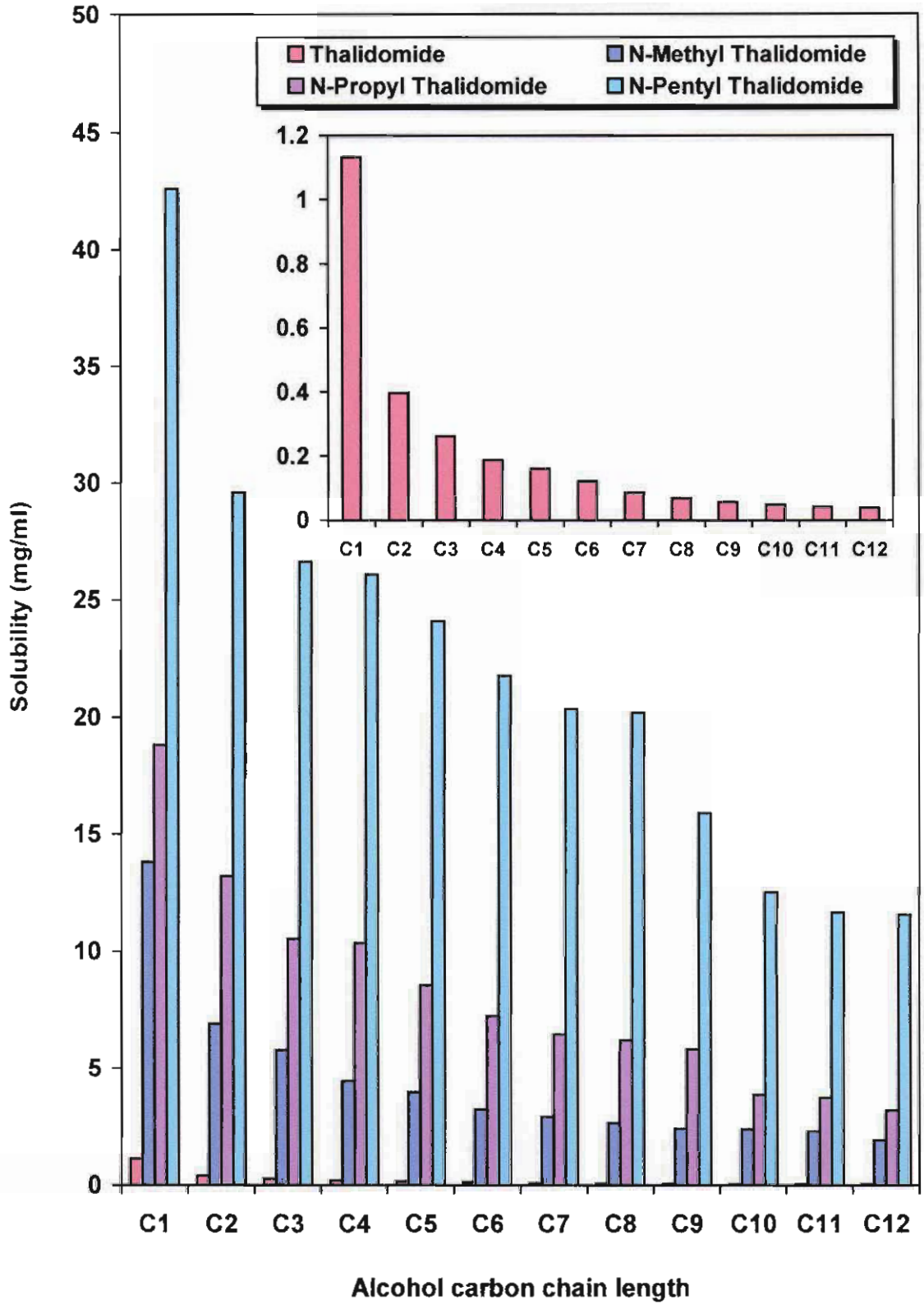



FIGURE 4-6: Solubilities of thalidomide and its N-alkyl analogues in n-alcohols at 32°C.

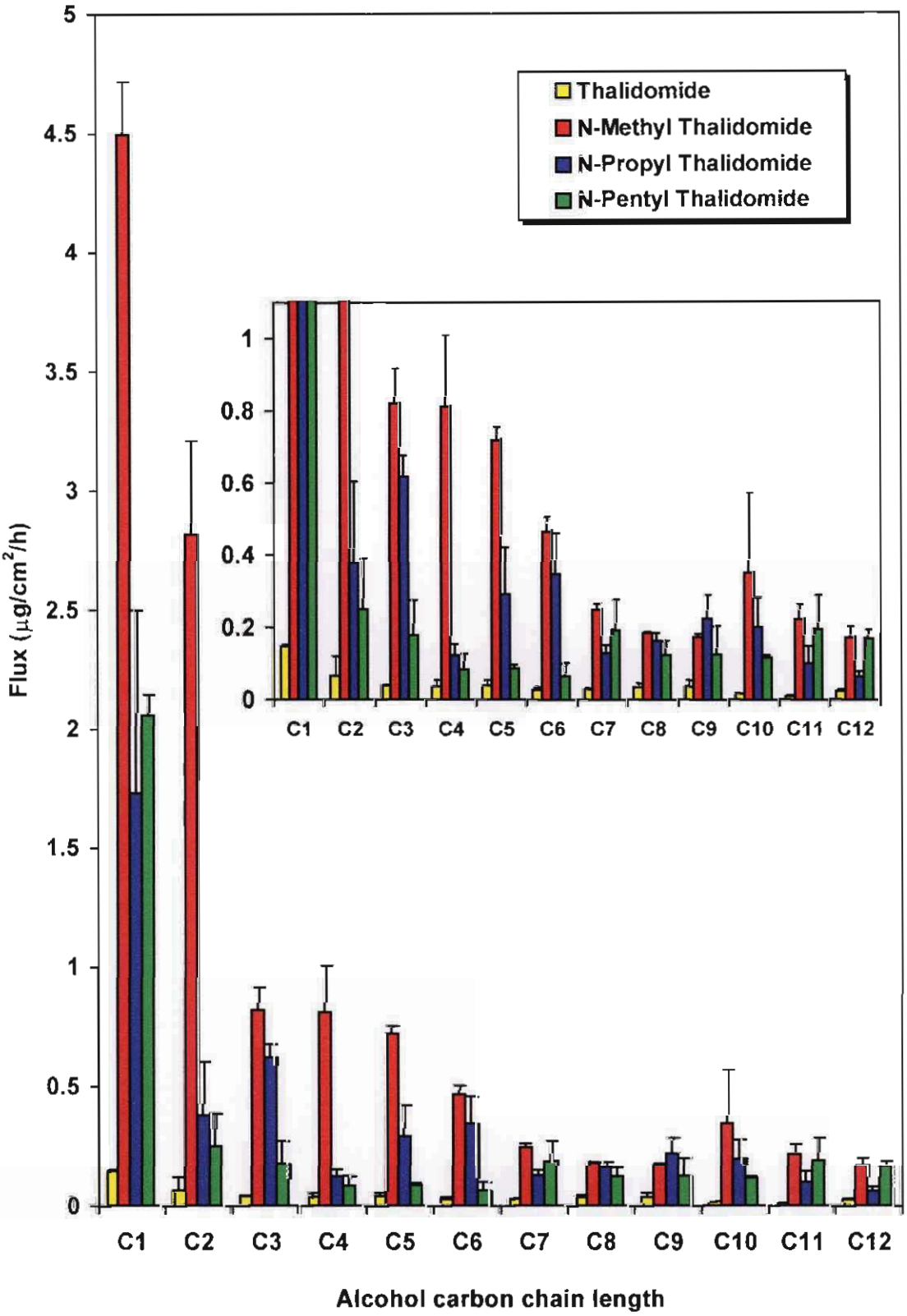



FIGURE 4-7: Steady-state fluxes of thalidomide and its N-alkyl analogues from saturated n-alcohol solutions plotted against the alcohol chain length. These data are the mean ± standard deviation of 3 diffusion experiments.

**TABLE 4-4:** Composition and ratios of formulations A-D.

| Formulation | Solvent / Enhancer | % Composition |
|---|---|---|
| A | Isopropanol | 70 |
| | NMP [a)] | 10 |
| | n-Octanol | 10 |
| | Citric Acid | 5 |
| | IPM [b)] | 5 |
| B | Ethanol | 80 |
| | n-Octanol | 10 |
| | Citric Acid | 5 |
| | IPM [b)] | 5 |
| C | Water [c)] | 57.5 |
| | Ethanol | 40 |
| | n-Octanol | 2.5 |
| D | Ethanol | 95 |
| | IPM [b)] | 5 |

