Studies on the Formulation of Topical Corticosteroids

by

Yuen Wah Yip

A thesis presented for the degree of

Doctor of Philosophy

of the

University of Aston in Birmingham

August 1982

Studies on the Formulation of Topical Corticosteroids

by

Yuen Wah Yip

Submitted for the degree of Doctor of Philosophy, 1982 in the University of Aston in Birmingham.

The stabilities of betamethasone-17-valerate and hydrocortisone-17-butyrate have been investigated. It has been shown that the 17-esters undergo acyl migration to the 21-isomers. These 21-isomers hydrolyse to the free alcohols which further degrade to other products. In order to elucidate the complex decomposition pathway, two normal phase and reversed phase HPLC systems were developed for assaying these two corticosteroids in the presence of their major decomposition products. The development and applications of these systems were discussed. The kinetics of decomposition were subjected to non-linear regression analysis. The rate constants for the various decomposition pathways were quantified.

Attempts were made to formulate a stable 0.1% w/w hydrocortisone-17-butyrate gel with various polymers. Carbopol gel was the most cosmetically acceptable base. Stability studies of the corticosteroid in semi-aqueous Carbopol gels revealed that the decomposition pathway parallel those in aqueous propylene glycol. Steroid gels with a shelf-life of about one year were formulated.

The percutaneous absorption of hydrocortisone and its 17- and 21-butyrates and 21-acetate were studied using three in vitro models which enabled simultaneous monitoring of more than one steroid. The studies included release from Carbopol gel into isopropyl myristate; penetration through mouse skin and 3-phase partitioning. Vehicle effects were related to the solubility of the corticosteroids in aqueous propylene glycol and to their partition coefficients between isopropyl myristate and aqueous propylene glycol.

The cutaneous biotransformation of betamethasone-17-valerate and hydrocortisone-17-butyrate were compared with their 21-isomers using esterases from hog liver and mouse skin homogenates. The results showed that the 21-esters were very sensitive to the esterases. Based on the quantitative data on the decomposition of these corticosteroids, it was clearly shown that the steroid-17-esters were resistant to the esterases. The resistance of beclomethasone-17,21-dipropionate and its 17-monopropionate to these enzymes further suggested that for steroids to be susceptible to enzymic degradation, the 17-hydroxyl group must remain free.

Key Words:
Stability
Formulation
Percutaneous absorption
Cutaneous biotransformation

CONTENTS

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. A. Li Wan Po and Dr. W. J. Irwin for their guidance and encouragement throughout the duration of this work.

Sincere thanks are extended to my research colleagues for their help, stimulation and discussion.

I am grateful to Professor M. R. W. Brown for making available to me the facilities of the Department of Pharmacy.

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

The term steroid refers to a group of compounds with a hydrogenated cyclopentophenanthrene-ring system (Figure 1). The adrenal cortex releases a large number of steroids into the circulation. These steroids are known as the corticosteroids. The corticosteroids may be classified, according to their functions, into three groups (1, 2). Mineral corticoids (eg. aldosterone) are formed in the glomerular (outer) layer of the cortex, exerting effects on electrolyte and water metabolism. Glucocorticoids such as cortisone and hydrocortisone are formed in the fascicular (middle) layer, having actions on gluconeogenesis, glycogen deposition and protein and calcium metabolism. The glucocorticoids and mineral corticoids exert some overlapping physiological effects. The sex corticoids (eg. oestrogens and androgens) are formed in the reticular (inner) layer.

The discovery that cortisone and cortisone-21-acetate were dramatically effective in the treatment of complicated rheumatic arthritis (3, 4) had led other investigators to search for other derivatives which might similarly improve anti-inflammatory activity. Hydrocortisone was thereby found. The search then, was concentrated on improving systemic activity and minimising unwanted side effects such as sodium retention by modification of hydrocortisone. However, such approaches only succeeded in enhancing activity but not in reducing side effects. Efforts were then shifted to searching for means of delivering the active agents directly to a desired site in higher concentrations. The skin was the first target. For topical application, the compound must reach the site of action in sufficient amounts. New steroids which were not only topically active but also sufficiently stable to allow pharmaceutical formulation were required.

The skin is not an inert organ, being capable of metabolising both endogenous and exogenous materials. Any new topical compound produced should not be readily inactivated by the skin enzyme and yet this rate should be fast enough to avoid systemic effects. A second approach is to improve the delivery system. It is known that the formulation in which the steroid is accommodated, affects the stability and skin permeability of the drug. The formulation must be designed so as to provide an optimal environment for the drug to show maximum stability and skin absorption.

An understanding of the structure-activity relationships of the steroids and their sensitivities to cutaneous metabolisms will therefore be a useful guide for choosing the right compound. This will also give some idea as to why some steroids are more potent than others. Prior to formulating a drug, we must have a knowledge of the possible decomposition mechanism of the compound, factors determining its skin permeability and its diffusion in the vehicle and the properties of the bases. Such knowledge will facilitate and expedite the development of a proper formulation. A reliable, selective and precise method of assay is essential for such studies. High-performance-liquid-chromatography is one of the methods of choice for assaying steroids and has been used throughout this work.

1.1 STRUCTURE-ACTIVITY RELATIONSHIPS OF TOPICAL CORTICOSTEROIDS

The basic structure which is essential for topical anti-inflammatory activity can be illustrated by examining features which are known to be essential for hydrocortisone to be active (Figure 1). The most critical feature is the perhydrocyclopentanophenanthrene ring system (Figure 1) containing the 11-β-hydroxyl group. Other

chemical groups necessary for activity are the C-4, C-5 double bonds together with the 3-ketone of ring A and the 17,21-dihydroxyacetone side chain (5). Slight modification on the structure may lead to large changes in biological activity. Figure 1 shows the structure of a few of hydrocortisone analogues. All of the chemical groups which appear to be essential for the anti-inflammatory activity of hydrocortisone are not necessarily critical for the topical activity of other synthetic steroids, and there is a discrepancy in the structural requirements for oral and topical effectiveness. For example, in fluorometholone (Figure 1), the essential side chain of hydrocortisone has been altered by deoxygenation at C-21, a change which characteristically lowers anti-inflammatory potency (6,7). However, three additional modifications are made to the hydrocortisone molecule to form fluorometholone: dehydrogenation at C-1 and C-2, 6α-methylation and 9α-fluorination. Together, these changes lead to a molecule with 40 times the topical potency of hydrocortisone (8,9). But, it should be noted that its oral potency is only up to twice that of hydrocortisone (10). Different enzyme systems in the liver and the skin may be responsible for these differences. Fluorometholone is more vulnerable to the hepatic enzymes (5).

Prednisolone differs from hydrocortisone only by the presence of an additional double bond between C-1 and C-2, which significantly increases the oral potency to about four times that of hydrocortisone (6) but topically, the two steroids are about equivalent (8,11). Triamcinolone, dexamethasone and betamethasone have similar structures, all possessing a double bond between C-1 and C-2, fluorination at C-9 and hydroxylation or methylation at C-16 (Figure 1). Triamcinolone is as effective as hydrocortisone topically (8) and about five times more effective orally (6). Betamethasone and dexamethasone are topically

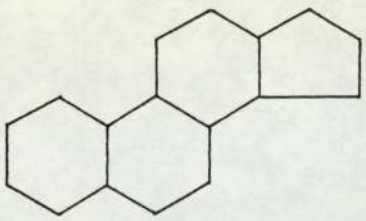
about ten times (12) and orally about thirty times as potent as hydrocortisone (13,14).

It appears that higher topical activity over the parent compound may be obtained with modifications which lead to an increase in lipophilicity. This is exemplified by the removal or masking of hydroxyl groups or by introduction of long carbon side chains, or both (15).

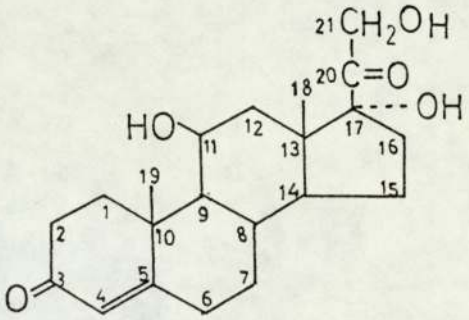
Acetonides: The 16,17-acetonides of 16α-hydroxycorticosteroids were among the first compounds shown to have improved topical efficacy, by masking of both the 16α and 17α-hydroxy groups. Triamcinolone acetonide (5, 16) and fluocinolone acetonide (17,18) are two commonly used examples. Fluocinolone acetonide is over 100 times as active as betamethasone in terms of vasoconstrictor activity (19). Simple 21-acetylation of fluocinolone acetonide gives fluocinonide (20,21) which is five times as active topically as fluocinolone acetonide (22).

21-esters: The simplest modification involves esterification of the 21-OH group and this leads to compounds like hydrocortisone-21-acetate which is 10 times as active as hydrocortisone in terms of vasoconstrictor activity (23). Improved stability of the ester linkage is obtained by using branched-chain acids for esterification. An example is flumethasone-21-pivalate (24) which also shows high local activity.

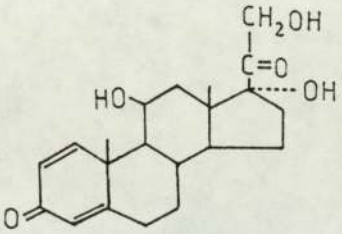
17-esters: In betamethasone-17-valerate and hydrocortisone-17-butyrate, their 17-hydroxyl group are masked by esterification with fatty acids and their therapeutic efficacy are comparable to fluocinolone acetonide (25). Betamethasone-17-benzoate shows similar vasoconstriction potency to betamethasone-17-valerate (26) but has relatively strong systemic activity (27).



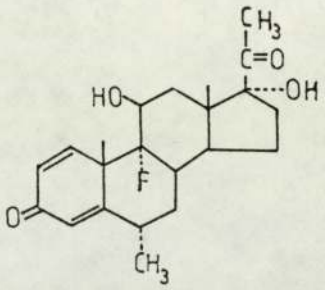
Perhydrocyclopentanophenanthrene



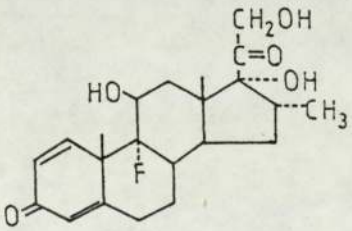
Hydrocortisone



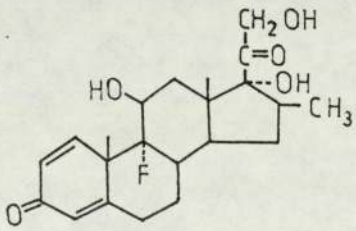
Prednisolone



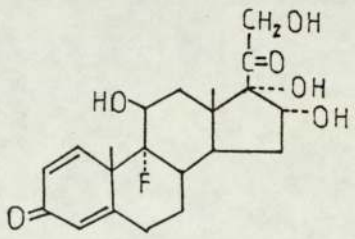
Fluorometholone



Dexamethasone

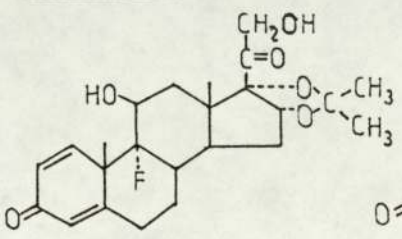


Betamethasone

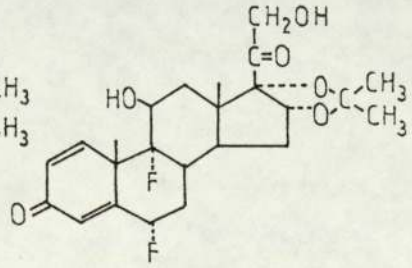


Triamcinolone

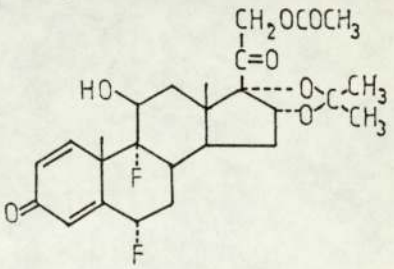
Acetonides:



Triamcinolone acetonide



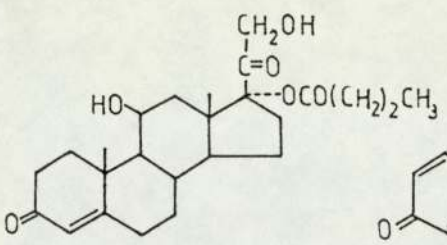
Fluocinolone acetonide



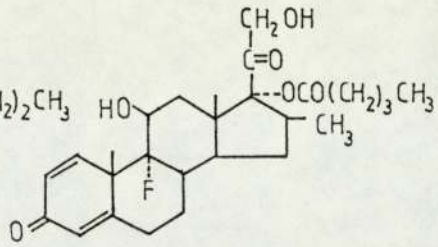
Fluocinolone acetonide acetate (Fluocinonide)

Figure 1. Structure of commonly used topical corticosteroids

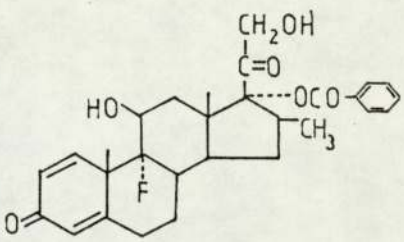
17-esters:



Hydrocortisone-17-butyrate

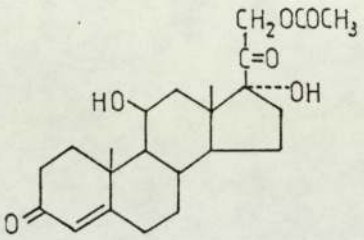


Betamethasone-17-valerate

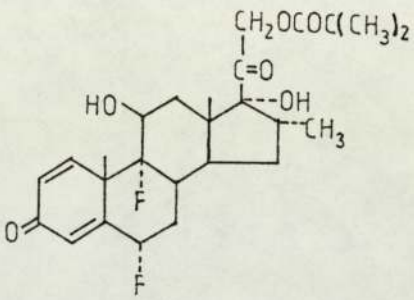


Betamethasone-17-benzoate

21-esters:

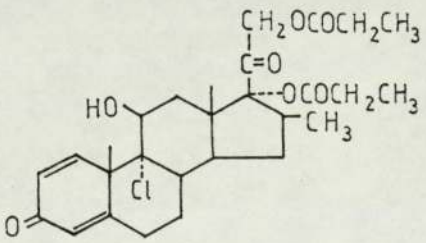


Hydrocortisone-21-acetate

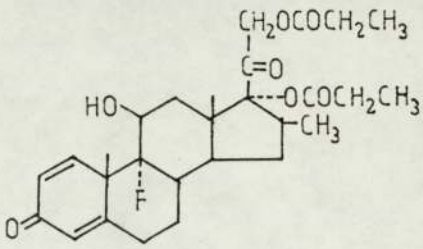


Flumethasone-21-pivalate

17,21-diesters:

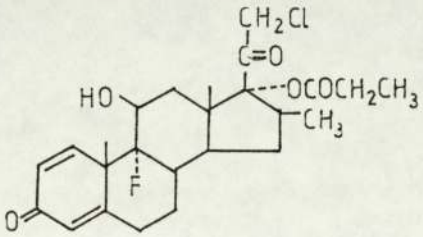


Beclomethasone 17,21-dipropionate



Betamethasone 17,21-dipropionate

21-halogenated-17-esters:



Clobetasol-17-propionate

Figure 1. cont'd

17,21-diesters: By vasoconstriction test, beclomethasone-17,21-dipropionate emerged as a useful topical agent (28,29) and betamethasone-17,21-dipropionate is also of value (26).

21-halogenated-17-esters: Clobetasol-17-propionate, with a chlorine atom in place of a 21-hydroxyl group, has proved to be particularly effective in clinical practice (30).

1.2 ASSAY METHODS FOR STEROIDS

1.2.1 The Isonicotinic Acid Hydrazine (INH) Reaction

The INH reacts with the Δ^4-3-ketone moiety of corticosteroids to form their hydrazone which is yellow and has maximum absorbance at 380 nm (31).

1.2.2 U V Spectrophotometry

This method is based on the U V absorption at about 247 nm by the Δ^4-3-ketone group in ring A of the steroids (32).

1.2.3 The Porter-Silber Reaction

Corticosteroids with a 17-dihydroxyacetone side chain react with phenylhydrazine in sulphuric acid to give a yellow chromophore (33,34). The acid catalysed rearrangement of the dihydroxyacetone moiety to a 17-deoxy-20-one-21-ol is followed by condensation with phenylhydrazine to form a 21-phenylhydrazone. Chafetz et al (34) claimed that this method is selective for betamethasone benzoate in the presence of its degradation product, the 21-benzoate which cannot be oxidized and so, does not interfere.

1.2.4 The Tetrazolium Reaction

The most widely used method involves a modification of the procedure proposed by Mader and Buck (35). This technique is used in the official United States Pharmacopoeia, 19th Rev. (36), National Formulary 14th Ed. (37) and British Pharmacopeia, 1973 (38) methods.

After extraction and separation by means of thin layer, paper or column chromatography, triphenyltetrazolium chloride or blue tetrazolium in a highly alkaline alcoholic solution is employed to detect the steroids and their impurities or break down products qualitatively as well as quantitatively.

1.2.5 Chromatography

Because of the sensitivity and specificity needed for monitoring steroid stability, a chromatographic technique appears to be a logical approach. Paper, thin layer, column and gas chromatography have been used extensively in the separation of steroids. However, each of these methods has limitations. Gas chromatography is a fast method with relative ease of quantitation and high resolution. The major limitation is that steroids are generally either of low volatility or too heat-labile for direct gas chromatographic analysis. High-performance liquid chromatographic methods have therefore been developed to overcome these problems. Derivatization is not necessary. By using narrow bore columns, small diameter supports, low dead volume injectors and detectors and pressurized eluent delivery to achieve desired flows, fast separation of closely related compounds can be achieved.

1.3 APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO CORTICOSTEROIDS

High-performance liquid chromatography (HPLC) is a powerful tool for the specific and precise analysis of corticosteroids. In a complex decomposition reaction, an analysis method which is able to monitor the parent compound and its major decomposition products is essential, so that a full kinetic profile of the decomposition can be obtained. A typical example is the decomposition of betamethasone-17-valerate. A previous thin layer chromatographic method of analysis

lacked the sensitivity and precision necessary for monitoring the rates of formation of the decomposition product (39). A normal-phase HPLC system was developed and was successfully applied to show that the steroid undergoes sequential first order isomerization, hydrolysis and condensation (A→B→C→D). HPLC is also particularly useful for the analysis of pharmaceuticals in formulated system, because of the presence of excipients which are potential sources of interference in the traditional colorimetric methods.

Numerous HPLC systems have been developed for analysing steroids (40 - 50). Since many of the more labile steroids are quite polar and elute relatively slowly using non-polar eluents, reversed-phase columns with aqueous methanol eluent were widely used in steroids. Table 1 gives a brief review of the reported HPLC systems for some commonly used steroids.

HPLC is also very useful for assaying steroids in biological fluids such as urine, plasma and blood (41,57,58). Petersen et al (46) reported rapid, precise, selective and sensitive HPLC systems for examining the time course of levels of betamethasone acetate which is administered and converted to betamethasone. Their effects on endogenous hydrocortisone was also simultaneously monitored. In the past, such detections were made by radio-immuno assay preceded by column chromatography to reduce cross-reactivity problems and a combination of a radioreceptor assay for total glucocorticoid activity and the corticosteroid-binding globulin isotope assay. However, these techniques cannot be used for simultaneous determination of steroids and required large numbers of samples. The time-involvement also limited their use in pharmacokinetic studies.

Table 1. HPLC systems for some commonly used corticosteroids

Steroid mixture	Column	Eluent	Detector & Wavelength	References
fluocinolone acetonide	Permaphase-ODS 1m x 2 mm i.d.	water-methanol (80:20)	UV 254 nm	52
flumethasone pivalate, dexamethasone, prednisolone	β,β-oxydipropionitrile on Zipax (Du Pont) 1m x 2.1mm i.d.	1% methanol in hexane	UV 254 nm	59
flumethasone pivalate	β,β-oxydipropionitrile on Zipax 1m x 2mm i.d.	5% ethyl acetate - 0.2% acetonitrile in hexane	UV 254 nm	59
hydrocortisone, cortisone	Sil-X (Perkin-Elmer) 0.5m x 3mm i.d.	chloroform-dioxane (100:5)	UV 254 nm	60
flucinolonide	cyanosilanes on silica 1m x 2mm i.d.	2,2,4-trimethylpentane 69% isopropanol 18.5% acetonitrile 12.5%	UV 254 nm	51
hydrocortisone, hydrocortisone acetate, cortisone, cortisone acetate	1% cyanoethyl silicone on Zipax 1m x 2.1mm i.d.	1% methanol in water	UV 254 nm	53
methandrostenolone methyltestosterone 6β-hydroxymethandrostenolone	Silica gel (Lichrosorb SI60) 25cm x 2.1mm i.d.	ethylene chloride 3% 2-propanol 15% in n-hexane	UV 254 nm	61

Table 1 cont'd

Steroid mixture	Column	Eluent	Detector & Wavelength	References
prednisone sodium phosphate, prednisolone sodium phosphate, 16β-methylprednisone sodium phosphate, betamethasone sodium phosphate, testosterone sodium phosphate, dichlorisone sodium phosphate	organosilane on porous silica particles 30 cm x 4 mm i.d.	methanol-0.09M KH_2PO_4 (1:1)	UV 254 nm	55
betamethasone sodium phosphate and its decomposition product		methanol-0.09M KH_2PO_4 (13:8)	UV 254 nm	55
betamethasone-17-valerate	Spherisorb-ODS (5 μ) or Hypersil-ODS (5 μ) (Shandon Southern) 100 mm x 5 mm i.d.	methanol-water (57.5 : 42.5)	UUV 236 nm	56
clobetasol-17-propionate, clobetasol-17-butyrate				
beclomethasone dipropionate, betamethasone-17-valerate			UV 239 nm	
hydrocortisone, beclomethasone dipropionate		methanol-water (45:55)	UV 240 nm	
betamethasone		methanol: pH 5 McIlvaines' buffer (45:55)	UV 240 nm	
betamethasone-21-disodium phosphate				

Table 1 cont'd

Steroid mixture	Column	Eluent	Detector & Wavelength	References
hydrocortisone, hydrocortisone hemisuccinate	Spherisorb-ODS 5 µ 250 mm x 3 mm i.d.	acetonitrile-water-glacial acetic acid (35:65:2)	UV 242 nm	41
methylprednisolone, methylprednisolone succinate	Spherisorb-ODS 5 µ 250 mm x 3 mm i.d.	acetonitrile-water-glacial acetic acid (40:60:2)	UV 242 nm	41
betamethasone valerate, phenylephrine HCl* lidocaine HCl*	Nucleosil C8 5 µ (Macherey Nagel, Duren, G.F.R.) 150mm x 4.65mm i.d.	0.005M Sodium dodecane sulphonate in methanol + 0.01M phosphate buffer pH 4.8 (7:3)	UV 260 nm	62
cortisol, corticosterone, deoxycorticosterone, 4-androstene,3,17-dione pregesterone	Hitachi gel 3011 500mm x 2 mm i.d.	n-hexane-methanol (5:95)	UV,254 nm or fluores-cence	63
corticosterone, cortisone, hydrocortisone, prednisolone	Zorbax-sil (Du Pont) 250mm x 2.1mm i.d.	7 ml of water + 12 ml of ethanol, made up to 500 ml with dichloromethane, the mixture was stirred for one hour, the separated organic phase was used	UV,254 nm or fluores-cence	63

* non-steroid

Table 1 cont'd

Steroid mixture	Column	Eluent	Detector & Wavelength	References
prednisone, cortisone, prednisolone, hydrocortisone, dexamethasone, prednisolone acetate, cortisone acetate, hydrocortisone acetate	octylsilane bonded to microsilica (5 μ)	25% tetrahydrofuran 12.5% methanol in water	UV 254 nm	45
hydrocortisone, hydrocortisone acetate betamethasone, betamethasone acetate	RP-8 column (Brownlee, Santa Clara, U.S.A.) (10 μ) 250 mm x 4.6 mm i.d.	60% methanol in water	UV 254 nm	46
desoximetasone	μ-Bondapak C18 (Water Assoc.) 300 mm x 3.9 mm	50% methanol 1% acetic acid in water	UV 254 nm	42
fluocortolone				
difluocortolone				

1.4 FORMULATION REQUIREMENT FOR TOPICAL PREPARATIONS

Some parameters have to be taken into account when formulating a topical preparations.

1.4.1 Compatibility

The components of the base must be compatible with each other or its efficiency as a donor of the active materials may be impaired, for example, cationic and anionic surfactants are incompatible and their interactions may lead to phase separation. The base should also be compatible with the active ingredients to provide the best possible environment.

1.4.2 Physiological Inertness

The vehicle itself should be free of irritation and sensitization to skin, and physiologically inert.

1.4.3 Sufficient Capacity

The vehicle must be able to maintain the drug in a homogeneous dispersion or solution.

1.4.4 Particle Size Distribution

In a dispersed system, the globule size or particle size of the dispersed phase is a significant factor in the rate of drug release and the physical stability of the product. If the particle is spherical (such as emulsion globule), its size is determined uniquely by its diameter. For non-spherical particles, the commonly used methods in pharmaceutical semi-solids has been reviewed (64). Microscopy is particularly useful for determining the particle size distribution of drug or disperse phase in semi-solid dosage forms.

1.4.5 Aesthetic Acceptability

The vehicle should be non-sticky, possess no unpleasant odour and be non-staining. In gel form, formation of a film on the skin which can be peeled off is undesirable. Whether a carrier base is

washable or water-resistant is the simplest initial matter to be considered.

1.4.6 Preservation

Conventional creams containing an aqueous and oily phase are particularly vulnerable to contamination by bacteria and suitable preservatives must be added to such systems. Poorly preserved topical steroid preparations can be especially hazardous to the patients because there is sufficient clinical evidence to show that steroids help the spread of infection. The effectiveness of a bactericide or fungicidal substance depends on its 'effective concentration', that is, the concentration on the germ's surface. This is not only determined by the amount of material added to the preparation but by various other factors such as the solubility of the preservatives in the respective medium. For example, in oil-water systems, bacteria are generally located in the aqueous phase. In addition, the preservatives must be in an unionized state to penetrate the bacterial membrane and must not be bound to other components of the medium.

1.4.7 Rheology

Topical steroid preparations are often formulated as ointments, gels and creams. Most pharmaceutical semi-solid products exhibit plastic or pseudoplastic flow, usually with thixotropy. The rheological consistency plays an important role in the patients' assessment of a topical product. The pharmaceutical elegance of all topical semi-solid preparations is most directly related to consistency (65). Products should easily spread when applied to the skin and yet be viscous enough at skin temperature. The consistency should be such as to allow convenient withdrawal of the product from the container. Good spreading will avoid high local concentrations of the drug. The effect of viscosity on the bioavailability has been mentioned repeatedly.

The effect of viscosity on drug release and absorption from semi-solids (66 - 69) can be rationalised on the basis of the Stoke-Einstein equation (70 - 72):

$$D = \frac{K\ T}{6\ \pi\ r\ \eta} \qquad \text{Eq. 1}$$

where K = the Boltzmann constant

T = absolute temperature

r = hydrodynamic radius of diffusing drug molecule

η = viscosity

From this equation, it is clear that an increase in viscosity will decrease the diffusion coefficient. Generally, when a poorly soluble drug is suspended in an ointment vehicle, relatively minor changes in vehicle composition such as a reduction in the viscosity may markedly increase the release rate (73). Giroux and Schrengel (74) however found no correlation between viscosity of the base and drug release.

1.4.8 Stability

The preparation must withstand storage over a considerable period of time, preferably, at least three years, over a temperature range of 5° to 30°C. A new product stability program usually involves storage under different combinations of heat, humidity and light as well as different types of containers and packaging materials. Because room temperature stability testing is very time consuming, accelerated stability testing is often resorted to.

1.4.9 Bioavailability

For a topical corticosteroid preparation, the steroid has to penetrate the stratum corneum, the protective barrier, of the skin to reach the dermis where it exerts its effect. The vehicles play

an important role on the penetration of the steroids. The vehicles may be required to lower the interfacial barrier if the dermis is intact or to soften the superficial epidermal tissues if excessively dry and thick. Thus, the goal of a topical formulator should be to design a dosage form which not only has good physical, chemical and cosmetic properties, but also provides the optimum environment for the release of the active ingredient. Although there can be no assurance that maximizing drug penetration into the skin means optimizing drug delivery, the permeability characteristics of the drug in the chosen formulation should ideally be known.

1.5 GEL PREPARATIONS

Gels are defined as semi-solid systems consisting of dispersion made up of either small inorganic particles or large organic molecules enclosing or interpenetrated by a liquid (75,76). The solid particle portion is referred to as disperse phase and the liquid portion is called the dispersion medium or continuous phase. Gels in which the macromolecules are distributed throughout the liquid in such a manner that no apparent boundaries exist between them and the liquid are called single phase gels, eg. tragacanth and carboxymethylcellulose gel. On the other hand, a two-phase gel mass consists of floccules of small distinct particles and is frequently called a magma or a milk, eg. bentonite magma and magnesia magma. Gels and magmas are considered colloidal dispersions because they contain particles of colloidal dimension (1 - 500 millimicrons). Gels owe their rigidity to an inter-twining network of the disperse phase which entraps and holds the dispersion medium. A change in temperature may cause certain gels to resume to liquid state. Some gels are thixotropic, i.e. they are semi-solids on standing and become liquids on agitation. In general,

gels formed by polymers in solution exhibit pseudoplastic flow (76) which is characterised by non-linear increases in the rate of shear and decreasing viscosity with increasing shearing stress. Gels composed of flocculated particles usually exhibit plastic flow (76). Such substances do not begin to flow until a shearing stress, corresponding to the yield value, is exceeded. Then, the rate of shear increases linearly with increasing shearing stress.

Clear gel preparations have become popular in pharmaceutics and cosmetics because they are washable and clear. Up to the twentieth century, formulations of gel were mainly dependent on waxes derived from insect or vegetable sources (77). Today, a much wider range of gelling agents are available.

Clear gels can be made in two ways. The first involves the use of hydrophilic surfactant, eg. polyoxyethylene fatty ethers and polyoxyethene fatty glycerides, at adequately high levels in aqueous solutions to form clear, rigid micro-emulsions. This type of gels will hold up to 13 - 14% of mineral oil and is mainly used in cosmetic preparations where maximum emolliency is desired. The other type is prepared by dissolving synthetic polymers in water or other solvents at sufficiently high concentrations to form gels. These gels are more commonly used in pharmaceuticals and also in cosmetics. Some commonly used polymers include celluloses, carboxypolymethylene (Carbopol) resins, methacrylate polymer (Eudispert) and polyethyleneglycol. A gel preparation must be non-irritating, easily spreadable, preferably water-soluble, non-sticky and free from unpleasant odours. The formation of a film on the skin and a tendency to drip are also undesirable. A gel that will resist dripping and yet be sufficiently fluid, must have a suitable yield strength. The yield value of a system is defined as its initial resistance to flow under applied

stress. Carboxypolymethylene resin, cellulose (78) and methacrylate polymer (79) gels are pseudoplastic systems with suitable yield values when properly formulated. All these polymers can form clear gels, but cellulose and methacrylate polymers tend to form dry films on skin after evaporation. Carbopol has many advantages such as clarity, high thickening efficiency, few incompatibilities with materials most frequently used in cosmetic and pharmaceutical formulations, high stability over a wide range of pH and temperature and resistance to bacterial and fungal attack. It has therefore been widely used as a thickening agent or suspending agent in many ointment, cream or lotion formulations (77,80), or combined with glycerine, as a lubricant (81). Carbopol is also a good base for systems such as surgical lubricant gels, where sterilisation (preferably autoclaving) is necessary (78,82). A wide range of cellulose derivatives, such as methyl cellulose, sodium carboxymethylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose are used in cosmetic and drug formulations. Some are only soluble in water, or aqueous alcohol while others (eg. hydroxypropylcellulose) are soluble in certain organic solvents such as acetone, propylene glycol. The gelling agents are available in various viscosity grades. Celluloses are liable to microbial attack and preservatives are necessary. Free methacrylic polyacids are insoluble in water, but they form viscous aqueous solutions or gels with alkalis, amines and alkaline earths. They are available in high, medium and low viscosity grades and have been used for formulating hydrocortisone (83).

1.6 DECOMPOSITION PATHWAY OF CORTICOSTEROIDS

Decomposition of corticosteroids apparently often takes place at ring A and/or the C-17-dihydroxy acetone side chain (32,34,84).

1.6.1 Decomposition at Ring A

It has been shown that the steroidal A ring is unstable particularly to light. Ring opening is the most likely decomposition pathway as shown in Figure 2 (32). This pathway is however, usually, much slower than decomposition at the C-17 side chain.

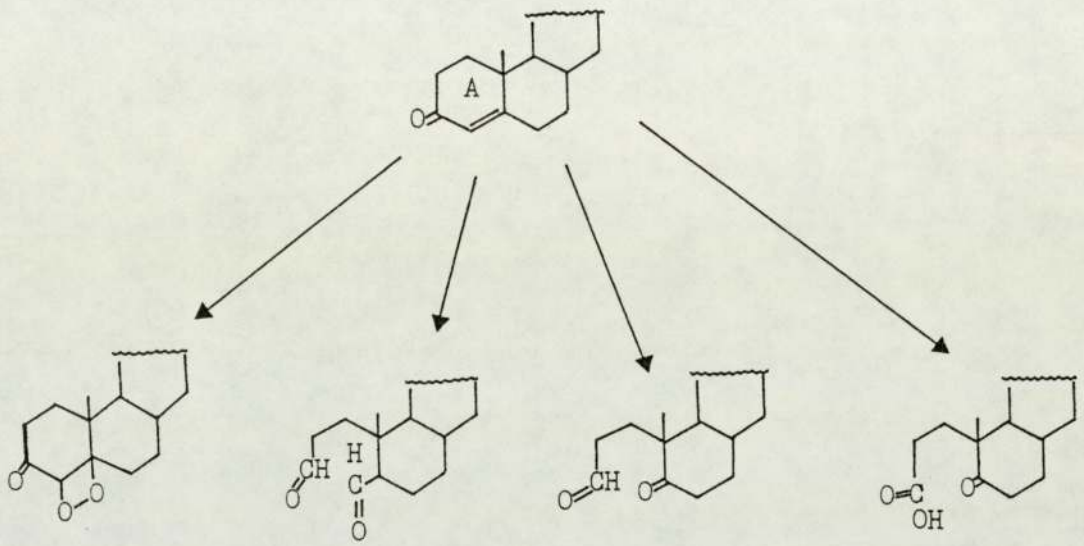


Figure 2 Possible degradation pathway of hydrocortisone at ring A.

1.6.2 Decomposition at C-17-dihydroxy-acetone Side Chain

1.6.2.1 Acyl Migration and Hydrolysis

The C-17-dihydroxyacetone side chain is susceptible to acid and base-catalysed decomposition (34,44,47,84 - 87), the esters at C-17 or C-21 being particulary vulnerable. Hydrolysis of the 21-ester to its corresponding free alcohol is common, eg. hydrocortisone-21-hemi-succinate (41), betamethasone-21-phosphate, 21-acetate (46) and 21-benzoate (34).

The corticosteroid-17-monoesters are known to undergo facile rearrangement to the corresponding 21-monoesters in basic media.

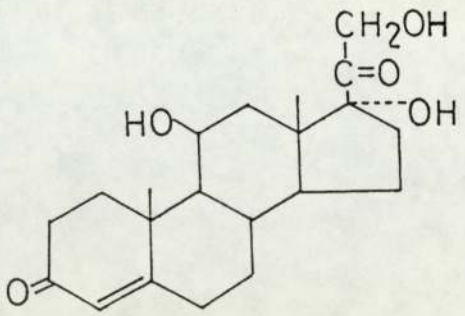
Prolonged exposure to the same conditions leads to the formation of the free alcohol (88). The 21-ester derivatives usually hydrolyse faster than the 17-esters. Direct hydrolysis of the 17-ester does not appear to be an important pathway. However, acyl migration of the 17-ester to the corresponding 21-ester is prominent, and the reaction is not only initiated by OH^- ion, but also by H^+ ion. The isomerisation of betamethasone-17-valerate to betamethasone-21-valerate in an aqueous pH 7 - 8 buffer solution has been reported to be about 80 times faster than the hydrolysis of the 21-valerate. Direct hydrolysis from betamethasone-17-valerate is however insignificant (44). Under very acidic conditions, pH 0.45 - 1.15, the hydrolysis rate of betamethasone-21-valerate is about 4 times faster than the isomerisation (44). Betamethasone-17-valerate is most stable at a pH of around 3.5. Another example is the hydrolysis of methylprednisolone-21-hemisuccinate and 17-hemisuccinate. The 21-hemisuccinate hydrolyses much faster than the 17-hemisuccinate (47) due to steric shielding at C-17. The 21-hemisuccinate is an ester of a primary hydroxy group while the 17-hemisuccinate, being an ester of a tertiary hydroxy group, is more hindered. The 17-hemisuccinate rearranges to the 21-hemisuccinate and is rapidly hydrolysed to methylprednisolone, but direct hydrolysis from the 17-hemisuccinate is insignificant. It is interesting that the 21-hemisuccinate also undergoes acyl migration to the 17-hemisuccinate in spite of the fact that the 17-OH group is in a sterically more hindered environment. The C-21 $\longrightarrow$ C-17 rearrangement is quite facile, and dominates at pH 3.6 - 7.4, although hydrolysis is a significant reaction at all pH values (47). A C-21 $\longrightarrow$ C-17 ester migration has also been shown in the decomposition of hydrocortisone-21-acetate (89).

1.6.2.2 Oxidative and Non-Oxidative Degradation

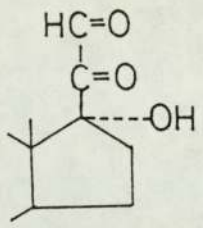
A typical example is the degradation of hydrocortisone. At least seven products (Figure 3) were identified from the decomposition of hydrocortisone in aqueous solutions over a broad range of pH (49,90). Two major decomposition pathways were observed: (i) an oxidative degradation leading to the formation of 21-dehydrohydrocortisone (I) which subsequently degraded to a 17-carboxylic acid (III) and a 17,20-dihydroxy-21-carboxylic acid derivative (II) and (ii) a non-oxidative reaction giving a 17-oxo (VI), 17-deoxy-21-aldehyde (IV) and 17-deoxy-20-hydroxy-21-carboxylic acid (V) and 17-deoxy-21-dehydro derivative (VII). The type and relative amounts of the products were strongly dependent on pH, type of buffers and trace metal impurities. Maximum stability occurred at pH 3.5 - 4.5 (91). 17-oxo-steroids have been found in marketed prednisolone, prednisone and dexamethasone tablets at a level of up to 2% (92). Prednisolone decomposed to derivatives similar to V and VI under basic, anaerobic condition and to III when oxygen was present (85). Similar oxidative degradation has also been found in cortisone (93).

1.6.3 Decomposition Involving the Fluorine Atom at C-6 and/or C-9

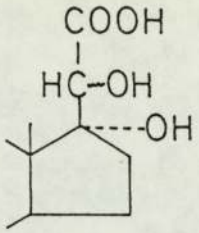
Many potent steroids are fluorinated derivatives, with a fluorine atom at C-6 or C-9 or both. Decomposition affecting the fluorine atoms has been little studied. Dekkers and Buijs (42), using three fluorinated-17-deoxyprednisolone derivatives — desoximetasone (with a fluorine atom at C-9), fluocortolone (at C-6) and diflucortolone (at C-6 and C-9) — for their studies, showed that the fluorine atom at C-6, in both fluocortolone and diflucortolone, is very stable. No splitting was detected after 16 hours at 120°C, under anaerobic conditions. The fluorine atom at C-9 in both of desoximetasone and diflucortolone, was much more labile, disappearing under the same



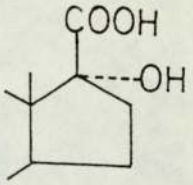
Hydrocortisone



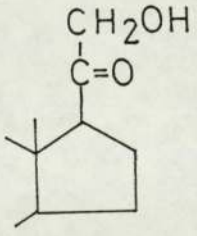
I



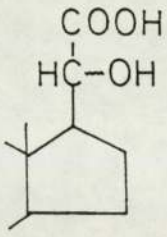
II



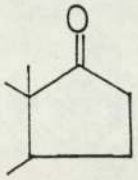
III



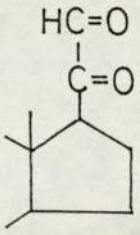
IV



V



VI



VII

Figure 3. Decomposition products of hydrocortisone (49)

conditions to form a compound with a double bond between C-11 and C-9 or between C-9 and C-8, and a 17-deoxy-17-carboxylic acid derivative. Addition of sodium edetate prevented the formation of the latter product but not the dissociation of the fluorine atom. However, only little decomposition was found. On the other hand, dexamethasone, which differs from desoximetasone by possessing a hydroxyl group at C-17, is deoxygenated to a major product — desoximetasone. Again decompositions affecting the fluorine atoms at C-6 or C-9 are negligible in comparison with the decomposition of the C-17 side chain.

1.7 PERCUTANEOUS ABSORPTION OF CORTICOSTEROIDS

1.7.1 Structure of Skin

For a topical corticosteroid to be effective, the drug must penetrate the skin to exert its effect. The human skin is a very good barrier to the external environment. It is made up of three basic layers: the epidermis, the dermis and a layer of subcutaneous fat.

The epidermis further consists of several layers of different cell types. The stratum corneum is the outermost layer of the body, it consists of dead horny cells and is composed of keratin, a protein which has remarkable physical strength and chemical stability. Just beneath the stratum corneum are clear translucent layers, making up the stratum lucidum, and a granular layer, the stratum granulosum. The term stratum corneum once embraced these two layers. The stratum corneum is continuously shed and replaced by new keratin from the deepest layer of the epidermis, the unicellular basal cell layer (stratum basale). In between the stratum granulosum and the stratum basal is a layer, several cells thick, called the prickle cell layer (stratum spinosum). Its constituent cells are daughter cells arising from the division of the cells of the basal layer. The dermis (corneum)

has a well-developed upper papillary layer and a deep reticular layer. The papillae are well supplied by capillaries entangled in collagenous fibres. The growth and differentiation of the epidermis are probably regulated by the papillae. The reticular layer is composed of coarse collagen bundles and elastic fibres. Further downwards, the reticular layer continues into the subcutaneous fat. The cutaneous appendages such as hair follicles, the sebaceous glands, the ecrine and apocrine sweat glands are derived embryologically from the epidermal cells and extend downwards to the dermis and often into the subcutaneous fatty layer.

The stratum corneum is very important in percutaneous absorption, being the main barrier against the external environment (94), and is also the site of a constant flow of liquid, nitrogenous substances, polysaccharides and so on. Besides these dam and filter functions, it may serve as a reservoir for steroids (95).

1.7.2 Factors Affecting Percutaneous Absorption

1.7.2.1 Skin Conditions

The 'barrier' function resides almost entirely in the stratum corneum (94). If the barrier is destroyed by trauma, as in cuts, chapping or ruptured blisters, absorption can be virtually 100% (96). Blank (97) developed a method for stripping the stratum corneum in successive layers and showed that the rate of water loss increased from 0.5 mg cm^{-2} h^{-1} to 8 mg cm^{-2} h^{-1} after complete stripping of the stratum corneum. Other authors claimed that the removal of the outer layer had little effect; it was actually the stratum lucidum which forms the main barrier (98). It is not however always clear in reported studies whether the stratum corneum used included the stratum lucidum. Damage to this layer also results in increased permeability to a variety of chemical agents (99,100). Skin with a disrupted epidermal barrier will

allow up to 80% of hydrocortisone to pass into the dermis while with intact skin only about 1% reaches the dermis (101). Skin conditions change with age. Preterm infants probably do not have a fully developed stratum corneum and have increased skin permeability (102). Skin of the elderly also undergoes change and this can influence absorption.

1.7.2.2. Skin Site of Application and Surface Area

Variation in absorption occurs depending on the thickness of the stratum corneum in different anatomic areas. The thicker the stratum corneum, the more impermeable is the barrier (103 - 106). Increasing the surface area of application, predictably increases absorption (107).

Backs of hands and feet, elbows and knees, palms and soles are much more resistant to penetration than such areas as the scrotum, eyelids and face. The penetration of hydrocortisone in several anatomic sites ranged from 1% on the forearm to 4% on the scalp, 7% on the forehead and 36% on the scrotum (103). Absorption through appendages eg. through hair follicles, sweat glands, is possible and may be important in areas where follicles are more numerous (such as the forehead and scalp). Increased absorption of hydrocortisone was found in hairy areas (93). The contribution of hair follicles and of sweat glands to percutaneous absorption has been investigated by a number of workers (99, 108 - 110). By using isotope-labelled compound, high concentrations of labelled material were found in the appendages (108). Evidence from in vitro study by Van Kooten and Mali (109) also supported the possibility of absorption through appendages, especially sweat glands.

1.7.2.3 Occlusion and Hydration of the Skin

Percutaneous absorption is increased if the site of application is occluded. Grab (111) was the first to use thin plastic films in

the treatment of skin diseases and claimed that it was more effective. Subsequently, Scholtz (112), and Sulzberger and Witten (113) reported the usefulness of this method on certain dermatoses, eg. psoriasis and chronic eczema. Hydrocortisone is 10 times better absorbed with plastic occlusion than when the skin is unoccluded (114). Shapiro (115) found that fluocinolone acetonide offered no advantage over its parent compound, triamcinolone acetonide, if both were used under occlusion and 0.01% and 0.025% of triamcinolone acetonide were equally effective if applied under occlusion. Hydrocortisone acetate, triamcinolone acetonide, fluocinolone acetonide and prednisolone when applied under occlusion produced vasoconstriction equivalent to response observed when a steroid concentration 100 times higher is applied without occlusion to the arms (116). Occlusion changes the temperature and hydration of the skin, consequently affects absorption. McKenzie and Stoughton (117) reported that the skin temperature rose to the internal body temperature after 16 hours occlusion. A ten-fold increase in penetration of some corticosteroids were found when the environmental temperature was raised from 10°C to 37°C (116). Occlusion causes air exclusion which in turn diminishes the evaporation of sweat and sebum. These hydrate the keratin of the horny layer and it is well recognised clinically that hydration of the skin enhances penetration as shown by Rothman (118), and Cronin and Stoughton (99). Soaking of the forearms of human volunteers has been shown to increase the absorption of triamcinolone acetonide five-fold (116, 119). Vickers has reported that the stratum corneum may serve as a reservoir for steroids and may hold them for up to two weeks if applied under occlusion for a few hours (95). Some fluorinated steroids may stay in the skin for up to 41 days (120).

1.7.2.4 Chemical Structure

Scheuplein and coworkers (121) have shown that chemical structure is of relevance in the absorption of steroids. Chemical structure is important because of its influence on lipid/water partition coefficient and on the interaction between the drug and the stratum corneum. They found that the penetration rates of steroids in vitro decreased with increasing polarity of the steroids in an aqueous solution. However, the lower permeability of the polar steroids is not mainly due to a limited solubility within the membrane. A strong chemical binding between the polar steroid with the stratum corneum was suggested to be the major contribution. This binding may explain the formation of steroid reservoirs in the stratum corneum. Contradictory to this finding, Ponc and Polano (122) reported increased penetration with increasing polarity of the steroids, hydrocortisone penetrating faster than hydrocortisone-17-butyrate. This finding is in agreement with those of Maibach (123).

1.7.2.5 Concentration of the Drug

Enhanced effect on vasoconstriction was observed with increasing concentration of steroids (117). Higuchi (70) emphasized the role of concentration of a drug on its skin penetration rate from suspension-type ointments, higher concentrations giving better penetration provided the release of drug from the suspended particles is the rate-limiting step.

1.7.2.6 Vehicle Effect

For topical preparations, the bioavailability of the drug is markedly affected by the nature of the vehicle in which it is applied. The choice of an optimum vehicle for a particular medicament depends on the physical and chemical properties of the drug.

(1) The solubility and affinity of the drug in the vehicle: Only the soluble drug fraction can diffuse out of the vehicle and across the barrier (73,124). If the drug is in suspension, dissolution may become the rate limiting factor. Ponec (125) reported that when hydrocortisone-17-butyrate was dissolved completely in the vehicle, penetration was enhanced, and only under this condition did an increase in drug concentration (range 0.05 - 0.2%) lead to an increase in penetration. Solubilization is required for higher diffusion. This may be achieved by maintaining the drug either in solution or in a homogeneous dispersion close to saturation (124,126).

(2) The nature of the base: It has been mentioned that hydration of the stratum corneum is an important factor in percutaneous absorption. Greases and oils are most occlusive vehicles and induce the greatest hydration. Water-in-oil type emulsions are somewhat less occlusive than grease (127). Aqueous vehicles hydrate the skin briefly but do not maintain hydration after the aqueous phase evaporates (127). Barry and Woodford's work (128,129) showed that betamethasone valerate ointment had a higher blanching activity than the corresponding cream. The major base ingredient in the ointment is white soft paraffin which reduces transepidermal water loss and in turn enhances percutaneous absorption.

(3) Penetration enhancers: A variety of organic solvents such as acetone, ethanol, propylene glycol, urea, dimethyl sulphoxide (DMSO). Feldman and Maibach (130) reported that hydrocortisone combined with 10% urea penetrated the skin about twice faster than without urea. Almeyda and Burt (131) claimed that in combination with urea, hydrocortisone (1%) was as effective as betamethasone valerate (0.1%) cream

in the treatment of atopic eczema due to its hydration of keratin.