[a)] N-Methyl Pyrrolidone. [b)] Isopropyl Myristate Ester. [c)] pH 6.0

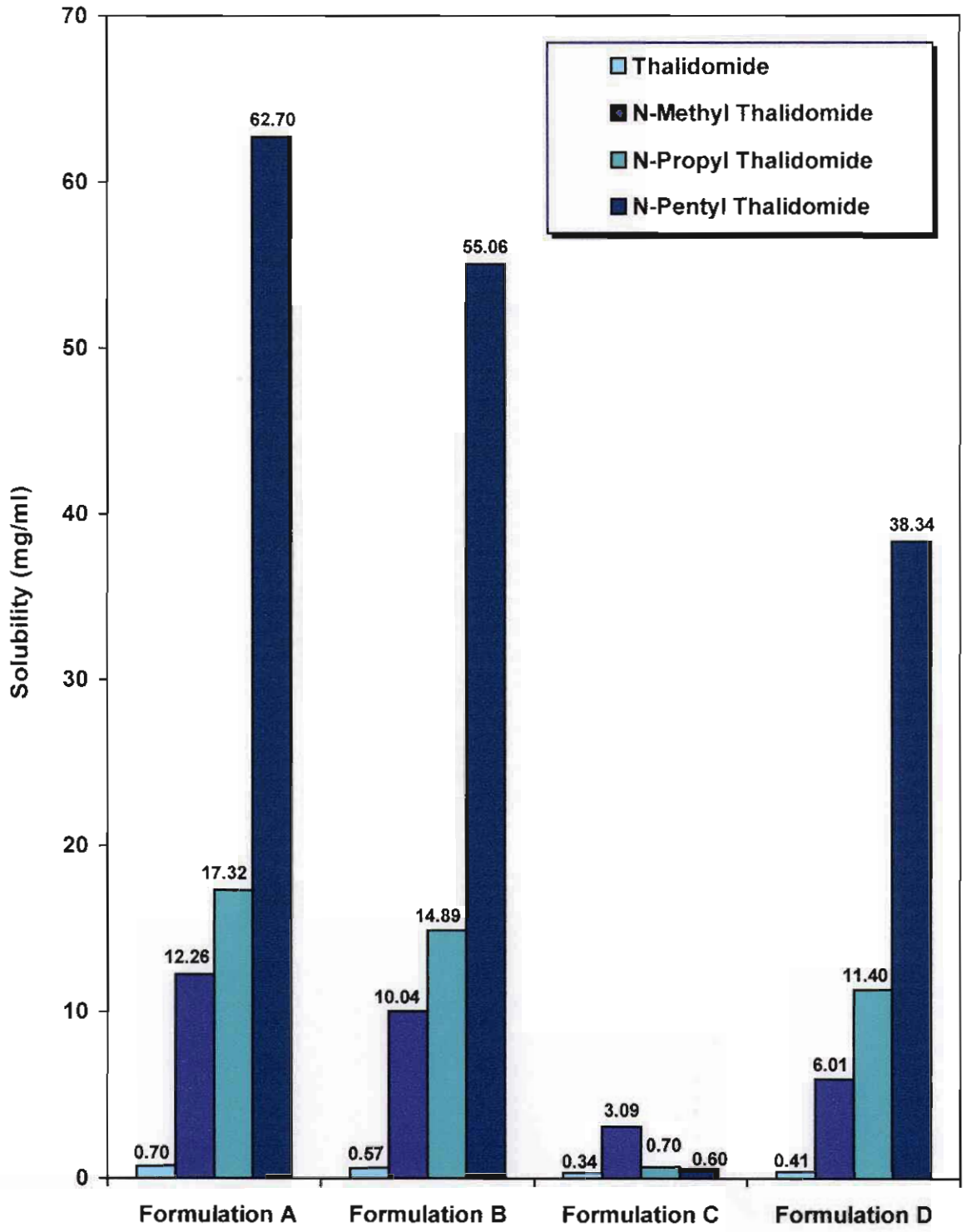



FIGURE 4-8: Solubilities of thalidomide and its N-alkyl analogues in different formulations.

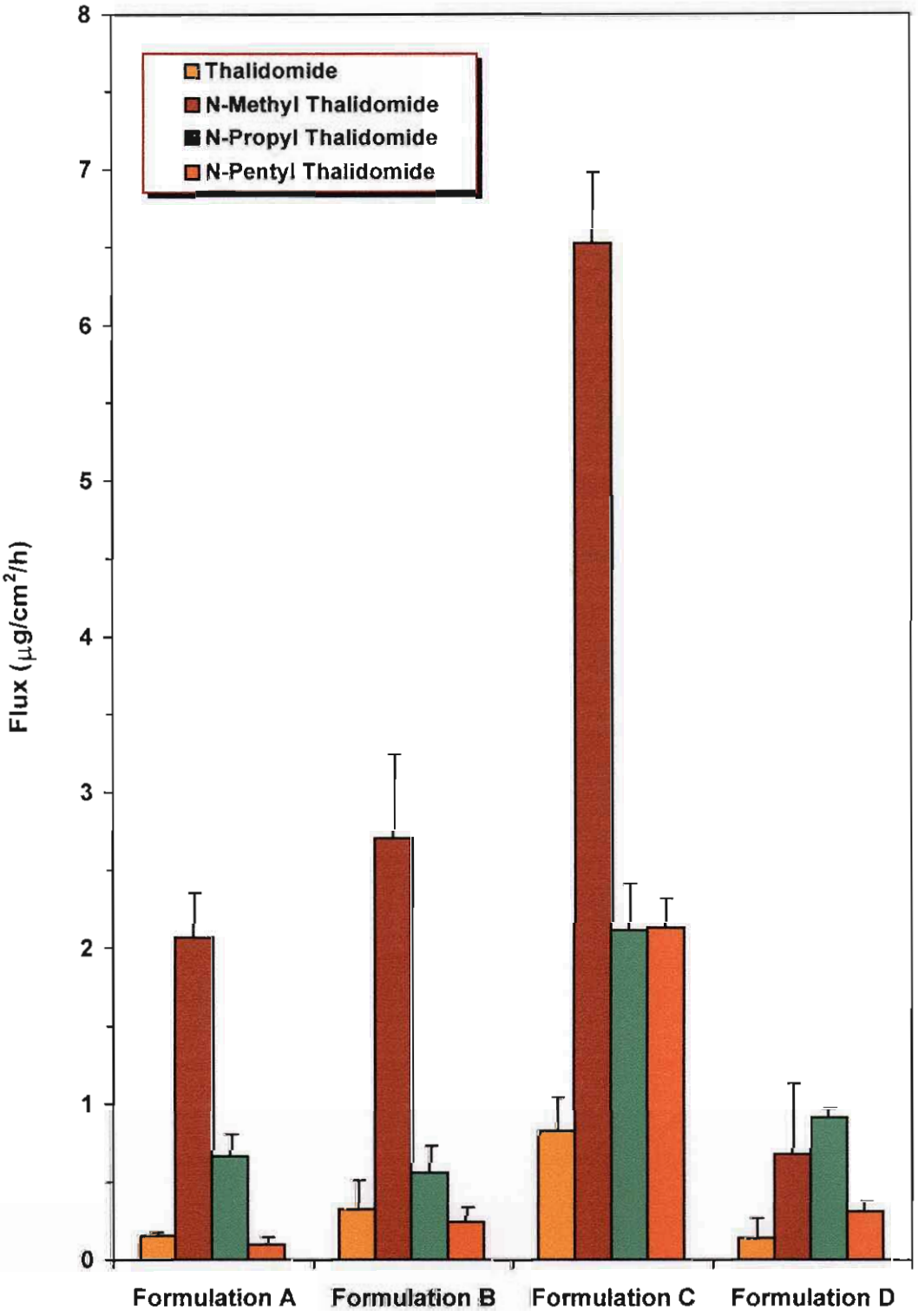



**FIGURE 4-9:** Bar plot showing a mean ($n$ = 3) steady-state flux ± standard deviation of thalidomide and its N-alkyl analogues from different formulations.

In Figure 4-10, N-methyl thalidomide's penetration curves (cumulative amount penetrated *versus* time) obtained using the four different vehicle compositions (Formulations A-D) is shown. These penetration curves depict the expected behaviour for passive diffusion through a membrane. There is a distinct lag time and eventually the penetration rate becomes constant. All the vehicle compositions were studied using membranes cut from the same skin specimen, so the resultant data should be comparable. The maximum mean (n = 3) steady-state flux obtained for N-methyl thalidomide (11.47 $\mu g/cm^2/h$) occurred with formulation C, as can be seen from Figure 4-11. Formulation C is statistically superior ($p<0.01$) as a delivery medium to formulations A, B and D, but there are no statistical differences ($p<0.5$) between formulations A, B and D.

It is known that thalidomide hydrolyses spontaneously in aqueous media. The rate of its hydrolysis accelerates with increasing pH and, of course, temperature (Schumacher ***et al.,*** 1965). To roughly determine whether the N-alkyl analogues hydrolyse to a comparable extent, the compounds were placed in solution in aqueous buffers at pH's 6.0, 6.4 and 7.4 and the stabilities of each was assessed at 25° and 32°C. Since the reactions are pseudo first order, the natural logarithms of concentration of thalidomide and its odd chain N-alkyl analogues were plotted as a function of time and the slopes of the linear curves were taken as the rate constants. These data can be seen in Figures 4-12 and 4-13 representing 25°C and 32°C data, respectively. The half-lives, listed in Table 4-5, were calculated by dividing the value of 0.693 by the individual rate constants.