The inclusion of propylene glycol in ointments has also been shown to enhance the clinical and vasoconstrictor efficacy (132 - 135) of topical steroids. High relative vasoconstrictor potencies were obtained when betamethasone valerate was formulated in an ointment base containing propylene glycol or 1,3-butanediol. The effect of the propylene glycol could also be enhanced by addition of lanolin (136); FAPG (fatty alcohol/propylene glycol) base is of this type. Whitefield and McKenzie (137) claimed that if 0.1% hydrocortisone is incorporated in a system consisting of propylene glycol with a critical proportion of an aqueous solution of sodium lauryl sulphate (Dioderm®), the same degree of vasoconstriction as more powerful synthetic and halogenated corticosteroids could be obtained. The penetration rate of diflorasone diacetate through propylene glycol pretreated skin was twice the rate that observed without pretreatment (138). The presence of propylene glycol in the vehicles enhanced the penetration of hydrocortisone-17-butyrate (125). Propylene glycol penetrated the skin rather easily (125,138), and decreases its diffusional resistance (138). However, the marked differences observed in the penetration rates of hydrocortisone-17-butyrate from Plastibase with and without propylene glycol could not be explained by the hygroscopic properties of propylene glycol (125).

DMSO has been found effective in enhancing the percutaneous absorption of many drugs (139 - 142). By using vasoconstriction as an index of percutaneous absorption, Stoughton and Fritsch (143) reported that the presence of 10% DMSO enhanced the absorption of fluocinolone acetonide five-fold. DMSO increased the penetration of testosterone and hydrocortisone three-and-half times (140). DMSO is known to pass through human stratum corneum rapidly (144).

There are several possible explanations for the effect of DMSO or similar accelerants on percutaneous absorption of drugs. DMSO passes rapidly through the stratum corneum (144). Consequently, the solvent drags the drugs into the skin. DMSO not only enhances percutaneous absorption but promotes the formation of a steroid reservoir in human skin (119). Baker (145) attributed the effect of DMSO to its strongly hygroscopic properties which increases the hydration and therefore the permeability of the skin. It is known that DMSO and some accelerants cause swelling (142,146) and dissolve lipoprotein or other structural materials (142, 147) from the stratum corneum. These changes are possibly reversible (145). According to these findings, Allen and coworkers (142) suggested that enhancers owe their effectiveness, at least in part, to their ability to lower the barrier function of the skin by modifying its natural structure.

1.7.3 Methods for Assessment of Percutaneous Absorption

The efficacy and toxicity of topical steroids is partly determined by their ability to penetrate skin. The ideal way to determine the penetration potential of a drug in human is do it in man. However, such in vivo tests may be hazardous and are costly. In vitro measurements are useful prior to in vivo tests. They are particularly useful in assessing the ability of a vehicle to liberate the drug and for investigating parameters which affect penetration.

1.7.3.1 In Vivo Techniques

(1) Disappearance technique: A known amount of non-volatile material is allowed to remain on the skin for a certain period of time and then removed quantitatively for analysis, the difference between the amount applied and the amount recovered is assumed to have penetrated the skin (148). This method has several disadvantages. Quantitative removal is difficult. Some chemicals bind firmly to the

stratum corneum and are not easily separated from it. The amount of drug penetrated is often so small that errors in analysis make such methods unreliable (149). This method can be improved with the use of radioactive substances. The drug loss from the surface can be determined from the decrease in radioactivity.

(2) Analysis of tissues and body fluids such as urine and sweat and blood: Percutaneous absorption is measured by analysis of the drug level in circulating blood (150,151) or urine (152) at different time intervals following the application of the drug on the skin of man or animals (153,154). Radioactive substances have been very useful in this method of study (152,155). Plasma levels of compounds are extremely low following topical application, making necessary the use of radioactive tracers. The difficulty of this method is that the rate of drug excretion cannot be assumed to be directly related to the rate of absorption. In some cases, the drug may be stored within the body or be subjected to chemical alteration before excretion.

(3) Systemic effect: A less sensitive indicator of percutaneous absorption of corticosteroids is the monitoring of the systemic activity by measuring the extent of depression of the pituitary-adrenal axis after application of the potent steroids to large areas of skin (156, 157).

(4) Autoradiography: Skin biopsies and observations are made at various intervals after topical application of labelled steroids. This can then be detected as silver grains on the nuclear track plates (158). This technique is useful in determining the presence of the drug in the various anatomical layers of the skin and in obtaining information on the relative concentration of the steroids.

(5) Vasoconstrictor test: Since McKenzie and Stoughton suggested that vasoconstriction, which is visible as blanching, can be

used as an index of percutaneous absorption for steroids (23,117), this method has been widely utilized as a screening technique for monitoring clinical efficacy (20,128,129, 159 - 168). The topical steroid preparations are applied to the skin of the flexor surfaces of the fore-arms. These are then wrapped with polyethylene tape for several hours, and the presence of pallor on the applied sites are noted. Parameters monitored should at least include the onset of action, duration of action and maximum intensity. It is dangerous to judge relative potencies by just comparing the intensity of action obtained at a particular time point. Some investigators have established improved interpretation of the vasoconstrictor data. Stoughton (110) used a 0 - 3 scale while Pepler et al (163) used a 0 - 4 scale. The number of readings has varied from a single determination (162) to four, over a 48 hours period (20). Recently, Barry and Woodford modified the technique and studied extensively the activity of many proprietary topical steroid preparations (128,129,164, 165). The procedure they used is a double-blind technique and took readings at appropriate intervals to obtain a complete blanching profile. A rank order arrangement of these formulations based on a biopharmaceutical parameter (i,e. area under the curve values) has been made which may well reflect their relative clinical efficacy. A pharmacokinetic model to describe the vasoconstrictor activity of the steroids has also been established by these authors (166,167). It has been said that vasoconstriction show a strong or at least a reasonably good correlation to clinical activity of the steroids (169,170). However, Stoughton (171) recently reported that there is no quantitative difference in penetration of skin between betamethasone versus betamethasone-17-valerate, fluocinolone versus fluocinolone acetonide and fluocinolone acetonide versus fluocinolone acetonide 21 acetate, whereas

dramatic differences in vasoconstrictor activity and clinical activity have been observed. The vasoconstrictor potency of a drug primarily reflects its inherent activity rather than its ability to penetrate skin although without absorption no vasoconstriction can be observed.

1.7.3.2 In Vitro Models

(1) In vitro cell diffusion techniques: The most common in vitro method for studying the release and skin penetration of drugs has been with diffusion cells using excised human skin (125, 172 - 176), mouse skin (138,177,178), other animal skins (106) or synthetic membranes (179). Although there are many variations in this basic system, they follow a general pattern. The specimen of skin is mounted in a hollow chamber so that it divides the chamber into two compartments. The drug in solution or the formulation is placed in the compartment of the epidermis side, the other compartment being filled with a suitable fluid acting as receiving phase. Penetration is measured either by the disappearance of the drug from one compartment or its appearance in the receiving phase or both. The penetration of steroid is usually very slow, steroids labelled with radioisotopes are employed in most cases for higher sensivity. The relative skin permeability of different species has been investigated (106,180,181). The skin of rabbit, rat and guinea pig are more permeable than human skin. The skin of the pig and monkey were closer to the permeability of human skin. Mouse skin was the most permeable, but hairless mouse skin was found to be very similar to human skin in the absorption of certain compounds (182). The techniques used and the species of skin will affect the results. In comparative studies, the methods and techniques used must be as close to each other as possible.

(2) Solvent systems: Systems which do not use skin membranes but which involves penetration of the drug into some model immiscible

solvent, designed to simulate the skin (124, 126, 136, 183, 184), are also used. For instance, a steroid gel was placed in a petri dish and immersed into the bottom of a beaker containing isopropyl myristate (IPM) to represent the skin and the rate of drug release into IPM was observed (124, 126).

Another device utilizing three layers of immiscible vehicles eg. ointment base, aqueous solution and chloroform, was also reported (136). The drug in the ointment base is released through the aqueous layer which simulates the skin, and then into the receiving phase. The distribution of the drug in each layers can be measured by assaying each layer individually. A similar design is based on a Schulman-type cell in which the test solution/formulation and the receiving phase are separated into two solid compartments. A layer of lipid fluid, which mimics the skin lipid, bridges the two compartments (183, 184).

1.8 BIOTRANSFORMATION OF STEROIDS IN SKIN

1.8.1 Skin as a Metabolizing Organ

One of the methods by which the body protects itself, is by metabolising of foreign substances and drugs. Besides the liver which is well known as the major site for drug metabolism, the lung and the skin also possess metabolic activity. The skin contains a number of enzymes and can carry out most of the metabolic drug biotransformation of the liver: oxidation, hydroxylation, reduction, hydrolysis, deamination, dealkylation and conjugation of sulphate and glucuronide (185, 186). The skin also contains the necessary complement of enzymes for the metabolism of carbohydrates, lipids and proteins (187). Although almost all the individual metabolic reactions are catalysed by separate enzymes, few of them are absolutely specific to the structure of the substrate. An example is the competitive inhibitory effect

on 5α-reductase of testosterone by progesterone, androstenedione, corticosterone and corticosterone acetate (188). A further example is the mutual competition of oestradiol and testosterone for the same dehydrogenase (189), skin also contains co-enzymes (eg. NAD, NADP) which resemble those of the liver (187,190).

1.8.2 Distribution of Enzymes and Variation in Activity with Body Site of the Skin

Reports have shown that most of the enzyme activity of the skin is localized in the epidermal layers (191). Different enzyme activities are observed at different sites of the skin (186,192,193).

3α- and 3β-hydroxy steroid dehydrogenase: 3α-OH steroid dehydrogenase is observed in the epidermis, hair follicles, sebaceous glands, apocrine sweat glands and fibrous dermis. 3β-OH steroid dehydrogenase activity is only observed in the sebaceous glands. 3β-OH steroid dehydrogenase Δ^{4-5} isomerase is located mainly in the sebaceous glands of facial skin (192).

5α-steroid reductase: Hydrocortisone 5α-reductase has been detected in human foreskin but not in skin from the thigh, the hand, the abdomen (193) or other anatomical sites (186). Testosterone 5α-reductase was found in foreskin, scrotum and abdominal skin (186, 193) but was less active in non-perineal area (193). Progesterone 5α-reductase activity is present in human foreskin, vaginal mucosa, and to a small extent in abdominal skin (186).

17β-OH steroid dehydrogenase: In facial and scalp skin the rate of conversion of 17β-hydroxy steroids into 17-oxo-steroid metabolites is much greater than the reverse reaction, whereas in axillary and pubic skin the opposite is found (194,195). There are two forms of this enzyme, one is NAD(H)-dependent and is mainly found in forehead skin and the other is NADP(H)-dependent and is associated with axillary skin (192).

Esterase: The skin contains large amounts of esterases. In body epidermis the entire malpighian layer (i.e. stratum basale and stratum spinosum) shows strong Tween esterase (which split Tween 60) activity, but the stratum granulosum and the stratum corneum do not. The epidermis of the palms and soles occupy an intermediary position (191). The malpighian layer of the epidermis has moderate amounts of α-esterases (191) while the stratum corneum shows variable amounts. The epidermis of the palms and soles has weak enzyme activity in the malpighian layer and no activity in the stratum granulosum and the stratum lucidum. The entire malpighian layer has indoxyl acetate esterase activity (191). All the nerves in the skin of the human embryo, have a high concentration of specific cholinesterase (191). In adult skin, only the small, presumably unmyelinated terminal nerves, have significant amounts of cholinesterase. Both 17-ester (196) and 21-ester (197) steroid esterase activities were found in human skin epidermis and dermis.

1.8.3 Significance of Steroid Metabolism in Skin

The presence of steroids or their metabolites in human skin was first reported by Duboive in 1954 (198) on the basis of positive Zimmerman test for 17-ketosteroids in lipids isolated from human skin. In 1960, the same author quantified the 17-ketosteroids, 3-hydroxy-steroids, 17-hydroxysteroids and glucocorticoids in human hair fat (199). By paper and thin layer chromatography, Julesz and coworkers showed that cholesterol and a few 17-ketosteroids were present in skin and hair (200) and dehydroepiandrosterone and 3-chloro-dehydroepiandro-sterone were identified (201).

Given the proper precursor, skin cells are able to synthesize biologically active steroids and activate or inactivate the endogenous and exogenous steroids. The epidermal cells are capable of synthesising

cholesterol from squalene and many of the intermediates can be detected (187). 7-Dehydrocholesterol, the precursor of vitamin D, appears to be formed in the epidermis and the photodynamic conversion of this material into the active vitamin occurs within the epidermal cells and not on the skin surface (187). The ability to activate and inactivate endogenous steroids allows the skin to have some control over its own hormonal environment to meet the requirements of the cells. Abnormality in these functions may lead to skin disorders. The transformations from cortisone to hydrocortisone (202,203,204), androstenedione to testosterone (205, 206), and oestrone to oestradiol (207) are reversible, with an equilibrium favouring the oxidative formation of the ketones. These ketones: cortisone, oestrone and androstenedione are the less active forms. Under certain conditions, the skin can transform the steroids into more active forms (193). Dehydroepiandrosterone (DHA) sulphate which is secreted by the adrenal cortex, can be activated to testosterone (208) and the more potent androgen, dihydrotestosterone (209,210) by the skin. Temporary age-related increases in the formation of dihydrotestosterone at specific skin sites causes the normal development of certain sexual characteristics, as well as androgen-dependent skin disorders such as acne vulgaris (209).

Most of the studies on the cutaneous biotransformation of corticosteroids has been centered on the metabolism of hydrocortisone and cortisone (202,204, 211 - 213). The skin can perform the reversible interchange between hydrocortisone and cortisone. After incubation with human skin slices, the major metabolite of hydrocortisone is cortisone (202,203). Other metabolites (203) include 4-pregnene-11β, 17α,20β,21-tetrol-3-one (Reichstein's E), 4-pregnene-11β,17α,20α,21-tetrol-3-one (Reichstein's epi-E), 4-pregnene-17α,20β,21-triol-3,11-dione (Reichstein's U), 4-pregnene-17α,20α,21-triol-2,11-dione

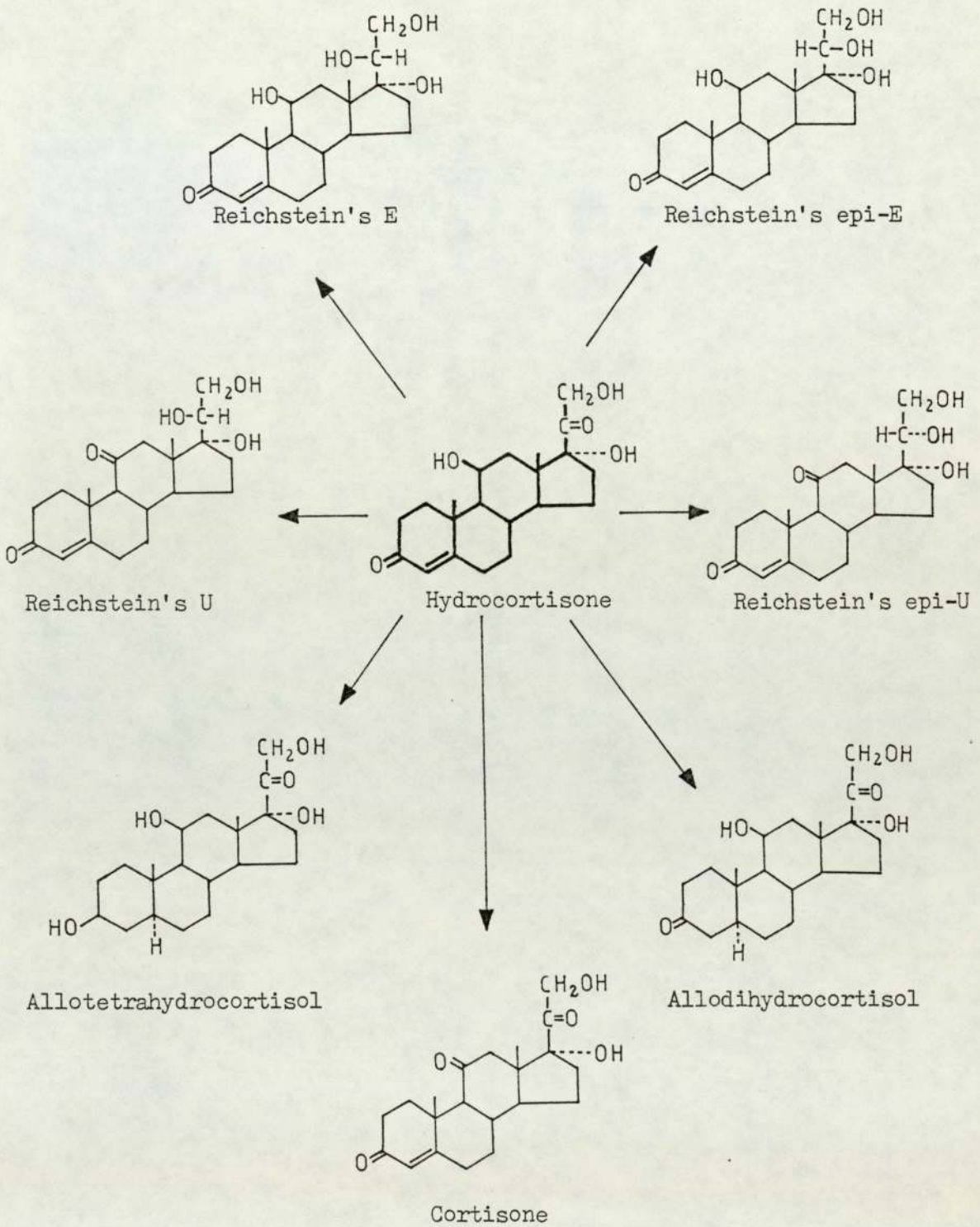


Figure 4. Hydrocortisone biotransformations by human skin (203)

(Reichstein's epi-U), allodihydrocortisol and allotetrahydrocortisol (Figure 4). Starting with cortisone, the major metabolite was hydrocortisone. Two minor metabolites were Reichstein's substances U and epi-U (204). It is known that cortisone is topically inactive while hydrocortisone is active, but both are systemically active. The general belief is that although the skin is capable of transforming cortisone to hydrocortisone, the amount of hydrocortisone formed is below the therapeutic level. An alternative explanation is the possibility that both steroids are biotransformed to more active metabolites by the skin but that such activation is less efficient with cortisone than hydrocortisone. This was based on the findings that only small amounts of hydrocortisone but large amount of other metabolites appeared in the urine after systemic administration of cortisone. Another possibility is that cortisone is inactivated much more rapidly than hydrocortisone by the skin. This was supported by the findings that transformation of cortisone was more rapid than that of hydrocortisone in vitro (202) and in vivo (155).

Comparative studies on the cutaneous metabolism of the 17-esters and 21-esters of hydrocortisone revealed that the 21-esters were more vulnerable to enzymic inactivation to form the free alcohols (214). The rates of ester cleavage vary with chain length, with maximum rates being obtained with a chain length of about 4 to 6 carbon atoms. Above 11, only relatively low rates were observed. The fact that some 17-ester steroids such as betamethasone-17-valerate are clinically more effective than their corresponding 21-esters or free alcohols may be related to their resistance to cutaneous transformation.

1.8.4 Methods for Cutaneous Metabolism Study

For in vitro study, the skin specimens are first cut into small pieces and suspended in a buffer solution chosen to optimise the

enzymic activity of the skin. Usually this is about pH 7.4. Then the mixture is homogenised and the tissue debris is removed by centrifugation at 0 - 4°C. The test compound and the co-enzymes (if needed) are incubated with the skin homogenates. The reaction is terminated by addition of extraction solvents such as methanol:chloroform (2:1) or methanol:methylene dichloride (2:1) or ethyl acetate, and then analysed. If separation of epidermis and dermis is desired, a strip of whole skin is stretched 1.5 to 2 times its original length, so that the surface at the dermo-epidermal junction is flattened and loosened. The epidermis can then be scraped off with a razor blade or gently teased off with foreceps (193).

In in-vivo studies, radioisotope-labelled steroids are applied to the skin surface and urine samples collected at appropriate intervals. The metabolites and original compound are then identified and analysed (213).

1.8.5 Enzyme Kinetics

In an enzyme-catalysed reaction, there is a very short lag before a steady rate of reaction is obtained. The lag is too short for detection (less than one minute). The rate of reaction begins to fall after a certain period of steady state, because of the fall in substrate concentration and/or the accumulation of products. The decrease of the reaction rate is difficult to predict mathematically. Therefore, it is usually the constant rate which is monitored (215).

1.8.5.1 Enzyme Concentration

The reaction rate is usually proportional to the concentration of enzyme, but deviations can occur. The presence of small amounts of some highly toxic impurity, of dissociable activator or coenzyme, or inhibitor. Alternatively, the enzyme may be unstable at low concentrations and lead to deviation from linearity (214a, 215)

1.8.5.2 pH Effect

Most enzyme have a characteristic pH at which their activity is maximal. The effect of pH on enzymes, may be due to changes in the state of ionization of the components of the system as the pH changes. The free enzyme, the enzyme-substrate complex and the substrate may all undergo such changes, since enzymes are proteins containing many ionizable groups (214a).

1.8.5.3 Temperature

The effect of temperature on the enzymic rate could be due to an effect on (i) the stability of the enzyme, (ii) the actual rate of breakdown of the substrate-enzyme complex which is determined by the heat of activation or (iii) an alteration in the pKa of the enzymes or substrates (214a). Most mammalian enzymes show little inactivation in the presence of their cofactors and substrates at 37°C (215).

1.8.5.4 Substrate Concentration

A distinctive feature of enzyme-catalysed reaction is the phenomenon of saturation with substrate. At low substrate concentration, the reaction velocity v is proportional to the substrate concentration and the reaction is thus first order with respect to the substrate. But at high substrate concentration, the rate becomes constant and independent on the substrate concentration. The enzyme is saturated with its substrate and the reaction becomes zero order with respect to the substrate. This relation can be expressed by:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \text{Eq.2}$$

where E is the enzyme, S the substrate, ES the enzyme-substrate complex, P the products and k_1, k_{-1} and k_2 are the rate constants. Then the constant steady-state velocity v is given by:

$$v = \frac{V\,[S]}{K_m + [S]} \qquad \text{Eq. 3}$$

where $K_m = \frac{K_{-1} + K_2}{K_1}$, V is the maximum velocity obtained at high substrate concentration, [S] the concentration of free substrate, and K_m , a quantity termed the 'Michaelis Constant'. Equation 3 was derived with the assumption that $k_{-1} \gg k_2$ and the free substrate concentration remains unchanged during the initial period of reaction, i.e. the total substrate concentration is much greater that the total enzyme concentration. K_m is equivalent to the substrate concentration at which the velocity is half the maximum velocity. The maximum velocity V is equal to $k_2[E]$ where [E] is the total enzyme concentration. Although K_m is independent of [S] and [E] , it usually changes with pH, temperature, substrate and the cofactor concentration.

1.8.5.5 Evaluation of K_m and V

K_m and V can be estimated by using one of the following plots (215):

(1) 1/v against 1/[S] will give slope = K_m/V; intercept = 1/V at ordinate and $-1/K_m$ at abscissa

(2) v against v/[S] will give slope = $-K_m$; intercept = V at ordinate and V/K_m at abscissa

(3) [S]/v against S will give slope = 1/V ; intercept = K_m/V at ordinate and $-K_m$ at abscissa

Three plots give straight lines if Equation 3 is obeyed.

1.8.5.6 Inhibition by Excess Substrate

In some cases, the enzyme-substrate complex can combine with a second substrate to form an inactive complex which, unless reversible to the original ES form, can only yield products slowly or not at all.

A symmetrical bell shape curve will be obtained when v is plotted against log [S].

1.8.5.7 Competitive Inhibition

Structural analogues can compete with each other for the same sites on the enzyme. Such inhibition can be reduced by increasing the concentration of substrate. This type of inhibition is most easily recognized by plots of 1/v versus 1/[S] at varying concentrations of inhibitor. The plots differ in slopes but have a common intercept on the 1/v axis, i.e. V is not altered by the presence of a competitive inhibitor.

1.8.5.8 Non-Competitive Inhibition

In this case, the amount of inhibition is only dependent on the concentration of inhibitor. The plots of 1/v versus 1/[S] in the presence of various concentrations of inhibitors will differ in slope but do not share a common intercept on the 1/v axis. The intercept on the 1/v axis is higher with higher concentrations of inhibition, i.e. V is decreased by the inhibition.

CHAPTER 2 DEVELOPMENT OF HPLC ASSAY SYSTEMS FOR CORTICOSTEROIDS

2.1 INTRODUCTION

The determination of specific steroids or other active compounds from their related degradation products in pharmaceutical preparations is a common problem (59). Developments in the early techniques for standard column liquid chromatography made possible the separation of closely related compounds but there were still many limitations. To overcome some of these, high-performance liquid chromatography (HPLC) was developed and its versatility both in terms of sensitivity and selectivity is demonstrated by its wide use for steroids analysis (41,46,47,55,56). Another advantage of HPLC is that some thermolabile steroids such as aldosterone can be handled without derivatization or exposure to heat, light and air. Liquid chromatography has greater potential for difficult separations than gas liquid chromatography because a wider range of sorption mechanism can be used in HPLC, eg. adsorption, partition, reversed-phase partition, chemisorption, gel permeation, ion exchange and ion-pair formation (40). This feature is useful for discriminating between the parent corticosteroid and its decomposition products which may be of similar polarities. Many thermolabile steroids are quite polar and elute relatively slowly using non-polar eluents. Reversed-phase columns with aqueous methanol or acetonitrile are widely used. It is comparatively easier to modify reversed-phase systems than normal-phase HPLC to obtain the required separation. Modification of the proportion of organic solvent present in the eluent is all that is often necessary. However, when the samples are non-aqueous, such as an organic extract of corticosteroid from its dosage form, normal-phase system may be preferable. Both techniques were used in this study. Betamethasone-

17-valerate is widely prescribed topical corticosteroid, which easily undergoes rearrangement to the less active isomer, the 21-valerate, under non-ideal conditions, and is further hydrolysed to betamethasone alcohol (216, 217). Previous work has shown that these three corticosteroids can be well-separated by thin layer chromatography, and the betamethasone-17-valerate quantitatively assayed by densitometry (39). However, in the systems studied, the quantitation of betamethasone-21-valerate and betamethasone were not successful. The procedure was tedious and numerous standards were required for acceptable results. The HPLC systems reported here can discriminate between these three corticosteroids and is useful for their simultaneous assay. The method was also found to be equally applicable to the separation of hydrocortisone and its 17- and 21-butyrates. This is essential for a full kinetic study.

2.2 MATERIALS AND METHODS

A high-performance liquid chromatography is constructed from an Altex 100A constant flow solvent-metering pump, a Rheodyne 7120 injector fitted with a 20 µl loop and a Pye LC3 variable wavelength ultraviolet monitor, equipped with an 8 µl flow-cell and operated at 250 nm.

2.2.1 HPLC Systems

System I (normal phase):-

Column: Spherisorb-Si (5 µm), 25 cm x 4.6 mm i.d.

Mobile phase: 1 - 3% methanol, 28 - 50% chloroform in ethyl acetate, and saturated with water

Flow rate: 1 ml/min

System II (normal phase):-

Column: Ultrasphere-Si (5 µm), 15 cm x 4.6 mm i.d.

Mobile phase: 0.3% ammonia, 0.5% water, 20% isopropanol in hexane

Flow rate: 1.5 ml/min

System III (reversed-phase):-

Column: Hypersil-ODS (5 μm), 10 cm x 4.6 mm i.d.

Mobile phase: 66 - 70% methanol in water

Flow rate: 1 ml/min

Detection wavelength: 250 nm

System IV (reversed-phase):-

Column: Hypersil-ODS (5 μm), 10 cm x 4.6 mm i.d.

Mobile phase: (a) 50% acetonitrile in water for hydrocortisone and its acetate and butyrate esters

(b) 55% acetonitrile in water for betamethasone and its 17- and 21-valerates

Flow rate: 1 ml/min for (a)

1.2 ml/min for (b)

Standard solutions of steroids were prepared, either separately or in combination, in chloroform for normal-phase HPLC and aqueous-methanol or aqueous-acetonitrile for reversed-phase HPLC analysis, with concentrations as indicated. 20 μl of the standard solutions were chromatographed.

2.2.2 Recoveries of Steroids from Cream, Ointment and Gel

2.2.2.1 Preparation of cream, test solutions and standard solutions

0.75 g of Betnovate® cream (Glaxo, nominally containing betamethasone-17-valerate equivalent to 0.1% of betamethasone) was weighed in glass-stoppered test tubes. 5 ml of 0.5% hydrochloric acid was added and shaked until dissolved. 10 ml of chloroform was added to extract the steroids. The chloroform extract was filtered through silicone-treated filter paper (Whatman 1SP), and to 5 ml of the filtrate was added 1 ml of 0.6 mg/ml caffeine in chloroform as internal standard. The final solution contained 0.1 mg/ml caffeine and 0.075 mg/ml

betamethasone-17-valerate theoretically. The recoveries of the mixture of betamethasone and its 17- and 21-valerates in Cetomacrogol cream B.P. formula A, was also studied. Accurately weighed amounts of these three steroids were dissolved in 10 ml chloroform, then shaken with the cream base dispersed in 5 ml of 0.5% hydrochloric acid and assayed as before. The standard solutions were treated in a similar way, i.e. partitioning the steroids between 5 ml of 0.5% HCl containing the same amount of Cetomacrogol cream as in the test and 10 ml of $CHCl_3$. The concentration used range from 0.02 - 0.075 mg/ml of steroid with 0.1 mg/ml caffeine as internal standard in the final solutions.

2.2.2.2 Preparation of ointment, test and standard solutions

Synthetic ointments containing 0.1% w/w of betamethasone-17-valerate were prepared by ultrasonically dissolving the steroid in propylene glycol (5%) and dispersing the product by rapidly stirring into molten white soft paraffin. This was followed by rapid cooling to produce even dispersion of the fine propylene glycol globules throughout the ointment. Commercial Betnovate® ointment (Glaxo), nominally containing betamethasone-17-valerate equivalent to 0.1% w/w betamethasone, was also used. The steroid contents of these ointments were determined by weighing 1 g into glass-stoppered test tubes and partitioning between 10 ml n-hexane and 10 ml dimethyl sulphoxide (DMSO). The DMSO extract was chromatographed. The standard solutions were prepared by shaking 10 ml of DMSO contained 0.02 - 0.1 mg/ml of steroid with 10 ml of n-hexane, standing for separation and the DMSO phases were used.

2.2.2.3 Preparation of gel, test and standard solutions

Synthetic carboxypolymethylene (Carbopol) gel containing 0.1% w/w hydrocortisone-17-butyrate was prepared according to the following formula:-

Carbopol	0.8	g
Propylene glycol	48.0	g
Hydrocortisone-17-butyrate	0.1	g
10% w/v NaOH solution	0.5	ml
Water to	100.0	g

Carbopol was gradually dissolved in 28 g of propylene glycol and about 49 g of water by stirring, then neutralized with sodium hydroxide. Hydrocortisone-17-butyrate was dispersed in the rest of propylene glycol and incorporated into the gel mass. More water was added to weight. 1.2 g of the gel was partitioned between 5 ml of 0.5% HCl and 10 ml of chloroform. The chloroform phase was filtered through silicone-treated filter paper. To 5 ml chloroform of the extract was added 1 ml of 0.6 mg/ml of caffeine in chloroform. The final solution contained 0.1 mg/ml of caffeine and hydrocortisone-17-butyrate. The standard solution (0.02-0.1 mg/ml) were analysed as the test solutions.

2.2.2.4 HPLC conditions

Column: Spherisorb-Si (5 μm), 25 cm x 4.6 mm i.d.

Mobile phase: (a) 1% methanol, 28% chloroform in ethyl acetate and saturated with water (for ointment and gel)

(b) 1% methanol, 50% chloroform in ethyl acetate and saturated with water (for cream)

Flow rate: 1 ml/min

Detection wavelength: 250 nm

Sensitivity: 0.64 AUFS

2.3 RESULTS AND DISCUSSION

For this project, two normal-phase and two reversed-phase systems have been developed for monitoring hydrocortisone, its 21-acetate, its 17- and 21-butyrates, and betamethasone and its 17- and 21-valerate esters. A successful HPLC separation depends on matching the right mobile phase to a given column and samples. The rational approach to solvent selection requires the knowledge of some of the important properties of the samples such as the solubility, polarity and ionization. The properties of the mobile solvent, such as solvent strength and selectivity, must also be considered. Valuable information could be obtained by searching of reported methods, which may reveal a suitable system. If an assay method is not available in the literature, a thin layer chromatographic separation is useful. Modifications can be made to optimize this preliminary separation. Previous work with thin layer chromatography showed that a chloroform: ethyl acetate (1:1) mixture is suitable for the separation of betamethasone and its 17-and 21- valerates on silica plates (39). Therefore, the same solvent mixture and a silica column were initially adopted for HPLC assay. As expected, no satisfactory separation was obtained without further modifications. Figure 5(a) shows the typical separation of these three steroids with caffeine being used as internal standard by a mixture of 28% chloroform and 72% ethyl acetate as mobile solvent. To improve the efficiency of the column, 1% methanol was added to moderate the activity of the surfaces of the solid stationary phase, silica, so that the adsorption of a relatively polar solute to the adsorbent is minimized. As shown in Figure 5(b), methanol was not powerful enough to produce an optimal chromatogram. Thus, another more polar and common moderator — water was used instead. When the solvent was saturated with water, significant improvement was

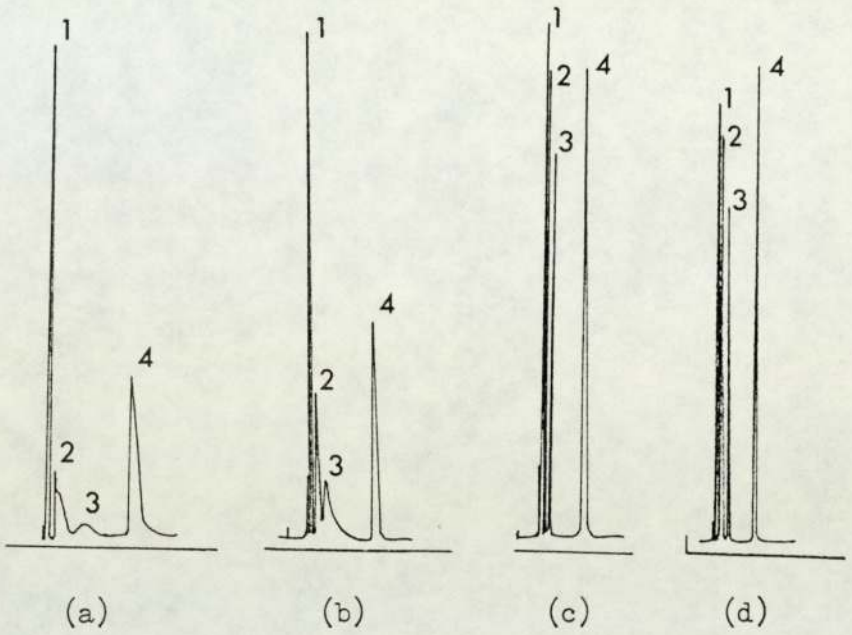


Figure 5. Development of an HPLC separation for steroids

Mobile phase composition:
(a) chloroform - ethyl acetate (28:72)
(b) chloroform - ethyl acetate (28:71) + methanol (1%)
(c) chloroform - ethyl acetate (28:72) — water saturated
(d) chloroform - ethyl acetate (28:71) + methanol (1%) — water saturated
Column: Spherisorb-Si (5 µm), 25 cm x 4.6 mm i.d.
Key:
1: betamethasone-21-valerate
2: betamethasone-17-valerate
3: betamethasone
4: caffeine

seen (Figure 5c). The combination of methanol and water showed even better resolutions (Figure 5d).

The amount of water present in the mobile phase is critical. The lower the polarity of the eluent, the bigger is the influence of small changes in water concentration (218). A typical example was demonstrated by Boehme and Engelhardt (219). On a column packed with alumina. With 'dry' n-heptane (water content $\leq$ 20 ppm) as eluent, five polynuclear aromatic hydrocarbon (naphthalene to chrysene) can be separated within 5 minutes. With 'moist' n-heptane (water content ca. 40 ppm), the separation is completed in about 1 minute. The increase of about 20 ppm in the water content results in a decrease in the K' value of chrysene from 11.9 to 2.2. As the water content is so critical, it is essential to standardize the amount of water in the eluent. However, it is almost impossible to store and preserve an eluent with defined water concentration. For example, opening the reservoir or decanting the eluent may introduce moisture, or the glass container may adsorb a considerable amount of water from the eluent. Snyder (220) recommended the use of eluents with the same percentage of water-saturation, eg. 50% water-saturation of the eluent may be optimal for silica columns (221). Theoretically, this partially saturated eluent can be made by mixing certain amount of 'dry' eluent and 100% water-saturated eluent. In practice, the so-called 'dry' eluent is also subject to the problems already mentioned. It is also very difficult to achieve complete water-saturation of the eluent just by shaking, even for systems which can hold a significant amount of water such as the chloroform-ethyl acetate-methanol mixture. Boehme and Engelhardt (219) suggested a closed-loop moisture control system for producing and maintaining the desired water content of an eluent. This system can be inserted between the detector outlet and the inlet

of the pump. The system is filled with silica or alumina impregnated with different amounts of water. For the methanol-chloroform-ethyl acetate system, adding fixed amounts of water to the eluent is convenient and reasonably reproducible. Working out the exact amount of water needed is a matter of trial and error. A known amount of water was put in the eluent and the system run for about 50 ml for equilibration. A sample solution is then chromatographed for several times. If the solvent contains too much water, the silica is inactivated and cannot hold the solute resulting in decreased retention times between injections. If the solvent is too dry, increasing retention times will be observed. Eventually constant retention times are obtained. A 0.5% v/v water content was optimum. Increasing the water content leads to decreased retention on the column and peak broadening is observed. An example is shown in Figure 6(a) and (b). This system is equally applicable to the separation of hydrocortisone 17-butyrate, 21-butyrate and hydrocortisone mixture (Figure 7). It is also useful in assaying prednisolone, prednisolone acetate, cortisone acetate and hydrocortisone acetate (Table 2).

Varying the relative proportion of the three components of the mobile phase permits modification of the retention time. Increasing the chloroform content prolongs the retention times, while increasing the methanol concentration has the opposite effect. For instance, when isopropyl myristate (IPM) is used as sample solvent, IPM itself showed a strong peak at the retention time of hydrocortisone-21-butyrate. Acceptable separations can be obtained by changing the mobile phase to methanol:chloroform:ethyl acetate (1:50:49). The nature of the sample solvent is another parameter which should be taken into account because of its effects on the peak heights, the peak shapes and even the separation, especially with mobile solvent and sample solvent of

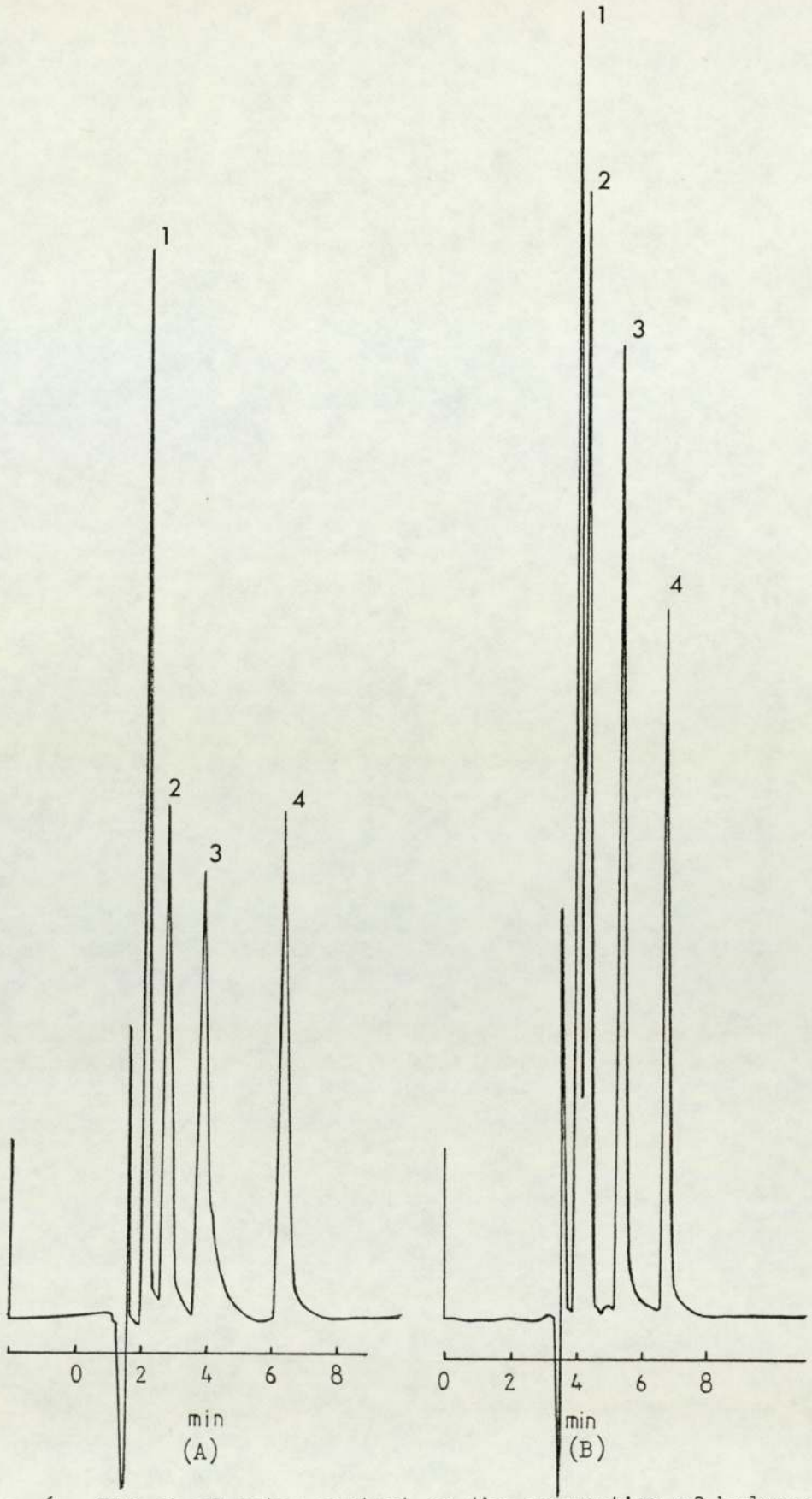


Figure 6. Effect of water content on the separation of hydrocortisone-21-butyrate, hydrocortisone-17-butyrate and hydrocortisone.
Column:Spherisorb-Si (5 μm), 25 cm x 4.6 mm i.d.
Mobile phase: 3% methanol, 35% chloroform in ethyl acetate with (A) 0.5% water (B) 1% water
Flow rate: 1 ml/min Steroid concentration: 0.1 mg/ml in $CHCl_3$
Key: 1:hydrocortisone-21-butyrate
2:hydrocortisone-17-butyrate
3:hydrocortisone
4:internal standard (caffeine)

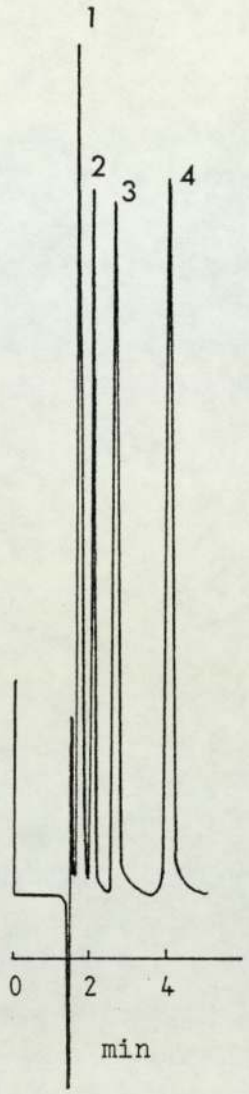


Figure 7. HPLC separation of hydrocortisone-21-butyrate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) and caffeine (4) as an internal standard.
Column: Spherisorb-Si (5 µm), 25 cm x 4.6 mm i.d.
Mobile phase: 1% methanol, 28% chloroform, 0.5% water, in ethyl acetate
Flow rate: 1 ml/min
Detection wavelength: 250 nm
Sample solvent: chloroform

Table 2. Retention times of various steroids.
Column: Spherisorb-Si (5 µm), 25 cm x 4.6 mm i.d.
Mobile phase: 1% methanol, 28% chloroform in ethyl acetate and saturated with water.

Compound	Retention time (min)
Betamethasone-21-valerate	1.6
Betamethasone-17-valerate	1.8
Betamethasone	2.2
Hydrocortisone-21-butyrate	1.8
Hydrocortisone-17-butyrate	2.1
Hydrocortisone	2.8
Hydrocortisone acetate	1.8
Cortisone acetate	1.8
Prednisolone acetate	2.0
Prednisolone	2.8

extreme high polarities (222). When a polar solvent such as propylene glycol is used as sample solvent instead of chloroform, the mobile solvent should be changed to 2% methanol, 35% chloroform, in ethyl acetate, saturated with water.

Betamethasone-17-valerate and hydrocortisone-17-butyrate are generally formulated as an ointment, a cream or a gel. In these dosage forms a large quantity of excipent must be removed before a reliable assay may be undertaken, is found. Typically, an ointment consists of propylene glycol (5%) dispersed in a soft paraffin base; a gel consists of a gelling agent such as carboxypolymethylene (Carbopol) resins or cellulose and Cetomacrogol cream B.P.C. for cream base. A useful preliminary treatment of these systems involves the partitioning of the ointment, cream or gel between two immiscible solvents. The partition coefficient for betamethasone-17-valerate between dimethyl sulphoxide and n-hexane exceeds 1500 (223) suggesting that this solvent system should yield adequate recoveries for assay purposes. Betamethasone-17-valerate and hydrocortisone-17-butyrate are water insoluble and Carbopol and Cetomacrogol cream are aqueous systems. Carbopol gel base showed no interference on the steroid assay (Figure 8d). At least one of the components of Cetomacrogol cream dissolves to some extent in chloroform and this is observed as a peak with a retention time which is too close to betamethasone-21-valerate when analysis is made using the original mobile solvent. This problem can be overcome by modification of the mobile phase to increase the retention time of the steroids. The optimum solvent was found to be 1% methanol, 50% chloroform and 49% ethyl acetate, and saturated with water (Figure 8a,b). The % recovery of steroids from creams, ointments and gels are tabulated in Table 3. Different recovery levels of the synthetic ointment (99.8±1.56%) and Betnovate ointment (97.2±0.52%)

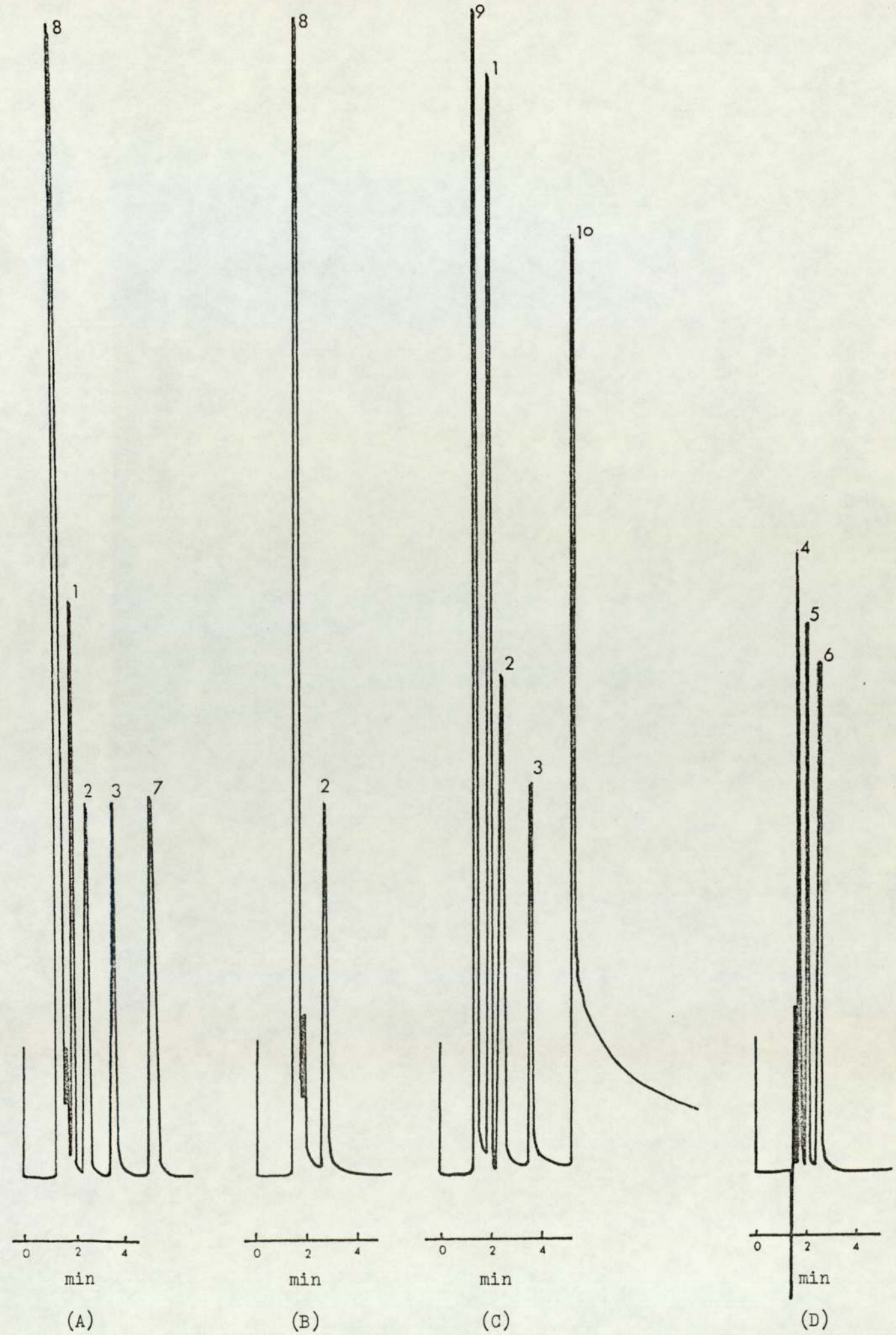

8
1
2
3
7
0
2
4
min
(A)
8
2
0
2
4
min
(B)
9
1
2
3
10
0
2
4
min
(C)
4
5
6
0
2
4
min
(D)

Table 3. Recovery levels of betamethasone esters and hydrocortisone esters from creams, ointments and gels.