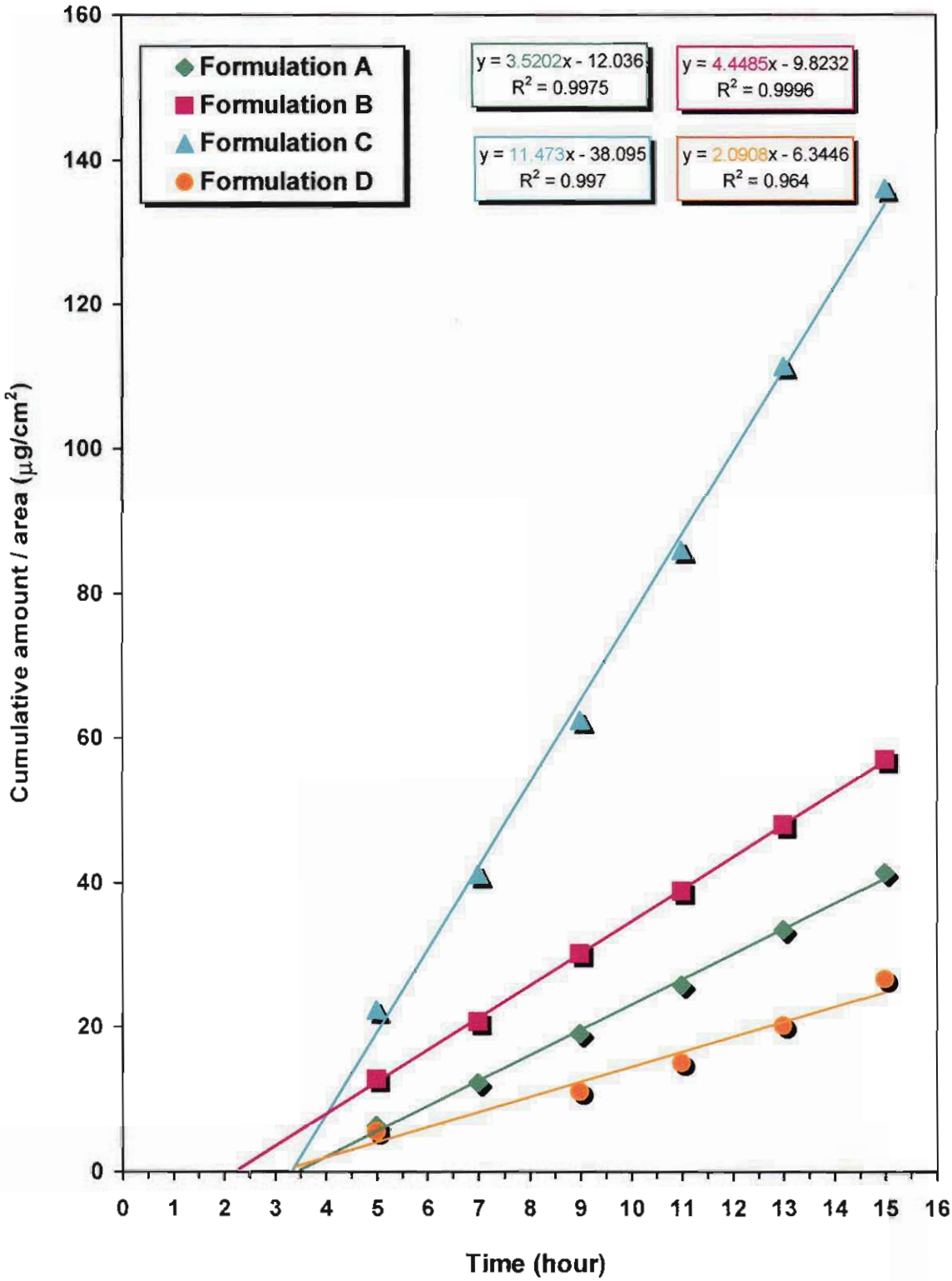



**FIGURE 4-10:** Representative permeation profiles of N-methyl thalidomide from formulation A-D.

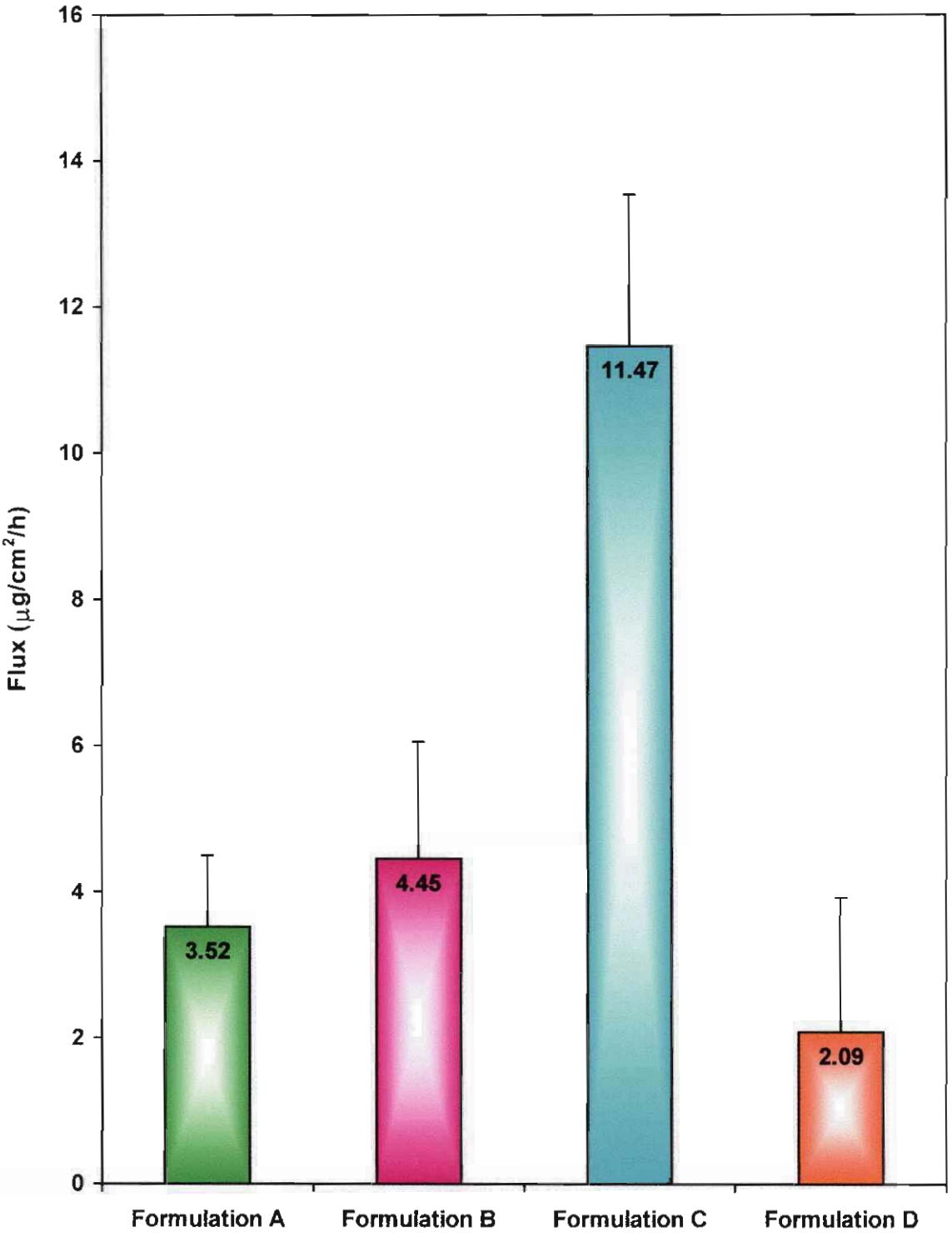
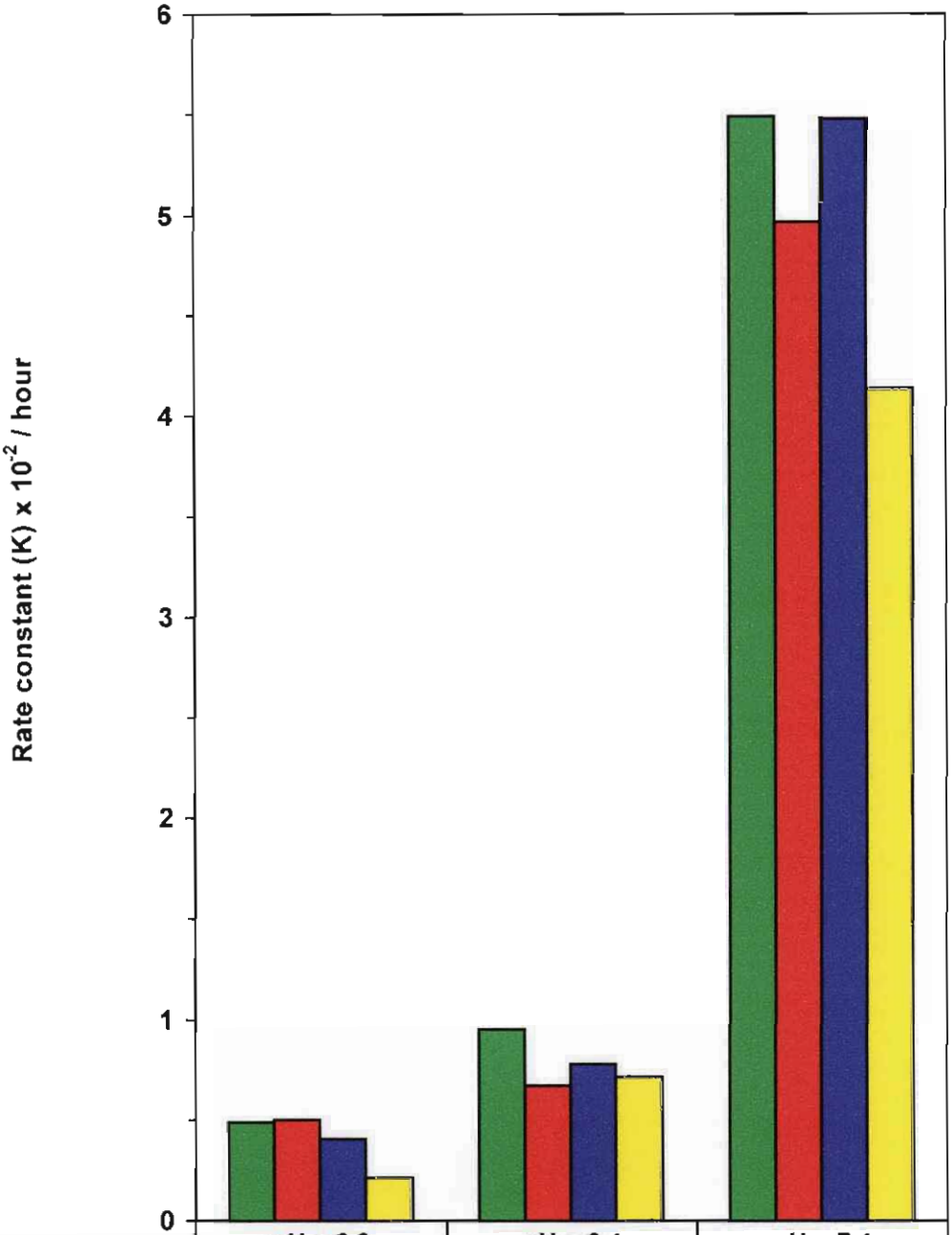
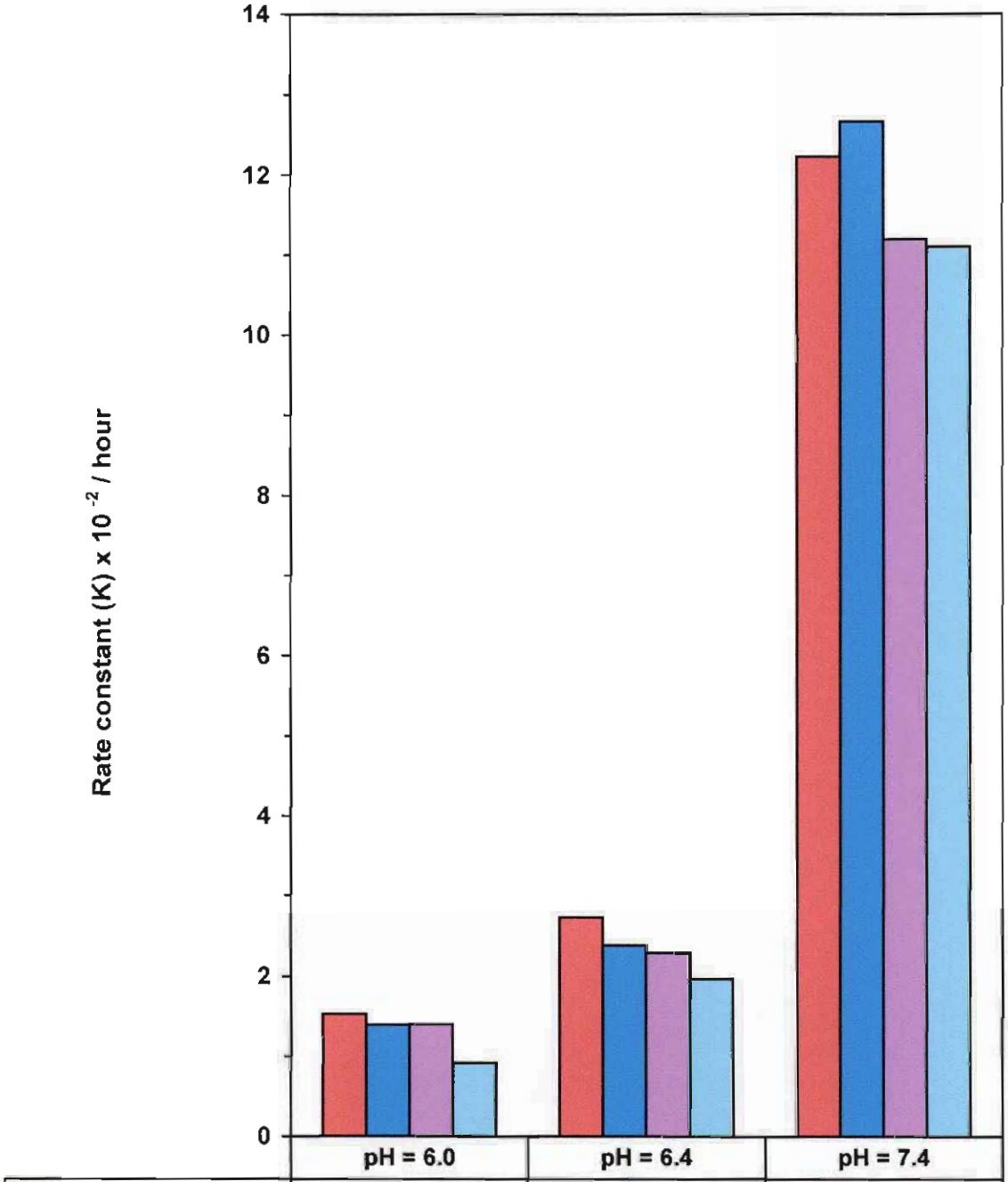



**FIGURE 4-11:** Mean steady-state flux of N-methyl thalidomide from different formulations.




| | pH = 6.0 | pH = 6.4 | pH = 7.4 |
|---|---|---|---|
| Thalidomide | 0.49 | 0.95 | 5.49 |
| N-Methyl Thalidomide | 0.51 | 0.67 | 4.98 |
| N-Propyl Thalidomide | 0.41 | 0.78 | 5.48 |
| N-Pentyl Thalidomide | 0.22 | 0.72 | 4.14 |

**FIGURE 4-12:** Chemical stability of thalidomide and its N-alkyl analogues in aqueous media (pH 6.0, 6.4 and 7.4) at 25°C.




| | pH = 6.0 | pH = 6.4 | pH = 7.4 |
|---|---|---|---|
| Thalidomide | 1.53 | 2.73 | 12.24 |
| N-Methyl Thalidomide | 1.39 | 2.39 | 12.67 |
| N-Propyl Thalidomide | 1.40 | 2.29 | 11.21 |
| N-Pentyl Thalidomide | 0.93 | 1.97 | 11.11 |

**FIGURE 4-13:** Chemical stability of thalidomide and N-alkyl analogues in aqueous media (pH 6.0, 6.4 and 7.4) at 32°C.

TABLE 4-5: Half-lives of thalidomide and its N-alkyl analogues.

| Compound | Half-lives, $t_{½}$ (hour) | | | | | |
|---|---|---|---|---|---|---|
| | 25°C | | | 32°C | | |
| | pH 6.0 | pH 6.4 | pH 7.4 | pH 6.0 | pH 6.4 | pH 7.4 |
| Thalidomide | 140.5 | 72.6 | 12.6 | 45.3 | 25.4 | 5.7 |
| N-Methyl Thalidomide | 137.2 | 102.8 | 13.9 | 49.7 | 29.0 | 5.5 |
| N-Propyl Thalidomide | 169.4 | 88.73 | 12.6 | 49.6 | 30.3 | 6.2 |
| N-Pentyl Thalidomide | 318.3 | 96.6 | 16.7 | 74.9 | 35.1 | 6.2 |

## 4.4 Discussion

We are working on the hypothesis that thalidomide or an analogue thereof can be delivered into tissues beneath the skin from topical applications in sufficient quantity to arrest the degenerative changes associated with rheumatoid arthritis. The purpose of this study was to determine the permeation parameters of thalidomide and its N-alkyl analogues from water, a series of n-alcohols and various other vehicles, to take measures of the intrinsic percutaneous permeabilities of these drugs as a step towards predicting their potentials for formulating into percutaneous therapeutic systems. The intent was also to establish a correlation between the physicochemical properties of these compounds and their percutaneous rates of absorption.

Compounds that are absorbed through skin *in vivo* are mainly taken up and cleared systemically by blood vessels directly beneath the epidermis. Thus, compounds do not have to penetrate the full thickness of the skin (epidermis and dermis) before entering the vasculature system. Accordingly, for *in vitro* permeation studies, the epidermis (including the ***stratum corneum***) is usually separated from the underlying dermis using a heat separation technique. This technique cannot confidently be applied to hairy skin, because the hair shafts are anchored firmly in and remain in the dermis, creating holes in the epidermal membrane as the dermis is pulled away. To obviate all concern here, only skin from female cadavers has been used in this study. Harrison ***et al.*** (1984) examined the integrity of the barrier layer following storage after various time periods. No differences were found between measurements of *in vitro* percutaneous penetration of tritiated water in skin stored at -20°C for up to 466 days *versus*

fresh skin stored at 10°C and used within 2-3 days after autopsy, indicating there is maintenance of the barrier properties inherent in skin under this circumstances that it remained frozen.

It is known that compounds with limited water solubility may seem to penetrate skin only slightly when, in fact, it is partitioning into an aqueous receptor phase (aqueous buffer) and not penetration *per se* which is limiting. This happens when the low aqueous solubility is directly attributable to solute hydrophobicity (as appeared to a high degree of crystallinity). Bronaugh & Stewart (1984) examined the effect of a variety of receptor fluids on the permeation of hydrophobic compounds. Cinnamyl anthranilate's absorption was enhanced (aqueous solubility = 0.23 µg/ml) when the saline receptor fluid was replaced with a 6% poly-ethylene glycol (PEG) 20 oleyl ether solution in water. The solubility and permeability properties of selected compounds were listed as a guide to indicate when the lack of water solubility may reduce the accuracy of an *in vitro* skin permeability study. Testosterone also appears to have borderline water solubility of about 10 µg/ml, and, as might be expected, a slight increased in its absorption was observed when the PEG 20 oleyl ether solution was substituted for water (Kou ***et al.,*** 1993). In the present study N-pentyl thalidomide, the most hydrophobic of the analogues, has an aqueous solubility of about 9 µg/ml at 32°C. While marginal, this still appeared high enough to use the same receptor fluid (phosphate buffer) for thalidomide and all its N-alkyl analogues. A factor of decision here was that the log (partition coefficient) was only 3.01.