Dosage form	Steroids	% Recovery* and standard deviation
Betnovate cream	Betamethasone-17-valerate	99.3 ± 1.1
Synthetic cream	Betamethasone-17-valerate	98.0 ± 1.2
	Betamethasone-21-valerate	99.3 ± 1.9
	Betamethasone	93.9 ± 1.5
Carbopol gel	Hydrocortisone-17-butyrate	99.2 ± 0.5
Synthetic ointment	Betamethasone-17-valerate	99.8 ± 1.6
Betnovate ointment	Betamethasone-17-valerate	97.2 ± 0.5

*mean of six replicates

may reflect the disparate storage conditions for this batch of samples but formulation differences cannot be excluded.

The three steroids, hydrocortisone and its 21-acetate and 17-butyrate, can be monitored by System I, if chloroform is used as sample solvent. However, when IPM is present, System I sometimes gave erratic results. Although quantitative results was obtainable, an alternative system (System II) was developed for subsequent studies. A typical separation is shown in Figure 9. System II is also applicable to other steroids listed in Table 4 which shows their retention times.

This system is a mixture of isopropyl alcohol, ammonia, water and hexane. The amounts of ammonia and water needed are small but essential. By using isopropanol/hexane as eluent, instead of three peaks, only one sharp peak and a small and broad peak were observed (Figure 10a). The peaks of hydrocortisone acetate and hydrocortisone-17-butyrate were not resolved. When 1% of water was added to the eluent, the hydrocortisone peak was much sharper but hydrocortisone acetate and hydrocortisone-17-butyrate were still eluting as a single broad peak (Figure 10b). Substituting 0.3% of water with 0.3% of strong ammonia solution leads to good separation (Figure 10c). Better resolution between hydrocortisone acetate and hydrocortisone-17-butyrate can be obtained by reducing the water concentration to 0.5%. The concentration of water affects the retention time and the peak height of each compound and consequently influences the number of theoretical plates, the capacity ratio (K') and the resolution (Table 5). High concentrations of water have an adverse effect on the resolution of hydrocortisone acetate from hydrocortisone-17-butyrate, as a result of the less marked retention of hydrocortisone-17-butyrate and hydrocortisone and the increased retention time of hydrocortisone

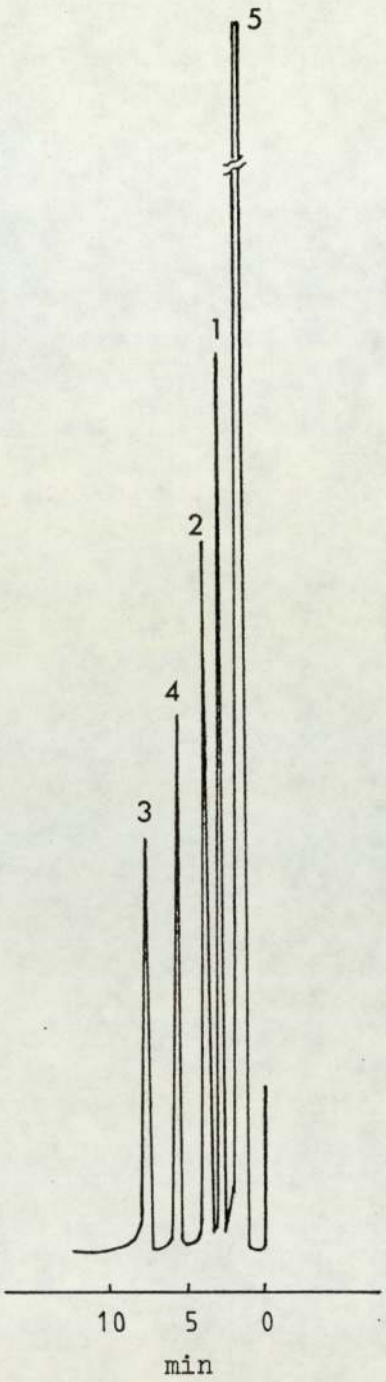


Figure 9. HPLC separation of hydrocortisone acetate, hydrocortisone-17-butyrate and hydrocortisone.

Column: Ultrasphere-Si (5 µm), 15 cm x 4.6 mm i.d.
Mobile phase: isopropanol-hexane-water-ammonia (20 : 79.2 : 0.5 : 0.3)
Flow rate: 1.5 ml/min
Detection wavelength: 250 nm
Sensitivity: 0.04 AUFS

Key: 1: hydrocortisone acetate (0.01 mg/ml)
2: hydrocortisone-17-butyrate (0.01 mg/ml)
3: hydrocortisone (0.01 mg/ml)
4: caffeine (internal standard; 0.025 mg/ml)
5: IPM

Table 4. The retention times of steroids.
Column, Ultrasphere-Si (5 µm), 15 cm x 4.6 mm i.d.; mobile phase, 20% isopropanol, 0.3% ammonia, 0.5% water in hexane.

Compound	Retention times (min)
Hydrocortisone-21-butyrate	1.75
Betamethasone-21-valerate	1.75
Hydrocortisone-21-acetate	2.40
*Phenacetin	2.40
Betamethasone-17-valerate	2.50
Prednisolone acetate	2.50
Cortisone acetate	2.50
Hydrocortisone-17-butyrate	3.30
*Caffeine	4.80
Betamethasone	5.30
Prednisolone	7.00
Hydrocortisone	7.00

*These two compounds are not steroids but were used as internal standards.

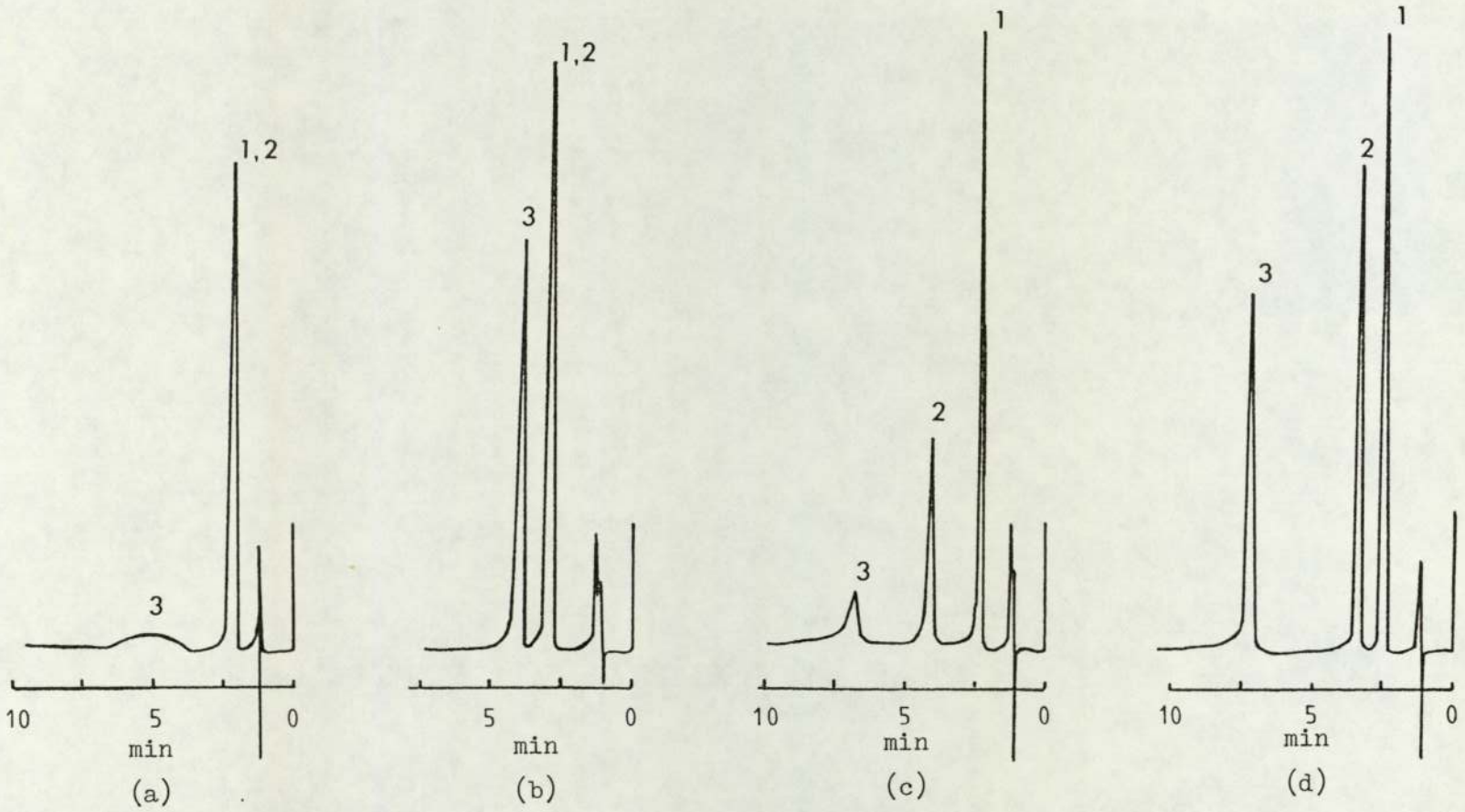


Figure 10. Development of an HPLC separation for steroids.

Column: Ultrasphere-Si (5 µm), 15 cm x 4.6 mm i.d.
Mobile phase composition:
(a) isopropanol - hexane (20 : 80)
(b) isopropanol - hexane - water (20 : 79 : 1)
(c) isopropanol - hexane - ammonia (20 : 79.7 : 0.3)
(d) isopropanol - hexane - ammonia - water (20 : 79.2 : 0.3 : 0.5)
Flow rate: 1.5 ml/min
Key: 1:hydrocortisone acetate 2:hydrocortisone-17-butyrate 3:hydrocortisone

Table 5. Effects of water content on the separation of the steroids on HPLC System II.

% H_2O	Retention Time (min)			K'[a]			N[b]			R_s[c] of HA, H17B
	HA	H17B	H	HA	H17B	H	HA	H17B	H	
0	2.5	4.25	8.0	1.0	2.4	5.0	1600	1156	1600	4.7
0.2	2.5	4.3	8.0	1.0	2.4	6.4	1537	2736	3240	6.3
0.4	2.5	3.6	7.5	1.0	2.0	5.3	1067	2304	2130	3.8
0.5	2.5	3.4	7.25	1.0	1.8	5.1	1024	2055	6960	3.3
0.6	2.5	3.25	7.15	1.2	1.7	5.0	1730	2704	5184	2.6
0.7	2.7	3.1	7.1	1.2	1.5	4.7	1936	2540	5735	1.6
0.8	2.75	3.0	6.75	1.3	1.6	4.6	1936	2033	5107	1.3

a. The capacity ratio $K' = \frac{t_A - t_o}{t_o}$

b. The number of theoretical plates $N = 16 \left(\frac{t_A}{W_A}\right)^2$

c. The resolution $R_s = \frac{2(t_B - t_A)}{W_A + W_B}$

Where t_o = retention time of unretained solute

t_A, t_B = retention times of compound A and B respectively

W_A, W_B = peak widths of compound A and B respectively

HA = hydrocortisone acetate

H-17B = hydrocortisone 17 butyrate

H = hydrocortisone

acetate. Increasing the proportion of isopropanol increases the peak height of each compound but reduces its retention time and capacity ratio (K') (Table 6). When as little as 15% of isopropanol is used, the hydrocortisone acetate and hydrocortisone-17-butyrate peaks are unresolved. The optimal conditions also depend on the sample solvent and sensivity used. The following is a useful guide to the optimal conditions for the separation of hydrocortisone from its 21-acetate and 17-butyrate:-

Sample solvent	Sensitivity	Mobile phase
$CHCl_3$:IPM (1:1)	0.08-0.16 AUFS	0.3% ammonia, 0.5% water, 20% isopropanol in hexane
$CHCl_3$:IPM (1:1)	0.64 AUFS	0.3% ammonia, 0.5% water, 25% isopropanol in hexane
$CHCl_3$ only	0.04-1.28 AUFS	0.3% ammonia, 0.5% water, 30% isopropanol in hexane

Reversed-phase HPLC with n-alkyl bonded (surface reacted) stationary phases has recently been widely used in drug analysis. It is well known that reversed-phase HPLC is an excellent method to separate substances based on size, or alkyl group structure, as a result of hydrophobic interactions (224,225,226), or substances based on polar group differences (227). The composition of the mobile phase play a significant role on retention, hydrophobic selectivity and polar group selectivity. Thus, the control of the mobile phase composition could be a powerful tool in optimizing the separation among related steroids.

The column used in system III and IV is octadecylsilane-bonded silica, 5 μm (Hypersil-ODS, Shandon Southern), which was packed in the laboratory with a Shandon column packing unit. After the column has been used for a certain period of time, decrease in column efficiency

Table 6. Effects of concentration of isopropanol on the separation of the steroids on HPLC System II.

% Isopropanol	Retention Time (min)			K'			N			R_s between HA, H17B
	HA	H17B	H	HA	H17B	H	HA	H17B	H	
18	3.0	3.5	8.1	1.2	1.8	5.6	1344	2177	5378	2.5
20	2.5	3.3	7.3	0.9	1.6	4.8	1820	2304	5329	3.6
25	2.0	2.8	5.2	0.5	1.3	3.2	924	2079	3532	3.5
30	1.7	2.5	4.0	0.4	1.0	2.1	740	1111	2635	2.9
35	1.5	2.2	3.0	0.2	0.8	1.4	711	1239	1600	2.9

K' = the capacity ratio = $\frac{t_A - t_o}{t_o}$

N = the number of theoretical plates = $16 \left(\frac{t_A}{W_A}\right)^2$

R_s = the resolution = $\frac{2(t_B - t_A)}{W_A + W_B}$

where t_o = retention time of unretained solute

t_A, t_B = retention times of compound A and B respectively

W_A, W_B = peak widths of compound A and B respectively

HA = hydrocortisone acetate

H17B = hydrocortisone 17 butyrate

H = hydrocortisone

leading to peak broadening and eventually spliting of peaks is observed. This is probably due to uneven distribution of pressures on the packing material between pumpings. It is nearly always possible to restore the column by removing the damaged top and refilling with the same packing material suspended in propan-2-ol.

The mobile phases used are aqueous-methanol (system III) and aqueous-acetonitrile (system IV). No attempt was made to control the pH of the eluent, since the steroids studied are all non-ionizable. Both systems are almost equally as good for analysing the steroids mentioned in system I and II although sharper peaks are usually obtained by aqueous-acetonitrile system. The only drawback of an aqueous-methanol mobile solvent is its viscosity. High pressures (about 1600 - 2000 psi) which may shorten the column life, are required. When aqueous-acetonitrile is used, the pressure is only about 500 - 700 psi. Applications of these two systems are shown in Figure 11 and Figure 12. They are also useful for separating prednisolone (same retention time as hydrocortisone) admixed with prednisolone acetate or cortisone acetate. The latter two have the same retention times as hydrocortisone acetate. Munson and Wilson (228) proposed that the separations by aqueous-methanol or aqueous-acetonitrile are controlled by different retention mechanisms.
A study of the effect of methanol or acetonitrile concentration on the capacity factor (K') of two non-polar steroids — hydrocortisone cypionate and cortisone cypionate, and three more polar steroids — hydrocortisone, hydrocortisone acetate and methyl-prednisolone acetate, suggested that mixed partition and adsorption mechanisms are responsible for retention of steroids on certain octadecylsilanes when acetonitrile is used in eluent. In methanol-water system, all solvent molecules are available for hydrogen-bonding, thus preventing

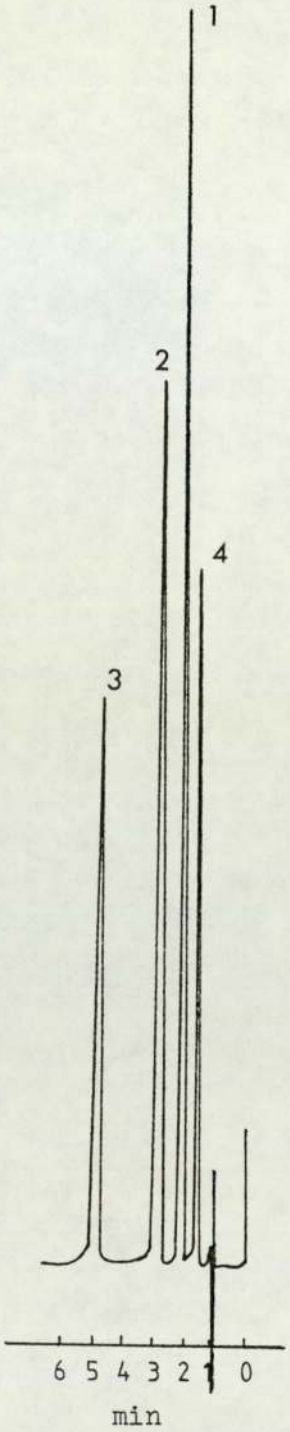


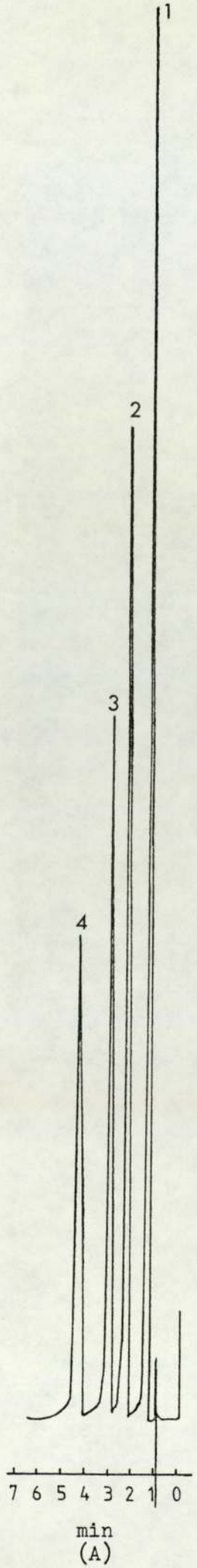
Figure 11. HPLC separation of hydrocortisone (1), hydrocortisone-acetate (2), hydrocortisone-17-butyrate (3), and phenacetin (4) was used as an internal standard.

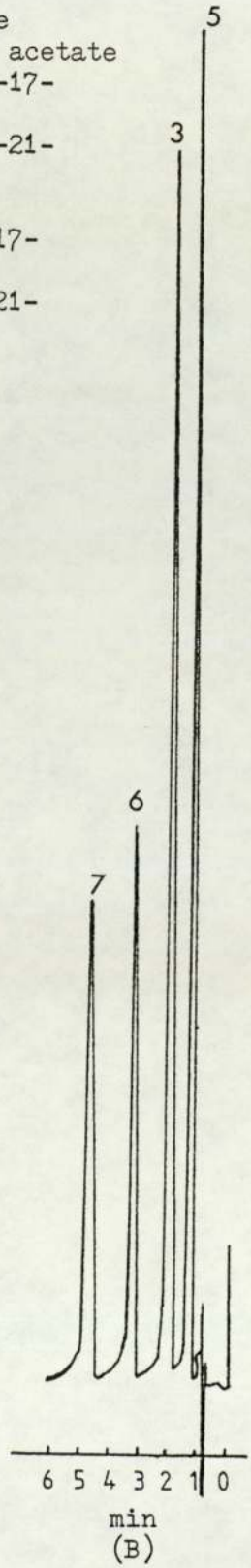
Column: Hypersil-ODS (5 µm), 10 cm x 4.6 mm i.d.
Mobile phase: 66% methanol in water
Flow rate: 1 ml/min

Figure 12. HPLC separation of hydrocortisone and its esters (A); and betamethasone and its esters (B).

Column: Hypersil-ODS (5 µm), 10 cm x 4.6 mm i.
Mobile phase:
(A) 50% acetonitrile in water
(B) 55% acetonitrile in water
Flow rate:
(A) 1 ml/min (B) 1.2 ml/min
Sensitivity: 0.16 AUFS
Steroid concentration: 0.03 mg/ml

Key:
1: hydrocortisone
2: hydrocortisone acetate
3: hydrocortisone-17-butyrate
4: hydrocortisone-21-butyrate
5: betamethasone
6: betamethasone-17-valerate
7: betamethasone-21-valerate





the drug molecules from interacting with the silane hydroxy group. The retention may therefore be entirely governed by partition mechanisms. On the other hand, acetonitrile does not participate in hydrogen-bonding. When the concentration of acetonitrile is high enough, the analyte-silane hydroxy group interaction, which is responsible for adsorption, cannot be overcome by the interaction with mobile solvent.

The injected sample solutions should be preferably prepared with the mobile phase used for the separation, or in a solvent that is weaker than the mobile phase. This is applicable to both normal and reversed-phase HPLC systems. In other words, the sample solvent must be less polar than the mobile phase in normal-phase liquid chromatography, whereas in reversed-phase liquid chromatography, sample solvent must be more polar, since solvent strength increases with solvent polarity in normal-phase and the reverse is true in reversed-phase liquid chromatography (221). If the sample solvent is stronger than the mobile phase, the shape and resolution of the early-eluting bands are usually adversely affected. The reason is that the sample solvent injected mixes with mobile phase and significantly increases its strength over a small region of the column. The 'plug' of enriched mobile phase then carries the early-eluting bands through the column in a stronger solvent, and decreases the average K' values of the sample bands during their elution (221). An example is shown in Figure 13. In Figure 13(a), the sample was prepared in 50% methanol-water which is more polar than the mobile phase (70% methanol-water). The resultant chromatogram is reasonably satisfactory. In Figure 13(b), the sample solvent is pure methanol, which is less polar than the aqueous methanol mobile solvent. Irregular peaks were observed for all three compounds. Since the retention times were

short, all the peaks were affected. A front formation rather than 'tailing' was seen. Such a front formation may also be seen in the separation of ionizable compounds with solvents of inappropriate pH values.

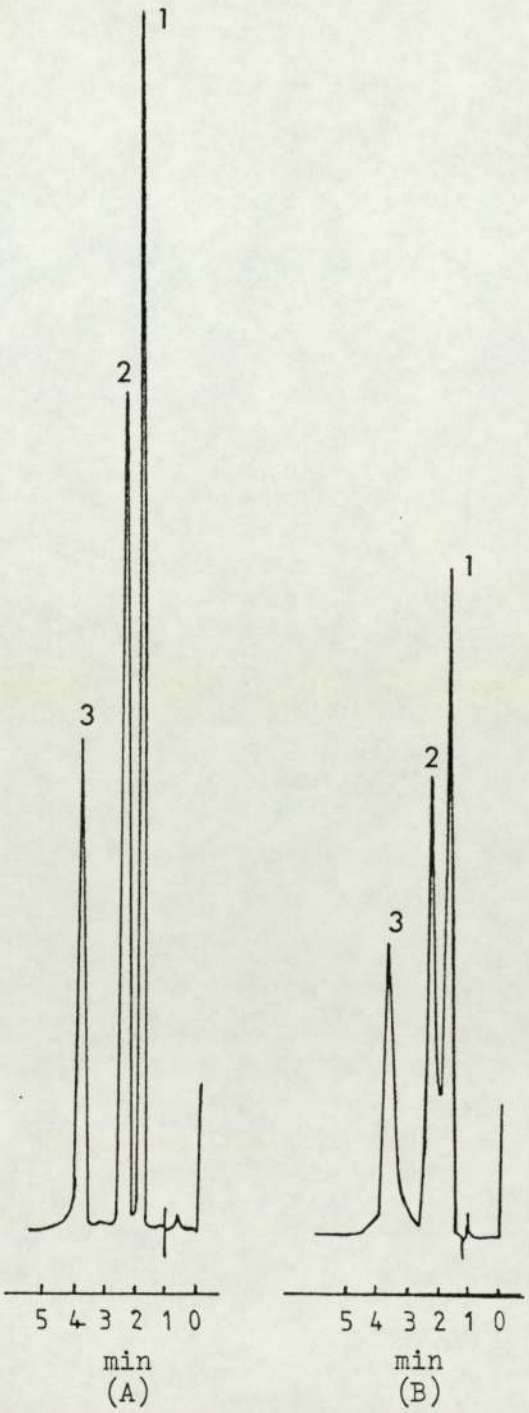


Figure 13. Effect of sample solvents on HPLC separation.

Column: Hypersil-ODS (5 μm), 10 cm x 4.6 mm i.d.

Mobile phase: 70% methanol in water

Flow rate: 1 ml/min

Steroid concentration: 0.1 mg/ml

Sample solvent: (A) 50% methanol in water
(B) 100% methanol

1. Hydrocortisone
2. Hydrocortisone-21-acetate
3. Hydrocortisone-17-butyrate

CHAPTER 3 FORMULATION AND KINETICS OF DECOMPOSITION OF TOPICAL CORTICOSTEROIDS

3.1 INTRODUCTION

The esters of corticosteroids are a widely prescribed group of drugs. Two of the widely used derivatives for topical applications are betamethasone-17-valerate and hydrocortisone-17-butyrate. Steroid-17-esters readily rearrange to the thermodynamically more stable but topically less active (19) 21-esters under non-ideal conditions (216). One possible source of problem is the widespread requests for dilutions of propietary systems (229). It has recently been shown that in some extemporaneously diluted ointments, the half-life of betamethasone-17-valerate may be less than one hour at room temperature (39). Hydrocortisone-17-butyrate also undergoes acyl migration to the 21-esters and hydrolysis to hydrocortisone in alkaline conditions.

Betamethasone-17-valerate and hydrocortisone-17-butyrate are available in various topical formulations and recently, a great deal of interest has been shown in transparent gels because of their cosmetic and patient acceptability. Semi-solid gels are easily applied to the skin and their transparency is appealing. The study was designed to investigate the decomposition kinetics of hydrocortisone-17-butyrate in such gels.

3.2 MATERIALS AND METHODS

3.2.1 Dilution of Betamethasone-17-Valerate Cream

3.2.1.1 Preparation of creams

35 g of betamethasone-17-valerate cream (Betnovate® cream, containing the equivalent of 0.1% w/w betamethasone) and an equal amount of diluent were accurately weighed, and transferred to a glass plate. These were thoroughly mixed with a stainless steel spatula and

stored in a glass jar at 25°C. Diluents used were Cetomacrogol cream (formula A) B.P.C., Aqueous cream B.P. and Boots E45 cream. Each g of the diluted cream contained 0.6 mg of betamethasone-17-valerate.

3.2.1.2 Preparation of test solutions

1 g of cream sample was weighed in stoppered-glass tube and then shaken with 5 ml of 0.5% hydrochloric acid until the cream base was homogeneously dispersed. 10 ml of chloroform was added to extract the steroid. The mixture was kept in an ice-bath or refrigerator overnight. The chloroform phase was filtered through silicone-treated filter paper (Whatman 1SP) and to 5 ml of the chloroform filtrate, was added 1 ml of 0.3 mg/ml caffeine in chloroform as internal standard.

3.2.1.3 Preparation of standard solutions

Standard solutions were treated in the same way as the test solutions. Cetomacrogol cream was used instead of Betnovate cream and diluted with an equal amount of the corresponding diluents. 1 g of the mixture was dispersed in 5 ml of 0.5% HCl, shaken with 10 ml of chloroform containing betamethasone-17-valerate and assayed as described for the test solutions. The concentrations of steroid in the final solutions ranged from 0.01 to 0.06 mg/ml in 0.01 mg/ml steps, and each contained 0.05 mg/ml of caffeine as internal standard. If the standard solutions were prepared in chloroform only, over-estimation of the concentration would be observed. A typical % recovery of a one to one dilution with Cetomacrogol cream is 105.57% on six replicates.

3.2.1.4 HPLC conditions

Column: Spherisorb-Si (5 μm), 25 cm x 4.6 mm i.d.

Mobile phase: 50% chloroform, 2% methanol in ethyl acetate and saturated with water

Flow rate: 1 ml/min

Detection wavelength: 250 nm

Sensitivity: 0.32 AUFS

3.2.1.5 Measurement of pH

Direct measurement was made with a PHM64 Research pH-meter (Radiometer). Indirect measurement was obtained by weighing 2 g of the cream in a beaker to which 30 ml of double distilled water was added. The beaker was heated in a water bath and the molten cream was stirred with a magnetic stirrer for 10 minutes, cooled and the pH of the aqueous phase measured.

3.2.2 Ethanolamine-Catalysed Decomposition of Betamethasone-17-Valerate and Betamethasone in Propylene Glycol

A solution of betamethasone-17-valerate (0.01% w/v) was prepared in propylene glycol-containing ethanolamine (0.128% v/v). The pH value of the solution was found to be about 10.8. Similarly, solutions of betamethasone (0.01% w/v) were prepared in propylene glycol-containing ethanolamine 0.16% (pH 10.82), 0.8% (pH 11.08) and 1.6% (pH 11.4). The solutions were maintained at 60°C and samples were withdrawn at intervals. 20 μl aliquots of these samples were injected directly into the chromatography. Chromatography was performed with system I using 1% methanol, 28% chloroform in ethyl acetate and saturated with water as mobile phase.

3.2.3 Formulation and Stability of Hydrocortisone-17-Butyrate Gel

3.2.3.1 Preparation of gels

(1) Klucel gel

Klucel HF (hydroxypropyl cellulose, Hercules Ltd.) is insoluble in hot water, but soluble in cold water and some organic solvent (either hot or cold). 1.7 g of Klucel was dispersed in 50 g of hot acidified water (0.0001% v/v H_3PO_4 , not exceed 60°C) by magnetic stirrer for about 20 minutes. 100 mg of hydrocortisone-17-butyrate in 48 g of propylene glycol was added and stirred until a gel was formed. The gel was adjusted to 100 g with water. The pH of the gel was found to be 5.45.

(2) Natrosol gel

1 g Natrosol (hydroxyethylcellulose, Hercules Ltd.) was dispersed in half of the final volume of the water with a magnetic stirrer. The remaining volume of water heated to about 90°C, and added to the Natrosal dispersion and stirred until a solution is obtained. 100 mg of hydrocortisone-17-butyrate dissolved in 48 g of propylene glycol was incorporated. The gel was adjusted to 100 g with water.

(3) Sodium carboxymethylcellulose hv (Hercules Ltd.) gel

2 g sodium carboxymethylcellulose hv was added gradually to boiling water and stirred until dissolved and 100 mg of hydrocortisone-17-butyrate dissolved in 48 g of propylene glycol was incorporated into the gel mass. The gel was adjusted to 100 g with water.

(4) Eudispert hv gel

2.5 g Eudispert hv (methacrylate polymers, Röhm Pharma) was dispersed in 50 ml of cold water, then heated to and maintained at 70°C for at least ten minutes. 7.5 ml of 2.5 N NaOH was added to form the

gel mass. 100 mg of hydrocortisone-17-butyrate dissolved in 48 g of propylene glycol was incorporated in the gel mass which was then adjusted to 100 g with water.

(5) Carbopol gel

Different gel formulations of hydrocortisone-17-butyrate were prepared according to the formulae shown in Table 7. The Carbopol 940 (carboxypolymethylene polymers, Goodrich Chem. Co.) was dissolved in most of the propylene glycol-water or propylene glycol-ethanol-water mixture by gentle stirring and then neutralized with sodium hydroxide solution or triethylamine. Disodium edetate was added to the vehicle where indicated. 0.1 g of hydrocortisone-17-butyrate was incorporated into the resultant gel using the remaining 20 g of propylene glycol. The gel was adjusted to 100 g with water to produce a 0.1% w/w hydrocortisone-17-butyrate gel for subsequent studies. Entrapped air was removed by vacuum suction in a desiccator.

3.2.3.2 Stability of hydrocortisone-17-butyrate gel

The hydrocortisone-17-butyrate gel, formula G and H, were stored at 25°C, 37°C and 50°C in water-baths for long-term stability studies. Gels of formula A to F were stored at 60°C in a water-bath. Accurately weighed samples of about 1.2 g were withdrawn at appropriate intervals and 5 ml of 0.5% v/v hydrochloric acid added to quench the reaction. The tubes were shaken until homogeneous and 10 ml of chloroform added. The chloroform extracts were assayed by HPLC as described under the previous section (Section 3.2.2). Six replicates were assayed initially to assess the extent of recovery. All subsequent assays were expressed as a percentage of this concentration.

Table 7. Formulae of hydrocortisone 17 butyrate gels

Formula	pH*	Composition								% residual H-17B after 2 weeks at 60°C
		H-17B (mg)	Carbopol (g)	Propylene glycol (g)	Ethanol (g)	NaOH 2.5 N (ml)	Triethylamine (ml)	EDTA (mg)	Water to (g)	
A	6.78	100	0.8	48	-	2.5	-	-	100	3.4
B	5.07	100	0.8	48	-	0.5	-	-	100	68.0
C	4.42	100	0.8	48	-	0.25	-	-	100	80.45
D	4.75	100	1.3	39	15	-	0.2	10	100	76.3
E	4.57	100	1.3	39	15	-	0.2	50	100	75.81
F	4.40	100	0.8	48	-	0.2	-	10	100	N.D.
G	5.50	100	0.8	97	-	0.2	-	10	100	N.D.

H-17B = hydrocortisone 17 butyrate

N.D. = not determined

* direct measurement

3.2.4 Kinetics of Decomposition of Hydrocortisone-17-Butyrate in Non-Buffered and Buffered Aqueous Propylene Glycol

3.2.4.1 Preparation of aqueous propylene glycol steroid solution

Both non-buffered and buffered aqueous propylene glycol solutions were used in the kinetic experiments. The steroid was firstly dissolved in the propylene glycol and then mixed with the aqueous portion. The final concentration of the steroid is 1 mg/ml in all cases. 0.05% w/v disodium edetate (EDTA) was added where indicated. 50% w/w aqueous propylene glycol containing 0.0005% w/v NaOH (pH 7.89) are referred to as non-buffered solutions although such solutions would be expected to possess some buffer capacity. The buffered aqueous propylene glycol solutions were prepared by mixing equal volumes of propylene glycol and McIlvaine's citrate buffer (230) and adjusted to constant ionic strength as required with potassium chloride (Table 8). These are referred to as single-buffered propylene glycol. As expected addition of propylene glycol to an aqueous buffer produced large changes in its pH. Typically, a buffer with an initial pH of 6.91 was altered to pH 7.60 upon dilution with an equal volume of propylene glycol (Table 8). The pH quoted in the stability studies are the final values in the aqueous propylene glycol systems. Those referred to as double-buffered propylene glycol were prepared by doubling the amount of solutes in the buffer solution such that the solutions possess double buffering but the same pH value, and then mixed with equal volume of propylene glycol.

3.2.4.2 Storage and assay of aqueous propylene glycol steroid solution

The solutions(1 mg/ml) were stored in a water-bath maintained at 60°C and 1 ml aliquots withdrawn at appropriate intervals for assay by reversed-phase HPLC. For hydrocortisone-21-butyrate, a 1 mg/ml

Table 8. Preparation of McIlvaine buffer solution

Buffer solution	Buffering capacity	$Na_2HPO_4 \cdot 12H_2O$ (g/l)	Citric acid (g/l)	KCl (g/l)	Ionic strength (M)		pH (20°C)	
					Buffer solution	Buffered PG*	Buffer solution	Buffered PG*
A	1	58.9 (0.165M)	3.7	5.44	0.5	0.25	6.906	7.598
B	1	58.9	3.7	27.79	0.8	0.4	6.810	7.471
C	1	58.9	3.7	42.70	1.0	0.5	6.772	7.434
D	1	58.9	3.7	57.59	1.2	0.6	6.734	7.437
E	2	117.8 (0.329M)	7.4	10.88	1.0	0.5	6.778	7.396

* Buffered PG consists of 50% of propylene glycol and 50% of buffer solution

product actually produced a suspension system. Homogeneous suspensions can be obtained by well shaking before sampling. The reaction was halted by the addition of 4 ml of, 50% acetonitrile in 0.024 M hydrochloric acid. Such diluted samples (pH 3.76) could be stored at 4°C for at least a week without measurable decomposition. The samples were diluted with a further 5 ml of the acetonitrile in hydrochloric acid mixture but this time also containing 0.16 mg/ml of hydrocortisone acetate as internal standard. Where hydrocortisone was studied on its own, an internal standard concentration of 0.32 mg/ml was used in order to obtain more precise data.

3.2.4.3 HPLC conditions

Column: Hypersil-ODS (5 µm), 10 cm x 4.6 mm i.d.

Mobile phase: Three systems have to be used for samples at different times

(1) 50% acetonitrile in water

(2) 40% acetonitrile in water

(3) 35% acetonitrile in water

Flow rate: 1 ml/min

Detection wavelength: 250 nm

Sensitivity: 0.64 AUFS for hydrocortisone-17-butyrate and 21-butyrate; 1.28 AUFS for hydrocortisone

3.3 RESULTS AND DISCUSSION

3.3.1 Dilution of Betamethasone-17-Valerate Cream

The dilution of commercially available topical steroid preparation is common practice in U.K. (229,231), especially when prescribing for children. Also the mixing of two or more creams are often prescribed in an attempt to simplify treatment. However, potential

problems may arise from such dilutions. Stability is one of the problems of clinical significance. The stability of diluted betamethasone-17-valerate ointment (Betnovate® ointment) has previously been studied (39) and it has been shown that the steroid may be unstable in such systems. For example, in a one to one dilution of Betnovate ointment with Emulsifying ointment B.P., the betamethasone-17-valerate had a half-life of less than one hour. Betamethasone-17-valerate isomerized rapidly to the 21-valerate which is only 1/15 as active as betamethasone-17-valerate (19) in terms of vasoconstrictor activity.

This section describes work done to investigate the stability of betamethasone-17-valerate in Betnovate creams diluted with some commonly used cream bases. Three diluents, Cetomacrogol cream (formula A) B.P.C., Aqueous cream B.P. and Boots E45 cream were studied. When Betnovate cream was diluted with an equal part of any one of these three diluents, the time required for 10% decomposition ($t_{10\%}$) was more than one month. The drug was most stable when diluted with Cetomacrogol cream. Only slight decomposition was observed when stored for 8 months at 25°C (Table 9). Cetomacrogol cream is recommended as a diluent for Betnovate cream by the manufacturers, although they advise against dilution. Cetomacrogol cream is pharmaceutically compatible with Betnovate cream, has an appropriate pH and contains the same preservative (232) as the commercial steroidal cream. Aqueous cream is also suggested as an alternative. Diluted product had a $t_{10\%}$ of 152.4 days and a half-life of 1002.4 days (Table 9). E45 cream contains a different preservative, has an inappropriately high pH and is not recommended as a diluent by the manufacturer. It is interesting to find that the diluted product with E45 cream has an acceptable shelf-life ($t_{10\%}$) which is 45 days

(Table 9), although the diluted product has a relatively high pH of 7.88 (Table 10). The results indicate that the decomposition of betamethasone-17-valerate is first-order reaction and is base-catalysed. Betnovate cream itself is slightly acidic. Cetomacrogol cream has a pH close to it and shows the least decomposition. E45 cream is the most alkaline (Table 10) and shows the fastest decomposition rate. The pH values of the cream (Table 10) were determined by indirect method which is necessary to overcome the unreliability of pH measurements in semi-solid systems.

As far as the stability is concerned, these creams provide acceptable shelf-lives, since the normal recommendation is that diluted products should be discarded after a month. However, apart from the stability problem arising from dilution, other factors should be considered. The bases for the preparation are normally chosen to give optimal release of the medicament. The relationship between dose and response is not necessarily linear and the bioavailability of the drug in the diluted product cannot be predicted. Diluting a preparation 1 to 4, does not always mean that a patient is getting a quarter of the effect. The original preparations are formulated to be resistant to micro-organisms. Dilution of the preparation may inactivate or dilute the preservatives to below their effective concentration and microbial contamination may be introduced at the time of dilution. Therefore, dilution or admixture are not encouraged and should be avoided whenever possible. When a milder product is needed, the less frequent applications or the use of a less active steroid preparation are recommended. An alternative to admixture with another product is to apply the two preparations at different times during the day.

Table 9 The decomposition of betamethasone-17-valerate in various cream systems, at 25°C.

Betnovate cream : Cetomacrogol cream (1:1)		Betnovate cream : Aqueous cream (1 : 1)		Betnovate cream : E45 cream (1 : 1)	
Time (days)	% B-17-val remaining*	Time (days)	% B-17-val remaining*	Time (days)	% B-17-val remaining*
0	$^{\Delta}$99.1 ± 0.97	0	$^{\Delta}$101.1 ± 1.24	0	$^{\Delta}$99.5 ± 0.94
15	99.0 ± 1.1	15	99.2 ± 0.9	10	98.9 ± 0.5
30	101.2 ± 1.04	26	100.2 ± 0.6	37	93.2 ± 1.3
64	97.4 ± 1.8	50	100.5 ± 1.4	45	89.7 ± 0.65
90	99.9 ± 0.4	80	95.8 ± 0.6	70	80.7 ± 1.85
120	100.7 ± 0.8	120	96.0 ± 1.6	90	81.4 ± 0
160	99.6 ± 0.73	145	92.5 ± 0.9	120	75.4 ± 1.2
200	100.5 ± 1.5	180	90.0 ± 0.6	140	70.4 ± 0.6
240	98.3 ± 2.5	220	86.0 ± 1.8	165	67.0 ± 1.33
		240	89.7 ± 1.8	200	62.9 ± 1.1
		270	83.7 ± 1.4	280	52.3 ± 0.94
		300	82.1 ± 0.7		
		350	79.7 ± 1.9		
		$K = 0.000692$ days^{-1}		$K = 0.00234$ days^{-1}	
		$t_{\frac{1}{2}} = 1002.4$ days		$t_{\frac{1}{2}} = 296.2$ days	
		$t_{10\%} = 152.4$ days		$t_{10\%} = 45$ days	

*average of three replicated

$^{\Delta}$average of six replicates

K = first order rate constant

B-17-val = betamethasone-17-valerate

$t_{\frac{1}{2}}$ = half-life = 0.6932/K

$t_{10\%}$ = time required for 10% decomposition = shelf-life = 0.10536/K

Table 10. pH values of betamethasone-17-valerate cream systems

Cream Systems	*pH
Betamethasone-17-valerate cream (Betnovate® cream)	5.44
Cetomacrogol cream (formula A), B.P.C.	6.12
Aqueous cream B.P.	7.39
Boots E45 cream	8.52
Betnovate cream:Cetomacrogol cream B,P.C. (1 : 1)	5.57
Betnovate cream:Aqueous cream B.P. (1 : 1)	6.01
Betnovate cream:E45 cream (1 : 1)	7.88

*the pH values are indirect measurement

3.3.2 Decomposition of Betamethasone-17-Valerate and Betamethasone

Analysis of the steroid by the HPLC system developed and by reference to authentic specimens enabled the construction of the full kinetic decomposition profile for the degradation of betamethasone-17-valerate. The method described also enables the calculation of all rate constants in one kinetic run. This is illustrated by following the ethanolamine-catalysed decomposition of betamethasone-17-valerate in propylene glycol solution. The reaction profile (Figure 14) indicates that betamethasone-17-valerate undergoes isomerisation to its 21-valerate which in turn hydrolyses to betamethasone. Betamethasone is further decomposed to another product. The reaction sequence in this system is recorded in Figure 15. The possible kinetics is an $A \longrightarrow B \longrightarrow C \longrightarrow D$ sequential first order reaction. This is confirmed by the good fit between the experimental and the theoretical data when subjected to non-linear regression analysis (233) using Model I (Table 16). Table 11 shows the kinetic parameters. The relevant expressions for the calculation of the composition of the mixture at time t are shown in Table 17(a). Although the final product is not eluted from the column under the conditions of this analysis, the appearance profile may be calculated from Equation 4 (Table 17a). Subsequent experiments showed that the isomerisation step is not irreversible. The reverse reaction is, however, small enough to be considered irreversible. This is shown by calculating the various rate constants using expressions for Model II as set out in Table 17(b). As can be seen in Table 11, the ratio of the forward to the reverse rate constants was about 31. Qualitatively, the stability of betamethasone-17-valerate in this system parallels that found in ointments (39) and creams (Section 3.3.1), with the most rapid degradation being the isomerisation of the 17- to the 21-ester, followed by a slower

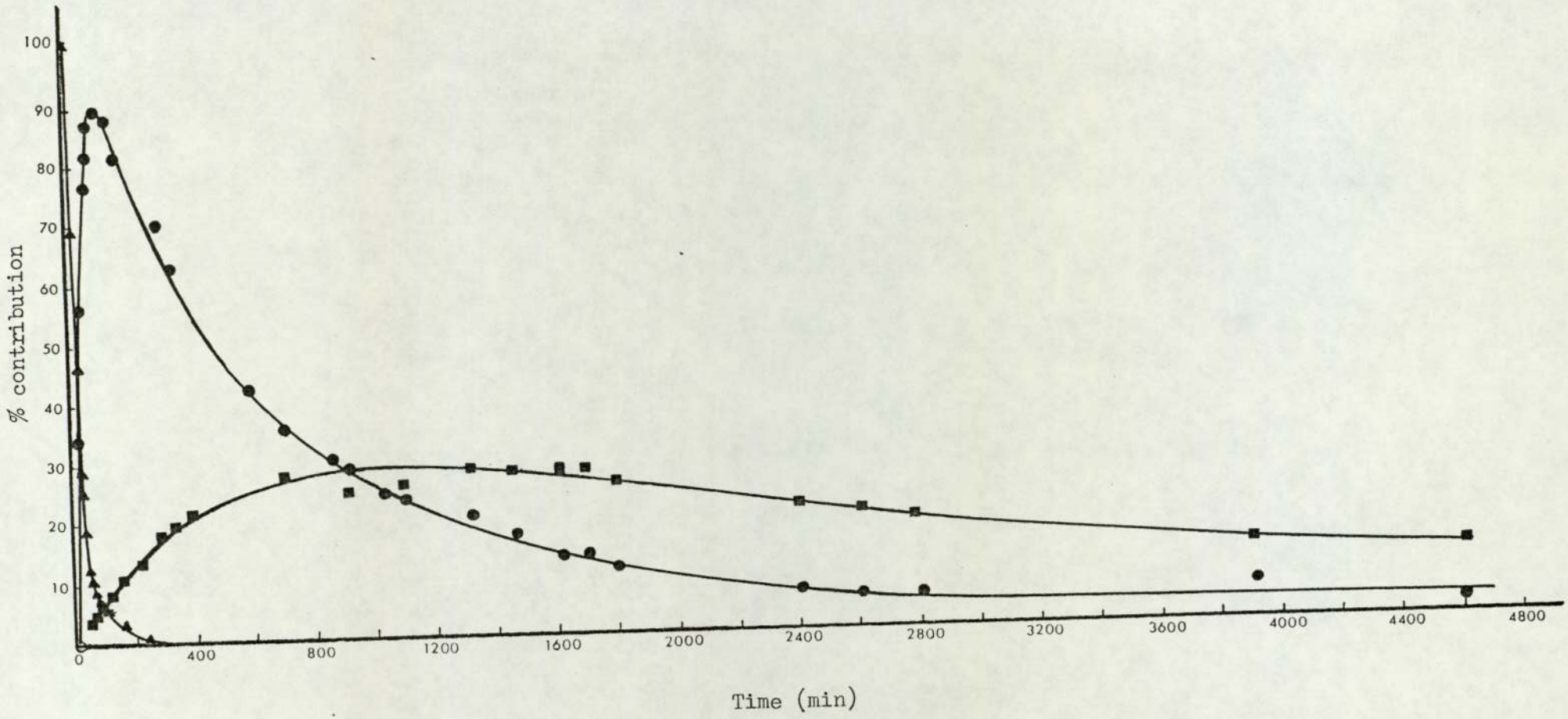


Figure 14. Reaction profile of the ethanolamine-catalysed degradation of betamethasone-17-valerate in propylene glycol, pH 10.8, at 60°C.

▲ Betamethasone-17-valerate
● Betamethasone-21-valerate
■ Betamethasone

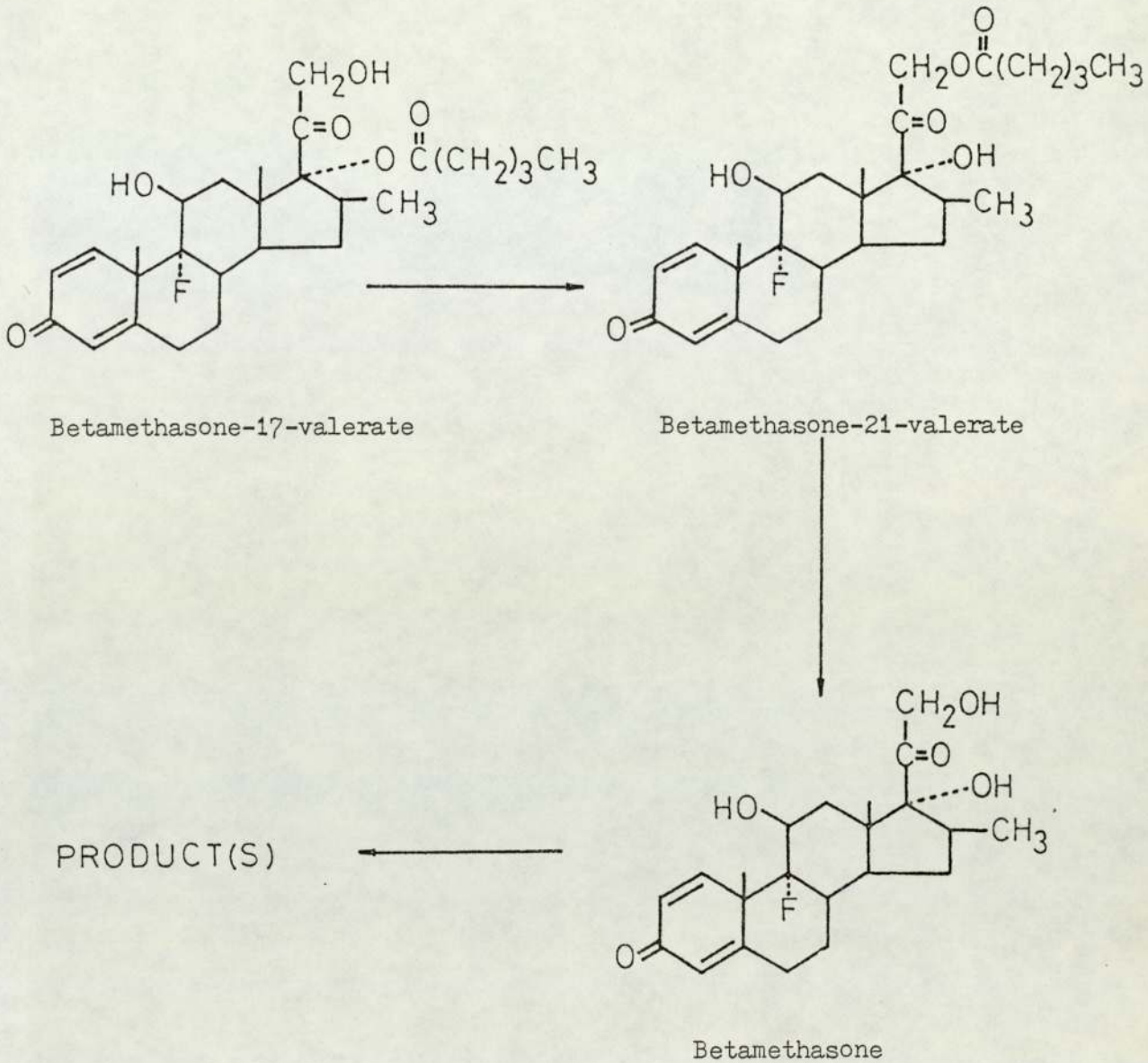


Figure 15. Decomposition of betamethasone-17-valerate with ethanolamine.

Table 11. Kinetic parameters for the ethanolamine-catalysed degradation of betamethasone-17-valerate in propylene glycol, pH 10.8, at 60°C.

Reaction	Rate constant (hr^{-1})	
	Model I	Model II
Rearrangement (I⟶II)	$K_1 = 2.41$	$K_1 = 2.52$
Hydrolysis (II⟶III)	$K_2 = 0.0718$	$K_2 = 0.072$
Decomposition (III⟶IV)	$K_3 = 0.0746$	$K_3 = 0.0756$
Reversed isomerisation (II⟶I)		$K_4 = 0.081$

Model I : $A \xrightarrow{K_1} B \xrightarrow{K_2} C \xrightarrow{K_3} D$

Model II : $A \underset{K_4}{\overset{K_1}{\rightleftharpoons}} B \xrightarrow{K_2} C \xrightarrow{K_3} D$

I = betamethasone-17-valerate
II = betamethasone-21-valerate
III = betamethasone
IV = product(s)

Table 12. The ethanolamine-catalysed degradation of betamethasone in propylene glycol, at 60°C

% ethanolamine	pH	Rate constants (hr^{-1})
0.16	10.82	0.0606
0.8	11.08	0.202
1.6	11.4	0.419

hydrolysis to yield betamethasone.

In order to ensure that the formed betamethasone did undergo decomposition, the investigation of decomposition of betamethasone in propylene glycol with different concentrations of ethanolamine was undertaken. Results showed that the decomposition was a first-order reaction and was dependent on the pH of the media (Table 12).

3.3.3 Formulation and Stability of Hydrocortisone-17-Butyrate Gel

Products commercially available indicate that a 0.1% w/w hydrocortisone-17-butyrate concentration would be a useful starting point when formulating a topical formulation of the steroid. In order to enhance percutaneous absorption of the steroid from the gel system, it was necessary to optimise the thermodynamic activity of the steroid in the formulation. Since a solution system was judged desirable, the vehicle used as the continuous phase should ideally be chosen in such a way that the concentration of steroid present is as close as possible to the saturation concentration. To do this quantitatively, a solubility profile of hydrocortisone-17-butyrate in aqueous propylene glycol, the chosen continuous phase, was constructed. This is shown in Figure 27 (p.152). On the basis of the data obtained, a 48% w/w propylene glycol in water was chosen for formulating the 0.1% w/w hydrocortisone-17-butyrate gel. The gelling agents studied included cellulose, methacrylate polymers, carboxypolymethylene polymers, and a biopolymer — Xanthan Gum. As far as cosmetic acceptability is concerned, only the gel formed by carboxypolymethylene polymers (Carbopol) was satisfactory. The desirable properties of a gel are: ease of spread on the skin, absence of stickiness and absence of film-formation on the skin following application. Hydroxyethylcellulose (Natrosal) does not dissolve in media with low water content, the

formed gel is not viscous enough. Hydroxypropylcellulose (Klucel) and sodium carboxymethylcellulose formed gels with acceptable consistencies. However, gels thickened by these three celluloses formed films upon application. These tended to roll up or to peel off when dried. The methacrylate polymer (Eudispert) is claimed to be suitable for hydrocortisone (83). Eudispert polymer has to be neutralized by alkali to form gels. Small deviations from neutrality produce gels with very loose textures, so that adjusting the pH for optimum stability of hydrocortisone-17-butyrate destroys gel consistency. The product was also of poor clarity and tended to form films upon application. Xanthan Gum is a heteropolysaccharide which is water soluble. It has been used as stabilizer or emulsifying agent in industrial, food and pharmaceutical preparations (234, 235). The texture of the gel formed by Xanthan Gum is very loose and the product is greyish and opaque. Carbopol was therefore chosen as the gelling agent for the subsequent studies.

Carbopol is an acid polymer which readily disperses in water to yield an acid solution of low viscosity and forms a clear, stable gel on neutralization with suitable alkalis. It is not an irritant, is heat-stable, and is not subject to hydrolysis or oxidation under normal use conditions (80,82). Carbopol resins are not attacked by and will not support mould and fungus growth. The presence of propylene glycol provides a specific inhibitory effect against bacteria and most moulds (82, 236). As little as 10% w/w propylene glycol in water was found to inhibit the growth of certain organisms. Apart from that, propylene glycol has been widely used as a humectant and lubricant. The Carbopol bases are stable over a fairly wide pH range. Maximum efficiency is obtained with Carbopol 934 at a pH within the range of 5.5 to 11. With Carbopol 940, the range is somewhat wider. This

means that gel bases with suitable consistency at a pH suitable for hydrocortisone-17-butyrate stability may be easily achieved. The optimal pH range for hydrocortisone-17-butyrate is 3 to 5. Partially neutralized Carbopol can itself act as a buffer. The most suitable pH must be sought by means of comparative stability studies for each gel, since exact measurements of pH in gel systems is difficult. pH also does not appear to be the only factor of importance since dilution of a betamethasone-17-valerate ointment with different ointment bases significantly altered the decomposition rate despite the fact that the product had a pH near that of the stable undiluted product (39). Monitoring the stability of hydrocortisone-17-butyrate in gels with various pH values showed that the disappearance of hydrocortisone-17-butyrate followed first order kinetics. Like betamethasone-17-valerate, it rearranged to the 21-butyrate and further hydrolysed to hydrocortisone. The isomerization was base-catalysed and the pH dependency is shown in Table 13. Kinetic analysis of the data from the gel (Figure 16) showed that the overall decomposition did not follow a sequential first order pattern similar to betamethasone-17-valerate. With the gels studied, the best shelf-life ($t_{10\%}$) observed at room temperature was about one year (Table 14). Gels containing high amounts of propylene glycol (97%) did not significantly influence the stability of hydrocortisone-17-butyrate (Figure 17). Direct measurements of the pH in the two systems gave a value of 5.5 for the system containing 97% propylene glycol while the corresponding value for the 48% propylene glycol system was 4.4. Indirect pH measurements however showed that in both systems the pH was 4.4. This shows the difficulty in comparing results from water to semi-aqueous systems. A plot of the logarithm of the observed rate constant against the reciprocal of the absolute temperature as seen in Figure 18 shows that the

Table 13. Effect of pH on the isomerisation of hydrocortisone-17-butyrate in a gel system, 60°C.

Formula No.	pH	Rate constant (hr^{-1})
A	6.78	0.0412
B	5.07	0.0115
C	4.42	0.00066

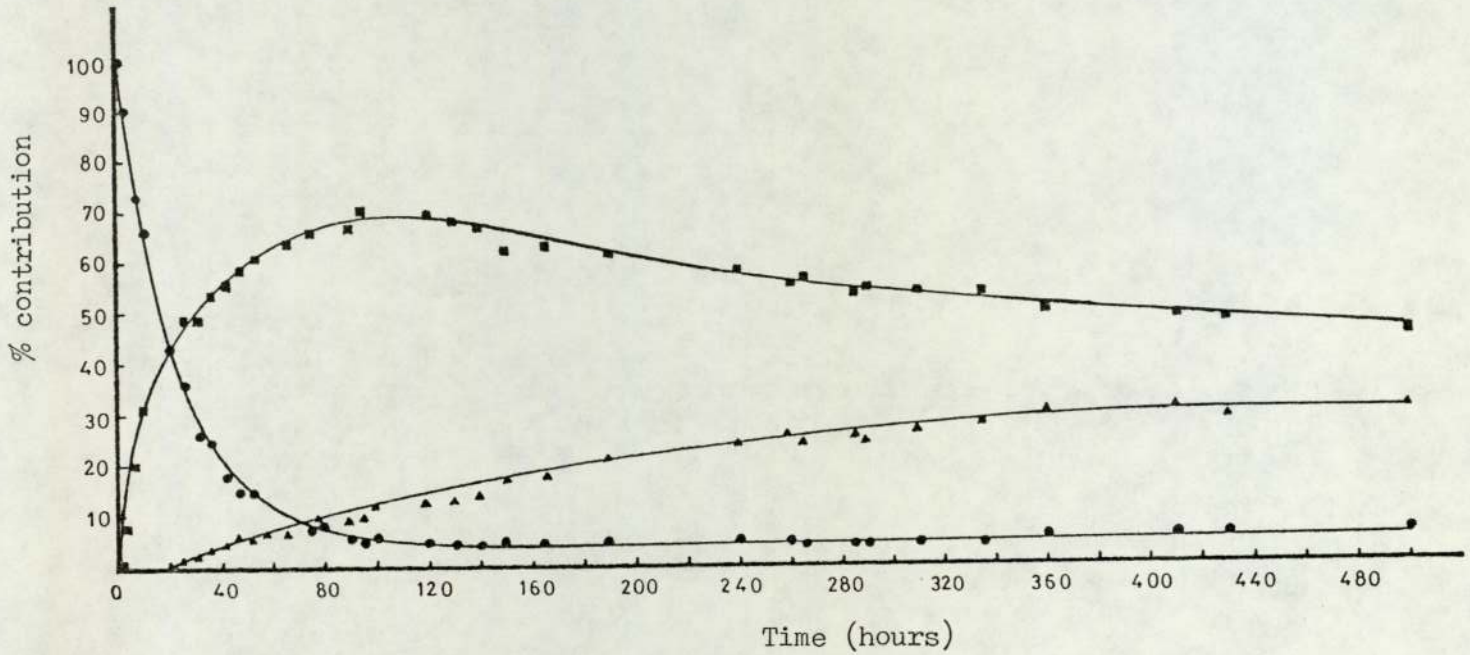


Figure 16. Profile for hydrocortisone-17-butyrate (●), hydrocortisone-21-butyrate (■) and hydrocortisone (▲) during the decomposition of the 17-butyrate in a gel system (pH 6.78 initially; 60°C)

Table 14. The shelf-life of hydrocortisone-17-butyrate in Carbopol gels.

Formula No.	% PG contained in gel base	*pH	Temperature (°C)	K ($month^{-1}$)	$t_{10\%}$ ($month^{-1}$)
F	48	4.4	25	0.00859	12.3
			37	0.0407	2.59
			50	0.174	0.61
G	97	5.5	25	0.0117	9.0
			37	0.0469	2.25
			50	0.184	0.57

*direct measurement

K = rate constant

$t_{10\%}$ = shelf-life (time required for 10% decomposition)

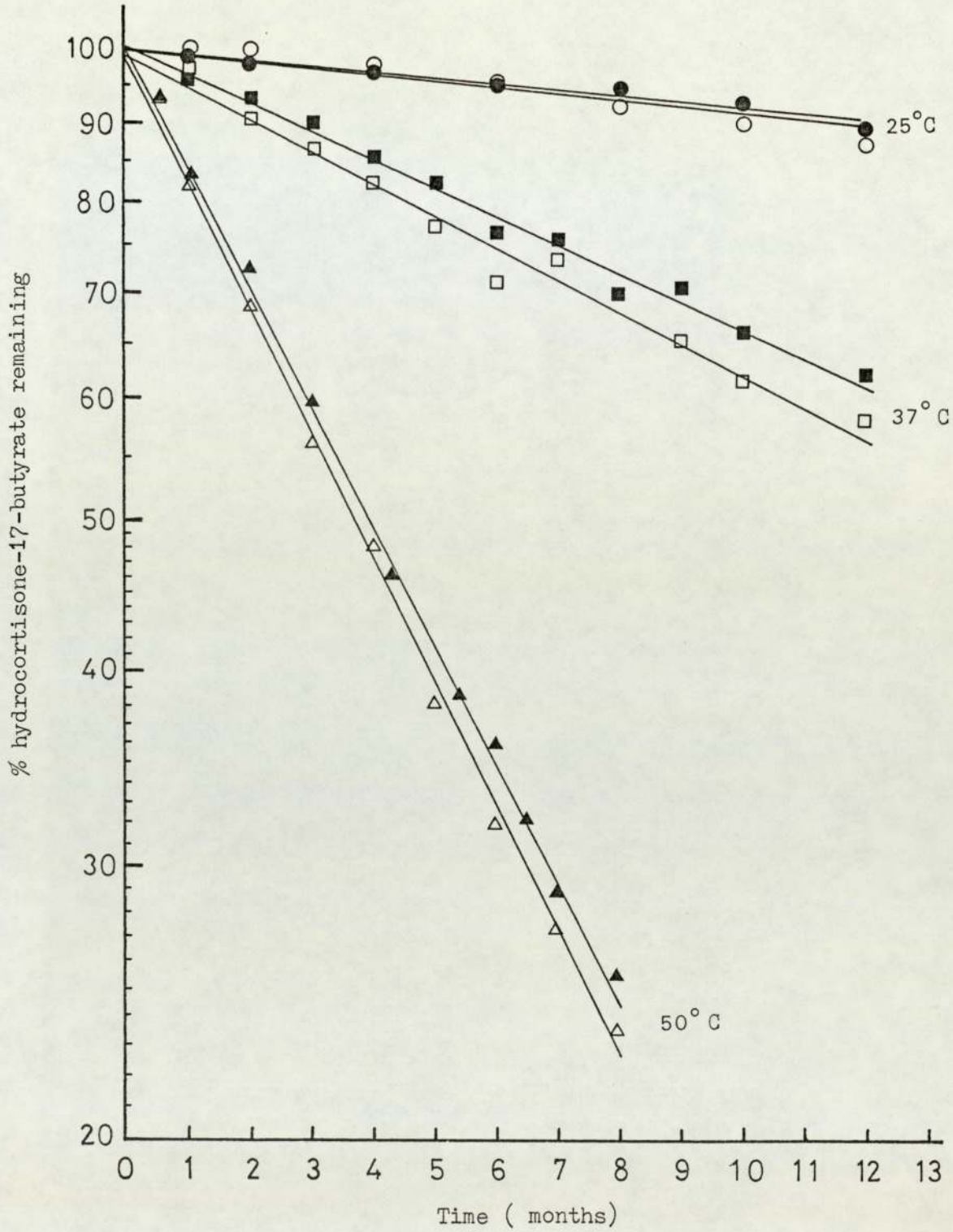


Figure 17. The effect of temperatures on the decomposition of hydrocortisone-17-butyrate in Carbopol gels.

▲ ■ ● gel contained 48% propylene glycol (formula F)

△ □ ○ gel contained 97% propylene glycol (formula G)

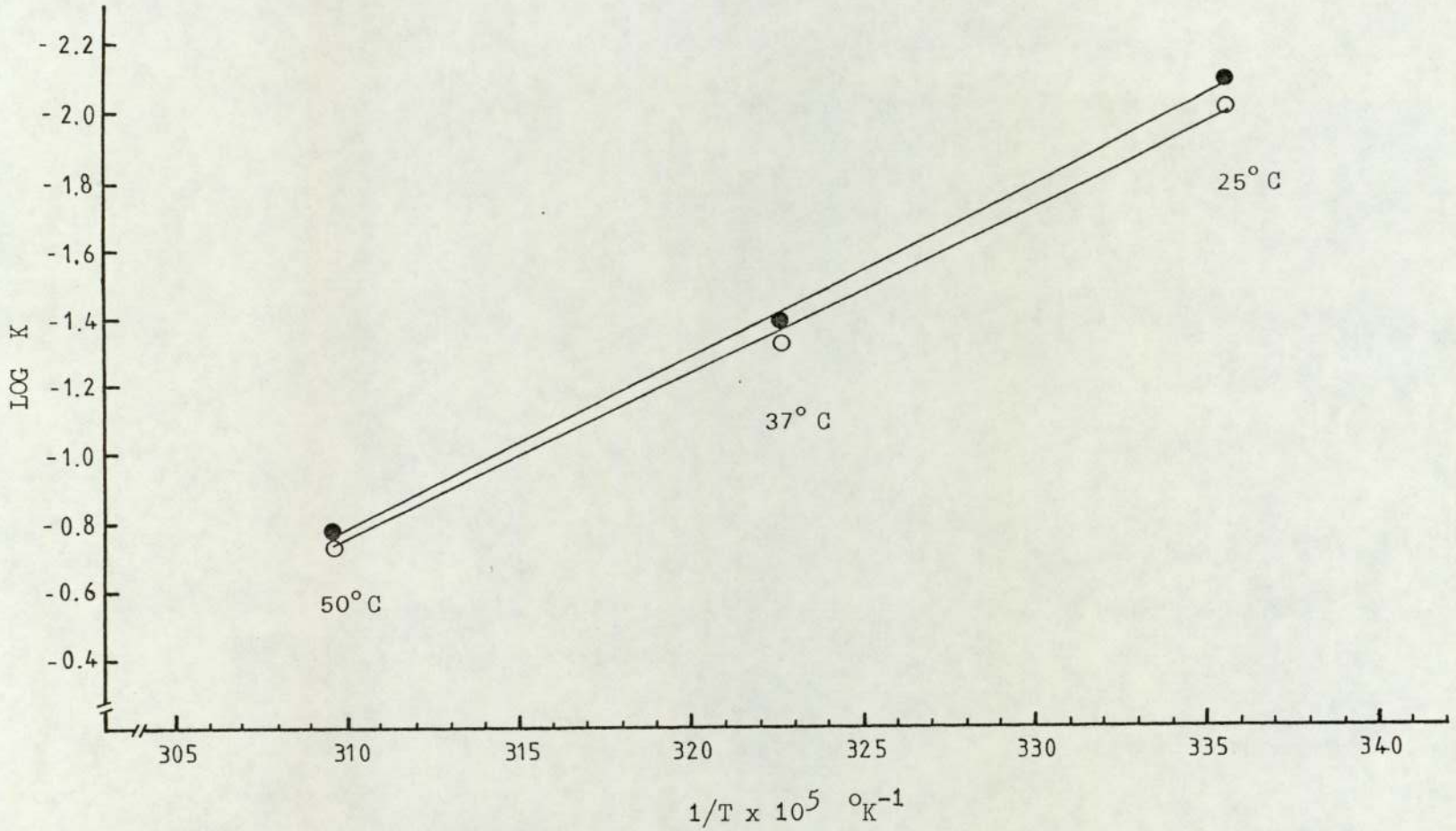


Figure 18. An Arrhenius plot showing the effect of temperatures on the decomposition of hydrocortisone-17-butyrate in Carbopol gels.

● gel contained 48% propylene glycol (formula F)

O gel contained 97% propylene glycol (formula G)

reaction follows the Arrhenius equation. The shelf-life of the steroid in both systems was about one year. To further prolong this value, alternative formulations were investigated. Further reduction in pH of the system was ruled out owing to interference with gel texture. Replacement of part of the propylene glycol with ethanol and substitution of the neutralizing agent by triethylamine showed no improvement (Table 7). The addition of EDTA to the Carbopol gels was then attempted (Formula D and E). EDTA did not appear to have any effect on the decomposition of hydrocortisone-17-butyrate. The steroid in Formula D and E showed similar decomposition rates despite the fact that the amount of EDTA in Formula E was five times that of Formula D (Table 7). EDTA also had no effect on the hydrolysis of the ester but only affected the decomposition of hydrocortisone to other products.

3.3.4 Kinetics of Decomposition of Hydrocortisone-17-Butyrate in Non-Buffered and Buffered Aqueous Propylene Glycol

In order to obtain adequate HPLC resolution of the steroids three aqueous/acetonitrile mixtures were found necessary. Quantitation of hydrocortisone-17-butyrate and 21-butyrate was carried out using a 50% v/v acetonitrile/water mixture. Under these conditions, hydrocortisone showed significant overlap with other minor decomposition products (Figure 19). Decreasing the acetonitrile concentration prolonged the retention times of all the steroids. The quantitative assay of hydrocortisone was possible with a mobile phase consisting of 35% v/v acetonitrile in water (Figure 19b). Although hydrocortisone-17- and 21-butyrates were resolved from the other steroids using this weaker solvent, their quantitation was not satisfactory because of peak broadening. In the initial stages of decomposition, a 40% v/v

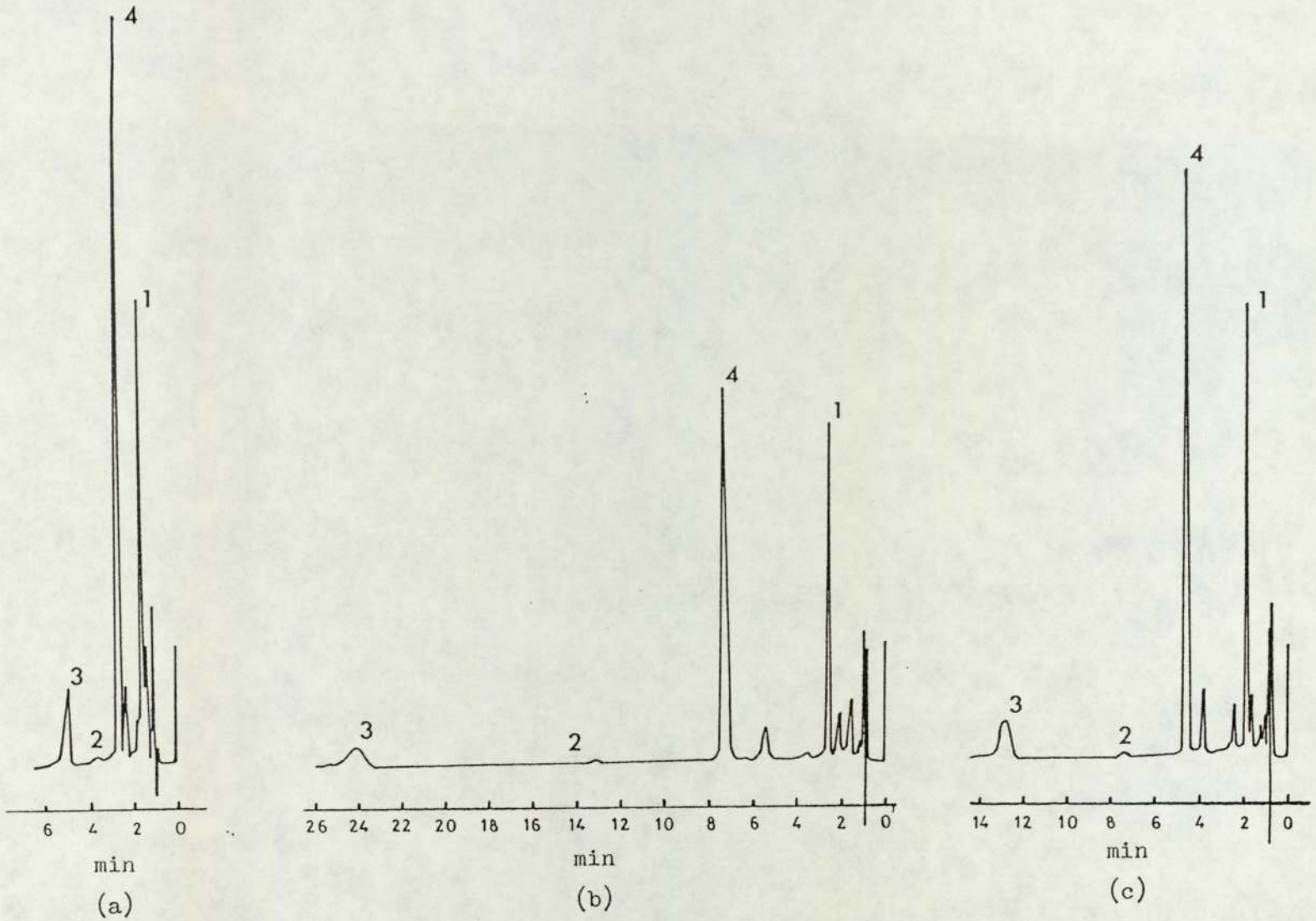


Figure 19. HPLC separation of hydrocortisone-17-butyrate from its decomposition products.
Mobile phase: (a) 50% v/v (b) 35% v/v (c) 40% v/v acetonitrile in water
Key: 1 : hydrocortisone 2 : hydrocortisone-17-butyrate 3 : hydrocortisone-21-butyrate
4 : internal standard (hydrocortisone acetate)

acetonitrile/water mixture was adequate in resolving hydrocortisone for quantitative assay (Figure 19c). Considerable time saving could be achieved using this system when compared with the 35% v/v aqueous acetonitrile mixture. Whenever possible therefore, this mixture was chosen for assaying hydrocortisone in the presence of the other steroids.

To dissociate any effects produced by the gelling agent from other effects the kinetics of decomposition of hydrocortisone-17-butyrate was followed in the absence of the vinyl polymer. The data obtained showed that the decomposition was again highly pH dependent (Table 15) but that unlike the gel system, even the rate of disappearance of the 17-butyrate in this non-buffered system did not follow first order kinetics. The profile for the three steroids in an aqueous-propylene glycol system adjusted to pH 7.9 with sodium hydroxide is illustrated in Figure 20. At this stage it was postulated that base was consumed during at least one of the reactions involved in the overall degradation of the 17-butyrate, since there was a pH change of about 2 units. As shown in Table 15, when higher amounts of sodium hydroxide were used, the decomposition rates of hydrocortisone-17-butyrate were too rapid to be followed, none being detectable, after 30 minutes. The decomposition was followed in buffered aqueous-propylene glycol systems. Under such conditions one would expect linearisation of the data if the reaction sequence was indeed a sequential first order (Model I, Table 16) pathway. The data obtained in fact gave a reasonable fit to the proposed model when subjected to non-linear regression analysis (233) using Equation 1 - 4 (Table 17a). For most stability predictions, such a fit would be adequate and the confidence limits of the data support this (Table 18). For precise modelling however, the computerised plot of the experimental data

against the predicted data using Model I shows that discrepencies are present (Figure 21). Both published data (49) and ours show that other products besides the 21-butyrate and the free alcohol are produced during the decomposition. The HPLC traces (Figure 19) of a decomposed sample clearly demonstrate the complexity of the pathways. Hansen and Bundgaard (49) have reported that at least seven products are formed during the decomposition of hydrocortisone.

If the decomposition of hydrocortisone to other products were all first order parallel processes then the sequential first order model would still hold because K_3 the rate constant for the decomposition would then equal the sum of the individual rate constants.

The possibility that experimental error accounted for the results was discounted by repeating the experiment and similar deviations were noted in both sets of data. There was good agreement between the estimates for the rate constants (Table 18).

Two alternative models to account for the discrepencies are possible. The first is that the isomerisation of hydrocortisone-17-butyrate to the 21-ester is reversible and the reverse rate constant is not negligible (Model II, Table 16). The second alternative is that hydrocortisone-17-butyrate hydrolyses to hydrocortisone directly and not solely via the formation of the 21-butyrate (Model III Table 16). To test these possibilities, the hydrolysis of hydrocortisone-21-butyrate was followed in samples initially free from the 17-butyrate. Any reversibility would be shown by the formation of the 17-ester. This was in fact the case and the kinetic parameters for this system under identical conditions are shown in Table 19. The improve fit to the model can be seen in Figure 22. Since K_1 is about fourteen times greater than K_4, imprecise data for these rate constants are to be expected when derived from experiments with the initial concentra-

tion of the 17-butyrate is zero. This is reflected in the wide confidence limits (Table 20). The experiment does not exclude reversibility in the hydrolysis of hydrocortisone-21-butyrate. Therefore, the decomposition of hydrocortisone was studied in the presence of an equimolar amount of sodium butyrate and in the absence of the esters. A control was carried out without the sodium butyrate. No hydrocortisone ester was detected throughout the run and the rate constants in the control and test systems (0.0096 and 0.0102 hr^{-1} respectively) were identical within experimental error. Having confirmed that the isomerisation of hydrocortisone-17-butyrate was reversible but the hydrolysis was not, the goodness of the fit to models II and III were compared, to exclude the possibility that hydrocortisone-17-butyrate is directly converted to hydrocortisone. The statistical data and the plots (Figure 22 and 23) show that this was likely although because of the small magnitude of K_5, the rate constant for the direct conversion of the 17-butyrate to hydrocortisone, relative to the other routes, the estimate for its value is poor as is evident in the wide span for the 95% confidence limits (Table 21). The correlation coefficients however showed that Model III probably describes the kinetics of decomposition of hydrocortisone in our system, better than the other alternative, although the improvement is only marginal and it can be argued that both models are equally acceptable.

Preliminary work on the effect of buffers on the decomposition indicated that by doubling the buffer concentration, the rates of decomposition of hydrocortisone-17-butyrate to the 21-ester and of the latter to hydrocortisone were not significantly affected. However, the rate of disappearance of hydrocortisone was increased. The first order rate constants in the single and double (bracketed terms) buffer

systems were 0.308 hr^{-1} (0.289 hr^{-1}) for the isomerisation, 0.016 hr^{-1} (0.016 hr^{-1}) for the hydrolysis and 0.017 hr^{-1} (0.032 hr^{-1}) for the degradation of hydrocortisone when subjected to non-linear regression analysis according to Model II (Table 22). Significant pH changes were not found at the end of the follow-up periods in both single-buffered and double-buffered solutions. It is possible that the buffer concentration and/or the ionic strength of the media may be responsible for the difference in K_3 , since the ionic strength of the single-buffered and double-buffered solutions were 0.25 M and 0.5 M respectively. The effect of ionic strength and buffer concentration on the degradation of hydrocortisone were therefore investigated. The decomposition of hydrocortisone in such systems were also found to be first order reactions. It seems possible that the decomposition of hydrocortisone are affected both by ionic strength and buffer concentration (Table 23). However, the differences were not statistically significant and were too small to account for the differences in K_3 observed for the decomposition of hydrocortisone-17-butyrate in the buffered-propylene glycol systems (Table 19 and 22).

Recent work has shown that the decomposition of hydrocortisone was metal catalysed and that sodium edetate significantly decreased the rate of decomposition (49). Indeed, buffer effects were rationalised on the basis of trace metal contaminants both for hydrocortisone (49) and for prednisolone (237). In our system the isomerisation and hydrolysis steps were unaffected and these are steps which would not be expected to be metal catalysed. Using different batches of the same grade of buffer salts, identical rate constants for the decomposition of hydrocortisone were obtained (Table 24) but this does not exclude metal catalysis. The effect of sodium edetate on the decomposition, was therefore investigated. In addition to helping in

explaining the mechanism of decomposition, the data obtained would also be expected to be useful in unveiling methods which would be suitable for stabilising the formulations used. Additionally, it has been suggested that some of the decomposition products were potentially immunogenic (238). When compared to control systems free from the chelating agent, a clear stabilising effect was observed when sodium edetate was added. As expected, the isomerisation and hydrolysis rate constants were not significantly altered (Table 25). Comparison of the chromatograms (Figure 24) for the decomposed solutions shows that the profile for hydrocortisone and the minor decomposition products (A - F) were markedly different when the system to which sodium edetate was added (Figure 24a) was compared with a control (Figure 24b). At 105 hours, the percentage residual concentrations of the steroids in the control system and the system to which EDTA was added (bracketed terms) were 1.2% (1.8%) for the 17-butyrate, 21% (24%) for the 21-butyrate and 32.6% (52%) for the free alcohol. No attempt was made to identify the minor decomposition products but published work suggests that oxidation of the side chain of hydrocortisone leading to the formation of steroid glyoxals, 17-oxo-steroid and glycolic acids were likely (49,90). However, doubling the buffer concentration did not significantly affect the rate of decomposition of hydrocortisone, with or without EDTA (Table 24). The rate constant for the disappearance of hydrocortisone (K_3) in systems initially composed of hydrocortisone-17-butyrate only were higher than those found in systems starting off with hydrocortisone itself (Table 25). The likely explanation is however that the computer generated K_3 values for systems consisting of hydrocortisone-17-butyrate only, initially, were obtained with less precise data and would hence lead to less accurate values.

Table 15. Effect of pH on the decomposition of hydrocortisone-17-butyrate in an aqueous-propylene glycol (50% v/v) mixture, at 60°C.

NaOH (% w/v)	pH of system	Steroid composition at time 30 minutes		
		% hydrocortisone -17-butyrate	% hydrocortisone -21-butyrate	% hydrocortisone
0.0005	7.89	87	8	0
0.005	9.95	4	57	37
0.02	11.17	0	0	77

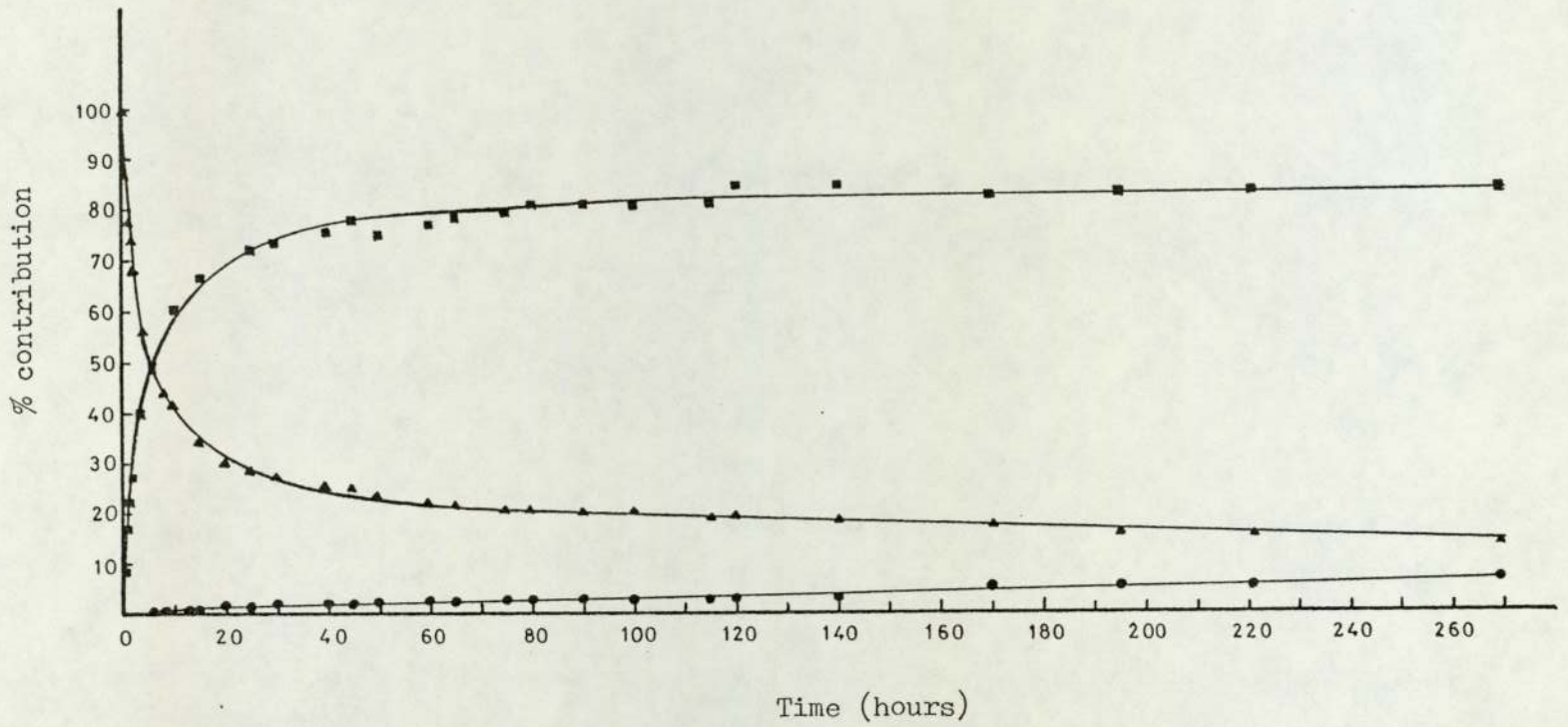
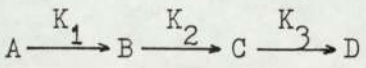
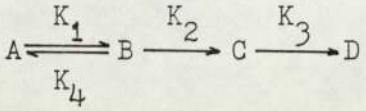
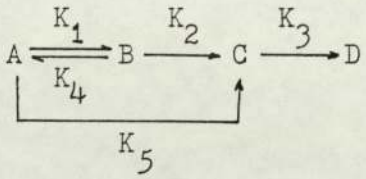


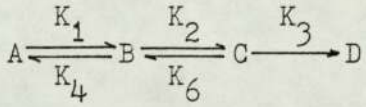
Figure 20. Profile of hydrocortisone-17-butyrate (▲), hydrocortisone-21-butyrate (■) and hydrocortisone (●) during the decomposition of the 17-ester in an aqueous propylene glycol solution (50% v/v, pH 7.9 initially; 60°C)

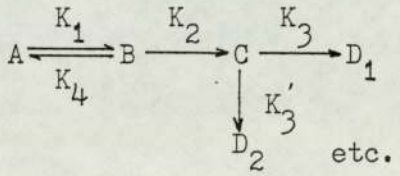
Table 16. Kinetic models for the decomposition of hydrocortisone-17-butyrate

I 

II 

III 

IV 

V 

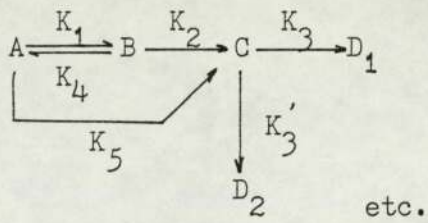
VI 

Table 17. Integrated rate equations for the different kinetic models and conditions used.

(a) Model I $A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$ sequential first order

Initial conditions: $[A]_o = 100\%$ $[B]_o = [C]_o = 0$

Integrated equations:

(1) $$[A]_t = [A]_o \, e^{-k_1 t}$$

(2) $$[B]_t = \frac{[A]_o \, k_1}{(k_2 - k_1)} [\, e^{-k_1 t} - e^{-k_2 t} \,]$$

(3) $$[C]_t = [A]_o [\frac{k_1 \, k_2}{(k_2 - k_1)(k_3 - k_1)} \, e^{-k_1 t} +$$

$$\frac{k_1 \, k_2}{(k_1 - k_2)(k_3 - k_2)} \, e^{-k_2 t} + \frac{k_1 \, k_2}{(k_1 - k_3)(k_2 - k_3)} \, e^{-k_3 t} \,].$$

(4) $$[D]_t = [A]_o \, [\, 1 - \frac{k_2 \, k_3}{(k_2 - k_1)(k_3 - k_1)} \, e^{-k_1 t}$$

$$- \frac{k_1 \, k_3}{(k_1 - k_2)(k_3 - k_2)} \, e^{-k_2 t}$$

$$- \frac{k_1 \, k_2}{(k_1 - k_3)(k_2 - k_3)} \, e^{-k_3 t} \,].$$

Table 17(b)

Model II $A \underset{k_4}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C \xrightarrow{k_3} D$ sequential first order

Initial conditions: $[A]_o = 100\%$ $[B]_o = [C]_o = 0$

$$[A]_t = [A]_o \left[\frac{(k_2 + k_4) - r_1}{(r_2 - r_1)} e^{-r_2 t} + \frac{(k_4 + k_2) - r_2}{(r_1 - r_2)} e^{-r_2 t} \right]$$

$$[B]_t = [A]_o \, k_1 \left[\frac{1}{(r_2 - r_1)} e^{-r_1 t} + \frac{1}{(r_1 - r_2)} e^{-r_2 t} \right]$$

$$[C]_t = [A]_o \, k_1 \, k_2 \left[\frac{1}{(r_2 - r_1)(k_3 - r_1)} e^{-r_1 t} \right.$$

$$+ \frac{1}{(r_1 - r_2)(k_3 - r_2)} e^{-r_2 t}$$

$$\left. + \frac{1}{(r_1 - k_3)(r_2 - k_3)} e^{-k_3 t} \right]$$

where $r_1 = \frac{1}{2} \left[(k_1 + k_2 + k_4) - \sqrt{(k_1+k_2+k_4)^2 - 4 k_1 k_2} \right]$

$r_2 = \frac{1}{2} \left[(k_1 + k_2 + k_4) + \sqrt{(k_1+k_2+k_4)^2 - 4 k_1 k_2} \right]$

Table 17(c)

Model II $A \underset{k_4}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C \xrightarrow{k_3} D$ sequential first order

Initial conditions: $[A]_o = 0 \quad [B]_o = 100\% \quad [C]_o = 0$

$$[A]_t = [B]_o\, k_4 \left[\frac{1}{(r_2 - r_1)} e^{-r_1 t} + \frac{1}{(r_1 - r_2)} e^{-r_2 t} \right]$$

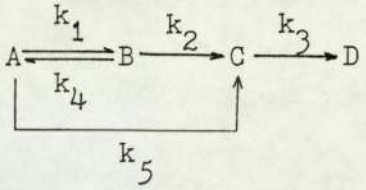
$$[B]_t = [B]_o \left[\frac{k_1 - r_1}{(r_2 - r_1)} e^{-r_1 t} + \frac{k_1 - r_2}{(r_1 - r_2)} e^{-r_2 t} \right]$$

$$[C]_t = [B]_o\, k_2 \left[\frac{k_1 - r_1}{(r_2 - r_1)(k_3 - r_1)} e^{-r_1 t} + \frac{k_1 - r_2}{(r_1 - r_2)(k_3 - r_2)} e^{-r_2 t} + \frac{k_1 - k_3}{(r_1 - k_3)(r_2 - k_3)} e^{-k_3 t} \right]$$

where $r_1 = \frac{1}{2}\left[(k_1+k_2+k_4) - \sqrt{(k_1+k_2+k_4)^2 - 4\, k_1\, k_2} \right]$

$r_2 = \frac{1}{2}\left[(k_1+k_2+k_4) + \sqrt{(k_1+k_2+k_4)^2 - 4\, k_1\, k_2} \right]$

Table 17(d)

Model III  sequential first order

Initial conditions: $[A]_o = 100\%$ $[B]_o = 0$ $[C]_o = 0$

$$[A]_t = [A]_o \left[\frac{k_4 + k_2 - r_1}{r_2 - r_1} e^{-r_1 t} + \frac{k_4 + k_2 - r_2}{r_1 - r_2} e^{-r_2 t} \right]$$

$$[B]_t = [A]_o k_1 \left[\frac{1}{r_2 - r_1} e^{-r_1 t} + \frac{1}{r_1 - r_2} e^{-r_2 t} \right]$$

$$[C]_t = [A]_o \left[\frac{k_1 k_2 + k_4 k_5 + k_2 k_5 - k_5 r_1}{(r_2 - r_1)(k_3 - r_1)} e^{-r_1 t} \right.$$

$$+ \frac{k_1 k_2 + k_4 k_5 + k_2 k_5 - k_5 r_2}{(r_1 - r_2)(k_3 - r_2)} e^{-r_2 t}$$

$$\left. + \frac{k_1 k_2 + k_4 k_5 + k_2 k_5 - k_3 k_5}{(r_1 - k_3)(r_2 - k_3)} e^{-k_3 t} \right]$$

where $r_1 = \frac{1}{2} \left[(k_1+k_2+k_4+k_5) - \sqrt{(k_1+k_2+k_4+k_5)^2 - 4(k_4k_5+k_1k_2+k_2k_5)} \right]$

$r_2 = \frac{1}{2} \left[(k_1+k_2+k_4+k_5) + \sqrt{(k_1+k_2+k_4+k_5)^2 - 4(k_4k_5+k_1k_2+k_2k_5)} \right]$

Table 18. Rate constants for the decomposition of hydrocortisone-17-butyrate to hydrocortisone-21-butyrate (K_1), the hydrolysis of the 21-butyrate to hydrocortisone (K_2) and of the latter to other products (K_3)[a,b]

Data set	Calculated rate constants (hr^{-1})	95% confidence limits	Correlation coefficient for function in brackets
1	$K_1 = 0.290 \pm 0.005$	0.281 - 0.300	0.999 (A)
	$K_2 = 0.016 \pm 0.0003$	0.016 - 0.017	0.995 (B)
	$K_3 = 0.0173 \pm 0.001$	0.016 - 0.019	0.979 (C)
2	$K_1 = 0.288 \pm 0.004$	0.260 - 0.277	0.998 (A)
	$K_2 = 0.016 \pm 0.0003$	0.015 - 0.016	0.995 (B)
	$K_3 = 0.015 \pm 0.0004$	0.014 - 0.016	0.987 (C)

[a]Model I of Table 16 with initial conditions Ao = 100% Bo = Co = 0

[b]Reactions in buffered aqueous propylene glycol (pH 7.6; 50% v/v) at 60°C

Table 19. The forward (K_1) and the reverse (K_4) rate constants for the isomerisation of hydrocortisone-17-butyrate to hydrocortisone-21-butyrate and the rate constants for the decomposition of the 21-butyrate to hydrocortisone (K_2) and of the latter to other products (K_3)[a,b]

Data set	Calculated rate constants (hr^{-1})	95% confidence limits	Correlation coefficient for function in brackets
1	$K_1 = 0.308 \pm 0.00485$	0.299 - 0.318	1.000 (A)
	$K_2 = 0.016 \pm 0.00026$	0.016 - 0.017	0.997 (B)
	$K_3 = 0.017 \pm 0.00057$	0.016 - 0.018	0.983 (C)
	$K_4 = 0.020 \pm 0.00253$	0.015 - 0.025	
2	$K_1 = 0.292 \pm 0.00321$	0.285 - 0.298	0.999 (A)
	$K_2 = 0.016 \pm 0.00015$	0.0157- 0.0164	0.999 (B)
	$K_3 = 0.015 \pm 0.00024$	0.015 - 0.016	0.989 (C)
	$K_4 = 0.025 \pm 0.00163$	0.021 - 0.028	

[a]Model II of Table 16 with initial conditions Ao = 100% Bo = Co = 0

[b]Reactions in buffered aqueous propylene glycol (pH 7.6; 50% v/v) at 60°C

Table 20. The forward (K_4) and the reverse (K_1) rate constants for the isomerisation of hydrocortisone-21-butyrate to hydrocortisone-17-butyrate and the rate constants for the decomposition of the 21-butyrate to hydrocortisone (K_2) and of the latter to other products(K_3)[a,b]

Calculated rate constants (hr^{-1})	95% confidence limits	Correlation coefficients for function in brackets
$K_1 = 0.348 \pm 0.195$	(-) 0.041 - 0.737	0.983 (A)
$K_2 = 0.015 \pm 0.00019$	(-) 0.258 - 0.051	0.999 (B)
$K_3 = 0.019 \pm 0.00038$	0.150 - 0.016	0.983 (C)
$K_4 = 0.024 \pm 0.0135$	0.018 - 0.020	

[a]Model II of Table 16 but initial conditions Ao = 0 Bo = 100% Co = 0

[b]Reactions in buffered aqueous propylene glycol (pH 7.6; 50% v/v) at 60°C

Table 21. The forward (K_1) and the reverse (K_4) rate constants for the isomerisation of hydrocortisone-17-butyrate to the 21-butyrate and the rate constants for the decomposition of the 17-butyrate (K_5) and the 21-butyrate (K_2) to hydrocortisone and of the latter to other products (K_3)[a,b]

Data set	Calculated rate constant (hr^{-1})	95% confidence limits	Correlation coefficient for function in brackets
1	$K_1 = 0.310 \pm 0.0077$	0.295 - 0.325	1.000 (A)
	$K_2 = 0.018 \pm 0.00079$	0.0163 - 0.019	0.998 (B)
	$K_3 = 0.015 \pm 0.00076$	0.0138 - 0.017	0.975 (C)
	$K_4 = 0.022 \pm 0.0042$	0.0134 - 0.030	
	$K_5 = 0.0001 \pm 0.0045$	(-) 0.009 - 0.009	
2	$K_1 = 0.292 \pm 0.0033$	0.286 - 0.299	0.999 (A)
	$K_2 = 0.016 \pm 0.00031$	0.215 - 0.280	0.999 (B)
	$K_3 = 0.015 \pm 0.00024$	0.0155 - 0.017	0.989 (C)
	$K_4 = 0.025 \pm 0.0017$	0.0146 - 0.0155	
	$K_5 = 0.0001 \pm 0.0018$	(-) 0.003 - 0.004	

[a] Model III of Table 16 with initial conditions Ao = 100% Bo = Co = 0

[b] Reactions in buffered aqueous propylene glycol (pH 7.6; 50% v/v) at 60°C

Table 22. The forward (K_1) and the reverse (K_4) rate constants for the isomerisation of hydrocortisone-17-butyrate to hydrocortisone-21-butyrate and the rate constants for the decomposition of the 21-butyrate to hydrocortisone (K_2) and of the latter to other products (K_3)[a]

Calculated rate constant (hr^{-1})	95% confidence limits	Correlation coefficient of function in brackets
$K_1 = 0.289 \pm 0.0052$	0.278 - 0.299	0.999 (A)
$K_2 = 0.016 \pm 0.00034$	0.016 - 0.017	0.998 (B)
$K_3 = 0.032 \pm 0.0014$	0.029 - 0.035	0.947 (C)
$K_4 = 0.025 \pm 0.0027$	0.020 - 0.031	

[a]Same model and conditions as set in Table 19 except that buffer concentration is doubled as described in the experimental section.