Intermolecular forces within the pure solid solute and within the solution phase ultimately determine the position of the solubility equilibrium. Equilibrium within a system is achieved when the maximum possible disorder for a system and its surroundings is attained. Of all the purely physical interactions, London's dispersion force and hydrogen bonding are usually of the greatest importance in solubility and solubilization, at least in so far as nonelectrolytes are concerned. London's dispersion force (also referred to as the induced dipole-induced dipole interaction) arises through the coordination of the electron motions of the countless atoms comprising a finite system. Hydrogen bonding is a unique interaction in which a proton covalently attached to one electronegative centre is shared with a second electronegative center. The bond is regarded as partly covalent and partly simple electrostatic. In water hydrogen bonding is the major contributor to the internal energy of cohesion but not so much so that the London's force is made insignificant (Flynn, 1984). In the solid solution equilibrium between a solid and its solution (saturated solution) two processes are involved which involve intermolecular interactions. First, the molecules that are highly interactive within the crystalline state, are being released and secondly, the released molecules interact with the solvent to form the solution. Both melting temperature and enthalpy of fusion measure the ease of releasing molecules from the crystalline state.

Figure 4-1 shows the relationship between water solubility and alkyl chain length. Though, it is not actually an alkyl homologue, thalidomide is plotted on the y-axis where *n*, the number of carbons in the alkyl chain, equals zero. Methylation of the thalidomide molecule enhanced the aqueous solubility about 6-fold but, as the alkyl chain length is extended from methyl to pentyl, the aqueous solubility decrease exponentially. The increase in aqueous solubility seen in going from thalidomide to its N-methyl analogue is attributable to a marked lowering of crystallinity with respect to the latter compound. Although the imido hydrogen (which favours water solubility through its ability to hydrogen bond) of thalidomide was replaced by a methyl group (which cannot hydrogen bond), the significant drop in melting point (from 275°C to 159°C) and enthalpy of fusion (from 8.61 to 4.33 kcal/mole) more than compensated for the loss of this specific interactive capacity and also the increased lipophilicity of N-methyl thalidomide. With respect to the latter, the more lipophilic a compound is the less able it is to favourably react with water. The drop in melting point and enthalpy of fusion was so extraordinary that the aqueous solubility of the N-methyl analogue was actually markedly enhanced. Increasing the alkyl chain length to three (propyl) makes the compound significantly more lipophilic than thalidomide and its N-methyl analogue. Consequently, there is a decrease in aqueous solubility of N-propyl thalidomide relative to the other two compounds, but its even lower melting point and enthalpy of fusion was still a factor mitigating the hydrophobic effects, so much so that the aqueous solubility of the N-propyl analogue is approximately the same as thalidomide (59.4 µg/ml). N-pentyl thalidomide on the other hand became so lipophilic that the aqueous solubility decreased to 9 µg/ml. Nevertheless, its still lower crystallinity is a factor of appreciable impact in that the drop in solubility is only a fraction of what would happen absent the changed crystallinity.

The solubility of a crystalline organic compound in any solvent is dependent in part on physicochemical properties of the crystal, for these relate to the energy of disengagement of molecules from the crystal. The temperature of melting and enthalpy of fusion are the simplest and most telling measures of the level of crystallinity. Thalidomide has a melting point of 275°C and an enthalpy of fusion of 8.61 kcal/mole. The level of crystallinity, which is reflected through these measures, is extraordinarily high. Alkylation of the thalidomide structure resulted in compounds (odd chain alkyl analogues up to pentyl) which melt at temperatures more than 100°C less than the temperature at which thalidomide melts and consume significantly less energy per mole in doing so. The destabilization of the crystalline structure with increasing alkyl chain length obviously also leads to an increase in lipophilicity (Figure 4-2). Some increase in solubility in non-polar media can be expected from the increasing lipophilicity, but it is the decreasing crystallinity across the series that mostly determines the solubility trends, except in water. The operative phenomena are played out in the data depicted in Figure 4-6 where

solubilities of the analogues in solvents, simple straight chain alkanols, which span the gourmet from highly polar (methanol and, to an extent ethanol) to highly non-polar (pentanol and higher). Thalidomide, clearly the most polar of the four test compounds, is the least soluble analogue in methanol. Moreover, solubilities in this solvent seem to defy the simplistic expectation of "like dissolve like" in that the order of solubility is N-pentyl thalidomide > N-propyl thalidomide > N-methyl thalidomide > thalidomide and not the inverse. It is the dominant impact of relative crystallinity that is reflected here. This same order holds across all twelve alkanol solvents irrespective of their relative hydrophobicities.

The solubilities of the individual analogues across the alkanol range are also telling. Thalidomide becomes systematically less soluble as the alkyl chain length of the solvents is increased in its case to the point where its solubilities appear vanishingly small on the scale of solubilities shown in Figure 4-6. This pattern is to be expected considering what's been learned about the compound's physicochemical properties. What's somewhat surprising is that the same type of pattern is seen not only for the N-methyl and N-propyl analogues but also for the N-pentyl analogue, albeit with decreasing loss of sensitivity to alkyl chain length extension as the solvents become more hydrophobic. One can read into this overall pattern of behaviour that the intramolecular interactions of these collective solvents with the highly polar thalidomide fraction of these molecules is most determinative of their solution phase activities. This may be the first time such behaviour is so clearly demonstrated.

With the exception of formulation C, the same increasing order of solubilities with extension of alkyl chain length of the compounds in the test vehicles is seen. Formulation C seems to be unique in that it is N-methyl thalidomide, which exhibited the greatest solubility of the four compounds in this vehicle. That this turned out to be the best delivery system of those tested could well be related to this observation.

For the clinical use of a percutaneous delivery system, two limiting factors, a long lag time and a low steady-state flux, must be overcome. The method for overcoming these obstacles is to select an optimal vehicle system that may or may not contain specific permeation enhancers. Formulations A–D were selected on the basis of previous experiences in order to formulate thalidomide in a suitable percutaneous application. Representative permeation profiles of thalidomide and three of its odd chain length N-alkyl analogues from formulation C is shown in Figure 4-5. These data are presented as mean (n = 3) cumulative micrograms per square centimeter penetrated through human skin as a function of time. The curves indicate that relative to thalidomide, there is initially an extraordinary increase in steady-state flux when the compound is methylated. However, with one exception, fluxes through skin drop off markedly when the chain length is extended to propyl and pentyl. The same trend is seen in fluxes from

formulations A-D (Figure 4-9). As can be seen from Figure 4-11, N-methyl thalidomide exhibited the highest flux from formulation C. Ethanol is one of the most commonly used skin permeation enhancers and its use as part of a cosolvent system with water has been observed to increase permeation of a wide range of drugs through human and animal skin either *in vivo* or *in vitro*. As already pointed out, it is used as a mediator of estradiol and fentanyl's absorption. It is also known that the degree of flux enhancement may increase upon adding a nonpolar long-chain enhancer to polar ethanol or isopropanol (Kim ***et al.,*** 1996). While the polar enhancers traverse the skin, the nonpolar enhancers are largely retained in the ***stratum corneum***, aspects that appears to make the combination a superior enhancer system to use of the enhancers individually. In terms of toxicology, ethanol and isopropanol are the most acceptable of the short-chain alcohols. However, to better understand the structural requirements for enhancement, studies were performed using all alkanols that are liquid at room temperature, e.g. alkanols up to a chain length of twelve, irrespective of toxicity. Truly extraordinary results were obtained upon doing so. Steady-state fluxes of thalidomide and its three N-alkyl analogues through human skin when each of the compounds was administered as saturated solutions in each of the alkanols are summarized in Figure 4-7. The first pattern that emerges upon viewing the data is that the alkanols become increasingly less effective delivering vehicles as their alkyl chain length is extended. Although there is variability in the data and some minor universals in the general pattern, declining permeabilities with increased alkyl chain length is seen with every one of the four test compounds. Not surprisingly, thalidomide is the poorest penetrant from every one of the alkanols. Its high level of crystallinity and resulting extremely low solubilities dictates this behaviour. What is totally surprising is that N-methyl thalidomide turns out to be the most facile penetrant (from saturated solutions) of the lot. Its behaviour runs against the grain of the solubility observations and the expectation set for the permeation of skin based on partitioning as well. And this is not a phenomenon strictly associated with alkanols as, with but one exception (Formulation D), the same happened when the four vehicles identified as A, B, C and D were employed. One is forced to accept that for what are as of yet inexplicable reasons, N-methyl thalidomide is a better skin penetrant than the other compounds considered.

Percutaneous products are designed to deliver drugs through the skin to achieve systemic effects hence; skin is not the target. Maximal absorption through the skin and minimal skin retention of the drug is the optimal attributes of a percutaneous product. Skin penetrants must have reasonable solubility in oil and water, but, given that the skin is purportedly a lipophilic barrier to a first good approximation, should favour the oil. A suitable drug for percutaneous delivery must ideally have a low molecular weight (i.e, < 350), a low melting point, and have a commensurately high lipid solubility. Dogma says the solubility parameter should be around 10,

and as described by Yalkowsky **et al.** (1983), the log of the partition coefficient should be between 1 and 2.5. N-propyl thalidomide is substantially a lipophilic nonelectrolyte; its molecular weight (300) and melting temperature are low (136°C). It has a log octanol/water partition coefficient of 2.11 and its solubility parameter is 11.5 $(cal/cm^3)^{1/2}$. N-pentyl thalidomide has a molecular weight of 328 and melts at 105°C. The log $K_{oct}$ appears slightly high for percutaneous delivery based on the criticism presented (3.01), whereas the solubility parameter is relatively close to ideal at 11.2 $(cal/cm^3)^{1/2}$. Overall, these compounds and not N-methyl thalidomide tend to have the best penetrant characteristics of the compounds in the study. Though, formulated in percutaneous systems at the upper limit of their thermodynamic activities (saturation), they permeated human skin at lower rates than N-methyl thalidomide, which exhibited a molecular weight of 272, a melting point of 159°C, a log $K_{oct}$ of 1.15 and a solubility parameter of 12.3 $(cal/cm^3)^{1/2}$. In the case of N-pentyl thalidomide, the low flux through human skin might be due to its relatively high lipophilicity and low aqueous solubility (9.0 µg/ml). We speculate it remains in the ***stratum corneum*** instead of partitioning out of it, into the aqueous viable epidermis. It's far more difficult to make the same argument for N-propyl thalidomide.