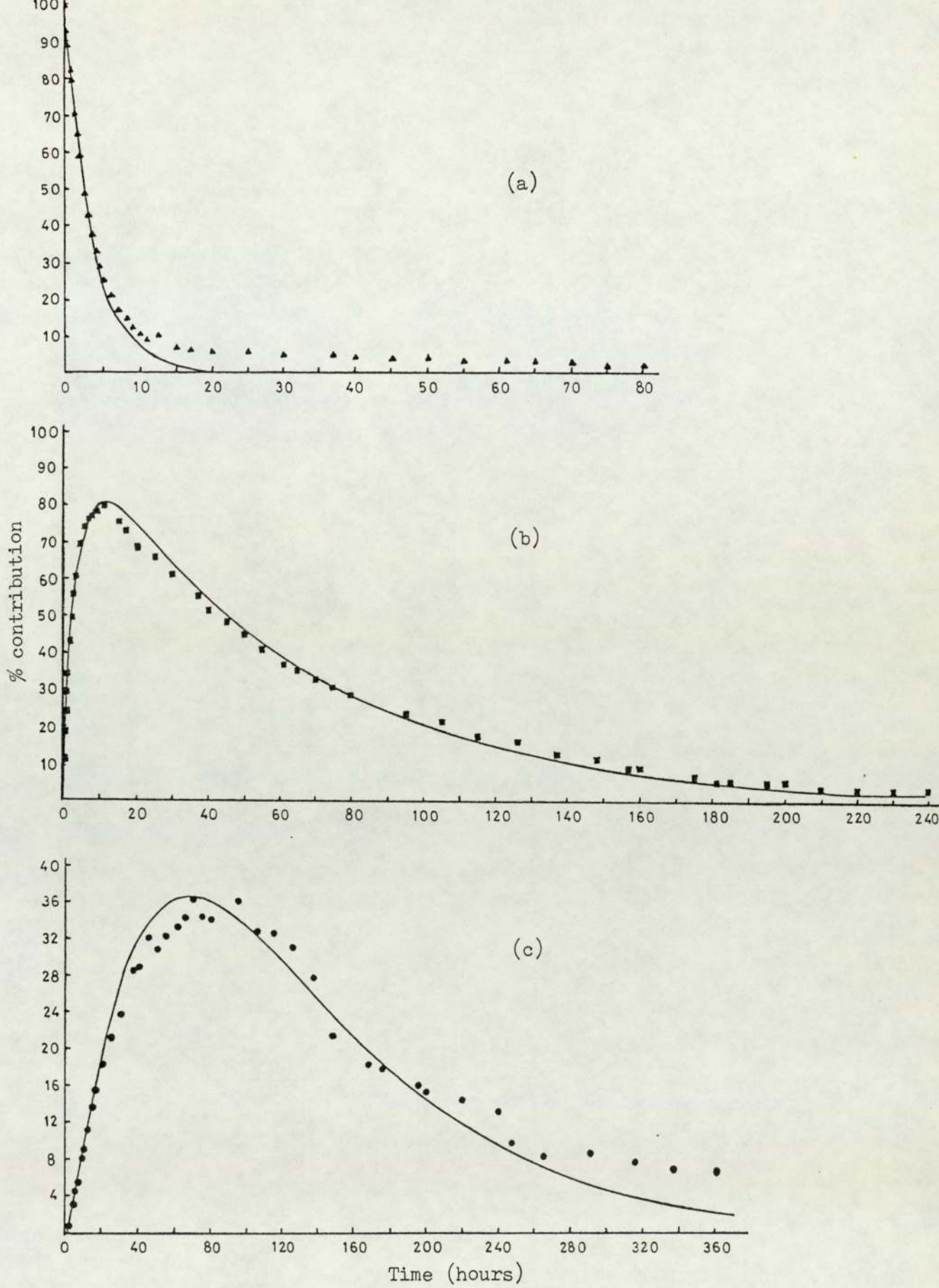


Figure 21. Profiles for hydrocortisone-17-butyrate (a), hydrocortisone-21-butyrate (b) and hydrocortisone (c) during the decomposition of the steroid-17-ester in a buffered 50% v/v aqueous propylene glycol solution (pH 7.6; 60°C).
Theoretical line using Model I ———
Experimental points ▲, ■ and ● .

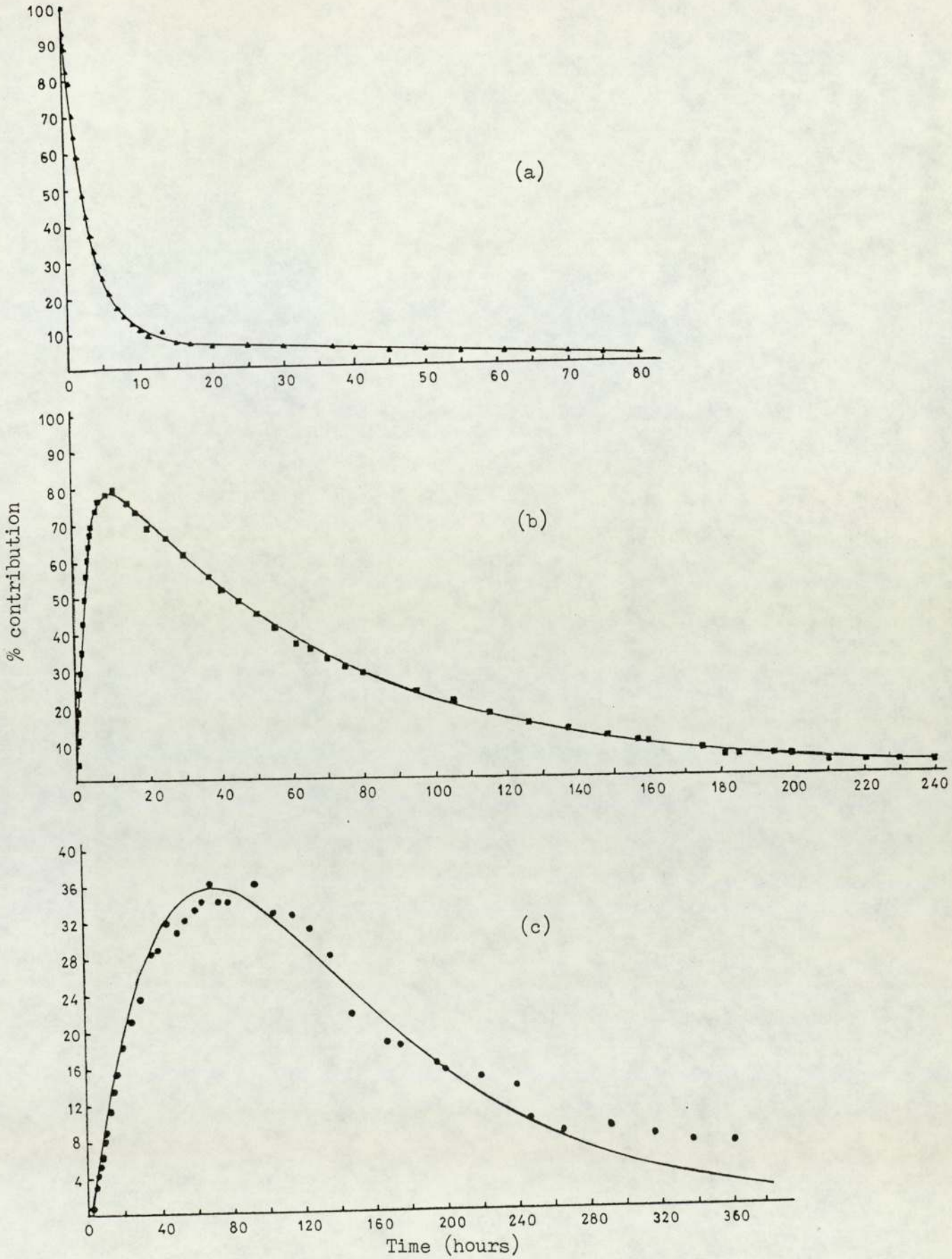


Figure 22. Profiles for hydrocortisone-17-butyrate (a), hydrocortisone-21-butyrate (b) and hydrocortisone (c) during the decomposition of the steroid-17-ester in a buffered 50% v/v aqueous propylene glycol solution (pH 7.6; 60°C).
Theoretical line using Model II ———
Experimental points ▲ , ■ and ● .

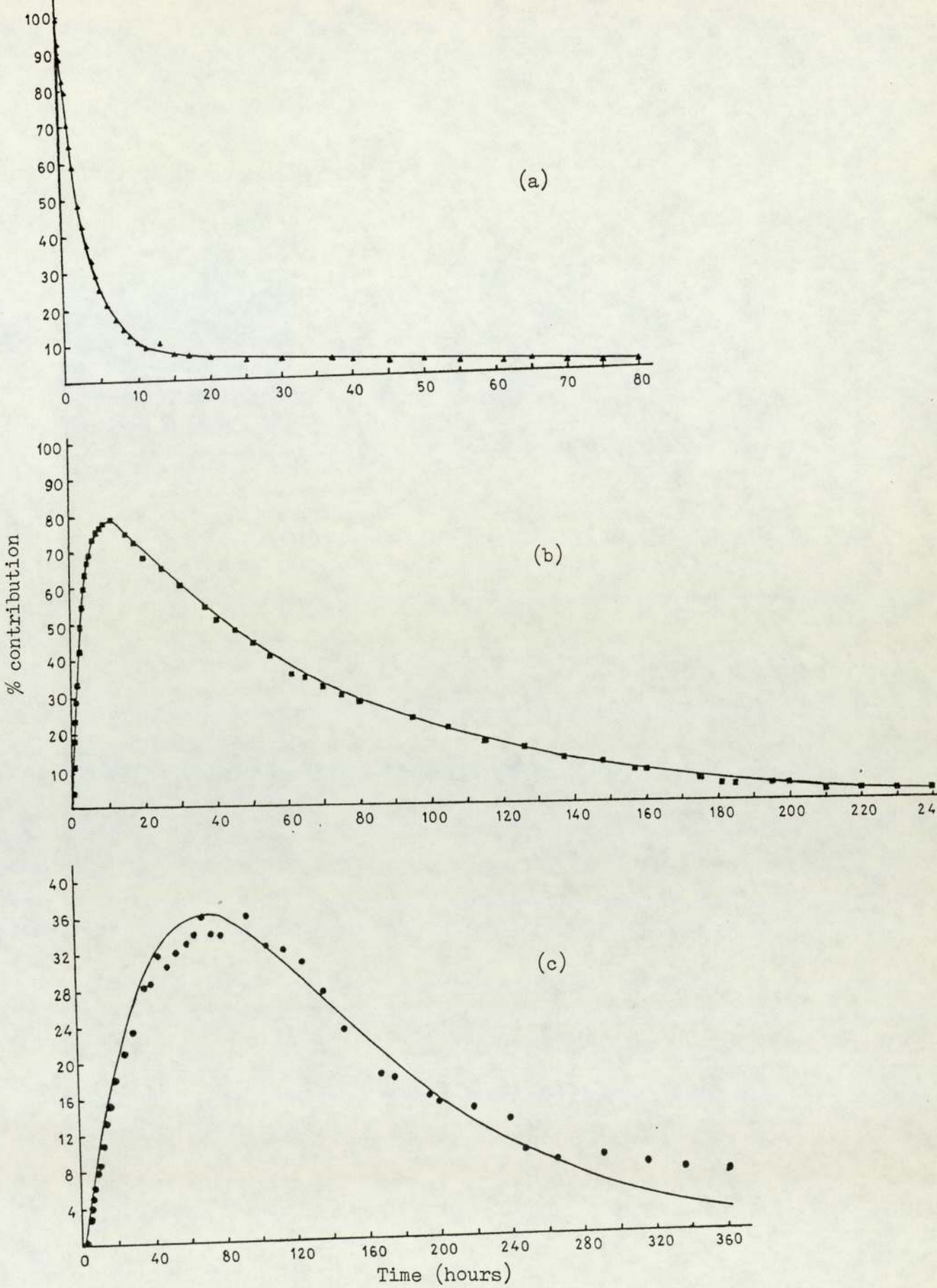


Figure 23. Profiles for hydrocortisone-17-butyrate (a), hydrocortisone-21-butyrate (b) and hydrocortisone (c) during the decomposition of the steroid-17-ester in a buffered 50% v/v aqueous propylene glycol solution (pH 7.6; 60 C).
Theoretical line using Model III ———
Experimental points ▲ , ■ and ● .

Table 23. The effect of ionic strength and buffering capacities on the degradation of hydrocortisone in aqueous propylene glycol (50% v/v)

Systems	*Buffering capacity	Ionic strength (M)	Rate constant (hr^{-1})
A	1	0.25	0.00926
B	1	0.4	0.00834
C	1	0.5	0.00764
D	1	0.6	0.00717
E	2	0.5	0.01182

*normalized to buffer capacity of the buffer solution used in system A.

Table 24. Rate constants for the decomposition of hydrocortisone in buffered solution using different batches of buffer salts, without EDTA

Batch No.	Buffering capacity*	Rate constant (hr^{-1})	Mean
1	1	0.00926	
2	1	0.00964	
2	1	0.01114	
			0.01001
3	2	0.0107	
3	2	0.01182	
			0.01126

*normalized to buffer capacity of the single-buffered propylene glycol

Table 25. Decomposition of hydrocortisone-17-butyrate in the presence and absence of sodium edetate (0.05% w/v)

Starting Compound	*Buffering Capacity	Decomposition Rate Constant x 10^3 (hr^{-1})	
		+0.05% EDTA	without EDTA
Hydrocortisone-17-butyrate	1	K_1 274	292
		K_2 14.4	16.1
		K_3 5.2	15.3
		K_4 24.4	24.6
Hydrocortisone	1	K_3 3.9	10.01
	2	K_3 4.42	11.26

*normalized to buffer capacity of the single-buffered propylene glycol

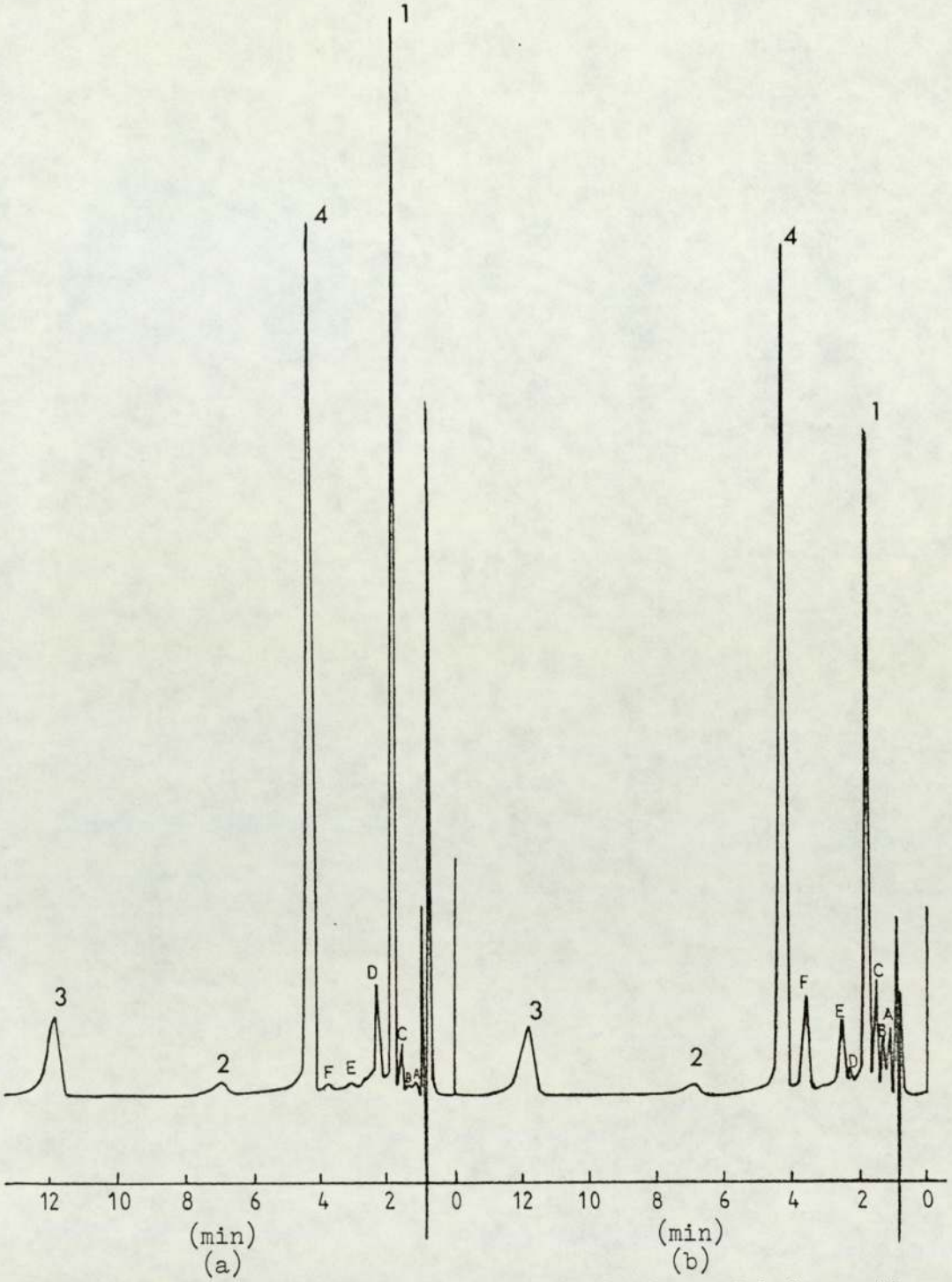


Figure 24. Chromatograms of decomposed solutions of hydrocortisone-17-butyrate in the presence (a) and absence (b) of sodium edetate.

Key: 1 : Hydrocortisone 2 : Hydrocortisone-17-butyrate
3 : Hydrocortisone-21-butyrate
4 : internal standard (hydrocortisone acetate)
A-F : decomposition products

CHAPTER 4 RELEASE AND PENETRATION OF TOPICAL CORTICOSTEROIDS

4.1 INTRODUCTION

Topical corticosteroids are widely used for controlling numerous dermatoses because of their marked anti-inflammatory activity and the relatively low risk of systemic adverse effects. For a drug to be effective, adequate amounts of the drug must reach the site of action. The ability of a drug to penetrate the skin and exert its effect depends on:

(1) its release from the formulated preparation

(2) its penetration through the skin barrier which resides almost entirely in the stratum corneum (94)

Either one of these two processes could be rate-limiting. Once the drug clears the barrier, it proceeds relatively rapidly through the remainder of the epidermis, dermis and into the circulation. The nature of the drug and the composition of the vehicle in which it is incorporated govern these two processes. The vehicles for topical drugs must provide an environment from which the drug is readily released when placed in contact with the skin. Improper formulation could render a topically potent drug essentially ineffective. It has been known that esterification of a given corticosteroid enhances its therapeutic efficacy and vasoconstrictor potency. For instances, in terms of vasoconstrictor activity, betamethasone-17-valerate is 400 times as active as betamethasone and 15 times as active as betamethasone-21-valerate (19), hydrocortisone-17-butyrate is 30 times as active as hydrocortisone-21-acetate (239) and hydrocortisone acetate is 10 times as active as hydrocortisone (23). One of the obvious possible explanations for the high potency is that esterification decreases the polarity of the steroid and this in turn enhances the skin

penetration of the steroid (240).

The ideal way to determine the penetration potential of a corticosteroid in man is to carry out the study in-vivo. However, this is not always possible and may be hazardous. In vitro techniques are therefore widely used. The most widely used technique involves a diffusion cell with animal or human skin. However, the permeability of the skin varies between subjects and it is difficult to obtain reproducible results even using the same skin specimen. An attempt has therefore been made to search for an in-vitro model to study the penetration rates of one or more steroids relative to a 'standard steroid' whose absorption profile is known. The idea was that by mixing one or more steroids with the 'standard steroid' in the chosen vehicle and their release or penetration profiles could be compared under identical conditions.

4.2 MATERIALS AND METHODS

4.2.1 Determination of Solubilities

4.2.1.1 Solubilities of hydrocortisone acetate, hydrocortisone-17-butyrate and hydrocortisone in chloroform at 25°C

Excess amounts of steroids were dispersed ultrasonically in 5 ml of chloroform. 2 ml were used for hydrocortisone-17-butyrate, since this steroid is very soluble in chloroform. The solution were kept at 25°C for 24 hours, then centrifuged, filtered through silicone treated filter paper (Whatman 1SP), diluted with chloroform appropriately and assayed by normal-phase HPLC. No partitioning into the silicone was detected during the filtration stage.

4.2.1.2 Solubilities of hydrocortisone acetate, hydrocortisone-17-butyrate and hydrocortisone in isopropyl myristate at 25°C and 37°C

Excess of the steroid was dispersed in 5 ml of isopropyl myristate (IPM) ultrasonically and maintained at 25°C or 37°C in a water bath for 24 hours. The mixture was centrifuged and replaced in the water bath for at least a few hours to readjust for any changes arising from the centrifuging. One ml aliquots were pipetted out and diluted with equal volumes of chloroform. Further dilution with 50% IPM in chloroform was needed for hydrocortisone-17-butyrate. Standard solutions were prepared in 50% IPM in chloroform. The concentration range was 0.02 to 0.1 mg/ml for hydrocortisone-17-butyrate and hydrocortisone and 0.005 to 0.05 mg/ml for hydrocortisone acetate. Analysis was done by normal-phase HPLC.

4.2.1.3 Solubilities of hydrocortisone acetate, hydrocortisone-17-butyrate, hydrocortisone-21-butyrate and hydrocortisone in aqueous propylene glycol at 25°C and 37°C

Excess amounts of steroids were dissolved ultrasonically in 5 ml of propylene glycol-water mixture (0 - 100% propylene glycol) for 15 minutes, then stored at 25°C or 37°C in a water bath and gently shaken for 24 hours. The solutions were centrifuged and re-equilibrated as before for at least a few hours. 1 ml supernatants were taken out and diluted with methanol and the corresponding propylene glycol-water mixture such that the final solutions consisted of 50% of this propylene glycol-water mixture and 50% of methanol containing phenacetin as internal standard. A typical concentration for the phenacetin used was 0.024 mg/ml at a sensitivity of 0.64 AUFS. In the presence of high amounts of propylene glycol, dilution with water instead of the corresponding propylene glycol-water was required so that the polarity

of the sample solvent was similar to the mobile phase. In order to simplify the preparation of standard solutions, an alternative way to prepare the test solutions was by diluting the supernatants with appropriate amounts of water or propylene glycol such that all test solutions contained the same amount of propylene glycol, eg. 20% propylene glycol in water and then diluted with an equal volume of methanol containing the internal standard. No matter how the test solutions were prepared, the important thing was that the composition of the solvent in both of the standard and the test solutions were the same. The samples were analysed by reversed-phase HPLC.

4.2.2 Determination of Partition Coefficient of Steroids Between Propylene Glycol-Water and IPM

The partition coefficient of hydrocortisone acetate, hydrocortisone-17-butyrate, hydrocortisone-21-butyrate and hydrocortisone between various propylene glycol-water systems and IPM were studied. The partition coefficient of each steroids were determined individually or as a mixture of hydrocortisone acetate, hydrocortisone-17-butyrate and hydrocortisone for comparison. The effect of initial concentration of the steroids on the partition coefficient was also investigated. Two different initial concentrations were used for comparison in the propylene glycol/isopropyl myristate system. The systems for study were 20%,40%, 60%, 80% and 100% propylene glycol in water. The propylene glycol-water and IPM were saturated with each other at 37^{o}C, and the presaturated propylene glycol-water or IPM were used for partition coefficient determination. The concentrations of steroids dissolved in the propylene glycol-water mixtures were as follows:-

100% propylene glycol : 0.5 mg/ml and 1 mg/ml

80% propylene glycol-water : 0.2 mg/ml

60% propylene glycol-water : 0.2 mg/ml

40% propylene glycol-water : 0.1 mg/ml

20% propylene glycol-water : 0.05 mg/ml for hydrocortisone-21-butyrate and 0.02 mg/ml for others

5 ml of these steroid sample solutions were shaken with 5 ml of IPM presaturated with the corresponding propylene glycol-water mixture and then stored at 37°C in a water bath overnight. The solutions were centrifuged, and re-equilibrated. 1 ml aliquots of the propylene glycol-water phase were diluted with methanol and the corresponding propylene glycol-water mixture such that the final solutions consisted of 50% of this propylene glycol-water mixture and 50% of methanol containing phenacetin as internal standard. 0.024 mg/ml of phenacetin can be used at a sensitivity of 0.64 AUFS. When the sample contained one steroid only, any one of the other steroids could be used as an internal standard. However, for the 20% propylene glycol-water system, due to the low initial concentration of steroid, no further dilution and no internal standard were added. The propylene glycol-water phase was monitored by reversed-phase chromatography. For the IPM phase, 1 ml aliquots were diluted with equal volumes of chloroform containing caffeine as internal standard. 0.01 mg/ml of caffeine was used at a sensitivity of 0.04 AUFS. The IPM phase was analysed by normal-phase HPLC.

4.2.3 In Vitro Release of Steroids from Carbopol Gel

100 g of steroid gel was prepared according to the following formula:-

Carbopol 940	0.8	g
propylene glycol	20, 40, 60, or 80	g
hydrocortisone-17-butyrate	0.1	g
hydrocortisone	0.1	g
hydrocortisone acetate or hydrocortisone-21-butyrate	0.1	g
EDTA	10	mg
1% w/v NaOH	2	ml
Water	to 100	g

The pH of the gel determined by direct measurement was 3.88. The steroids were dispersed in the propylene glycol portion or dissolved in 50 g of propylene glycol if more than 50 g propylene glycol was used in the gel. The Carbopol and EDTA were dissolved in water and the remainding propylene glycol, then neutralized with the sodium hydroxide solution. The steroid mixture was incorporated into the gel mass and well mixed. Water was then added to adjust to weight. Entrapped air was removed by vacuum suction in a desiccator. About 75 g of gel was placed on a glass petri dish of 9 cm in diameter, and the surface smoothed with a spatula. The amount of gel transferred was accurately weighed. The thickness of the gel was 1.2 cm. The dish was placed in a 1 litre beaker and the beaker was partially immersed in a water bath of 37°C. 300 ml of IPM preheated to 37°C was carefully layered over the gel and stirred at a rate of 30 r.p.m. The whole apparatus was protected from light. 2 ml aliquots were sampled at intervals and was replaced with 2 ml fresh IPM. The sample solutions were diluted with equal volumes of chloroform and assayed by normal-phase HPLC.

4.2.4 Three-Phase Partitioning Model

A Schulman cell (184) (Figure 25) consisting of two 100 ml capacity compartment A and C, which can be used to accomodate the aqueous or semi-aqueous donor and receiving phases respectively. 100 ml of a lipoidal solution was layered on top to bridge these two phases, to mimic the lipid layer of skin and to serve as a barrier between the drug dosage form and the body. Solutions used as the donor or receiving phase were presaturated with IPM at 37°C. The steroids were dissolved in the donor solution and put in compartment A of the cell, 100 ml of receiving solution were placed in the C compartment. 100 ml of the presaturated IPM were carefully layered on top of the A and C phases. Both sides were stirred at 100 r.p.m. with double bladed stirrers without disturbing the lipid/propylene glycol interface. 1 ml aliquots from each phase were taken at appropriate time intervals without replacement of fresh solvent. The following systems were studied:-

System No.	A Phase (100 ml)	B Phase (100 ml)	C Phase (100 ml)
I	Hydrocortisone acetate, hydrocortisone-17-butyrate, and hydrocortisone, 1 mg/ml of each, in PG	IPM	Propylene glycol (PG)
II	Hydrocortisone-17-butyrate, 1 mg/ml in PG	IPM	PG
III	Hydrocortisone-21-butyrate, hydrocortisone-17-butyrate, and hydrocortisone, 0.1 mg/ml of each, in PG	IPM	PG

System No.	A Phase (100 ml)	B Phase (100 ml)	C Phase (100 ml)
IV	Cortisone acetate, hydrocortisone-17-butyrate, and prdenisolone, 0.2 mg/ml of each, in PG	IPM	PG
V	Hydrocortisone acetate, hydrocortisone-17-butyrate, and hydrocortisone, 1 mg/ml of each, in 80% PG-H_2O	IPM	80% PG-H_2O
VI	Hydrocortisone acetate, hydrocortisone-17-butyrate, and hydrocortisone, 1 mg/ml of each in PG	IPM	5% DMSO-PG
VII	Hydrocortisone acetate, hydrocortisone-17-butyrate, and hydrocortisone, 1 mg/ml of each, in 5% DMSO-PG	IPM	PG

1 ml aliquots of A and C phases were diluted with 3 ml of a 0.016 mg/ml phenacetin solution in 80% methanol-water for separation of System IV; with 4 ml of a 0.02 mg/ml hydrocortisone acetate solution in 80% methanol for System III; and 9 ml of a 0.024 mg/ml phenacetin solution in 80% methanol-water for all other systems. Standard solutions were made in a solvent of identical composition at appropriate concentrations. These samples were analysed by reversed-phase HPLC. 1 ml aliquots of the B phase were diluted with 1 ml of a 0.04 mg/ml solution of caffeine in chloroform as internal standard for the separation of hydrocortisone-21-butyrate, hydrocortisone-17-butyrate and

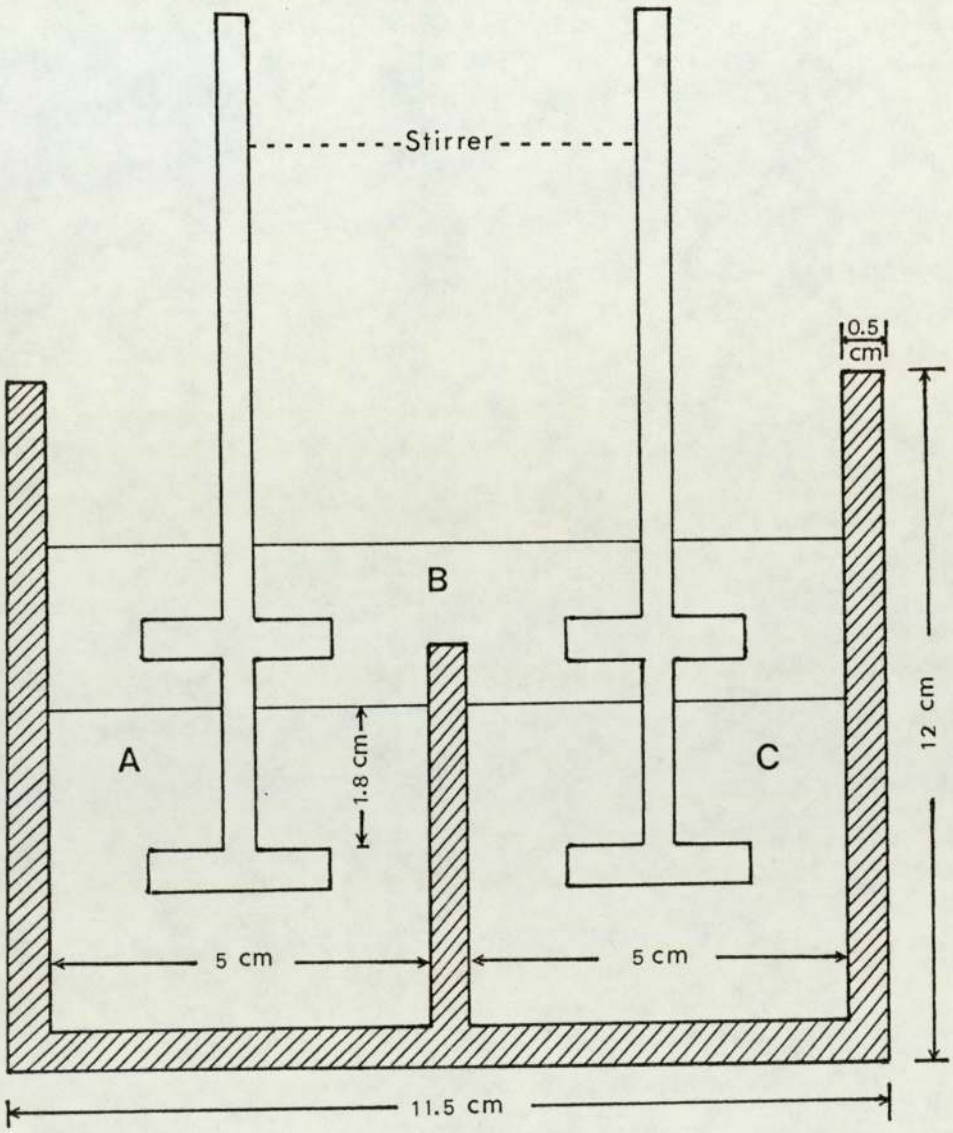


Figure 25. Three-phase partitioning system

hydrocortisone; 1 ml of a 0.02 mg/ml caffeine solution in chloroform for the separation of cortisone acetate, hydrocortisone-17-butyrate and prednisolone ; and 0.05 mg/ml caffeine solution in chloroform for the separation of the other mixtures.

4.2.5 In Vitro Penetration Study of Steroids through Mouse Skin

A mixture containing 100 mg quantities of hydrocortisone-17-butyrate, hydrocortisone acetate and hydrocortisone were dissolved or dispersed in 100 ml of propylene glycol-water (40% and 100% of propylene glycol were used) and put in an ointment jar. The hair of the mouse skin was removed by plucking and the skin mounted on the opened end of the jar with the epidermal side facing inwards. This membrane was securely fastened by the screw cap which was specially opened to expose a definite area of the skin. This diffusion cell was clamped and immersed in a container which contained 350 ml of chloroform layered with about 100 ml distilled water to prevent evaporation. The skin should be below the chloroform surface (approximately 0.5 cm below). The receiving phase was stirred with a magnetic stirrer and the level of chloroform carefully marked so that constant volume could be maintained. The whole apparatus was protect from light and at ambient temperature. 2 ml aliquots were taken out with replacements at appropriate intervals. Sample solutions were assayed by normal phase HPLC.

4.2.6 HPLC Analysis

4.2.6.1 Normal Phase HPLC

Two normal phase HPLC systems were used. System I (see Chapter 2) was used for (i) the determination of the solubility of steroids in chloroform and IPM (ii) the release studies from Carbopol gels

and (iii) diffusion of the steroids through mouse skin.

System II (see Chapter 2) was used for analysing the B phase aliquots in the three-phase partitioning studies. In the case of separation of hydrocortisone-21-butyrate, hydrocortisone-17-butyrate and hydrocortisone mixture, a mobile solvent consisting of 15% isopropanol, 0.3% ammonia, 0.5% water in hexane, delivering at a rate of 2 ml/min, should be used instead of the original system.

System I was developed before System II. Because the latter was better at resolving the steroids, it was used whenever possible in the later studies.

4.2.6.2 Reversed-phase HPLC

This was used for determining the solubility of the steroids in propylene glycol-water and the analysis of the A and C phases in the three-phase partitioning studies.

Column: Hypersil-ODS (5 μm), 10 cm x 4.6 mm i.d.

Mobile phase: 70% methanol in water for analysis of mixture of hydrocortisone and its 17- and 21-butyrates

67% methanol in water for all other steroids

Flow rate: 1 ml/min

For the three-phase partioning study, an aqueous methanol must be used as mobile phase rather than acetonitrile-water system because the solvents for the A and C phases were presaturated with IPM which is not miscible with acetonitrile but is miscible with methanol. The polarity of the sample solvents must be considered and the one chosen must be of similar or higher polarity than the mobile phase whenever possible. For diluting the samples from the A and C phases in the three-phase partitioning study the choice of solvent (80% aqueous methanol) was dictated by the solubility of IPM.

4.2.7 Viscosity Determination of Aqueous Propylene Glycol

A U-tube viscometer, size C, was used for the measurements. The viscosities of various concentrations of propylene glycol in water were calculated relative to water, 10 cps silicone fluid and 20 cps silicone fluid by the expression $\frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2}$

where η_1, η_2 are the viscosities of the standard and test sample, ρ_1 and ρ_2 are the respective densities and t_1 and t_2 are the respective times in seconds.

4.3 THEORETICAL

4.3.1 Solubility and Partition Coefficient

The solubility of a substance which is practically insoluble in water can usually be increased significantly by addition of a cosolvent in which the substance is more soluble. Literature data indicate that the solubility of many drugs in such binary aqueous systems can be represented by the following expression (138,241,242):-

$$S_f = S_w e^{\alpha f} \qquad \text{Eq. 4}$$

or written in logarithm form, Equation 4 becomes:

$$\log S_f = \log S_w + \alpha f \qquad \text{Eq. 5}$$

where S_f is the solubility of the solute in a binary aqueous system and S_w is its solubility in water, f is the volume fraction of the non-aqueous cosolvent and α is a constant that is dependent on the polarity of the substance and the cosolvent. Equation 5 means that the logarithm of the solubility of certain compounds is directly proportional to the volume fraction of the cosolvent. The linear relationship of a plot of log S_f versus f make possible a useful estimation of solubilities in the binary systems, provided the polarity of the solute is significantly less than that of either solvents.

And this relationship is adequately general (242).

Similarly, the partition coefficient of a drug between a vehicle (binary systems) and a skin (or an immiscible solvent) can be expressed as (242):-

$$\log (PC)_f = \log (PC)_w + \beta f \qquad \text{Eq. 6}$$

where $(PC)_f$ is the partition coefficient of a substance between a binary system with volume fraction f of a given cosolvent (vehicle) and an immiscible solvent (or skin); $(PC)_w$ is the partition coefficient between water and the immiscible solvent, β is a constant.

4.3.2 Case where the Rate Controlling Process is in the Skin

A simple but very important model involves the study of a drug transfer across a homogeneous, non-porous membrane with thickness h. The concentration of the drug on the vehicle side is C_1 while that on the receptor side is C_2 ($C_1 > C_2$) (Figure 26).

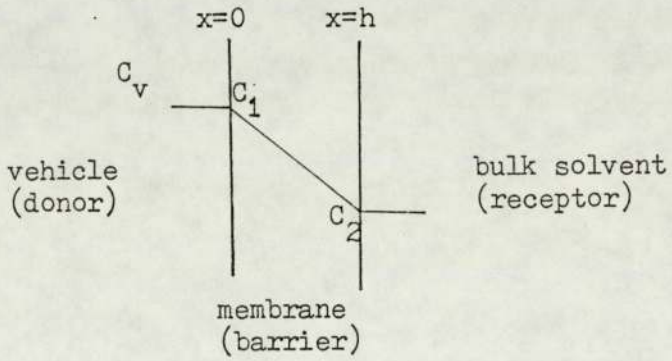


Figure 26. Diagram showing the simple steady state diffusion across a membrane (partition effects are ignored)

During the initial stages of drug transfer from the vehicle to the receptor, the rate of flow and the concentration at any point within the membrane varies with time, this is called the unsteady state period. After sufficient time, a steady state is established, in which

the concentration gradient within the membrane is constant. The time required for establishment of a quasi-stationary state is called the lag time. In the steady-state condition, the rate of penetration dQ/dt, is then given by Equation 7 (70):-

$$\frac{dQ}{dt} = \frac{D\ (C_1 - C_2)}{h} \qquad \text{Eq. 7}$$

where Q is the amount transferred per unit area, D is the diffusion ceofficient of the drug in the barrier. Since the concentration C_1 is related to the concentration of drug in the vehicle C_v, by $PC = C_1 / C_v$, PC is the partition coefficient of the drug between the barrier and the vehicle, and if the receptor side is maintained in a perfect sink, i.e. $C_2 = 0$, such that the concentration gradient for diffusion, dC/dx is equal to C_1/h, then Equation 7 becomes:

$$\frac{dQ}{dt} = \frac{D\ (PC)\ C_v}{h} \qquad \text{Eq. 8}$$

This equation only applies to steady state period, and is restricted to cases where binding to the skin does not occur. Experimentally, dQ/dt can be obtained by calculating the slopes from plots of amount transferred versus time in the steady-state. PC can be determined by partitioning the drug between the vehicle and the skin barrier or a representative solvent. C_v is either the concentration of the drug in the vehicle, if it is in a solution form or the solubility of the drug in the vehicle if it is in a suspension form. D can be calculated from Equation 8 if the true PC is known, or estimated from the lag time L:

$$L = \frac{h^2}{6D} \qquad \text{Eq. 9}$$

L is calculated by extrapolation of the steady-state portion from plots

of penetrated amount versus time to the time axis (174).

4.3.3 Case where Rate-Controlling Process is the Release from Vehicle

T. Higuchi (70, 243) and W. Higuchi (244) have proposed two equations for predicting the release profile of a drug from vehicles, with the drug presented as a solution or as a suspension. In both cases, a linear relationship between the amount released and the square root of time can be obtained.

4.3.3.1 Release from suspension

When a drug is present as suspended particles in a vehicle, the release of drug from the vehicle may be rate-limiting and can be described by Equation 10 (243):-

$$Q_1 = \sqrt{(2C_o - C_s)\, C_s\, D\, t} \qquad \text{Eq. 10}$$

where Q_1 is the amount released at time t per unit area of exposure, C_o is the initial concentration of drug in the vehicle, C_s is the solubility of the drug in the vehicle. Equation 10 is valid for all times before complete depletion of the suspended phase, and the total drug concentration C_o is only a few (3 - 4) times greater than the solubility in the vehicle, C_s. Differentiating Equation 10 with respect to time t, the instantaneous rate of release at time t, dQ_1/dt, can be obtained:-

$$\frac{dQ_1}{dt} = \frac{1}{2}\sqrt{\frac{(2C_o - C_s)\, D\, C_s}{t}} \qquad \text{Eq. 11}$$

Equation 10 is derived under the assumption that (i) the suspended drug is in a fine state such that the particles are much smaller in diameter than the thickness of the applied vehicle layer (ii) the initial concentration of drug C_o is substantially greater than its

solubility C_s, (iii) the applied surface is immiscible with the vehicle of the preparation and perfect sink condition exists. When C_o is much greater than the solubility C_s, Equation 10 can be simiplified to:-

$$Q_1 = \sqrt{2\ C_o\ D\ C_s\ t} \qquad \text{Eq. 12}$$

and

$$\frac{dQ_1}{dt} = \sqrt{\frac{C_o\ D\ C_s}{2\ t}} \qquad \text{Eq. 13}$$

The diffusion coefficient D can be calculated from the slopes, K, of plots of Q versus $t^{\frac{1}{2}}$, with units of cm^2/unit of time, when Equation 10 applies,

$$D = \frac{K^2}{(2C_o - C_s)\ C_s} \qquad \text{Eq. 14}$$

when Equation 12 applies, then,

$$D = \frac{K^2}{2\ C_o\ C_s} \qquad \text{Eq. 15}$$

4.3.3.2 Release from solution

Under sink condition: The simplest system of this type is presented when the penetrating substance is initially uniformly dissolved in a homogeneous vehicle. If sink conditions on the receptor site exist, then the amount of drug released per unit area, Q_2, is given by Equation 16 (245, 70):-

$$Q_2 = h\ C_o \left[1 - \frac{8}{\pi^2} \sum_{m=0}^{\infty} \frac{1}{(2m+1)^2} \exp\left(\frac{-D\ (2m+1)^2\ \pi^2\ t}{4\ h^2} \right) \right] \qquad \text{Eq.16}$$

where D is the diffusion coefficient of the drug in the vehicle, h is the thickness of the vehicle, C_o is the initial drug concentration, m is an integer and t is time. Equation 16 is a solution of Fick's law

of diffusion. The main assumptions involved in Equation 16 are that (i) only one single drug species is important in the vehicle (ii) D must be constant with respect to both time and position in the vehicle (iii) only the drug is able to diffuse out of the vehicle layer (iv) the diffused drug in the receptor side is removed rapidly, i.e. sink condition exists, W. Higuchi (244) proposed that Equation 16 can be simplified to:-

$$Q_2 = 2\ C_o\ (D\ t\ /\pi)^{\frac{1}{2}} \qquad \text{Eq. 17}$$

because for small t, only the first term of the sum in Equation 16 is significant. Equation 17 holds for up to 30 - 50% drug release. Differentiating Equation 17 with respect to time t, the instantaneous rate of release at time t, dQ_2/dt is:

$$\frac{dQ_2}{dt} = C_o\ \left(\frac{D}{\pi\ t}\right)^{\frac{1}{2}} \qquad \text{Eq. 18}$$

From the slope, K, of plot of Q_2 versus $t^{\frac{1}{2}}$ (Equation 17), the diffusion coefficient D can be obtained by:

$$D = \frac{\pi}{4}\ \left(\frac{K}{C_o}\right)^2 \qquad \text{Eq. 19}$$

The mathematics derived for predicing the release profile is so far based on the assumption that sink conditions exist in the receptor side. The assumption that in vivo the drug is removed rapidly by the circulation, to provide sink conditions may be true for many cases. However, the skin does not always provide sink conditions (124) and in vitro it is not always possible to have sink condition due to experimental constraints. Guy and Hadgraft (246) have derived equations for drug release into sink and nonsink condition and it is assumed that the drug is released from a vehicle at a diffusion-controlled rate into a

well-stirred receptor volume. An equation which is equivalent to Equation 16 was derived for sink condition:

$$M_t = M_\infty \left[1 - \frac{8}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{(2n-1)^2} \exp\left(\frac{(2n-1)^2 \pi^2 T}{4} \right) \right] \quad \text{Eq.20}$$

where $M_\infty = A\ h\ C_o$ and is the initial total amount of drug

$T = (D\ t)/h^2$ and is a normalized variable for simplifying the solution of diffusion equations

A = the area across which release takes place

h = the thickness of the vehicle layer

n = an integer

M_t = the amount released at time t

Simplified expressions obtained by approximating the hyperbolic term in Equation 20 were also derived:-

For short times, $T < 1$:

$$M_t = 2\ M_\infty\ T^{\frac{1}{2}} \pi^{-\frac{1}{2}} \quad \text{Eq. 21}$$

For long times, $T > 1$:

$$M_t = M_\infty \left[1 - \exp(-3T) \right] \quad \text{Eq. 22}$$

Equation 21 is equivalent to Equation 17.

Under non-sink conditions, the drug concentration is allowed to build up in the receptor phase and the partition coefficient PC between receptor to the vehicle becomes important. A unique equation is not possible for describing the release profile under non-sink conditions and approximations were made for short and long times release. Let $R = (A\ h)/(V_r\ PC)$, V_r is the volume of receptor phase, then for short

times $T<1$:

$$M_t = M_\infty R^{-1} \left[1-\exp(R^2 T)\ \mathrm{erfc}(R T^{\frac{1}{2}})\right] \qquad \text{Eq. 23}$$

For long times $T>1$:

$$M_t = M_\infty (1 + R)^{-1} \left[1 - \exp [-3 (1 + R) T]\right] \qquad \text{Eq. 24}$$

4.3.4 Three-Phase Partitioning Model

The kinetics of the drug transfer in this three-phase system involves a consecutive reversible first-order reactions and is represented by the model:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C$$

A, B and C represent the concentration of the drug in the donor, lipid (barrier) and receptor compartments respectively, k_1 and k_3 are the forward rate constants and k_2, k_4 the backward rate constants. The rate of concentration changes in each phase is expressed by the following differential equations:

$$\frac{dA}{dt} = - k_1 A + k_2 B \qquad \text{Eq. 25}$$

$$\frac{dB}{dt} = k_1 A - k_2 B - k_3 B + k_4 C \qquad \text{Eq. 26}$$

$$\frac{dC}{dt} = k_3 A - k_4 C \qquad \text{Eq. 27}$$

The concentration of the drug in each phases at time t can be obtained by solving these three differential equations according to the operator method described in Capellos and Bielski, 1972 (247) and the solutions are:-

$$A_t = A_o \left[\frac{k_2\, k_4}{r_1\, r_2} + \frac{r_1^2 - r_1\,(k_2 + k_3 + k_4) + k_2\, k_4}{r_1\,(r_1 - r_2)}\; e^{-r_1 t} + \frac{r_2^2 - r_2\,(k_2 + k_3 + k_4) + k_2\, k_4}{r_2\,(r_2 - r_1)}\; e^{-r_2 t} \right] \quad \text{Eq. 28}$$

$$B_t = A_o\, k_1 \left[\frac{k_4}{r_1\, r_2} + \frac{k_4 - r_1}{r_1\,(r_1 - r_2)}\; e^{-r_1 t} + \frac{k_4 - r_2}{r_2\,(r_2 - r_1)}\; e^{-r_2 t} \right] \quad \text{Eq. 29}$$

$$C_t = A_o\, k_1\, k_3 \left[\frac{1}{r_1\, r_2} + \frac{1}{r_1\,(r_1 - r_2)}\; e^{-r_1 t} + \frac{1}{r_2\,(r_2 - r_1)}\; e^{-r_2 t} \right] \quad \text{Eq. 30}$$

where $r = \frac{1}{2}\left[(k_1+k_2+k_3+k_4) - \sqrt{(k_1+k_2+k_3+k_4)^2 - 4(k_1k_3+k_2k_4+k_1k_4)} \right]$

$r = \frac{1}{2}\left[(k_1+k_2+k_3+k_4) + \sqrt{(k_1+k_2+k_3+k_4)^2 - 4(k_1k_3+k_2k_4+k_1k_4)} \right]$

When A and C are identical solvent systems, the kinetic are simpler. For such cases, $k_1 = k_4$ and $k_2 = k_3$ and have the solutions for A_t, B_t and C_t are as follows:

$$A_t = A_o \left[\frac{k_2}{r_2} + \tfrac{1}{2}\, e^{-r_1 t} + \frac{r_1}{2\, r_2}\, e^{-r_2 t} \right] \quad \text{Eq. 31}$$

$$B_t = A_o\, k_1 \left[\frac{1 - e^{-r_2 t}}{r_2} \right] \quad \text{Eq. 32}$$

$$C_t = A_o\, k_1\, k_2 \left[\frac{1}{r_1\, r_2} - \frac{1}{2\, k_1\, k_2}\; e^{-r_1 t} + \frac{1}{2\, k_2\, r_2}\; e^{-r_2 t} \right] \quad \text{Eq. 33}$$

where $r_1 = k_1$

$r_2 = k_1 + 2\, k_2$

The values of the rate constants k_1, k_2, k_3 and k_4 are first estimated by a curve-fitting method using an analog computer (184). By means of a digital computer, the accurate rate constants can be calculated by non-linear regression analysis (233).

4.4 RESULTS AND DISCUSSION

Steroid molecules diffuse slowly through normal skin. Thus, development of formulations that increases steroid penetration are desirable. There are two general approaches to this problem. One approach involves the use of an accelerant in the vehicle to alter the skin's resistance to diffusion of the steroid. The second approach involves changing the physicochemical properties of the vehicles. Satisfactory absorption of a drug from a topical preparation is not only dependent on the drug's ability to penetrate the skin but also on its ability to diffuse out of the vehicle. From the diffusion equations (Equations 8, 12 and 17), the parameters which can be modified to facilitate drug penetration are the partition coefficient between skin and vehicle (PC), the concentration of drug in solution in the vehicle (C_v), and the diffusivity of the drug in the barrier or the vehicle (D). C_v is important because only the fraction of drug which is solubilized can readily diffuse out of the vehicle. In the case of release from suspension, the solubility (C_s) can be increased by addition of cosolvent or complexing agent. The parameter PC can be optimized by decreasing the drug's solubility in the vehicle. When the rate-controlling step is the diffusion of the drug through the vehicle, the PC can play no role if perfect sink conditions exist. However, the skin normally does not provide sink conditions for release (124), and under such conditions the PC is certainly important. The diffusivity in the vehicle is inversely proportional to the microscopic viscosity of the

vehicle and penetration rates can therefore be varied by modifying this parameter.

4.4.1 Release Study from Carbopol Gel

Release studies have long been used as criteria for predicting vehicle effects on skin penetration. Optimizing the release rate also provides environments for optimal penetration even though the rate-limiting step is in the skin barrier. To study the effect of vehicles on drug release, a similar model to Poulsen and coworker's (126) was used. Steroid mixture were formulated in Carbopol gels with various amounts of propylene glycol in water, and the release of the steroids into IPM was observed. IPM is immiscible with water and propylene glycol, and separation of phases by a membrane is not essential. IPM was chosen to represent the skin barrier because its lipophilicity is said to be similar to that of the skin (172). Low molecular weight polyols are used extensively as humidity conditioners in dermatological hydrogels. Propylene glycol is usually introduced in these dosage forms to prevent undesirable formation of films after application of the gel to the skin and to promote absorption. For example, the penetration rate of diflorasone diacetate through propylene glycol-pretreated skin has been shown to be twice that observed with a control (138). Since the solubility of the drug in the vehicle and the partition coefficient of the drug between skin and vehicle are important parameters in controlling the efficacy of the preparations, it is useful to determine or estimate these two variables. Although it is difficult to measure the real solubility of the drug in a semi-solid base, reasonable estimations can be made by determining the solubility of the drug in the solvent component(s) of the vehicle. The solubilities of the steroids studied were therefore measured in

aqueous-propylene glycol solutions. A plot of log solubility versus the volume fraction of propylene glycol (Figures 27 and 28) shows a biphasic phenomenon with a transition point at about 80% propylene glycol. The reason for this biphasic behaviour is not known but each portion of each of the curves obeys Equation 5, i.e. with two different a values for each curve. The partition coefficient between IPM and propylene glycol-water were determined (Figure 29) and served as a useful index of the relative activity coefficients for the steroids in these vehicles (126), although they will not duplicate that between human skin and the vehicles. Figure 29 shows that the relationship between the partition coefficient of the steroids and % propylene glycol obeys Equation 6 in a biphasic manner similar to that for solubility.

In order to compare the relative release rates of hydrocortisone, hydrocortisone-17-butyrate and hydrocortisone-21-butyrate or 21-acetate in the same dosage form, the studies were conducted with mixtures of these three corticosteroids. The release profiles are shown in Figures 30 and 31. It is reasonable to assume that the release of each steroid is not affected by the presence of the others, provided that the partition coefficients of a given steroid in the solvent systems used are independent of the presence of other steroids. This was verified by determining the partition coefficients of these three steroids between propylene glycol-water and IPM individually and in combination (Table 26). The partition coefficient of each steroid in a given system was also unaffected by different initial concentrations (Table 26) provided that the concentration was less than the sum of its solubilities in both phases. The release patterns of hydrocortisone and its 17-butyrate were the same irrespective of whether hydrocortisone-21-butyrate or hydrocortisone acetate were added to them although the

latter two showed marked differences in their release behavior (Figures 30a and 31a; and Figures 30b and 31d). Based on the independence of the steroids in their solubilities and partitioning behavior when admixed, Poulsen and others (248) suggested that the use of corticosteroid mixtures provided a new concept for improving topical drug bioavailability. They found that the penetration produced by a mixture of three fluocinolone acetonide esters containing a given total amount of steroids and in a ratio based on their solubilities, was the same as the sum of the amounts absorbed from the three single-steroid systems at equivalent concentration. In the present study, as shown in Figure 32, the effect of altering vehicle composition on the % of steroid released was dependent on the steroid used. The initial concentration of each steroid in each vehicles were the same, 0.1%, in all cases. From the solubility profile (Figure 28), the minimum amount of propylene glycol required to completely dissolve hydrocortisone, hydrocortisone-17-butyrate, hydrocortisone-21-butyrate and hydrocortisone acetate are 19, 41, 68 and 70% respectively. The results show that the maximum release of a specific steroid is observed in a steroid-saturated vehicle. Hydrocortisone shows maximum release in a 20% propylene glycol-water gel. At this concentration, the system is approximately saturated with the steroid. Excess propylene glycol increases its affinity for the vehicle and the release drops dramatically (Figure 32). 0.1% w/v hydrocortisone-17-butyrate produces a saturated solution at about 40% propylene glycol, at which point maximum release is observed. Excess propylene glycol decreased its release due to unfavourable partition coefficient. With a decrease in propylene glycol one would have expected a decrease in the % released because of the lower concentration in solution. However, the data show that the release from the 20% and the 40% propylene glycol-water

gels were comparable. This may be due to an increase in the diffusion coefficient as the propylene glycol concentration is reduced. Additionally, the release is into a non-sink system and an increase in the partition coefficient (IPM/propylene glycol) of the steroid with a reduction in the % propylene glycol will also work towards compensating for any reduction in the concentration of drug in solution. Hydrocortisone acetate shows maximum release from 60% propylene glycol-water gel with the available data, but it is to be expected that the release would be equal or higher if 70% propylene glycol-water gel had been used. The decrease in the release with a reduction in the amount of propylene glycol is probably due to a decrease in concentration of hydrocortisone acetate not totally compensated for by changes in diffusion coefficients and partition coefficients. The solubility of hydrocortisone-21-butyrate in aqueous propylene glycol is about equivalent to that of hydrocortisone acetate, but its partition coefficient between IPM and propylene glycol is higher than the other steroids. In all cases, whether the steroid was totally dissolved or in suspension, a linear relationship between the amount released and the square root of time is obtained (Figures 33 and 34), indicating that the rate-controlling step was the release from vehicle. The data appear to obey the Higuchi's models for diffusion-controlled release from suspension or solution (Equations 12 and 17). However, those equations carry the assumption that perfect sink exits. Obviously the experimental conditions were such that for hydrocortisone and its 21-butyrate and acetate, sink conditions were not provided. If perfect sink conditions do exist, then according to Equations 12 and 17, the release from ointment bases is independent of the partition coefficient. In other words, the releases of a drug from different vehicles, in which the drug is in solution, should be the same if the initial

concentrations of the drug are the same and the viscosities of the vehicles are similar. Hydrocortisone-17-butyrate has relatively high solubility in IPM (Table 27) and it is experimentally possible to provide sink conditions without going beyond the HPLC detection limits. The hydrocortisone-17-butyrate in aqueous-propylene glycol systems fall into this group. Yet under such conditions, a dependence of partition coefficient on release of hydrocortisone-17-butyrate was still observed, indicating a diffusional rate-limiting step. A similar phenomenon has been reported by Poulsen et al (126) and Weiss and Sciarrone (249). The Higuchi model (Equation 17) assumes the diffusion coefficient is constant, the drug alone diffuses out of the base and the drug is rapidly cleared from the interface. The latter assumption in turn can be related to the earlier stipulation that sink conditions exist. Finally, the model also requires that only a single drug species is important in the base. This can be generalised to the requirement that drug present within the same system do not affect each other's activity. Partition coefficient determinations (Table 26) have shown no interaction between the component steroids and data reported by Poulsen et al (126) showed no changes in the macroscopic viscosities of Carbopol gels with a change in the percentage of propylene glycol added. Despite these conditions, when $Q/(C_o\ t^{\frac{1}{2}})$ was plotted against PC, the straight line with zero slope expected from Equation 17 was not obtained. This suggested a change in the diffusion coefficient with the experimental set up. To test for this, the diffusion coefficient for the steroids in the gels were calculated by non-linear regression analysis (233), using equations developed by Guy and Hadgraft (246) for release into both sink and non-sink receptors. These equations only apply to systems with all the drug in solution. Table 28 lists the diffusion coefficients of hydrocorti-

sone and hydrocortisone-17-butyrate, derived from Equation 23, as a function of propylene glycol concentration; and Figure 35 shows the goodness of fit between the experimental data and Equation 23. The values of the diffusion coefficients for the two steroids at any given propylene glycol concentration are not very different as can be expected from their similar molecular size and shape. There was however a clear decrease in the diffusion coefficient of each steroid with an increase in propylene glycol concentration. A possible explanation for this observation is that the microscopic viscosities of the gels changed significantly with changes in propylene glycol concentration. This was confirmed by viscosity measurements as shown in Table 29. The effects can therefore be rationalised using the Stoke-Einstein Equation (70 - 72):

$$D = \frac{K\,T}{6\pi\,r\,\eta}$$

where K = the Boltzmann constant

T = absolute temperature

r = hydrodynamic radius of diffusing drug molecule

η = viscosity

Equation 23 is only applicable when the drug is in solution form in the vehicle. No expressions have been derived for the case in which the drug is in suspension form in the semi-solid bases. In these cases, the release profiles would be dependent on whether dissolution of the steroids was rate-limiting.

It may be concluded that the releases of the steroids studied from the propylene glycol-water gels are highly dependent on the composition of the continuous phase of the gel and maximum release may be obtained from vehicles saturated with drug.

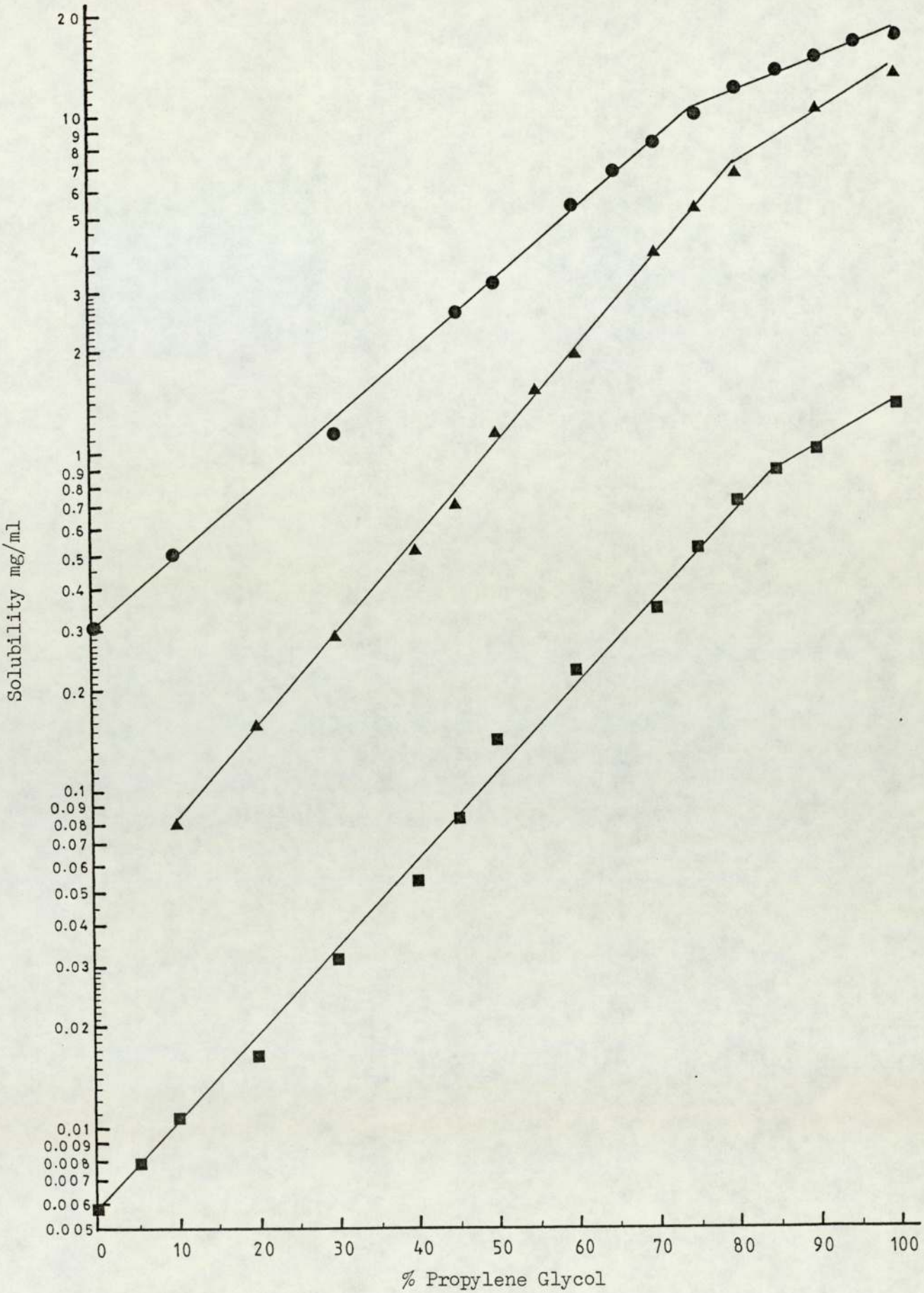


Figure 27. Solubility of hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) as a function of % propylene glycol in aqueous propylene glycol mixtures at 25°C

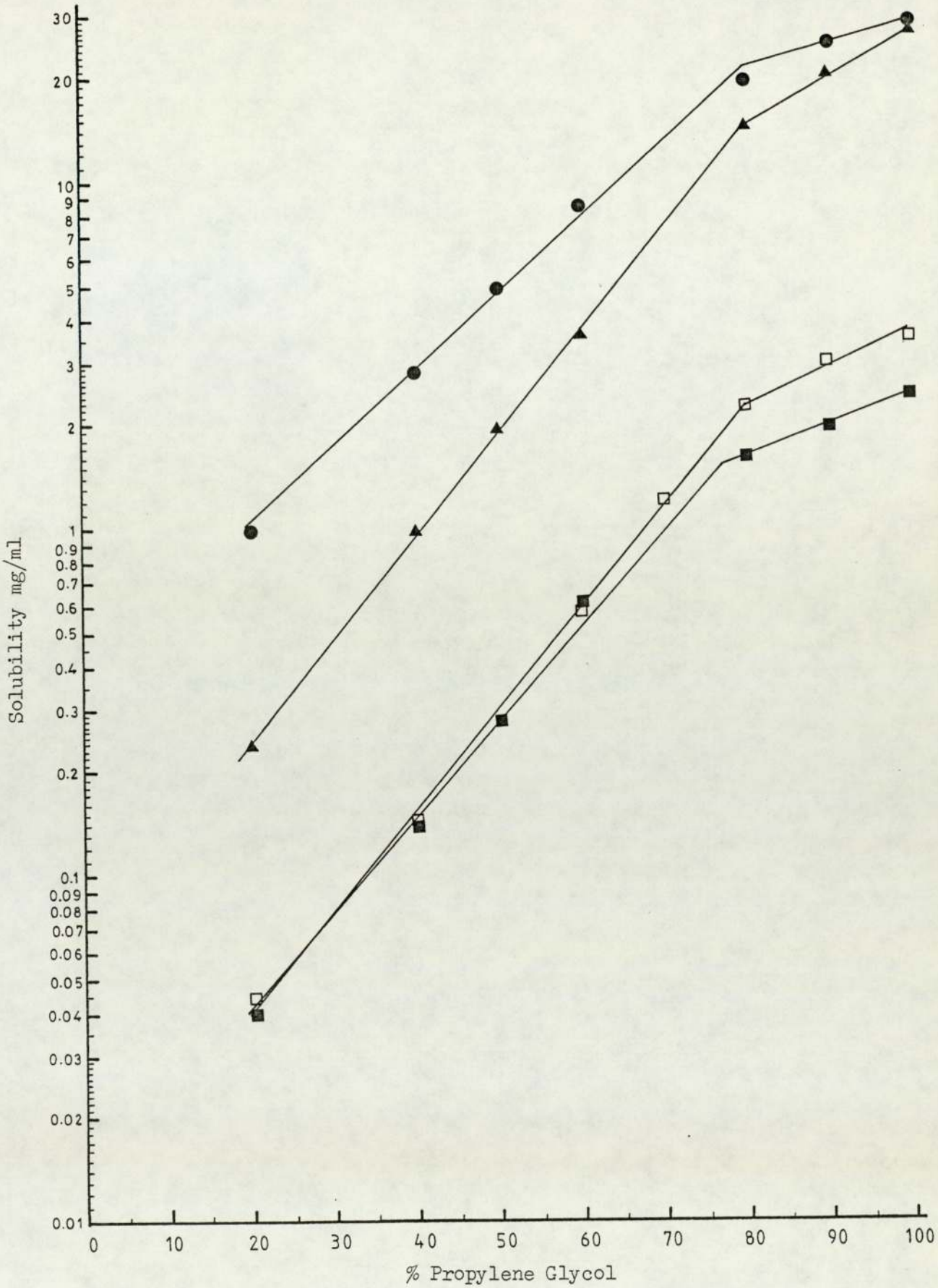


Figure 28. Solubility of hydrocortisone acetate (■), hydrocortisone-21-butyrate (□), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) as a function of % propylene glycol in aqueous propylene glycol mixtures at 37°C

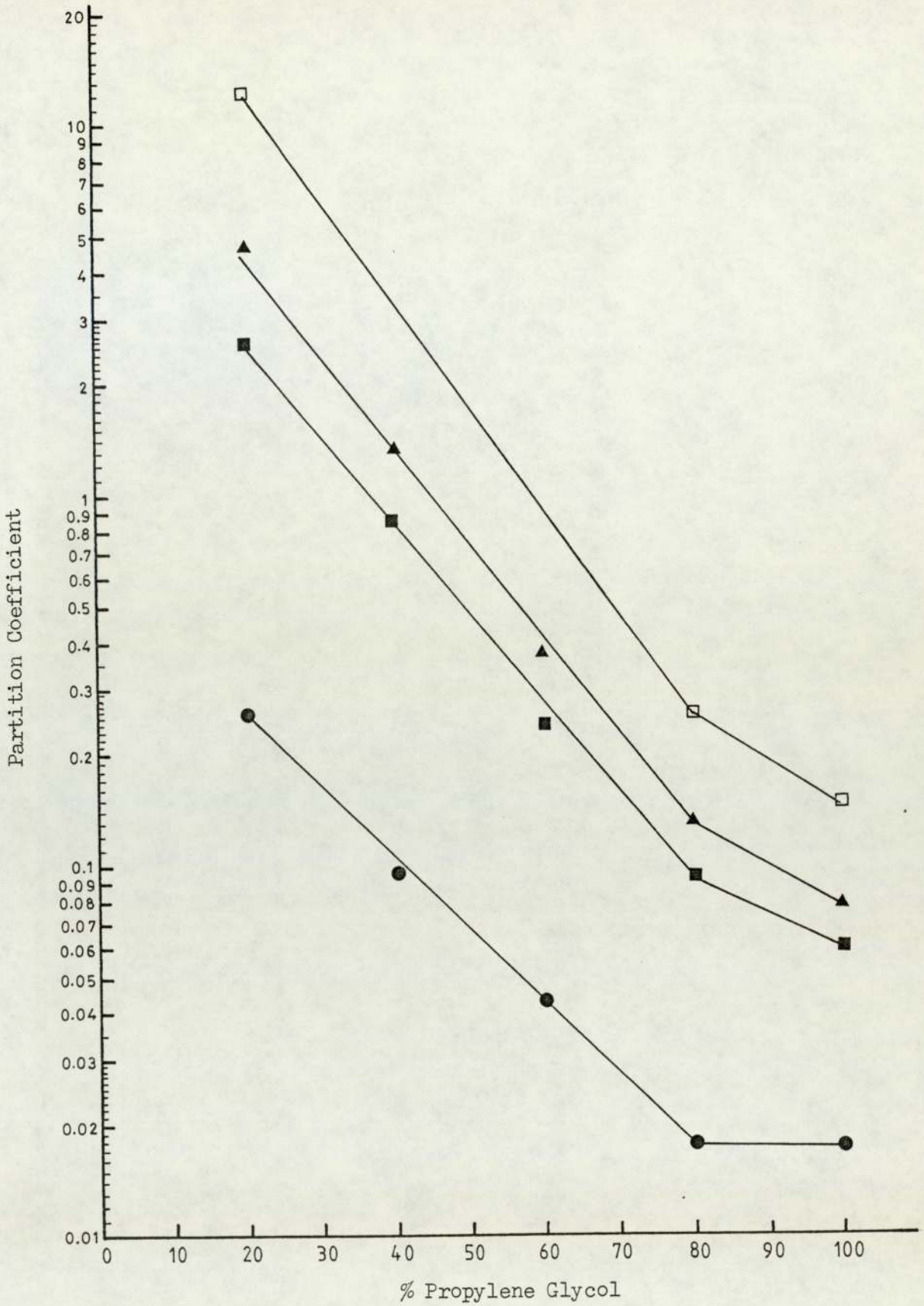


Figure 29. Partition coefficient of hydrocortisone acetate (■), hydrocortisone-21-butyrate (□), hydrocortisone-17-butyrate (▲) and hydrocortisone (●), as a function of % propylene glycol, between isopropyl myristate and propylene glycol-water, at 37°C.

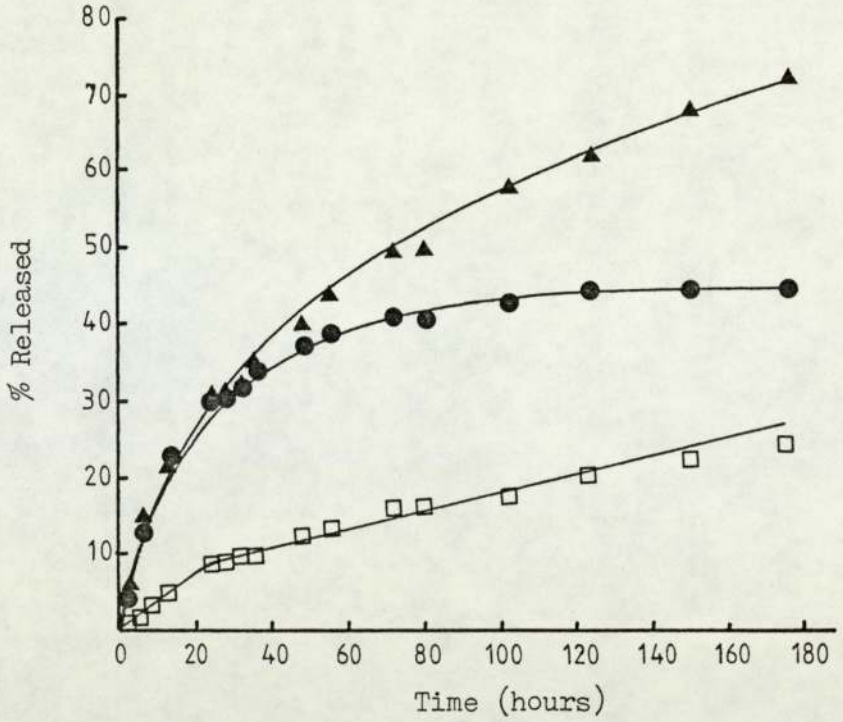
Table 26. Partition coefficient of hydrocortisone, hydrocortisone acetate, hydrocortisone-17-butyrate, and hydrocortisone-21-butyrate between aqueous-propylene glycol and isopropyl myristate at 37°C

Steroids	% P G	Initial conc. (mg/ml)	Partition Coefficient		
			Determined individually*	Determined in mixture*	Determined by kinetic method$^{\Delta}$
H	20	0.02	3.92	4.08	
	40	0.10	10.65	10.25	
	60	0.20	23.62	23.20	
	80	0.20	56.40		52.6; 86.0
	100	0.50	51.70		
	100	1.00	56.50	52.30	34.0; 47.4
HA	20	0.02	0.43	0.40	
	40	0.10	1.18	1.18	
	60	0.20	4.11	4.25	
	80	0.20	10.69	11.10	10.6; 10.7
	100	0.50	18.71	16.41	
	100	1.00		16.18	14.4; 15.3
H-17-B	20	0.02	0.21	0.21	
	40	0.10	0.74	0.75	
	60	0.20	2.67	2.78	
	80	0.20	7.37	7.80	7.0; 7.14
	100	0.50	14.10	12.79	
	100	1.00		12.86	11.6; 13.0
H-21-B	20	0.05	0.083		
	80	0.40	3.9		
	100	0.50	6.5		7.79

* average of 3 replicates
Δ ratio of rate constants (k_2/k_1) from the three-phase partitioning model studies

H = hydrocortisone
HA = hydrocortisone acetate
H-17-B = hydrocortisone-17-butyrate
H-21-B = hydrocortisone-21-butyrate

(a)



(b)

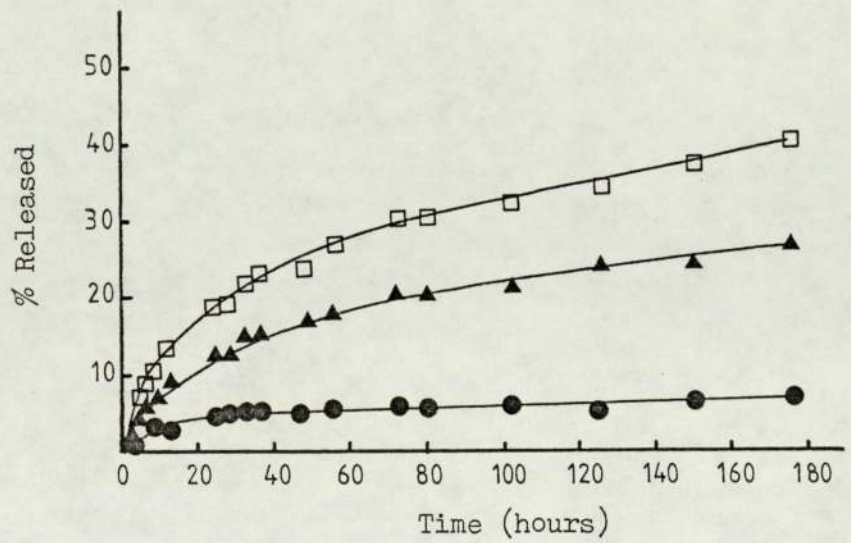


Figure 30. Release profiles for hydrocortisone-21-butyrate (□), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from aqueous propylene glycol Carbopol gels containing 0.1 %w/w of each steroid, at 37°C, under non-sink conditions.

(a) 20% propylene glycol-water

(b) 80% propylene glycol-water

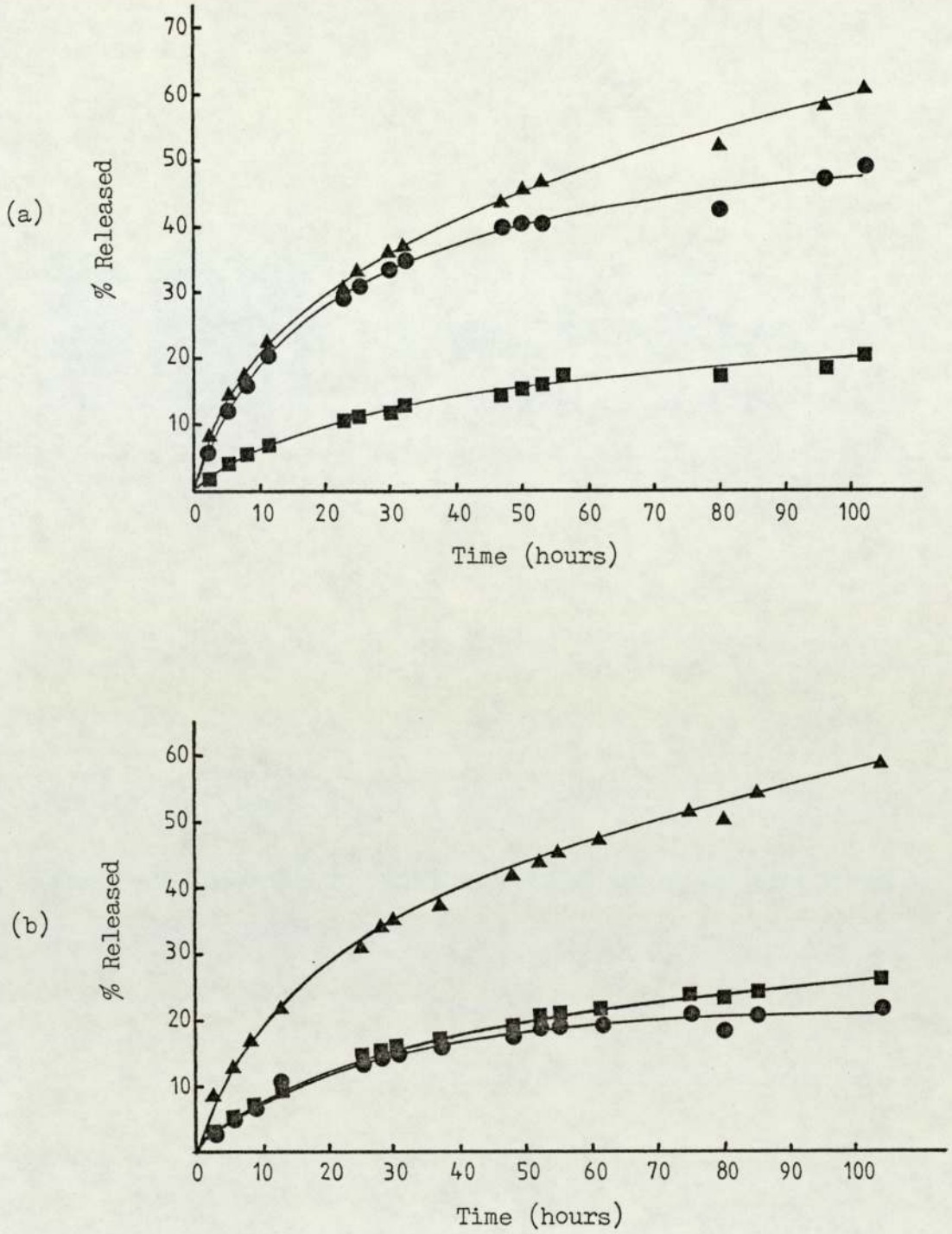
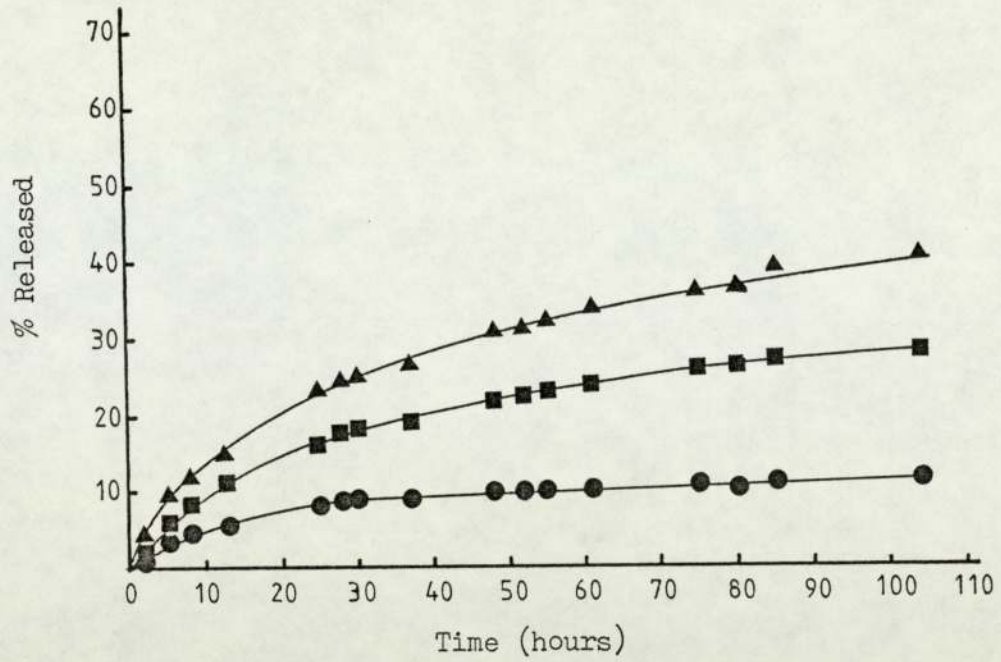


Figure 31. Release profiles for hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from aqueous propylene glycol Carbopol gels containing 0.1% w/w of each steroid, at 37°C, under non-sink conditions.

(a) 20% propylene glycol-water
(b) 40% propylene glycol-water
(c) 60% propylene glycol-water
(d) 80% propylene glycol-water

(c)



(d)

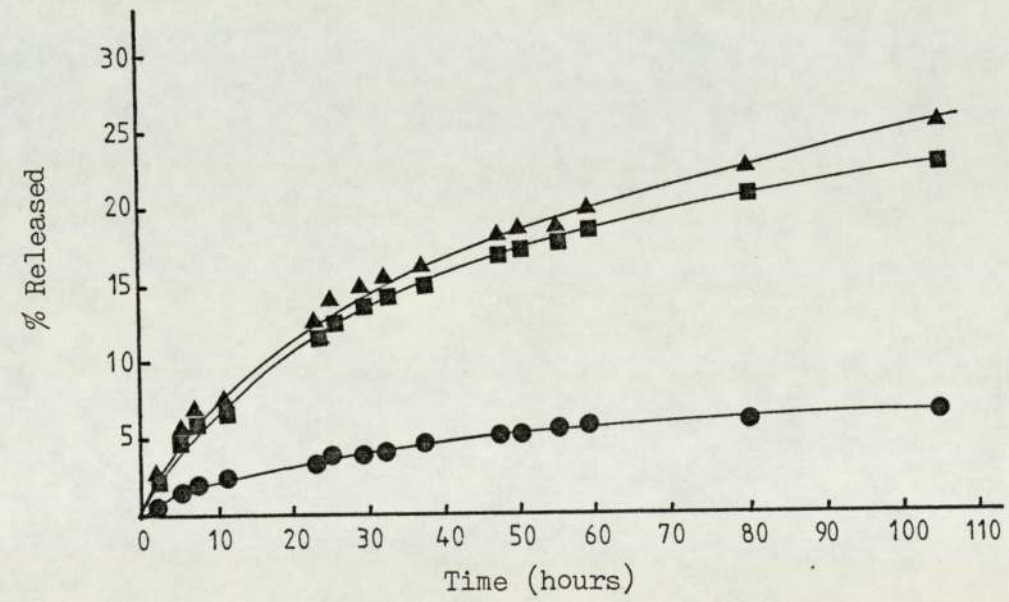


Figure 31. cont'd

(c) 60% propylene glycol-water

(d) 80% propylene glycol-water

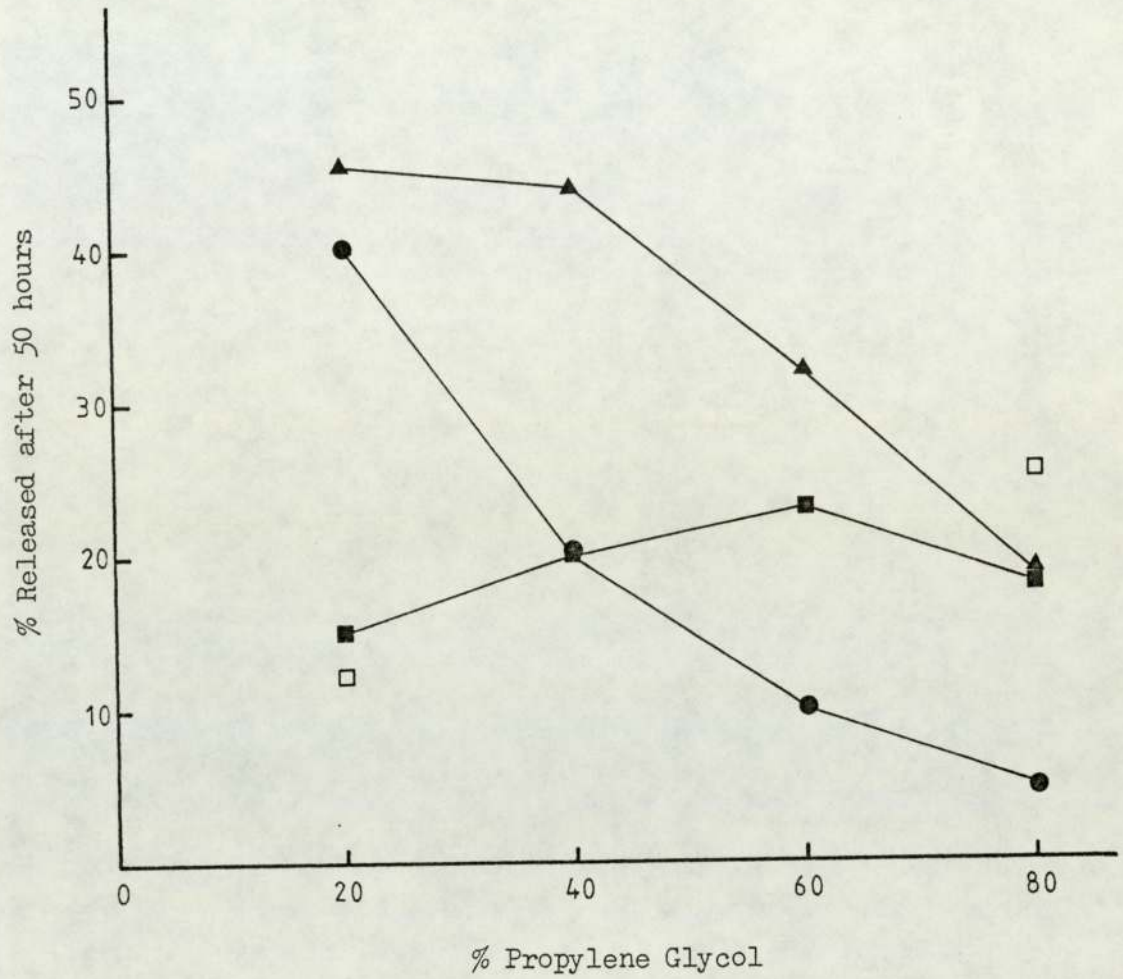


Figure 32. Effect of propylene glycol concentrations on the release of hydrocortisone acetate (■), hydrocortisone-21-butyrate (□), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from aqueous propylene glycol Carbopol gels, at 37°C, under non-sink conditions.

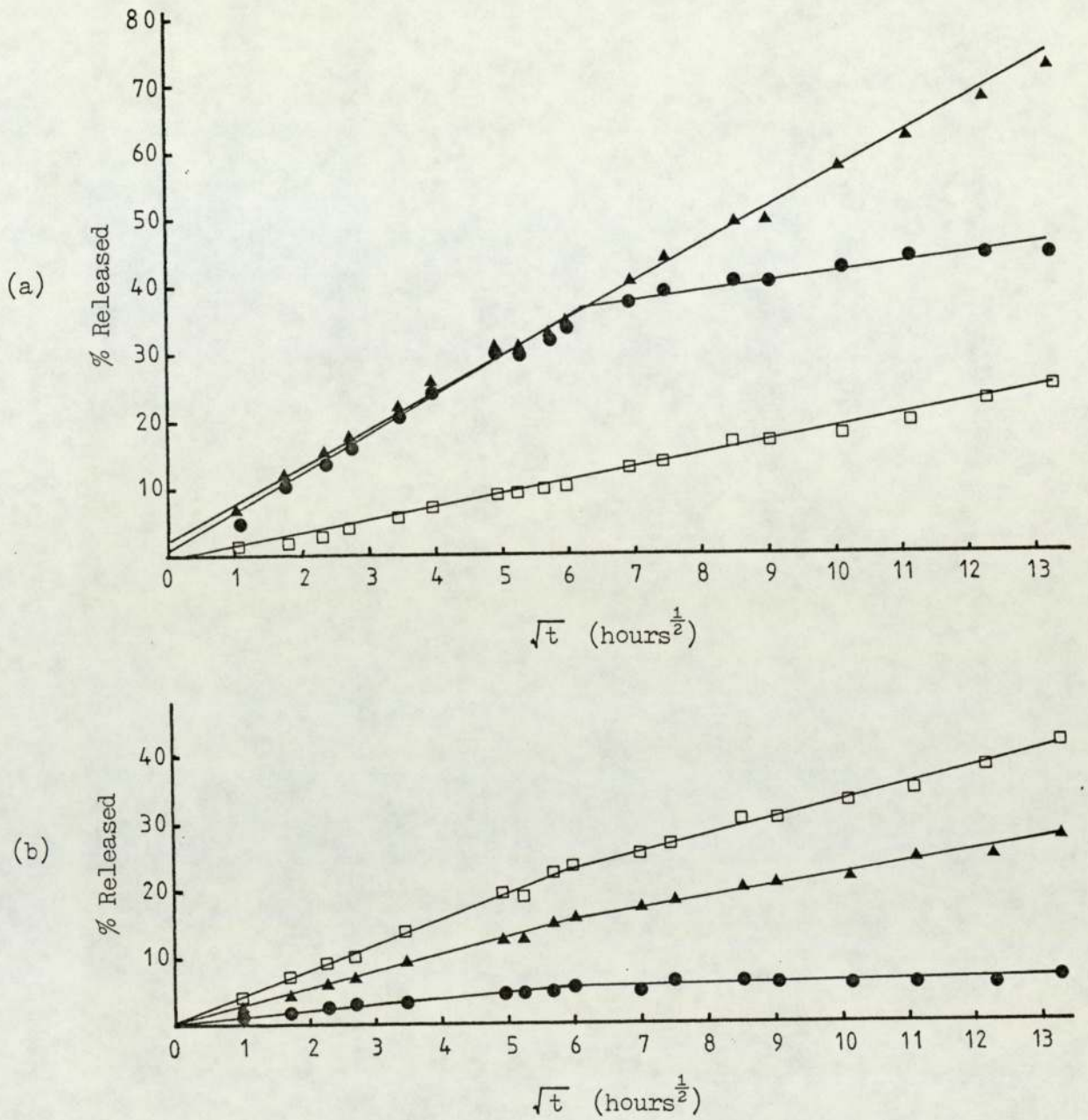
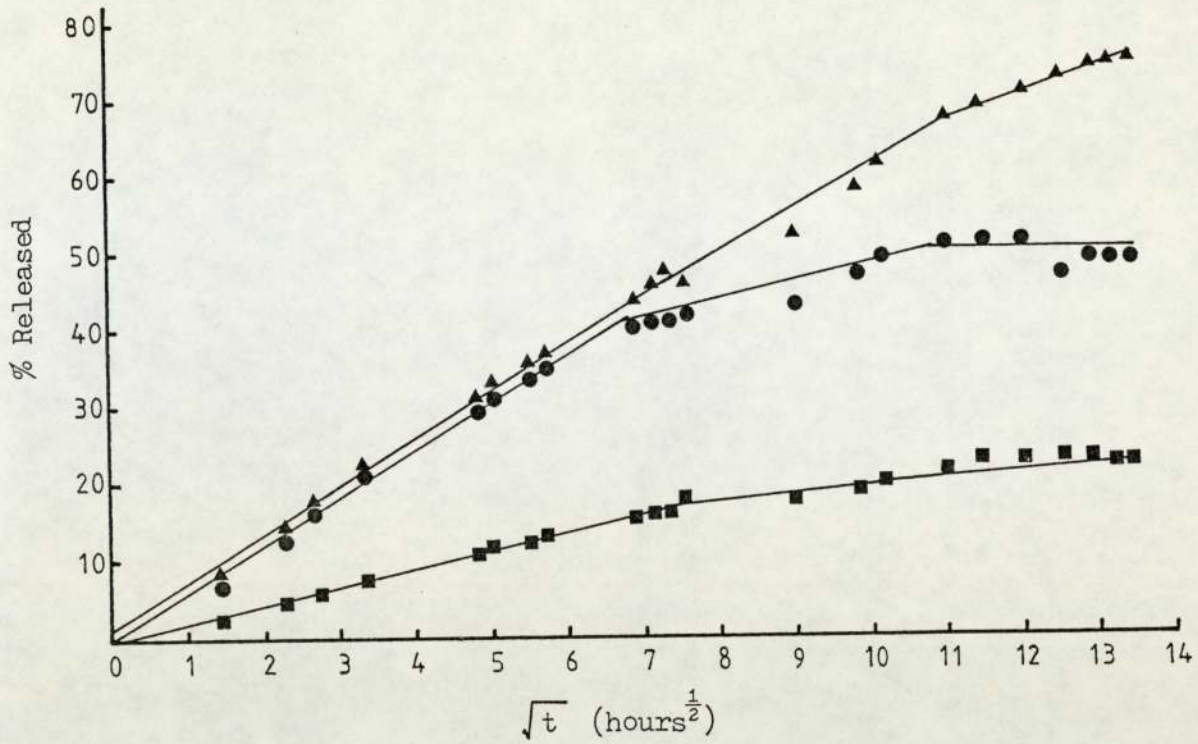


Figure 33. Plot of % released versus square root of time, t, for hydrocortisone-21-butyrate (□), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from aqueous propylene glycol Carbopol gels containing 0.1% w/w of each steroid, at 37°C.

(a) 20% propylene glycol-water

(b) 80% propylene glycol-water

(a)



(b)

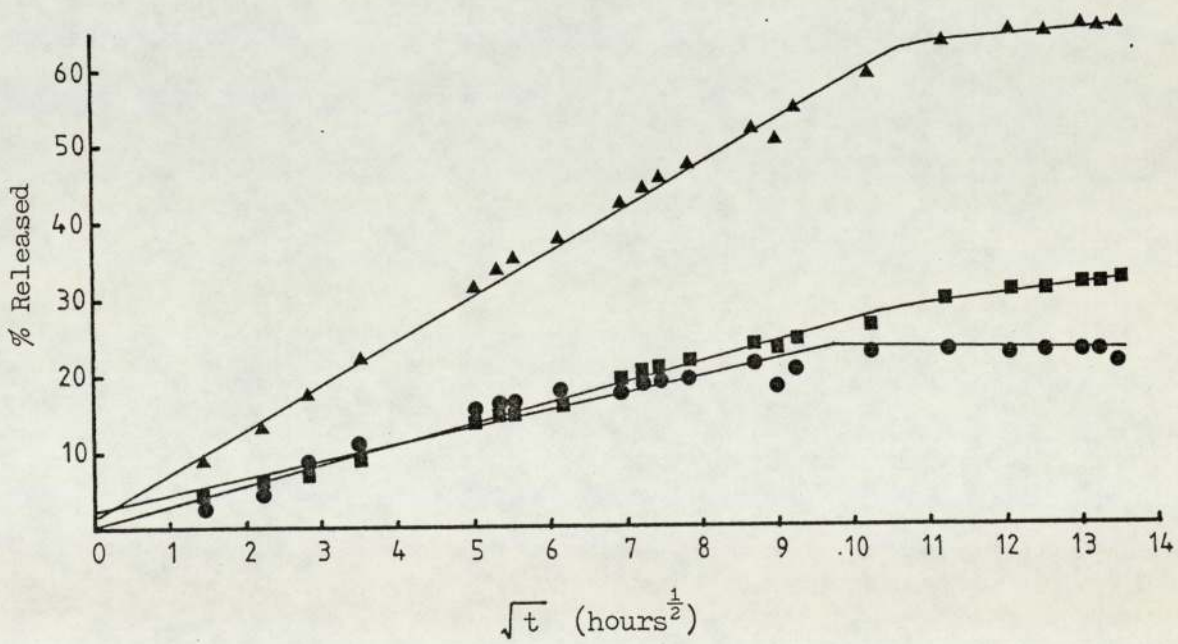
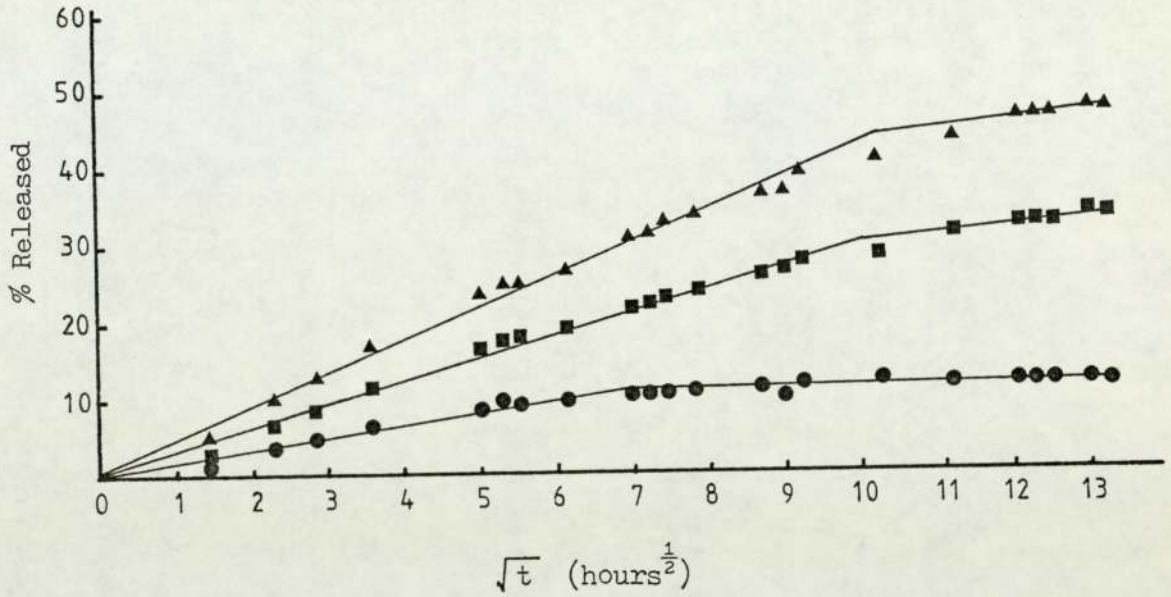


Figure 34. Plot of % released versus square root of time, t, for hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲), and hydrocortisone (●) from aqueous propylene glycol Carbopol gels containing 0.1% w/w of each steroid, at 37°C.