Thalidomide is sparingly soluble in aqueous solution (± 50 µg/ml at 25°C) and is broken down in the body mainly by hydrolytic mechanisms. Schumacher **et al.** (1965) studied the spontaneous hydrolysis of thalidomide in solution and found that, at pH values above 6, thalidomide undergoes spontaneous hydrolysis, the rate of which accelerates with increasing pH. While all the substituted amide bonds of the molecule are sensitive to hydrolysis, from pH 6 to 7 only the phthalimide ring undergoes cleavage. At pH 7 and above the glutarimide moiety also suffers measurable hydrolytic splitting. Theoretically, 12 different products can be formed from thalidomide by hydrolysis of one or more amide bonds. The spontaneous rate of hydrolysis of thalidomide and its N-alkyl analogues has been compared in this study. Thalidomide and its N-alkyl analogues are hydrolysed at very similar rates with half-lives ranging from 25 to 35 hr at 32°C at pH 6.4. The average rate constant was 2.35/hr. The rate of hydrolysis was dependent on temperature and pH, in expected ways as can be seen in Figures 4-12 and 4-13.

## 4.5 Conclusion

The higher percutaneous permeability through human skin has been suggested to correlate with the greater lipophilicity and lower melting points of the drugs studied. From all the permeability data of thalidomide and its odd chain N-alkyl analogues, using water, a series of n-alcohols and various combinations of solvents and enhancers as vehicles, it is clear that N-methyl thalidomide is the best permeant of this series. It can be concluded that, not only do lipophilicity and crystallinity play important roles in skin permeability, but other factors, perhaps aqueous

solubility also have to be taken into account. In the following chapter, the biological activity of thalidomide and its N-alkyl analogues will be assessed and discussed.

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# Chapter 5

# Biological Activities of Thalidomide and its N-Alkyl Analogues

## 5.1 Introduction

Thalidomide (α-*N*-phthaloyl-glutamic-acid-imide) was widely prescribed as a sedative in the late 1950's but was withdrawn from the market in 1961 due to its teratogenic effects on developing fetuses. Thalidomide has a relatively simple chemical architecture (Figure 5-1), but exhibits a multitude of physiological activities on mammals. In addition to its sedative and teratogenic effects (Mellin & Katzenstein, 1962), thalidomide possesses significant and unique immunomodulatory and antiinflammatory activities. During the last few years there has been a resurgence of interest in thalidomide due to the numerous reports on the successful use of thalidomide in the treatment of type 2 (erythema nodosum leprosum) lepra reactions (Sheskin, 1965 and Sampaio ***et al.,*** 1993), chronic graft-versus-host disease (Vogelsang ***et al.,*** 1986), discoid lupus erythematosus (Barnhill & McDougall, 1982), aphthous ulcers in patients with AIDS (Peterson ***et al.,*** 1995) and rheumatoid arthritis (Gutiérrez-Rodríguez, 1984). The effectiveness has been attributed to its ability to inhibit the synthesis of tumour necrosis factor-alpha (TNF-α). Studies have shown that thalidomide is an inhibitor of TNF-α produced by monocytes *in vitro* (Sampaio ***et al.,*** 1991 and Moreira ***et al.,*** 1993).

The observation that overexpression of TNF-α in transgenic mice results in chronic arthritis suggests that TNF-α plays a pivotal role in rheumatoid arthritis (Keffer ***et al.,*** 1991). Elliott ***et al.*** (1994) observed beneficial responses in patients with active rheumatoid arthritis after open-label administration of a chimeric monoclonal antibody to TNF-α. Tumour necrosis factor was also readily detected in both synovial fluid and serum of patients with rheumatoid arthritis (Tetta ***et al.,*** 1990 and Saxne ***et al.,*** 1989). When rheumatoid arthritis patients are treated with thalidomide, the levels of TNF-α in synovial fluid and serum fall appreciably as the result of the drug's inhibitory activity. The observation here is not the inhibition *per se* but the lowering of TNF-α levels, the result of inhibition of TNF-α production in synovial fluid and serum.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease associated with high levels of TNF-α in synovial fluid and synovial tissue (Saxne ***et al.,*** 1989). TNF-α is a major inflammatory mediator which acts positively, along with other important cytokines, to recruit and coordinate the activities of macrophages and other cells in the acute stages of inflammation arising from injury, parasitic or other infection, or tumour formation. Though inflammation is the normal immune system response to infection or injury, chronic or high levels of TNF-α lead to either a persistent or an overly robust inflammatory response. In RA, inflammation is localized in the synovium, a monolayer of synovial cells that lines diarthroidal joints. The synovium becomes markedly thickened due to synovial cell proliferation and the mass of infiltrated inflammatory cells. This proliferative mass, the pannus, invades and destroys articular cartilage and bone, leading to irreversible destruction of joint structure and function (Fries, 1986). TNF-α has been detected in the synovium and synovial fluid of RA patients (Chu ***et al.,*** 1991). Normal synovium does not express TNF-α. It still remains unknown what causes the accumulation of TNF-α in the joints, where it participates in chronic inflammatory processes and resorption of bone and cartilage. Bone matrix destruction induced by TNF-α results from the activation of osteoclasts, with bone synthesis also being inhibited (Bertolini ***et al.,*** 1986).

Oral therapy of thalidomide has proven to be effective in RA, but unacceptable side effects such as drowsiness, constipation, eosinophilia, swelling of the lower limbs and the most significant, of all peripheral neuropathy are easily provoked. Administration of thalidomide or a compound sharing its inhibitory activity *via* the dermal route, if possible, would bypass liver metabolism and, optimistically, provide high local tissue drug levels without systemic complications. There is every reason to believe thalidomide's action is on TNF-α's expression at the local tissue level. TNF-α has been detected in rheumatoid synovium and synovial fluid samples, which suggests that this cytokine is produced locally in inflamed tissue (Chu ***et al.,*** 1991). Therefore, if the drug's delivery can be targeted, it should be feasible to treat localized inflammation selectively. A localized delivery system should enable rheumatoid arthritis to be treated since it is the joints in the extremities that are most effected. Local applications of the drug over the affected joints will allow us to down-regulate TNF-α production in and around the joints and this without raising the systemic blood level to a problematical level.

Thalidomide has some serious physicochemical limitations to overcome in order to be delivered percutaneously (See Chapter 3). Based on thalidomide's physicochemical properties, it is unlikely that it can be delivered percutaneously at a dose required for rheumatoid arthritis. It is for this reason that we have embraced the idea of using N-alkyl analogues of thalidomide that exhibit better physicochemical properties for percutaneous delivery. In Chapter 4, we proved that the N-alkyl analogues of thalidomide are delivered percutaneously far easier than

thalidomide itself. However, a still open question is whether or not these analogues are themselves active compounds. Therefore, in this chapter, the TNF-α inhibitory effects of the N-alkyl analogues were investigated, by stimulating peripheral blood mononuclear cells *in vitro* with lipopolysaccharide (LPS). It is already known that thalidomide inhibits LPS induced TNF-α production and thus this compound was used as a control. Our investigation was aimed at establishing whether the N-alkyl analogues of thalidomide are also active as TNF-α inhibitors and to what extent relative to the thalidomide which was synthesized. The thalidomide which was obtained commercially was also placed in the study as a secondary control.

# 5.2 Experimental

## 5.2.1 Materials and methods

### 5.2.1.1 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by Ficoll Hypaque (Sigma Chemical Co., St. Louis, MO, USA) density centrifugation. The cells were washed three times in cell culture medium (RPMI 1640) (Bio Whittacker, USA) and centrifuged for 10 min at 1,200 rpm at 4°C. The supernatant was discarded and the pellet (PBMCs) was resuspended in R-10 (89% RPMI 1640, 10% sterile Human serum and 1% 100U/ml PCN-penicillin). The cells were counted on a hemacytometer, using Trypan Blue (Sigma Chemical Co., St. Louis, MO, USA) and the final volume of cell suspension was adjusted with R-10 to obtain $2 \times 10^6$ cells/ml. One hundred µl of the cell suspension ($2 \times 10^6$ cells/ml R-10) were added to wells of a 96-wells flat bottom microtiter plate.

### 5.2.1.2 TNF-α inhibition

Thalidomide and its N-alkyl analogues were synthesized (College of Pharmacy, University of Michigan, USA) as described previously (Chapter 3). The compounds were dissolved in DMSO (Sigma Chemical Co., St. Louis, MO, USA) and further dilutions were done in 0.06% acidified R-10 (0.1M HCL + R-10). Final solutions (50 µg/ml) contained 0.5% DMSO. Fifty µl of each drug solution was added separately to the wells containing 100-µl cell solution. Each drug was tested in triplicate from six donors. The positive and negative controls were also run in triplicates and received 50 µl of 0.5% DMSO in R-10 instead of the drug solution. The plate was covered with a plate cover and incubated at 37°C in a humidified 5% $CO_2$ incubator for 1 hr.

### 5.2.1.3 TNF-α induction

PBMCs were stimulated with *Salmonella minnesota* R592 (LPS) (Sigma Chemical Co., St. Louis, MO, USA) to produce TNF-α. A stock solution of LPS (2 mg/ml sterile water) was stored

at -20°C. LPS was diluted in R-10 and used at 2 μg/ml for the assay. After the plate was incubated for 1 hr, 50 μl of the LPS solution (2 μg/ml) was added to each well (with exception of the negative control). The negative controls received 50 μl of regular R-10. The final volume of the cultures was 200 μl. The plate was covered and incubated for 16-18 hr at 37°C in a humidified 5% $CO_2$ incubator.

### 5.2.1.4 TNF-α determination

After incubating the plate for 16-18 hr, it was centrifuged for 2 min at 2,000 rpm at 4°C. One hundred μl of the supernatants were harvested and TNF-α concentrations were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's specifications. The absorbance (492 nm) of each well was determined with an automated plate reader. The optical density was read in triplicate samples. The values for the standards were plotted *versus* the concentration of the same standards, and the best curve was obtained. The data were linearized using regression analysis and presented as pg/ml. A regression analysis equation was used to determine the TNF-α concentration for each well. Percentage TNF-α inhibition was calculated as: 100 × [1-(TNF-α experimental/TNF-α control)]; where TNF-α experimental represents TNF-α secretion by stimulated PBMCs that were cultured in the presence of the drug, and TNF-α control represents TNF-α secretion by stimulated PBMCs that were cultured in the absence of the drug. PBMCs cultured in medium containing equivalent amounts of DMSO in the presence or absence of the stimulating agent were used as controls for drug treated cells.

### 5.2.1.5 Principle of the TNF-α determination method

The BioSource International Cytoscreen™ (BioSource International, USA) Human Tumour Necrosis Factor-Alpha (hTNF-α) ELISA was used for the *in vitro* quantitative determination of hTNF-α in human serum.

The BioSource Cytoscreen™ hTNF-α kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). An antibody specific for hTNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known hTNF-α content, control specimens, and unknowns are pipetted into these wells.

During the first incubation, the hTNF-α antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated antibody specific for hTNF-α is added. During the second incubation, this antibody binds to the immobilized hTNF-α captured during the first incubation.

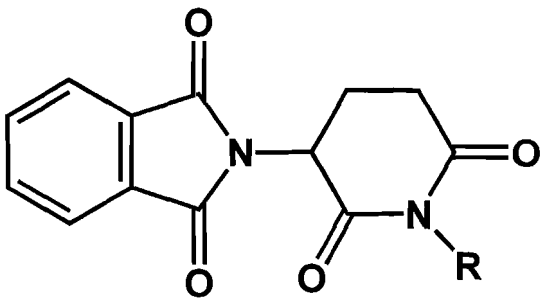
After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove the unbound enzyme, a substrate solution is added which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of hTNF-α present in the original specimen.

## 5.3 Statistical analysis

The concentration of TNF-α (pg/ml) in the supernatant of three replicate cultures of human mononuclear cells stimulated with LPS and containing drug were compared to the TNF-α concentration (pg/ml) in the supernatants of three replicate cultures stimulated with LPS and not containing drug (positive control). Student's *t* test was used to determine statistical significance and *p* values of $<0.05$ were considered significant.

## 5.4 Results

The TNF-α inhibitory effects of thalidomide and its N-alkyl analogues were measured in the supernatant of human peripheral blood mononuclear cells (PBMCs) stimulated with LPS. Cultures containing $10^6$ human mononuclear cells were incubated with thalidomide or one of the N-alkyl analogues for 1 hr and then stimulated with 2 μg/ml of LPS for 16 hr. The data on the TNF-α effects of thalidomide and its N-alkyl analogues are summarized in Table 5-1. Thalidomide has been shown in previous studies to partially inhibit TNF-α production by PBMCs stimulated *in vitro* with LPS (Sampaio ***et al.,*** 1991). The addition of N-alkyl groups to the glutarimide ring of the thalidomide molecule (Figure 5-1) rendered the compounds capable of inhibiting TNF-α production at a level similar to that observed with thalidomide as shown in Figure 5-2. Data represent mean ± S.D. (standard deviation) of three replicate cultures from six donors. There were no statistical significant differences between thalidomide and its N-alkyl analogues at a 95% confidence level ($p<0.05$). Like thalidomide, the N-alkyl analogues in this series inhibit an average of 60% of the TNF-α synthesis in LPS-stimulated PBMCs.




R = H → Thalidomide

R = $CH_3$ → N-Methyl Thalidomide

R = $CH_2CH_2CH_3$ → N-Propyl Thalidomide

R = $CH_2CH_2CH_2CH_2CH_3$ → N-Pentyl Thalidomide

**FIGURE 5-1:** The structure of thalidomide and its N-alkyl analogues.

**TABLE 5-1:** The effect of Thalidomide and its N-alkyl analogues on the synthesis of TNF-α.

| Compound | TNF-α (pg/ml) Mean[c] ± S.D. (% of Control) | | | | | |
|---|---|---|---|---|---|---|
| | Donor # 1 | Donor # 2 | Donor # 3 | Donor # 4 | Donor # 5 | Donor # 6 |
| Positive Control | 553 ± 167 | 380 ± 14 | 450 ± 27 | 570 ± 9 | 754 ± 103 | 768 ± 72 |
| Thalidomide[a] | 320 ± 33 (58) | 206 ± 83 (54) | 178 ± 28 (40) | 117 ± 42 (21) | 268 ± 34 (36) | 159 ± 47 (21) |
| Thalidomide[b] | 255 ± 23 (46) | 240 ± 77 (63) | 192 ± 91 (43) | 84 ± 11 (15) | 191 ± 80 (26) | 200 ± 92 (26) |
| N-Methyl Thal. | 280 ± 16 (51) | 204 ± 10 (54) | 254 ± 87 (56) | 194 ± 20 (34) | 331 ± 38 (44) | 315 ± 54 (41) |
| N-Propyl Thal. | 358 ± 80 (65) | 166 ± 32 (44) | 148 ± 20 (33) | 120 ± 57 (21) | 250 ± 75 (33) | 116 ± 38 (16) |
| N-Pentyl Thal. | 292 ± 19 (53) | 206 ± 45 (54) | 293 ± 26 (65) | 158 ± 19 (28) | 191 ± 67 (26) | 356 ± 71 (46) |