(a) 20% propylene glycol-water
(b) 40% propylene glycol-water

(c)



(d)

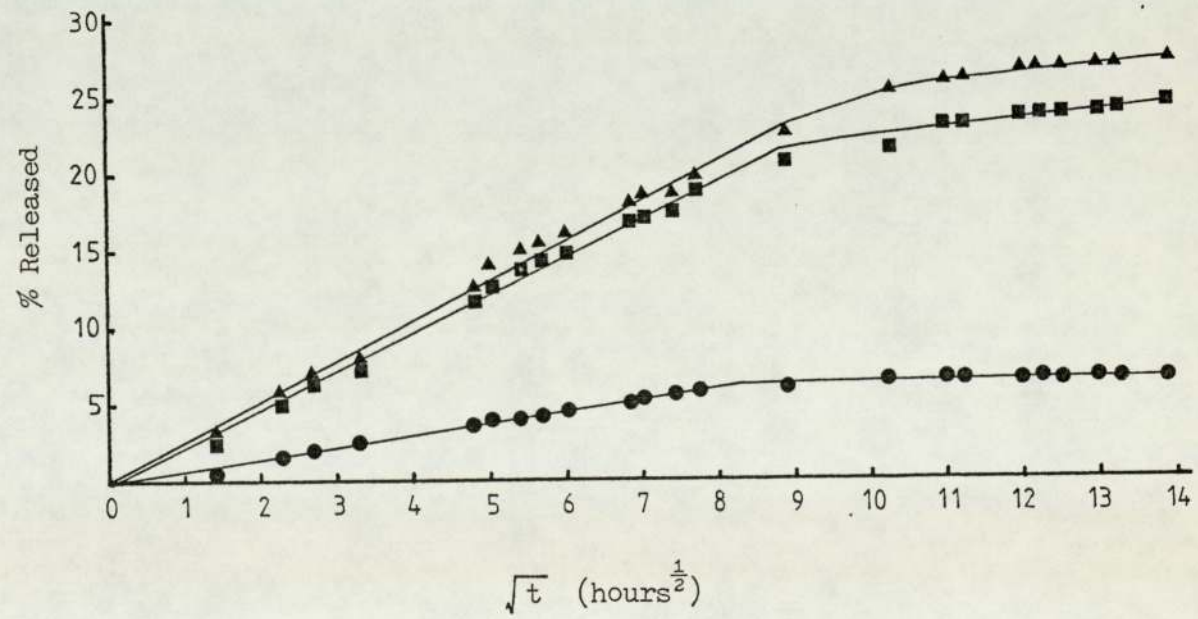


Figure 34. cont'd

(c) 60% propylene glycol-water

(d) 80% propylene glycol-water

Table 27. Solubility of steroids in isopropyl myristate.

Steroids	*Solubility (mg/ml)	
	25°C	37°C
Hydrocortisone	0.223 ± 0.007	0.241 ±0.009
Hydrocortisone acetate	0.0766 ± 0.0012	0.083 ± 0.002
Hydrocortisone-17-butyrate	0.814 ± 0.01	1.154 ± 0.007

* mean of three replicates

Table 28. Diffusion coefficients of steroid mixtures in Carbopol gels under non-sink condition.

% Propylene glycol contained in gels	Diffusion Coefficient (cm^2/hr)	
	Hydrocortisone	Hydrocortisone-17-butyrate
20	77.0×10^{-4}	-
40	20.2×10^{-4}	48.6×10^{-4}
60	7.03×10^{-4}	31.5×10^{-4}
80	2.23×10^{-4}	14.5×10^{-4}

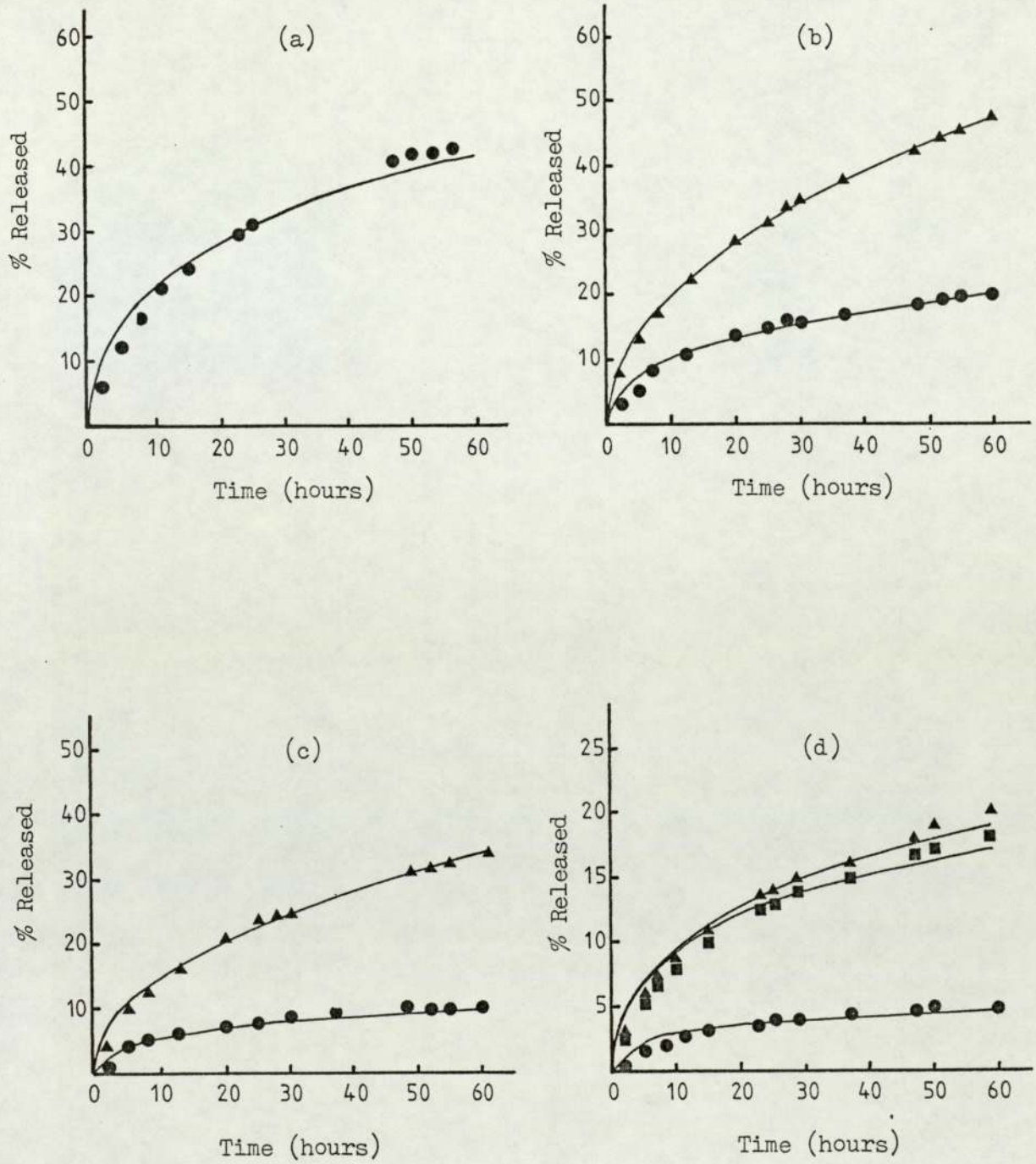


Figure 35. Release profiles for hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲), and hydrocortisone (●) from aqueous propylene glycol Carbopol gels, at 37°C, under non-sink conditions.

Theoretical line using Equation 23 ——

Experimental points ■, ▲, ●

(a) 20% propylene glycol-water
(b) 40% propylene glycol-water
(c) 60% propylene glycol-water
(d) 80% propylene glycol-water

Table 29. Viscosity of aqueous propylene glycol at 23°C.

% PG	Relative Viscosity			
	vs. water	vs. silicone-10 cs	vs. silicone-20 cs	Mean±standard deviation
20	1.908	1.839	2.039	1.93 ± 0.1
40	4.071	3.924	4.350	4.12 ± 0.22
60	8.052	7.762	8.603	8.14 ± 0.43
80	19.127	18.44	20.43	20.43 ± 0.1
100	24.75	23.86	26.44	25.02 ± 1.3

PG = propylene glycol

4.4.2 Three-Phase Partitioning Model

This model was developed to provide an in-vitro model for comparing the transport properties of the steroids used in the present study. It is hoped that such a model may help to overcome the problem presented by the variability of skin in the widely used skin penetration model. It is a modification of the in vitro systems which employs a liquid lipid phase to simulate the skin. IPM was used to simulate the skin lipid and its thickness can be varied or controlled by the volume added. The drug released into the lipid is removed by the receiving phase so that the lipid barrier serves as a transfer-barrier. By choosing suitable vehicle combinations in these three phases, the model can be made useful for studying skin penetration and factors such as vehicle effects, which influence it.

The steroids studied are water insoluble and propylene glycol a commonly used cosolvent was chosen for dissolving steroids. Various aqueous propylene glycol mixtures were used for studying vehicle effects. For the receiving phase, propylene glycol was chosen due to the relatively high solubilities of the steroids in it and its immiscibility with IPM. Unfortunately, in an experiment using 80% propylene glycol-water as donor phase (A phase) and 100% propylene glycol as receiving phase (C phase), a transfer of the vehicles between the donor and the receiving phases was observed. At the end of the experiment (about 195 hours), it was found that the C phase was much lower than A phase. In order to confirm that the transfer of solvents between phase A and C took place the water content of both phases was determined by the Karl Fischer Method. Results showed that C phase (pure propylene glycol originally) contained 7.8% water and A phase (80% propylene glycol initially) contained 10.9% water. This indicated that both propylene glycol and water cross-transferred.

Identical solvents must therefore be used for both A and C phases. If non-identical solvent were employed, it must be ascertained that no transfer took place or that the solvents used for the A and C phases were totally insoluble in each other. There is ample evidence to show the importance of the partition coefficient of a drug between the skin and vehicle in percutaneous absorption. The transfer rate of the drug in this model is also dependent on the partitioning of the drug between the vehicle and the lipoidal barrier. The kinetics of transfer can be shown by the following scheme:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C$$

The reaction profiles are shown in Figures 36 - 42 and the solvent systems, steroid mixtures, computated rate constants and statistical parameters are summarized in Table 30. When A and C phases are identical, equal amounts of the drug are as expected, observed in the A and C phases at equilibrium (Figures 36 - 40); k_1 is then equal to k_4 and k_2 is equal to k_3. The concentration of the steroid in the B phase is less than 5% for hydrocortisone-17-butyrate, hydrocortisone acetate, hydrocortisone-21-butyrate and cortisone acetate, and about 1% for hydrocortisone and prednisolone. The reproducibility of the results can be seen in Figure 36 and Figure 40, in which duplicates were run at the same time. The presence of the other steroids does not affect the transfer rate. The transfers of hydrocortisone-17-butyrate on its own (System II) and in the presence of hydrocortisone acetate and hydrocortisone (System I) were compared (Table 30). In the presence of hydrocortisone acetate and hydrocortisone, the k_1 and k_2 values (0.0465 and 0.540 hr^{-1} respectively) for hydrocortisone-17-butyrate are practically identical to those (0.0439 and 0.571 hr^{-1}) obtained in a system containing only the 17-butyrate. This is further supported

by the fact that the partition coefficient was not affected by the presence of other steroids.

The rate constants were obtained by non-linear regression analysis using the integrated form of the rate equations (Equations 28 - 33). The ratios of k_2/k_1 and k_3/k_4 give the partition coefficients for A/B, and C/B phase respectively. In fact, apart from the determination of drug transfer, this model can be used for determining partition coefficient (183). The partition coefficients calculated by the ratios of the rate constants were compared to those measured experimentally (Table 30). For hydrocortisone-21-butyrate, the partition coefficients calculated by kinetic rate constants are virtually the same as the measured ones. For hydrocortisone, there are big variations between experiments. Since the concentration of hydrocortisone in IPM is so small, slight differences will cause large changes in the ratios of the rate constants. The problems associated with the precision of the assay for hydrocortisone, is reflected in the partition coefficient obtained by both the kinetic and static methods. In all solvent systems studied, the transfer rate (k_1) of hydrocortisone-17-butyrate is slightly higher than hydrocortisone acetate, but much higher than hydrocortisone, an observation which is related to their affinities for the lipid barrier. Hydrocortisone-17-butyrate and hydrocortisone acetate have similar partition coefficients (12.8 and 16.4 respectively between propylene glycol and IPM; and 7.8 and 11.1 between 80% propylene glycol-water and IPM) whereas the partition coefficients of hydrocortisone are much larger (56.5 for propylene glycol/IPM; and 56.4 for 80% propylene glycol-water/IPM).

System III is a comparison of the transfer of hydrocortisone and its 17- and 21-esters. It is known that the 17-ester is the most active form and one possibility is that the 17-ester is capable of penetrating the skin faster. However, in this study, it was found that the 21-ester partitioned into the receiving phase faster than the other two steroids (Table 30). The difference between hydrocortisone-17-butyrate and hydrocortisone-21-butyrate are quite significant (Figure 38). To gain more insight into the kinetics of partitioning of topical steroids, their behaviours were compared with steroids which are usually formulated for oral administration. Cortisone acetate and prednisolone were chosen as representative samples of the latter group. From this study, cortisone acetate showed the fastest transfer rate. Prednisolone transferred to the C phase very poorly. In drawing conclusions from these data it is important to note that although the rate of skin permeation is clearly an important determinant in determining activity, other factors such as the drug's molecular structure and its rate of biotransformation by the skin enzymes are also crucial. Additionally, increasing permeation rate does not necessarily increase activity. A too rapid clearance from the skin will reduce the drug's efficacy. The value of the model lies in enabling comparisons to be made so that optimum in-vitro profiles can be defined.

It has been suggested that DMSO enhanced drug penetration (143, 250). Using the vasoconstrictor test, the absorption of fluocinolone acetonide was shown to be enhanced by DMSO (143). In vitro studies with human skin showed that DMSO was about seven times better than water in promoting the penetration of hydrocortisone through skin. Another study showed that the penetration of hydrocortisone into the

skin of the forearm was increased approximately four fold with DMSO (140). One likely mechanism by which DMSO increases penetration of drugs is that DMSO penetrates easily into skin and by 'solvent drag' enhances the penetration of steroids (251). Therefore, an experiment with 5% DMSO incorporated into the receiving phase (System VI) was carried out and compared with that in which 5% DMSO was contained in the donor solvent (System VII). The k_1 values of each steroid in System VI appear to be slightly higher than that in System I (Table 30). The transfer profiles of the three steroids show significant differences (Figures 36 and 41). When DMSO was placed in the donor phase (System VII), the k_1 values of each steroids was similar to that in System I. The transfer profiles of each steroids in both systems were essentially the same (Figures 36 and 42). One would have expected that a lower rate would have been obtained due to the higher affinity of the steroids for DMSO. The k_1, k_2, k_3 and k_4 values in System VI were expected to be comparable to k_4, k_3, k_2 and k_1 in System VII respectively. However, k_3 and k_4 in System VI were nearly twice k_2 and k_1 in System VII respectively, while k_1 and k_2 in System VI are comparable to k_4 and k_3 in System VII. One possible explanation for this is that, apart from the dependence of the partition coefficient between A/B phase, k_1 also depends on the nature of the receiving phase. It is also evident in System V (80% propylene glycol-water/IPM/80% propylene glycol-water). Higher rate constants in System V than that in System I (propylene glycol/IPM/propylene glycol) would be expected due to the lower affinities of the steroids for 80% propylene glycol-water relative to 100% propylene glycol. The results show that the opposite was true (Table 30, Figures 36 and 40). In fact, the main problem, in this model for studying skin absorption, is the reversibility of the reaction between A and C phases. In the skin situation, only the

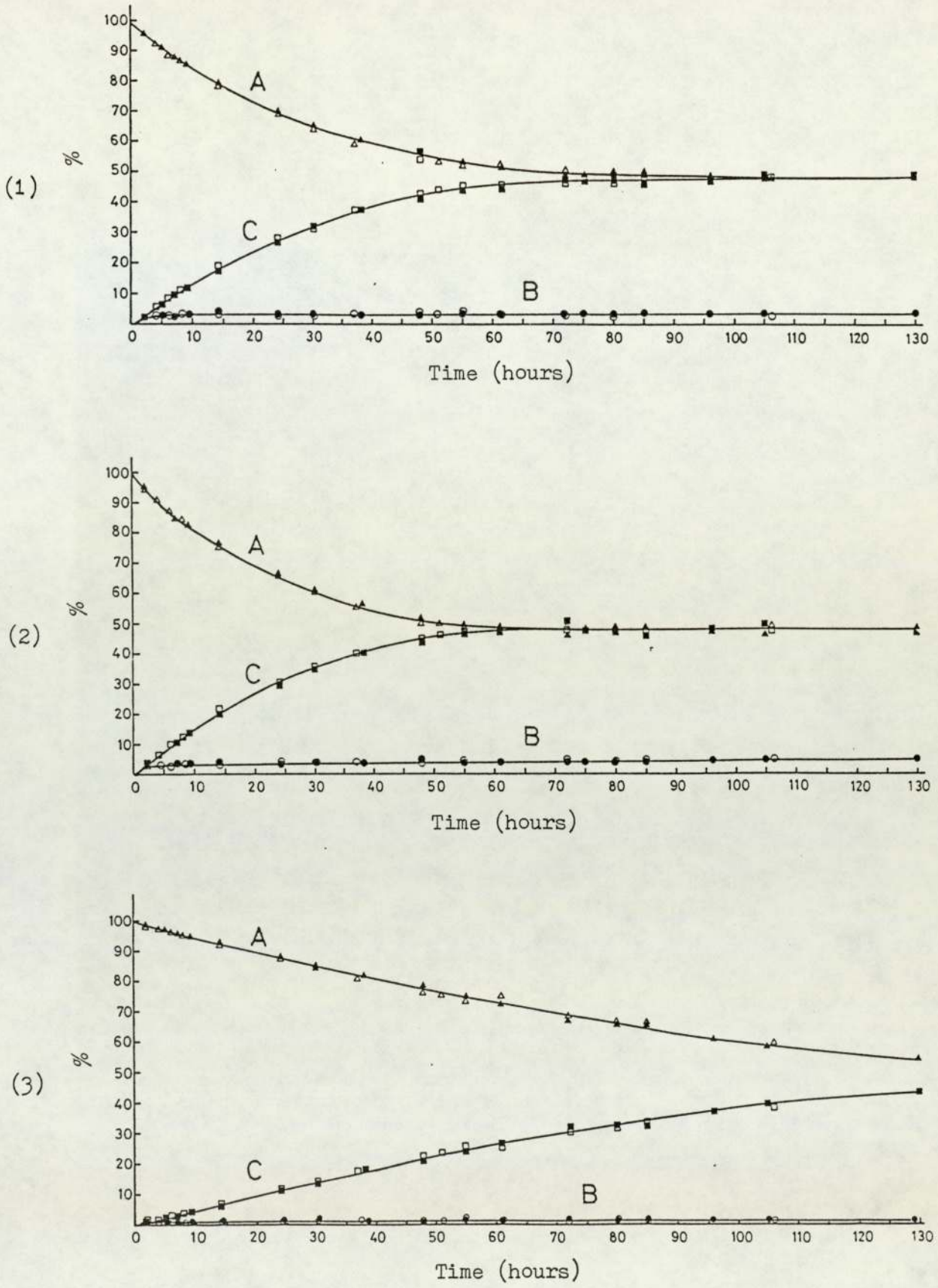


Figure 36. Distribution of hydrocortisone acetate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) in a single three-phase system consisting of propylene glycol (A)/isopropyl myristate (B) /propylene glycol (C), at 37°C.
Initial concentration of each steroid in phase A was 1 mg/ml
Key: ▲, ■, and ●: sample 1 △, □, ○ and : sample 2

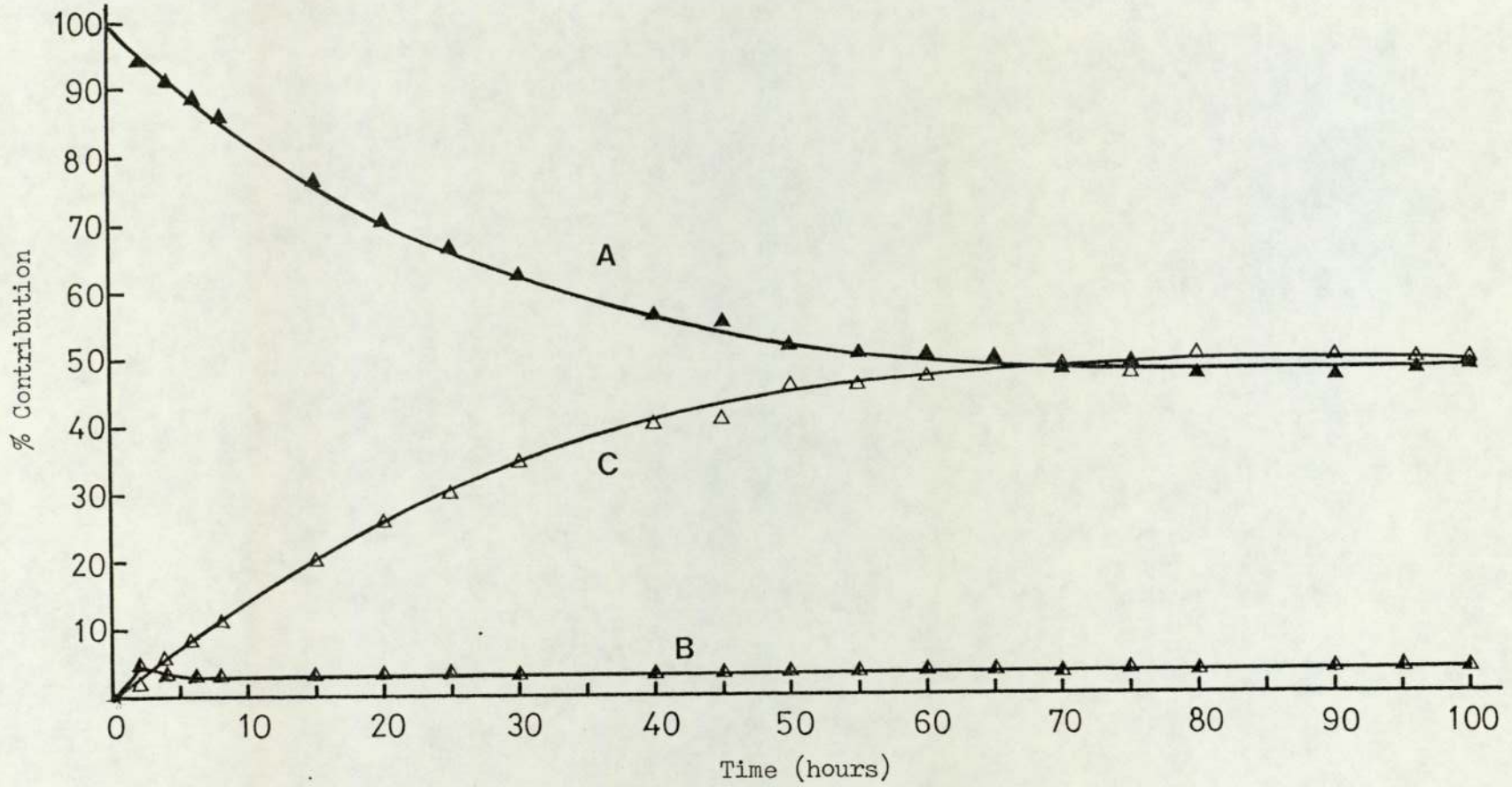
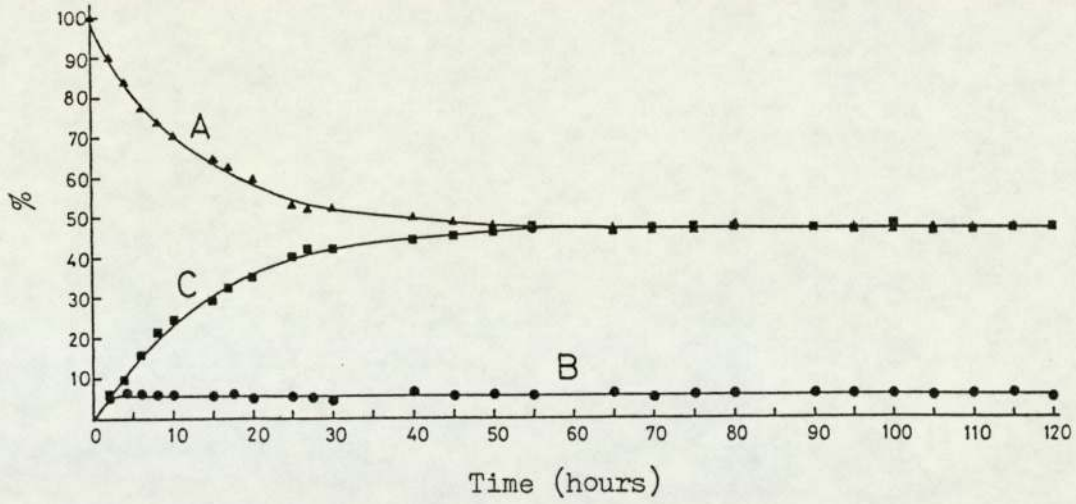
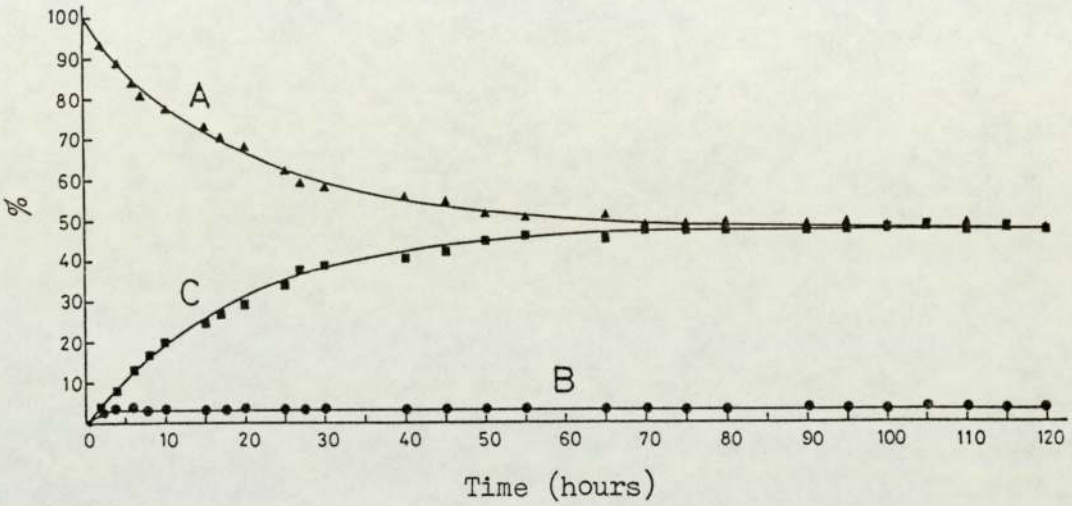


Figure 37. Distribution of hydrocortisone-17-butyrate in a single three-phase system consisting of propylene glycol (A)/isopropyl myristate (B)/propylene glycol (C), at 37°C. Initial concentration of the steroid in phase A was 1 mg/ml.

(1)



(2)



(3)

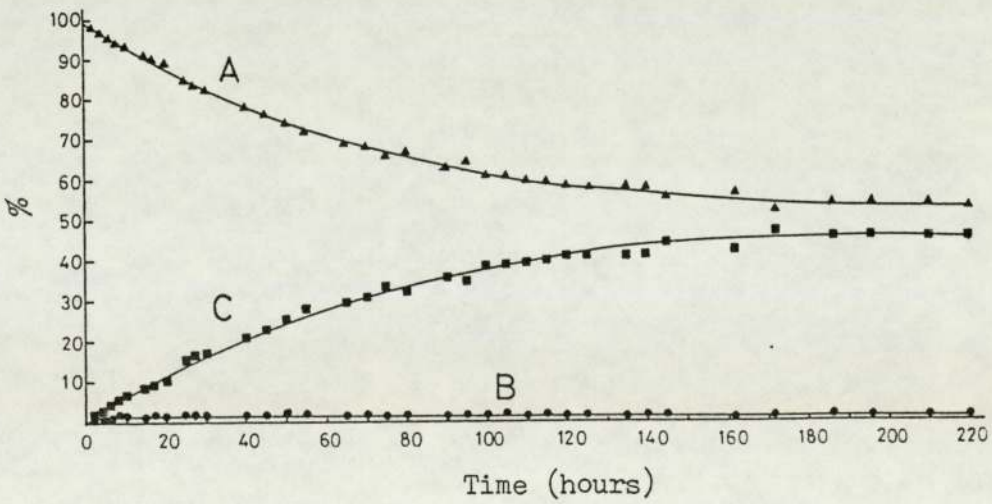


Figure 38. Distribution of hydrocortisone-21-butyrate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) in a single three-phase system consisting of propylene glycol (A)/isopropyl myristate (B) /propylene glycol (C), at 37°C.
Initial concentration of each steroid in phase A was 0.1 mg/ml

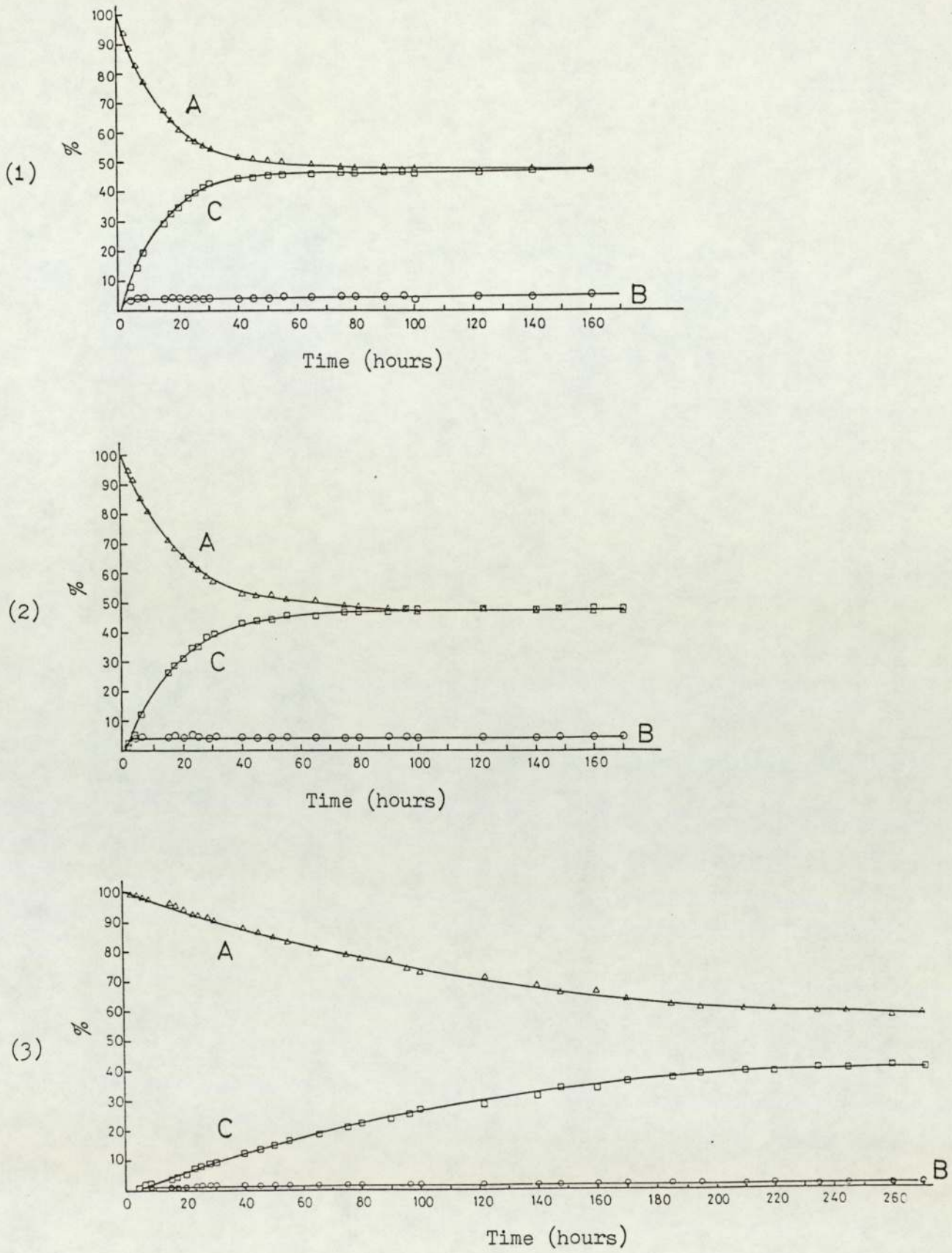
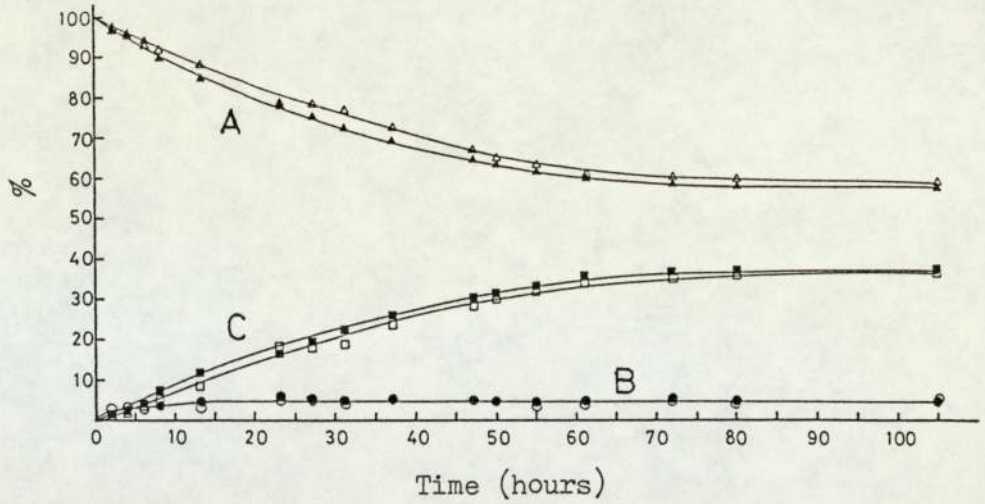
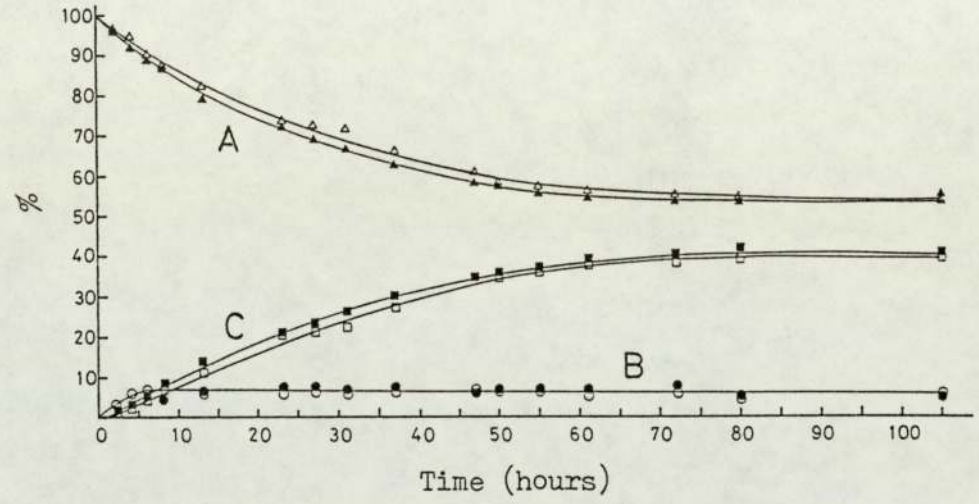


Figure 39. Distribution of cortisone acetate (1), hydrocortisone-17-butyrate (2) and prednisolone (3) in a single three-phase system consisting of propylene glycol (A)/isopropyl myristate (B)/ propylene glycol (C), at 37°C.
Initial concentration of each steroid in phase A was 0.2 mg/ml

(1)



(2)



(3)

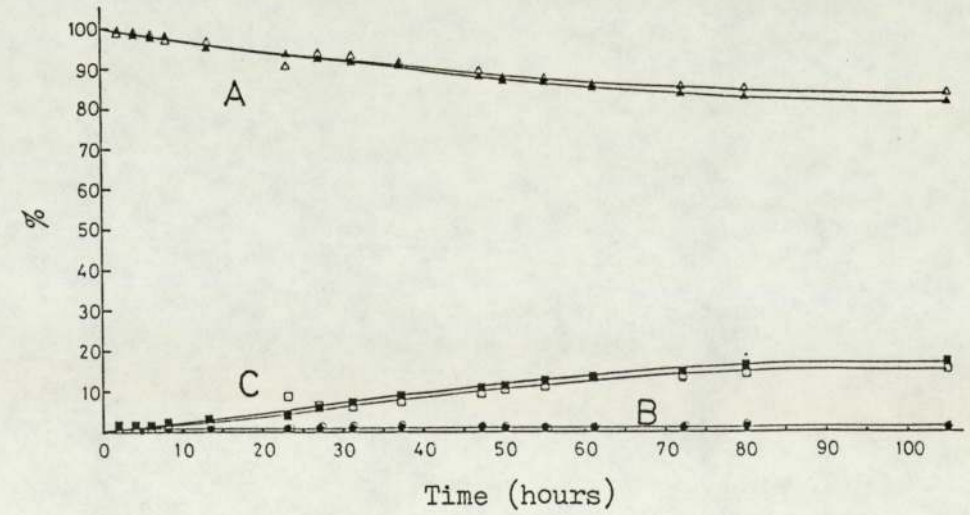
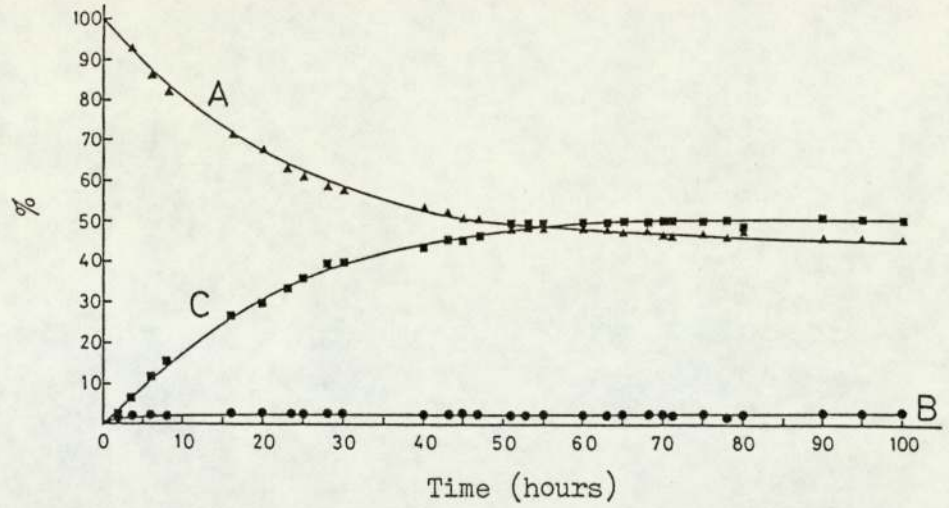
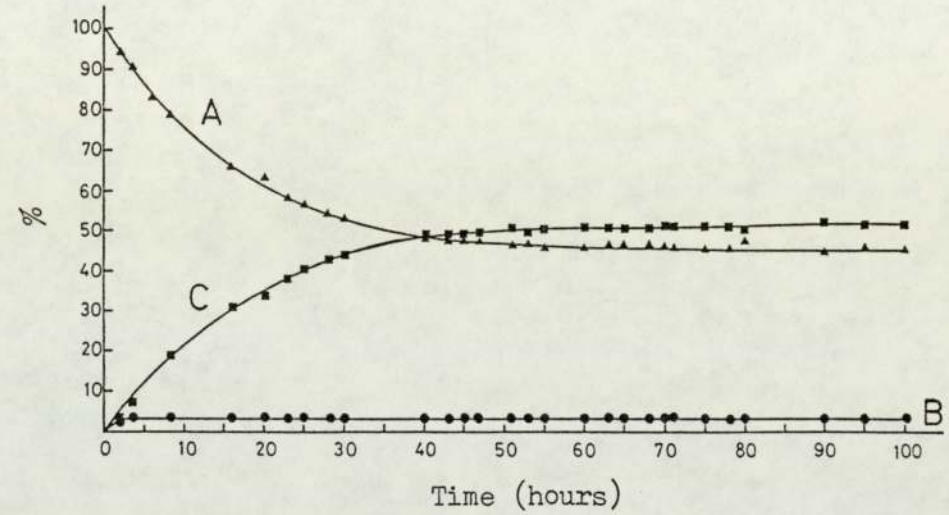


Figure 40. Distribution of hydrocortisone acetate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) in a single three-phase system consisting of 80% propylene glycol-water (A)/isopropyl myristate (B)/80% propylene glycol-water (C), at 37°C. Initial concentration of each steroid in phase A was 1 mg/ml. Key: ▲, ■, and ● : sample 1 △, □, and ○ : sample 2

(1)



(2)



(3)

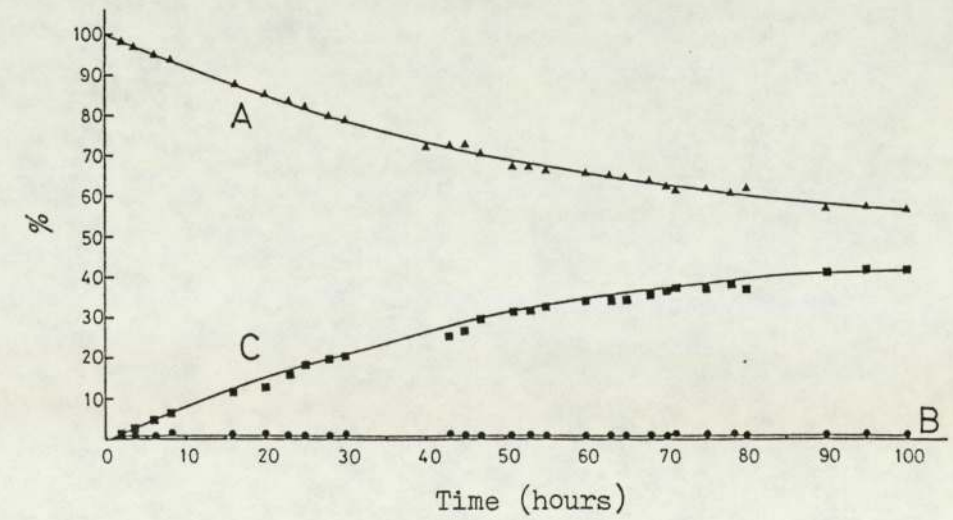
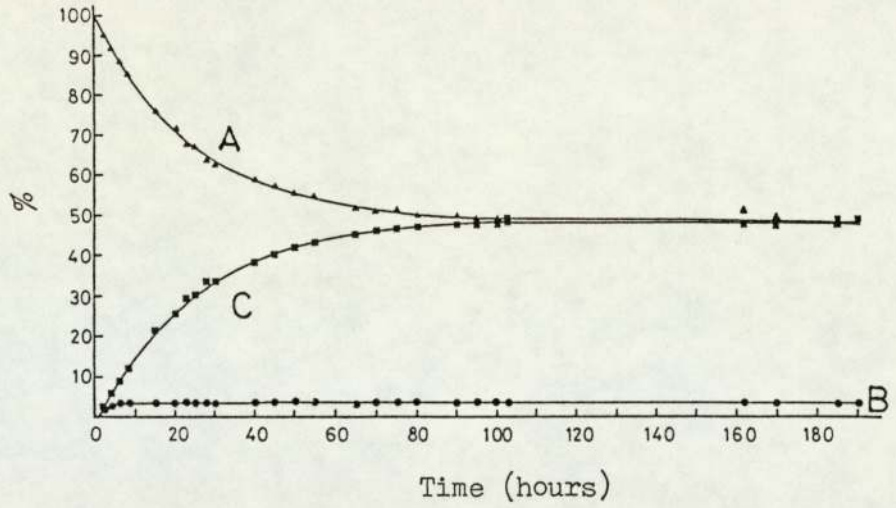
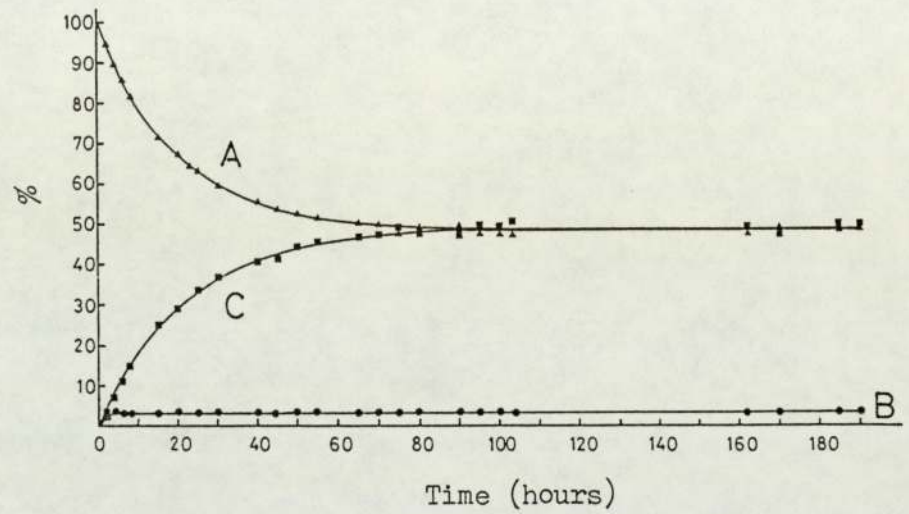


Figure 41. Distribution of hydrocortisone acetate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) in a single three-phase system consisting of propylene glycol (A)/isopropyl myristate (B)/5% DMSO in propylene glycol (C), at 37°C.
Initial concentration of each steroid in phase A was 1 mg/ml.

(1)



(2)



(3)

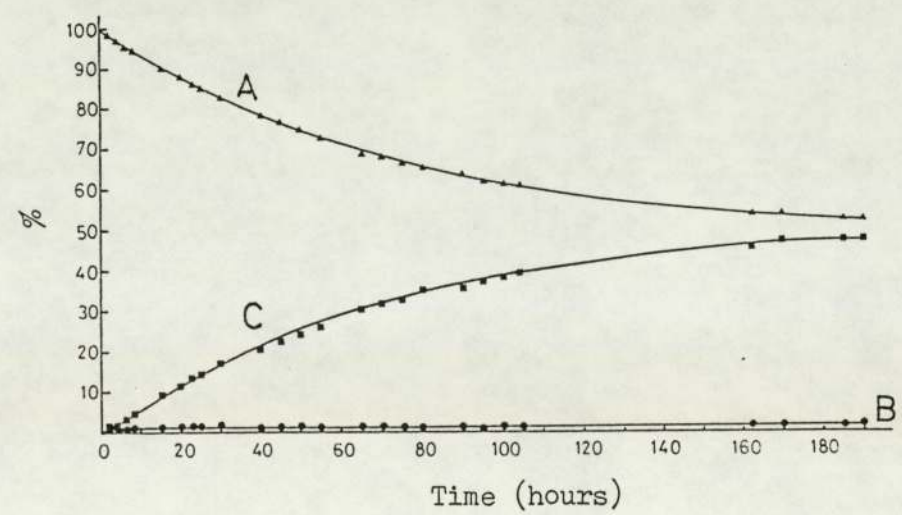


Figure 42. Distribution of hydrocortisone acetate (1), hydrocortisone-17-butyrate (2) and hydrocortisone (3) in a single three-phase system consisting of 5% DMSO in propylene glycol (A)/isopropyl myristate (B)/propylene glycol (C), at 37°C.
Initial concentration of each steroid in phase A was 1 mg/ml.

Table 30. The transfer rate constants of hydrocortisone (H), hydrocortisone-17-butyrate (H17B), hydrocortisone-21-butyrate (H21B), hydrocortisone acetate (HA), cortisone acetate (CA) and prednisolone (P) in the three-phase partitioning model.