a) Commercial Thalidomide

b) Synthesized Thalidomide

c) Three replicate cultures of mononuclear cells

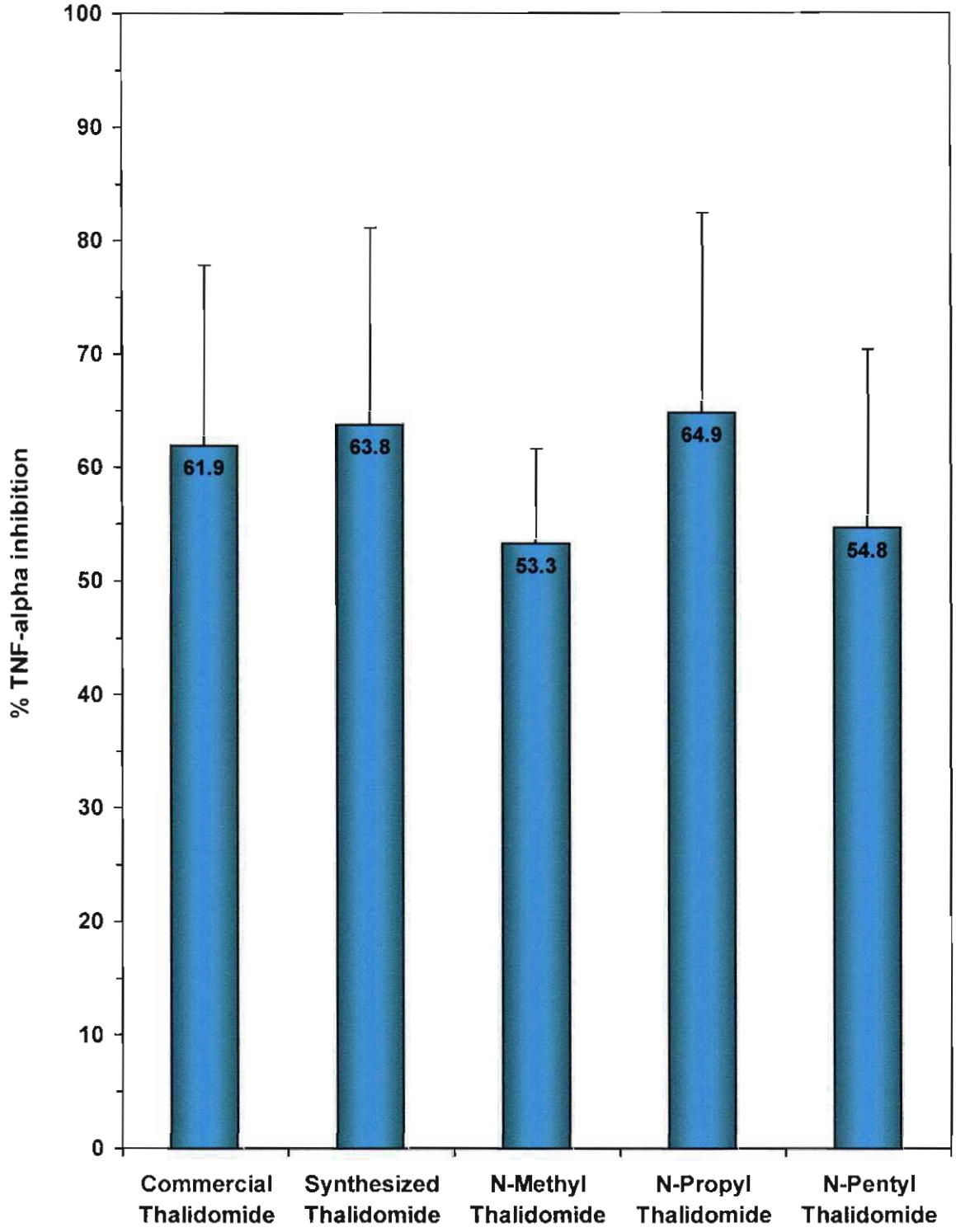



**FIGURE 5-2:** Percentage TNF-α inhibition of thalidomide and its N-alkyl analogues.

## 5.5 Discussion

We have studied the biological activity (TNF-α inhibition) of thalidomide and its N-alkyl analogues by stimulating peripheral blood mononuclear cells (PBMCs) with lipopolysaccharide (LPS). First, we showed that the synthesized thalidomide inhibits the production of TNF-α in the same manner as the commercial thalidomide purchased from ICN, USA. Like thalidomide, its N-alkyl analogues also suppressed the synthesis of TNF-α in PBMCs stimulated with LPS at a concentration of 50 μg/ml. The activities of all four compounds were, for all practical purposes, identical. This strongly suggests that the structural features of thalidomide responsible for its activity were conserved upon making the analogues. The N-alkyl fragments added to the thalidomide molecule spanned a range from one carbon to five. One might expect a five carbon chain to sterically inhibit receptor association at the imide position if this position was somehow involved in the occupation of the receptor. It appears, therefore, that the imide nitrogen is not an essential "lock-and-key" position for activity. Moreover, the alkyl analogues are incrementally, exponentially more hydrophobic than thalidomide. The similar inhibitions of the compounds indicate that they are not driven out of solution and onto the receptor sites by their hydrophobicities. This suggests comparable structuring of water takes place around the molecules when bound as when "free" (or there is a truly remarkable cancellation of two opposite effects). These cultures contained $10^6$ human mononuclear cells. Thalidomide's ability to suppress TNF-α in cultures of PBMCs is also in agreement with the finding of Shannon & Sandoval (1996). They demonstrated a significant reduction in TNF-α produced by PBMCs stimulated with LPS and treated with 50 μg/ml thalidomide.

Thalidomide and its N-alkyl analogues inhibit only an average of 60% of TNF-α produced by PBMCs. This may be in part attributed to the instability of the compounds in aqueous solution (See Chapter 4). Thalidomide is labile to $OH^-$ ions and undergoes spontaneous hydrolysis in solution at a pH $\geq$ 6.0. At physiologic pH, hydrolysis of thalidomide occurs at both the phthalimido and glutarimide rings (Schumacher ***et al.,*** 1965).

We have shown in Chapter 3 that alkylation of the thalidomide molecule resulted in compounds with enhanced physicochemical properties for percutaneous delivery and, from the *in vitro* permeability data (Chapter 4), it is evident that the N-alkyl analogues of thalidomide are better delivered percutaneously than thalidomide itself. From all the permeability data of thalidomide and its N-alkyl analogues, using water, a series of n-alcohols and combinations of solvents and enhancers as vehicles, N-methyl thalidomide ranked number one in its ability to penetrate skin. Not only did we enhance the physicochemical properties and consequently the skin permeability

of the analogues, but we also proved that all the N-alkyl analogues of thalidomide are active as TNF-α inhibitors.

## 5.6 References

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# Chapter 6

# *Summary and final conclusions*

Thalidomide (α-*N*-phthaloyl-glutamic-acid-imide) was widely prescribed as a sedative in the late 1950's. Somewhat later, it became popular as a tranquilizer and anti-emetic for pregnant women. It was only in use for this purpose a few years when it achieved world-wide notoriety for a completely unsuspected, devastating side-effect, teratogenicity. It was summarily withdrawn from the market in the early 1960's. However, in 1963 thalidomide was shown to be effective in the treatment of erythema nodosum leprosum (ENL), a localized skin reaction commonly manifested in patients with leprosy. Since this discovery, thalidomide has been used selectively and effectively to treat a number of inflammatory and auto-immune conditions.

The expressions of inflammatory diseases such as rheumatoid arthritis (RA) and ENL appear to bear some relationship to local, continuous presence of tumour necrosis factor-alpha (TNF-α). This has been convincingly demonstrated by long term remissions in patients with RA, treated with monoclonal antibody to TNF-α. So it is interesting that thalidomide, a drug which was initially developed as a sedative, offers us a means of achieving profound relief to the symptoms of RA. However, at the systemic levels which must be achieved, its side effects are easily provoked and, in general, seem to be unacceptable in degree. Consequently, topical administration of thalidomide is considered here. To the extent that the drug's action can be confined to an inflamed joint, one can expect to achieve its beneficial effects, the prevention of wasting of tissue in the joints, with substantially less risk, if any, of systemic toxicity. Percutaneous delivery of thalidomide or a congener with comparable activity, whereby systemic absorption is minimized, thus is an exciting therapeutic alternative. Unfortunately, cursory examination reveals that, for thalidomide to be delivered percutaneously at doses that might be required to suppress the symptoms of RA, some serious physicochemical limitations have to be overcome. The literature revealed that there were active thalidomide analogues which don't share its marked polarity and high melting properties. Several of these, all N-alkyl analogues, were therefore studied.

The overriding goal of the research was to learn enough about thalidomide and select analogues to eventually allow the design and development of a percutaneous delivery system for treatment of RA.

The specific aims of the study were to:

- synthesize thalidomide and select analogues;
- characterize those physicochemical properties of test compounds that are relevant to their percutaneous delivery;
- determine how thalidomide and its N-alkyl analogues permeate in human skin from saturated aqueous solutions and from solvents which might eventually act as vehicle components;
- develop relationships which exist between the physicochemical properties of the selected compounds and their percutaneous delivery, and
- as a matter of choice of compounds for further study, determine the comparative biological activities (TNF-$\alpha$ inhibition) of the selected compounds.

Thalidomide and three N-alkyl analogues, all of odd chain length, namely, the N-methyl, N-propyl and N-pentyl analogues, were synthesized and purified (Chapter 3). Their identities and purities were confirmed using elemental analysis and spectrophotometric (NMR, EI-MS), thermal (DSC) and other techniques (HPLC, melting behaviours). As was initially suspected from structural requirements, N-alkylation of the glutarimide ring in the thalidomide molecule resulted in compounds with physicochemical properties that appear to be better suited for percutaneous delivery than thalidomide's (Table 3-1). The N-alkyl analogues of thalidomide melt at lower temperatures and consume less energy per mole in doing so than does the reference compound. They are also considerably more lipophilic than thalidomide as indicated by their higher octanol/water partition coefficients. Their absolute solubilities in non-polar solvents were demonstrably higher. All these trends are in the direction of improving percutaneous delivery over that which is possible with thalidomide. From these physicochemical properties it was hypothesised that the rank order of skin permeability would be N-pentyl > N-propyl > N-methyl >> thalidomide. Inexplicably, a different order was observed with the N-methyl analogue being the best penetrant. However, thalidomide's position, last in the rank order, was found to be quite secure. In the light of the unexpected change in rank order, the influence of alkylating thalidomide on aqueous solubilities seems interesting. The

methyl analogue was substantially more water soluble than thalidomide, but on extension of the alkyl chain length to five, the aqueous solubility dropped to one-sixth of that of thalidomide.

Based on *in vitro* skin permeation data obtained for thalidomide and its three N-alkyl analogues, using water, a series of n-alkanols, and also various combinations of solvents and putative enhancers as vehicles, it became evident that the N-alkyl analogues were far superior penetrants when compared to the reference drug. But as suggested above, it was surprising that the N-methyl analogue ranked number one with respect to ability to penetrate skin (Chapter 4). This alone made it clear that not only do lipophilicity and crystallinity, an independent solubility determining factor, play important roles in skin permeation, but, in the instance of these compound, so must another or other factors. We speculate, with no real convincing foundation for doing so, that the aqueous solubilities of the compounds may in some way have influence on their transport.

The work discussed in Chapter 4 establish that one could markedly improve the ability of thalidomide-like compounds to penetrate skin by modulating their crystallinities and adjusting their lipophilicities. The unresolved issue was: was this done with retention of biological activity or, more hopefully, with potentiation of biological activity? Therefore, the biological activities of thalidomide and its N-alkyl analogues were assessed by stimulating peripheral blood mononuclear cells (PBMCs) to produce TNF-$\alpha$ with lipopolysaccharide, taking note of the dependencies of suppression of the cytokine on concentrations of the drugs (Chapter 5). It was shown that alkylating thalidomide, as was done, had little effect on the ability of the compounds to inhibit the production of TNF-$\alpha$ in these cell cultures. On the basis of this observation, all of the compounds put to test seem to have some, and perhaps comparable, therapeutic potential.

In this study:

- thalidomide and three of its N-alkyl analogues were successfully synthesized, purified and identified;
- the physicochemical properties relevant to percutaneous delivery were determined for these compounds;
- the permeation parameters of thalidomide and its N-alkyl analogues were determined in human skin from different vehicles;
- a correlation between the physicochemical properties of these compounds and their percutaneous delivery was establish and
- the biological activities of thalidomide and its N-alkyl analogues were determined.

Unresolved issues and future work:

- the lead compound, N-methyl thalidomide, needs to be formulated into a suitable topical vehicle. Based on the data in Chapter 4, a hydro-alcoholic vehicle would likely be an appropriate choice as an initial choice;
- the lead compound, N-methyl thalidomide, will have to undergo toxicity testing. This minimally would involve topical irritation assessment in a limited number of subjects over limited skin area prior to the drug being used in a small clinical trial for the purpose of evaluation of proof of concept (early, physician-directed evaluation of its clinical potential);
- if the proof of concept test is at all encouraging, then the lead compound, N-methyl thalidomide, will have to be subjected to extensive toxicity evaluations, including evaluations of all its systemic actions. Work as this is enormously expensive and thus will require a commitment from and the resources of an industrial partner;
- the efficacy of the lead compound will have to be unequivocally proven in the clinic, providing of course it passes all the hurdles which lead up to this point of examination, and
- because far more active compounds than thalidomide and its N-alkyl analogues are known because some appear to suppress the production of TNF-$\alpha$ by means other than the means employed by thalidomide, a number of these potent compounds, those with the best physical chemistries, should be prepared and entered into the developmental scheme as secondary and tertiary therapeutic candidates.