Model: $\text{Phase A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Phase B} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{Phase C}$

Solvent system (A/B/C)	Steroid mixture	Initial conc. (mg/ml)	Data set	Steroids	k_1 (hr^{-1}) Mean	k_1 (hr^{-1}) 95% confidence limit	k_2 (hr^{-1}) Mean	k_2 (hr^{-1}) 95% confidence limit	k_2/k_1	Measured partition coefficient (A/B)	Figure No.
I. PG/IPM/PG	HA, H17B, and H	1	1	HA	0.0388	0.0373 - 0.0402	0.561	0.469 - 0.654	14.46	16.2	36
			2		0.0407	0.0394 - 0.0419	0.623	0.530 - 0.716	15.31		
			1	H17B	0.0465	0.0443 - 0.0487	0.540	0.452 - 0.628	11.61	12.8	
			2		0.0490	0.0467 - 0.0513	0.636	0.518 - 0.754	12.98		
			1	H	0.0135	0.0130 - 0.0140	0.459	0.251 - 0.667	34.0	56.5	
			2		0.0130	0.0127 - 0.0133	0.616	0.384 - 0.847	47.38		
II. PG/IPM/PG	H17B only	1		H17B	0.0439	0.0420 - 0.0458	0.571	0.473 - 0.669	13.00	12.8	37
III. PG/IPM/PG	H21B, H17B, and H	0.1		H21B	0.0770	0.0753 - 0.0786	0.600	0.573 - 0.628	7.79	6.5	38
				H17B	0.0515	0.0504 - 0.0525	0.829	0.753 - 0.905	16.10	12.8	
				H	0.0139	0.0137 - 0.0141	0.892	0.599 - 1.185	64.17	56.5	
IV. PG/IPM/PG	CA, H17B, and P	0.2		CA	0.0675	0.0658 - 0.0691	0.716	0.662 - 0.769	10.61	N.D.	39
				H17B	0.0542	0.053 - 0.0553	0.691	0.634 - 0.747	12.75	12.8	
				P	0.00718	0.00705 - 0.00732	0.559	0.286 - 0.883	77.86	N.D.	
V. A= 80% PG-H_2O B= IPM C= 80% PG-H_2O	HA, H17B, and H	1	1	HA	0.0212	0.0203 - 0.0221	0.224	0.186 - 0.263	10.57	11.1	40
			2		0.0194	0.0186 - 0.0202	0.207	0.172 - 0.242	10.67		
			1	H17B	0.0292	0.0279 - 0.0304	0.204	0.179 - 0.229	6.99	7.8	
			2		0.0259	0.0250 - 0.0269	0.185	0.164 - 0.207	7.14		
			1	H	0.005	0.0048 - 0.00519	0.263	0.151 - 0.374	52.6	56.4	
			2		0.0045	0.00424 - 0.00483	0.387	⊖0.009 - 0.783	86.0		

N.D. = not determined

IPM = isopropyl myristate

PG = propylene glycol

DMSO = dimethyl sulphoxide

Table 30. (cont'd)

Model: Phase A $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ Phase B $\underset{k_4}{\overset{k_3}{\rightleftharpoons}}$ Phase C

Solvent system (A/B/C)	Steroid mixture	Initial conc. (mg/ml)	Steroids	k_1 (hr^{-1})		k_2 (hr^{-1})		k_3 (hr^{-1})		k_4 (hr^{-1})		k_2/k_1	k_3/k_4	Measured partition coefficient		Figure No.
				Mean	95% confidence limit	Mean	95% confidence limit	Mean	95% confidence limit	Mean	95% confidence limit			A/B	C/B	
VI.			HA	0.0408	0.0311 - 0.0504	0.678	0.464 - 0.892	1.203	0.727 - 1.680	0.0638	0.0356 - 0.092	16.62	18.86	16.2	N.D	
A = PG	HA, H17B, and H	1	H17B	0.0507	0.0399 - 0.0616	0.692	0.501 - 0.882	1.253	0.777 - 1.730	0.0811	0.0476 - 0.115	13.65	15.45	12.8	N.D.	41
B = IPM																
C = 5% DMSO in PG			H	0.0144	0.00451 - 0.0244	0.709	0.0907 - 1.508	1.410	0.132 - 2.953	0.0311	0.0128 - 0.0751	49.24	45.34	56.5	N.D.	
VII.			HA	0.0375	0.0298 - 0.0452	0.641	0.474 - 0.807	0.722	0.565 - 0.879	0.0424	0.0319 - 0.0530	17.1	17.03	N.D.	16.2	
A = 5% DMSO in PG	HA, H17B, and H	1	H17B	0.0469	0.0386 - 0.0551	0.679	0.532 - 0.827	0.740	0.602 - 0.879	0.0504	0.0399 - 0.0610	14.48	14.68	N.D.	12.8	42
B = IPM																
C = PG			H	0.0119	0.00125 - 0.0225	0.582	(-)0.256 - 1.419	0.961	(-)0.133 - 2.056	0.0225	(-)0.0120 - 0.0570	48.91	42.71	N.D.	56.5	

N.D. = not determined

IPM = isopropyl myristate

PG = propylene glycol

DMSO = dimethyl sulphoxide

forward reaction is of significance since the drug going through is rapidly removed by the circulation. It has also been pointed out (252) that in this model there is no concentration gradient in the middle phase since all phases are stirred.

4.4.3 Penetration through Mouse Skin

While the assessment of the permeation behaviour of steroids using models such as the Schulman cell described in the previous section is useful, it is desirable to include diffusion through skin in the overall assessment of the steroids. Permeation of the steroids through skin was therefore studied. To overcome problems associated with sample to sample variation in the skins used, the steroids compared were studied simultaneously. Solutions were initially used in this part of the study so as to overcome problems associated with vehicle effects. The aim was therefore to investigate the relative permeabilities of the steroids through mouse skin.

Figure 43 shows the penetration profile for hydrocortisone, and its 21-acetate and 17-butyrate through mouse skin under identical conditions. It can be seen that following a lag period, there was steady-state permeation for all three steroids. The permeation profile indicates that the rate-controlling step was the skin barrier and the rate equation can therefore be expressed by:

$$\frac{dQ}{dt} = \frac{D\ (PC)\ C_v}{h} \qquad \text{Eq. 8}$$

where Q = amount transferred per unit area

D = the diffusion coefficient of the drug in the barrier

C_v = concentration of the drug in the vehicle

PC= partition coefficient of the drug between skin/vehicle

h = thickness of skin

t = time

For the equation to hold, only the initial portion of the curve should be analysed. Chloroform was used as the sink receptor, since the steroids used are quite soluble in it (Table 31). Hydrocortisone acetate clearly permeated more rapidly than the 17-butyrate which in turn crossed the skin faster than hydrocortisone. From Equation 8 it is clear that the partition coefficient should be the determinant if the equation is obeyed. Examination of the partition coefficients of these three steroids between IPM and propylene glycol-water showed that the slower permeation rate of hydrocortisone could be expected. The relative rates between the 17-butyrate and 21-acetate were different to those expected if one assumes that the IPM and aqueous propylene glycol partition coefficients can be directly related to the skin-propylene glycol coefficients. This of course is not necessarily the case and the results are therefore not incompatible with existing theories. The diffusion coefficients of these three steroids can be taken as being constant owing to the small variation in their molecular weights. As indicated by Higuchi (70), the relationship between the diffusion coefficient and the molecular weight is a cube root relationship.

To optimise release profiles, it is essential to have reliable solubility values for the steroids in the bases used. This is illustrated by the data shown in Figure 44. The same three steroids (hydrocortisone and its 17-butyrate and 21-acetate) were investigated in a donor system consisting of 40% aqueous propylene glycol. Unlike the 100% propylene glycol system, in this case, mixed solution/suspension systems are present. Hydrocortisone acetate remains a suspension throughout the study period. Hydrocortisone remains as a solution in the entire period while the 17-butyrate changes from a suspension to a solution system at mid-point during the release. The dramatic

effects are seen as the reversal in the relative profiles of hydrocortisone and its acetate (Figure 44). These experiments therefore clearly demonstrate the critical effects which formulation can exert on the permeation of drugs through skin.

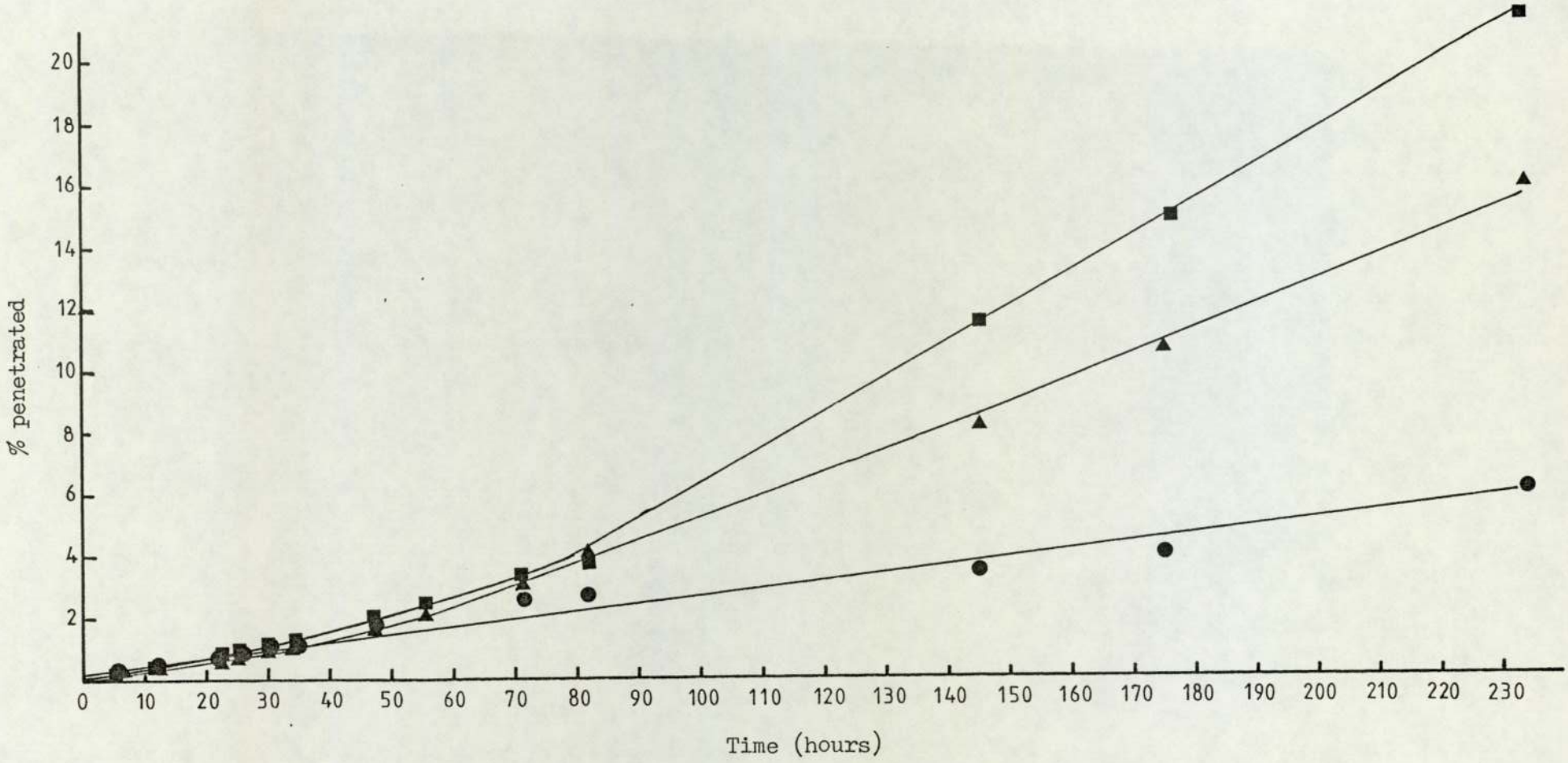


Figure 43. Penetration of hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from propylene glycol (0.1% w/v of each steroid), through mouse skin into chloroform, at ambient temperature.

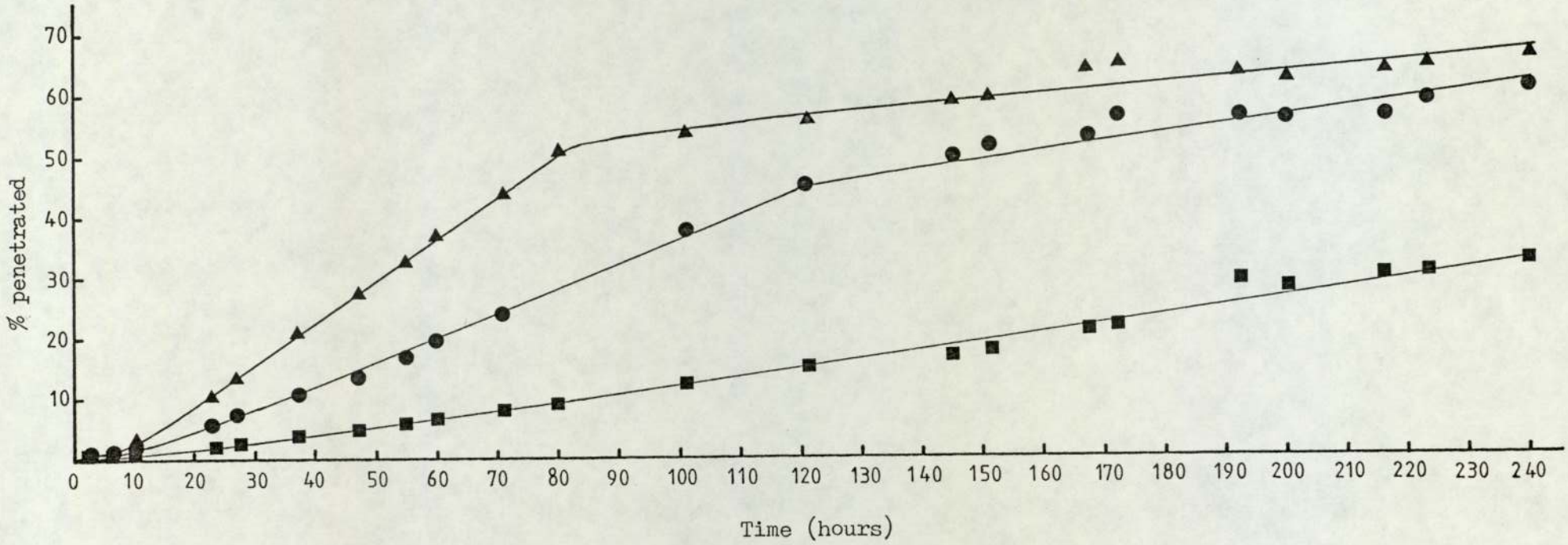


Figure 44. Penetration of hydrocortisone acetate (■), hydrocortisone-17-butyrate (▲) and hydrocortisone (●) from a 40% propylene glycol-water mixture (0.1% w/v of each steroid) through mouse skin into chloroform, at ambient temperature.

Table 31. Solubility of steroids in chloroform at 25°C.

Steroids	*Solubility (mg/ml)
Hydrocortisone	3.27 ± 0.036
Hydrocortisone acetate	3.723 ± 0.06
Hydrocortisone-17-butyrate	512.2

*mean of three replicates

CHAPTER 5 METABOLIC TRANSFORMATION OF CORTICOSTEROIDS

5.1 INTRODUCTION

The skin was for a long time considered as an inert organ. However, recent work has shown that it possesses significant metabolic activities (203, 204, 209, 211, 212, 253 - 255). It is now known to be an important site for the metabolism and biosynthesis (256, 283) of steroids. It may also serve as a reservoir for steroids (93, 120). Abnormalities in these functions are related to inflammation, sebum production, acne, hirsutism and testicular feminization syndrome (185). This biotransformation ability of the skin is very important as far as cutaneous drug bioavailability is concerned.

It is known that hydrocortisone (cortisol) is a topically active anti-inflammatory corticosteroid. Its analogue, cortisone, is inactive when applied locally to the skin but effective systemically. This striking difference cannot be explained by differences in cutaneous absorption profiles since cortisone is absorbed to the same degree as hydrocortisone (139). Malkinson et al (202) suggested that the skin metabolizes cortisone or cortisone metabolites more readily and perhaps more rapidly than it does hydrocortisone. Consequently, the anti-inflammatory effect of cortisone is drastically reduced. There is adequate evidence to show that interconversion of hydrocortisone and cortisone takes place in man after systemic administration (257, 258, 259). This led to the claim that the inactivity of cortisone when applied topically was due to the inability of the skin to metabolise it to hydrocortisone. However, Hsia and Hao (204) studied the metabolism of cortisone in human skin and found that cortisone was transformed to hydrocortisone, but it is still possible that insufficient amounts are formed during transit through the skin. It has also been suggested that the enhanced

potency of fluocinolone acetonide relative to hydrocortisone when applied topically was due to the fact that its side chain could not be cleaved by skin enzymes whereas hydrocortisone was metabolised to 11-hydroxy-17-oxosteroid (213).

After the efficacy of hydrocortisone was firmly established, attempts were made to improve its efficacy by preparing its derivatives. More potent and now widely prescribed, halogenated or esterified derivatives quickly followed. Esterification is usually made at the C17 or C21 positions of the steroid's molecule. Such esterification greatly enhances potency. For instance, betamethasone was not very active topically but by forming the 17-valerate, a compound with over 300 times the activity of betamethasone topically, was produced (19). Fluorination of the steroid molecule is not essential for high potency. For example, the non-fluorinated topical corticosteroid —— hydrocortisone-17-butyrate 0.1% is as effective as triamcinolone acetonide 0.1%, fluocinolone acetonide 0.025% or betamethasone-17-valerate 0.1% (239). The potency of the esterified derivatives depends on the nature and the position of the side chain. Betamethasone-17-valerate has fifteen times the activity of the 21-isomer (19). The fatty acid used to esterify the steroid alcohols also has an influence on activity. Engel et al (260) have compared hydrocortisone and seven of its 17-esters by the McKenzie's Skin-Blanching Test and found that the 17-valerate and 17-butyrate were superior to its 17-acetate and other esters tested. Three factors may be of importance in explaining the differences in topical potency of the parent steroid alcohol and its derivatives:

(a) the derivatives possess stereochemical features which improve on those of the parent compound.

(b) the derivatives penetrate human skin better.

(c) the derivatives are less susceptible to metabolic inactivation in the skin

The first of these possibilities can only be adequately tested by characterising the binding of the various steroids to the recently identified cutaneous steroid receptor (261 - 263). Changes in lipophilicity by derivatisation must inevitably affect the overall activity of the parent steroid. The partitioning and the three-phase transfer data (Chapter 4) show that these differences are likely to be important. The third possible explanation for the observed differences in activity has recently been experimentally investigated (196, 214).

Human skin contains a large number of enzymes. Esterases if present would be the enzymes most likely to modulate the activity of the esters of hydrocortisone and betamethasone. This has been shown by Rawlins et al (196). Using [^{3}H]-betamethasone-17-valerate incubated with human skin for 3 hours, at 37°C, pH 7.5, these workers found that betamethasone-17-valerate was metabolised to a compound having the same chromatographic mobility as betamethasone. Similar studies have been reported by O'Neill and Carless (214) who have studied the influence of the side chain on the hydrolysis of hydrocortisone esters. These studies were carried out by incubating a series of straight chain esters of hydrocortisone with hamster and guinea pig skin homogenates as well as pure carboxyl esterase and human plasma. They found that the hydrolysis rate varied with chain length and maximum hydrolysis was observed with steroids possessing a 4 to 6 carbon long side chain and the 21-esters were much more sensitive to hydrolysis than its 17-esters. The rate slowed down as the carbon number increased. However, these reports did not give the full kinetic profile of the steroids and failed to quantify the difference in the transformation rate constant between the 17- and 21-esters. This part of the work was initiated to explore these further. In this part of the study, the enzymic transformation

of corticosteroid-17 and 21-esters by hog liver and mouse skin enzymes are investigated in an attempt to rationalise the well known difference in activity between the 17- and 21-esters when applied topically.

5.2 MATERIALS AND METHODS

5.2.1 Enzymic Hydrolysis by Esterase

Source of enzyme: The enzyme used was a carboxylic ester hydrolase from hog liver, suspended in 3.2 M $(NH_4)_2SO_4$ solution, pH 8, purchased from Sigma Chemical Co. The claimed activity was that one unit hydrolyzed 1 µmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8, 25°C. The enzyme concentration was 8 mg protein/ml, 120 units/mg protein, (i.e. 1 ml = 960 units). The term 'esterase' is used through the text for this preparation.

Diluted enzyme solution: The original enzyme solution was diluted to 1/20, 1/50, 1/100 and 1/200 of its original strength by addition of appropriate amounts of 3.2 M $(NH_4)_2SO_4$.

Tris buffer solution: 6.0575 g of tris[hydroxymethyl]aminomethane was dissolved in water, 279 ml of 0.1N HCl was added, more water added to 1 litre, pH = 8.094.

Tris buffer solution, concentrated: Each litre of this solution contained 9.692 g tris[hydroxymethyl]aminomethane and 446.4 ml of 0.1N HCl in water, pH = 8.25.

Acidified acetonitrile solution is used to describe a 0.024 N HCl in 50% v/v aqueous acetonitrile solution.

Internal standard solutions: All internal standard solutions were prepared in acidified acetonitrile. The internal standards and the concentrations used were as following:-

Steroids studied	Initial concentration of steroid (mg/ml)	*Internal standard
Hydrocortisone acetate	0.03	0.02 mg/ml hydrocortisone-17-butyrate
Hydrocortisone and its 17- and 21-butyrates	0.1	0.06 mg/ml hydrocortisone acetate
Betamethasone and its 17- and 21-valerates	0.02	0.012 mg/ml
	0.06	0.06 mg/ml
	0.1	0.06 mg/ml hydrocortisone-17-butyrate
Beclomethasone, its 17- and 21-monopropionates, and its 17,21-dipropionate	0.1	No internal standard

* noted that the concentrations indicated are not concentrations in the final test solutions.

Test solutions and procedure: Experiments were carried out in 20% propylene glycol in tris buffer solution pH 8.094. The final pH was 8.136. For studying the beclomethasone esters, 50% propylene glycol in tris buffer solution, concentrated, was used due to the relatively small solubility of beclomethasone-17,21-dipropionate in more aqueous systems. The pH of the 50% propylene glycol-tris buffer solution in this case was 8.038. The same buffer salt and acid concentration was used. The temperature was 37°C in all cases. Since the steroids were unstable

in alkaline solutions and were difficult to dissolve in the medium used (especially hydrocortisone-21-butyrate which was in crystalline form), the steroids were firstly dissolved in propylene glycol of appropriate concentration such that the final concentration after mixing with tris buffer solutions were as required. For hydrocortisone and betamethasone esters, 41.6 ml of the tris buffer solution was placed in an amber coloured glass bottle and warmed to 37°C. When this was achieved, 10.4 ml of steroid-propylene glycol solution were added and well shaken. For beclomethasone esters, 26 ml of tris buffer solution, concentrated, were mixed with 26 ml steroid-propylene glycol solution. 2 x 1 ml aliquots were taken immediately as the initial samples in all cases, to leave 50 ml of solution. 0.1 ml of the enzyme solution was introduced with a syringe and the resulting solution thoroughly mixed and timing started simultaneously. 1 ml aliquots were taken out at appropriate time intervals. 1 ml of the appropriate internal standard solution (see section under title 'internal standard solutions') or 1 ml of the acidified acetonitrile solution (for those no internal standard used) was added to quench the enzymic reaction. From preliminary studies, it was found that addition of these solutions stopped the enzymic hydrolysis and the mixed solutions were stable for at least 1 week at 4°C. Samples were analysed by HPLC. Control experiments were also carried out without the enzymes. Since the enzyme solution contained ammonium sulphate, control experiments with 0.1 ml of 3.2 M $(NH_4)_2SO_4$ added instead of 0.1 ml of enzyme solution were also carried out for comparison. Table 32 summarizes the steroids studied, the reaction conditions and the composition of the test solutions.

Table 32. Compositions of the test solutions used in the enzymic esterase study.

Steroid studied	Initial concentration (mg/ml)	Incubation medium	Enzyme concentration %	△Strength	x volume#
Hydrocortisone-21-acetate	0.03		0.2	1	x 0.1 ml
Hydrocortisone-17-butyrate	0.1	20% Porpylene glycol-tris buffer	0.2	1	x 0.1 ml
	0.1		0.01	1/20	x 0.1 ml
Hydrocortisone-21-butyrate	0.1		0.2	1	x 0.1 ml
	0.1		0.01	1/20	x 0.1 ml
Hydrocortisone	0.1		0.01	1/20	x 0.1 ml
Betamethasone-17-valerate	0.1		0.2	1	x 0.1 ml
	0.06		0.2	1	x 0.1 ml
	0.02		0.004	1/50	x 0.1 ml
Betamethasone-21-valerate	*0.1		0.2	1	x 0.1 ml
	0.02		0.01	1/20	x 0.1 ml
	0.02		0.004	1/50	x 0.1 ml
	0.02		0.002	1/100	x 0.1 ml
	0.02		0.001	1/200	x 0.1 ml
Betamethasone	0.02		0.004	1/50	x 0.1 ml
Beclomethasone-17,21-dipropionate	0.1	50% propylene glycol-tris buffer, concentrated	0.4	1	x 0.2 ml
Beclomethasone-17-propionate	0.1		0.4	1	x 0.2 ml
Beclomethasone-21-propionate	0.1		0.4	1	x 0.2 ml

* suspension

△ strength 1 means the undiluted enzyme solution, 1/20 means the original enzyme solution was diluted to 20 times, and so on.

The volume used is for each 50 ml sample solution.

Standard solutions: It is important that the solvent of each standard solution was the same as that of the test solution. Since the steroids studied are non-ionizable, it is not necessary to adjust to pH of the solvent and both propylene glycol-water or propylene glycol-tris buffer can be used. The sample solvent used was a 1:1 mixture of 20% propylene glycol-water (or 50% propylene glycol-water for the beclomethasone esters) and acidified acetonitrile, with concentration range and steroids contained as following:-

Steroid studied and initial concentration (mg/ml)	Compounds contained in standard solutions	Concentration range (mg/ml)	Internal standard and concentration (mg/ml)
Hydrocortisone-acetate 0.3	Hydrocortisone-acetate and hydrocortisone	0.002 - 0.015	Hydrocortisone-17-butyrate, 0.01
Hydrocortisone Hydrocortisone-17-butyrate Hydrocortisone-21-butyrate 0.1	Hydrocortisone, hydrocortisone-17-butyrate and hydrocortisone-21-butyrate	0.005 - 0.05	Hydrocortisone acetate, 0.03
Betamethasone Betamethasone-17-valerate 0.1 Betamethasone-21-valerate 0.02	Betamethasone, betamethasone-17-valerate and betamethasone-21-valerate	0.005 - 0.05 0.002 - 0.01	Hydrocortisone-17-butyrate 0.03 0.006
Beclomethasone-17,21-dipropionate Beclomethasone-17-propionate 0.1 Beclomethasone-21-propionate	Beclomethasone, beclomethasone-17,21-dipropionate, beclomethasone-17-propionate and beclomethasone-21-propionate	0.005 - 0.05	No internal standard

HPLC analysis

Column: Hypersil-ODS (5 μm), 10 cm x 4.6 mm i.d.

Mobile phase: (a) 50% acetonitrile in water — for hydrocortisone and its esters and beclomethasone and its esters

(b) 55% acetonitrile in water — for betamethasone and its esters

Flow rate: 1 ml/min for mobile phase (a)

1.2 ml/min for mobile phase (b)

Detection wavelength: 250 nm

Sensitivity: 0.04 - 0.32 AUFS

5.2.2 Ethanolamine-catalyzed decomposition of beclomethasone-17, 21-dipropionate

10 mg of beclomethasone-17,21-dipropionate was dissolved in 100 ml of 50% propylene glycol-water, the degradation was initiated by the introduction of 0.15 ml of ethanolamine and stored at 37°C in a water bath. 1 ml aliquots were taken at appropriate time intervals and the reaction was quenched by the addition of 1 ml of 0.06 mg/ml of betamethasone-17, 21-dipropionate as internal standard in acidified acetonitrile. The internal standard solution was prepared on alternate days. The test solutions were chromatographed as described in Section 5.2.1.

5.2.3 Metabolic transformation by mouse skin homogenates

5.2.3.1 Preparation of mouse skin homogenates

Freshly killed mice were skinned and the hair was removed. The whole skin was weighed (about 3.5 g to 5 g), cut into tiny pieces and suspended in about 20 ml of iced Krebs-Ringer buffer solution. The pH 7.51 Krebs-Ringer buffer contained 6.9 g NaCl, 0.35 g KCl, 0.29 g $MgSO_4 \cdot 7H_2O$, 0.16 g KH_2PO_4, 1.8 g $NaHCO_3$ and 1.2 ml of $CaCl_2 \cdot 2H_2O$ 1M

solution in each litre. The mixture was twice homogenized by passing through a cooled French Press (Aminco). The French Press was refrigerated overnight before use in order to minimize the rise in temperature during processing. The specimens were kept in an iced-bath whenever possible as high temperatures may reduce the enzyme activity. The homogenates were further diluted with iced-buffer solution where necessary and centrifuged at 12,000 r.p.m., 4°C for one hour. The supernatant was filtered through MF-milipore 1.2 μm membrane and diluted to volume (usually the equivalent of three mouse-skins in 200 ml). The skin homogenates were frozen at -15°C until used.

5.2.3.2 Effect of coenzyme on the enzyme activity and stability of the skin homogenates

In order to test whether a coenzyme was necessary for restoration of enzyme activity and to optimise storage conditions, the following samples were examined:-

(1) 10 ml freshly prepared homogenates and 5 ml Krebs solution

(2) 10 ml freshly prepared skin homogenates and 5 ml NADPH generating solution*

(3) 10 ml skin homogenate and 5 ml NADPH generating solution* and then stored at 0°C for one day

(4) 10 ml skin homogenate, stored at 0°C for one day, and 5 ml NADPH generating solution* added just before the start of the experiment

(5) 10 ml skin homogenate, stored at 0°C for one day, followed by the addition of 5 ml Krebs solution

(6) 10 ml skin homogenate, stored at 0°C for two days, followed by the addition of 5 ml Krebs solution

(7) 10 ml skin homogenate, stored at 0°C for three days, followed by the addition of 5 ml Krebs solution

*The NADPH generating solution was a mixture of 25 mg glucose-6-phosphate, 15 mg NADP and 50 µl of glucose-6-phosphate dehydrogenase in 25 ml Krebs solution.

To each of these mixtures, 15 ml of 0.12 mg/ml betamethasone-21-valerate in propylene glycol (preheated to 37°C) was added just before the experiment started and the solution well mixed before storage at 37°C. 1 ml aliquots were taken at appropriate time-intervals and 1 ml acidified acetonitrile solution was added to quench the enzymic reaction. Before analysis by HPLC, 1 ml of 0.036 mg/ml of hydrocortisone-17-butyrate in acidified acetonitrile as internal standard was added, followed by filtration through glass fibre filter paper (Whatmann, GF/C 2.5 cm). The filtrate was monitored by reversed-phase HPLC as mentioned in Section 5.2.1. In calculating the % metabolite or decomposition product formed, the initial concentration must be corrected for molecular weight changes.

5.2.3.3 Effect of metabolites on enzyme activity

Betamethasone-21-valerate was metabolized to betamethasone and valeric acid. Each of these alone or in combination could act as competitors for the enzyme. The enzymic hydrolysis of betamethasone-21-valerate in the presence of betamethasone, or valeric acid or a mixture of both were therefore compared:-

(a) Betamethasone-21-valerate 0.06 mg/ml, with 0.06, 0.02, 0.015, 0.01, 0.005 and 0 mg/ml of betamethasone

(b) Betamethasone-21-valerate 0.06 mg/ml, with 0.02 mg/ml valeric acid

(c) Betamethasone-21-valerate 0.06 mg/ml, with 0.02 mg/ml valeric acid and 0.02 mg/ml betamethasone (duplicates have been done) incubated in 50% propylene glycol and 50% skin homogenates, at 37°C. The general procedure as mentioned in Section 5.2.3.2 followed.

5.2.3.4 Effect of propylene glycol on enzyme activity

To test the effect of the presence of propylene glycol in the incubation media on the activity of the enzyme, the following samples were used:

(a) 25 ml skin homogenates mixed with 23 ml of propylene glycol at 37°C for 12 hours

(b) 25 ml skin homogenates mixed with 23 ml of propylene glycol at 37°C for 5 hours

(c) 25 ml skin homogenates mixed with 23 ml of propylene glycol, then frozen at -15°C and preheated to 37°C before use

(d) 25 ml skin homogenates was frozen at -15°C, thawed and 23 ml of propylene glycol was added just before experiment started, and heated to 37°C (control standard)

The metabolic reactions were initiated by the introduction of 2 ml x 1.5 mg/ml betamethasone-21-valerate in propylene glycol to each mixture. The general procedures described in Section 5.2.3.2 were then followed. The initial concentration of betamethasone-21-valerate in all the test solutions was 0.06 mg/ml at the start of each experiment, and the actual incubation media consisted of a 1:1 dilution of skin homogenates and propylene glycol. Each 150 ml of skin homogenates contained about 12.6 g of mouse skin. For the control sample, Krebs solution was used instead of skin homogenates, and the non-enzymic decomposition rate constant was calculated from this solution.

5.2.3.5 Enzymic decomposition of betamethasone-17-valerate

25 ml of 0.12 mg/ml of betamethasone-17-valerate in propylene glycol were mixed with 25 ml skin homogenates and incubated at 37°C. 1 ml aliquots were taken at appropriate time intervals. The reaction

was quenched by the addition of 1 ml acidified acetonitrile solution. 1 ml x 0.036 mg/ml of hydrocortisone-17-butyrate was added as internal standard, and the whole filtered through glass fibre filter paper before being chromatographed as previously described.

5.2.3.6 Comparison of the metabolic transformation of 17- and 21-steroid esters by mouse skin

Since under the conditions used the pH value of the Krebs solution was observed to be unstable, tris buffer solution was substituted in the following studies. The mouse skin homogenates was prepared as previously described except that the buffer solution used was pH 8.05 tris buffer. Two mouse skins were homogenised in 350 ml of buffer solution. The steroid was firstly dissolved in propylene glycol, heated to 37°C, then mixed with the skin homogenate solution preheated to the same temperature, to initiate the reaction. The test solution was 0.02 mg/ml of steroid in 20% propylene glycol-tris buffer. 1 ml aliquots were taken at appropriate intervals and the reaction was quenched by the addition of 1 ml of 0.012 mg/ml hydrocortisone acetate (for hydrocortisone esters) or hydrocortisone-17-butyrate (for betamethasone esters) in acidified 50% acetonitrile-water. The test solutions were chromatographed as before.

5.3 RESULTS AND DISCUSSION

To investigate the enzymic biotransformation profiles of hydrocortisone acetate, and 17- and 21-butyrates, and betamethasone-17- and 21-valerates, a pure carboxylic ester hydrolase extracted from hog liver has been used as a model enzyme. This was chosen because of its low specificity and because it is commonly found in animal tissues. The resistance of hydrocortisone-17-esters and betamethasone-17-esters

to this esterase were compared with that of their 21-isomers. The optimal pH for this enzyme is 8. The medium used in this study was therefore buffered to pH 8 with tris buffer solution. 20% propylene glycol was incorporated to increase the solubility of the corticosteroids. Propylene glycol was chosen as co-solvent because it is commonly used in the formulation of topical dosage forms of corticosteroids. The enzymic and base-catalyzed reactions were stopped by the addition of acidic solvent such that the final pH was around 2 - 4. Under these conditions, the test solutions could be kept for a week at $4^{\circ}C$, but the samples were analysed within three days. The esterase acts on the ester bond and liberates the free steroid alcohol. The disappearance of the parent steroid as well as the formation of the free alcohol and that of the 21-isomers were monitored. The reactions were shown to be first order under the conditions used in this study.

Table 33 summarises the results giving both the enzymic and the non-enzymic reaction rate constants. The findings indicates that the 21-esters —— hydrocortisone acetate, betamethasone-21-valerate and hydrocortisone-17-butyrate —— are very sensitive to the esterase when compared with their 17-esters. Among the three of them, hydrocortisone acetate had the shortest chain length and was most resistant to the enzyme. From preliminary studies, using 0.2% of enzyme and 0.1 mg/ml of betamethasone-21-valerate and of hydrocortisone-21-butyrate, and 0.03 mg/ml of hydrocortisone-21-acetate, it was found that virtually complete hydrolysis of betamethasone-21-valerate and hydrocortisone-21-butyrate occurred within one minute. The reactions were too fast for precise monitoring of the rate constants. With hydrocortisone-21-acetate, the reaction was slower having a rate constant of 0.165 min^{-1} (Table 33), and the reaction profile is shown in Figure 45. The enzymic hydrolysis is clearly first order (Figure 46). These results are in

Table 33. Enzymic and non-enzymic transformation rate constants of steroids in 20% propylene glycol-tris buffer, pH 8.14, at 37°C.

Steroid	Initial Concentration (mg/ml)	Enzymic Hydrolysis		Non-Enzymic Degradation (Control)	
		% v/v Esterase	Observed Rate Constant[a] (hr^{-1})	Observed Rate Constant[b] (hr^{-1})	Real Rate Constant[c] (hr^{-1})
Hydrocortisone-21-acetate	0.03	0.2	9.87 ($0.165\ min^{-1}$)	No change after 30 minutes	
Hydrocortisone-17-butyrate	0.1	0.2	0.059	0.0567	
	0.1	0.01	0.057	0.0514	0.0573
	0.1			0.0473	0.0524
Hydrocortisone-21-butyrate	0.1	0.2	instantaneously	No change after 30 minutes	
	0.1	0.01	9.59	0.0110	0.00602
Betamethasone-17-valerate	0.02	0.004	0.0838	0.0722	0.0839
	0.02			0.0760	0.0798
	0.06	0.2	0.0867		
	0.1	0.2	0.0840	0.0792	
	0.1	0.2	0.0837		
Betamethasone-21-valerate	0.1	0.2	instantaneously	No change after one hour	
	0.02	0.01	26.6	No change after one hour	
	0.02	0.004	13.022	0.0362	
	0.02	0.002	6.62		
	0.02	0.001	2.775		
Betamethasone	0.02	0.004	no change after 40 hours	No change after 40 hours	
Hydrocortisone	0.1	0.1	no change after 43 hours	No change after 43 hours	

[a] Rate constants calculated according to the disappearance of the starting compound, have not been corrected for the non-enzymic decomposition.

[b] Rate constants calculated according to the disappearance of the starting compound.

[c] True non-enzymic rate constant, calculated by non-linear regression analysis. With correction for the isomerization of the 21-esters back to the 17-esters.

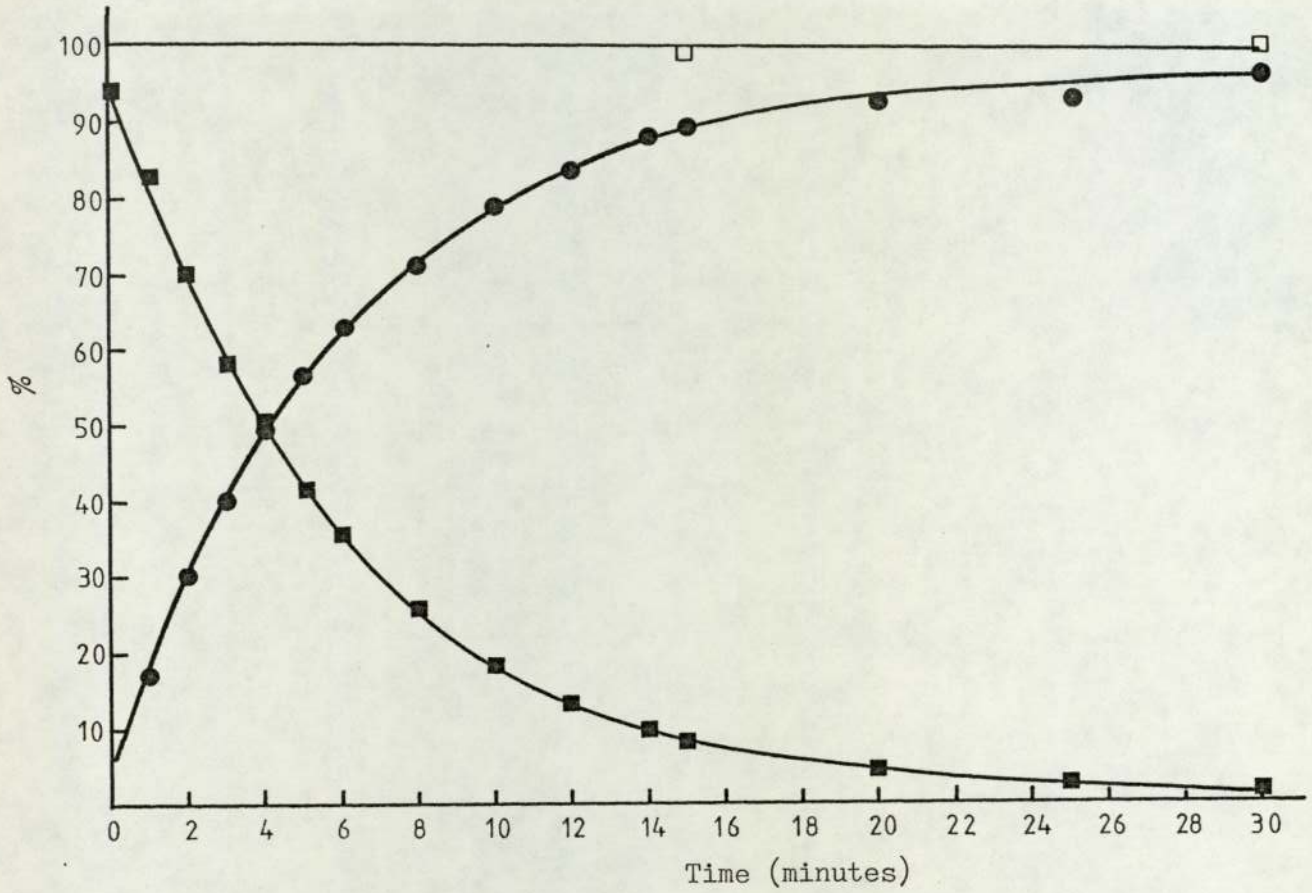


Figure 45. Hydrolysis of hydrocortisone-21-acetate 0.03 mg/ml by 0.2% esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

key: ■ hydrocortisone-21-acetate remaining
● hydrocortisone formed
} in the presence of 0.2% esterase

□ hydrocortisone-21-acetate remaining in the control

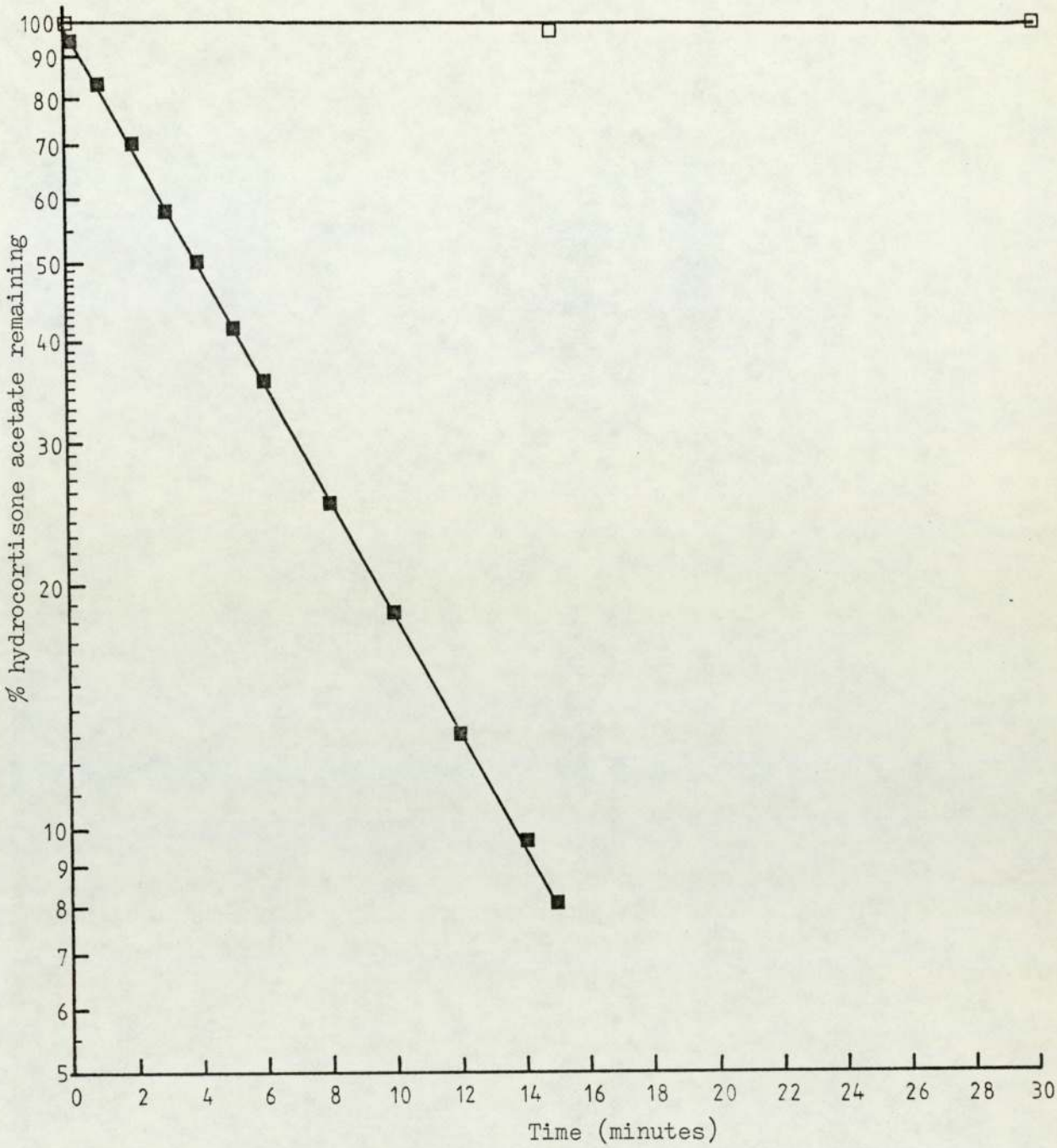


Figure 46. Hydrolysis of hydrocortisone-21-acetate 0.03 mg/ml by 0.2% esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

key: ■ hydrocortisone acetate remaining in the presence of 0.2% esterase

□ hydrocortisone acetate remaining in the control

agreement with those of O'Neill and Carless (214) who showed that maximum hydrolysis rate was observed with 21-esters having a 4 or 5 carbons side chain.

In the study of enzyme kinetics, the substrate concentration is usually chosen to be in excess such that the enzyme is saturated and the order of reaction is zero. In our studies, the objective was to observe the intrinsic enzymic hydrolysis rates of the drugs rather than the characteristics of the enzyme itself. The enzyme merely served as a model esterase. Therefore, the kinetics of reaction were first followed under conditions where the enzyme was in excess. The observed order of reaction would then be first rather than zero.

In order to compare the rate of enzymic hydrolysis of the 21- and 17-esters, a measurable and reproducible rate constant is required. To achieve this, the original enzyme was diluted with 3.2 M ammonium sulphate and the concentration of betamethasone-21-valerate was reduced to 0.02 mg/ml to maintain the drug in solution. A comparison between hydrocortisone-17 and 21-butyrates at a steroid concentration of 0.1 mg/ml each and an esterase concentration of 0.01%, the 21-isomer was found to be over 160 times more sensitive to the esterase than its 17-ester (Table 33). Similarly, the enzymic hydrolysis rate constant of betamethasone-21-valerate is over 150-fold higher than that of the 17-valerate (Table 33). This rate difference was based on the disappearance of the starting steroid, and has not been corrected for non-enzymic decomposition. It has been known that under the neutral or alkaline condition, betamethasone-17-valerate and hydrocortisone-17-butyrate easily isomerize to the 21-esters and further hydrolyse to their free alcohols which subsequently degrade to other products (Chapter 3). Therefore, in evaluating the enzymic hydrolysis, a correction should be made for these non-enzymic decomposition pathways.

Control samples without enzyme were therefore run simultaneously. If the isomerization rate of hydrocortisone-17-butyrate to the 21-butyrate and that of the betamethasone-17-valerate to the 21-valerate are compared with their corresponding enzymic hydrolysis rates (Table 33), it is found that the enzymic hydrolysis rate constants are only about 10% higher than the isomerization rate constants.

To establish the range of enzyme concentrations over which first order kinetics are observed, the reactions were monitored in the presence of different concentrations of the enzyme. One would have expected no difference in the first order rate constants as long as saturation kinetics was not observed. This was not so, as shown in Figures 47 and 48, first order kinetics were maintained. Yet, a linear dependence of the rate constants for the disappearance of the 21-esters on the enzyme concentration range of 0.001 - 0.004% was observed (Figure 49). No significant difference was observed in the enzymic hydrolysis of the 17-esters with different concentrations of enzyme. For example, the hydrolysis rate of 0.1 mg/ml hydrocortisone-17-butyrate with 0.2% of enzyme (0.059 hr^{-1}) is essentially the same as that with 0.01% enzyme (0.057 hr^{-1}). Similarly, for betamethasone-17-valerate, despite different initial concentrations (0.02, 0.06, 0.1 mg/ml), similar hydrolysis rate constants (0.0838, 0.0867, 0.0840 and 0.0837 hr^{-1}) were found for 0.2% enzyme and 0.004% enzyme (Table 33). In the absence of enzyme, the isomerisation rate constants under the same conditions were 0.0567, 0.0514 and 0.0473 hr^{-1} for hydrocortisone-17-butyrate to the 21-butyrate; and 0.0722, 0.0760 and 0.0792 hr^{-1} for betamethasone-17-valerate to the 21-valerate (Table 33). This suggests that the 17-esters are resistant to esterase. But once they are converted to the 21-esters, the enzymic hydrolysis can then proceed rapidly. When high concentration (0.2%) of enzyme was used, no

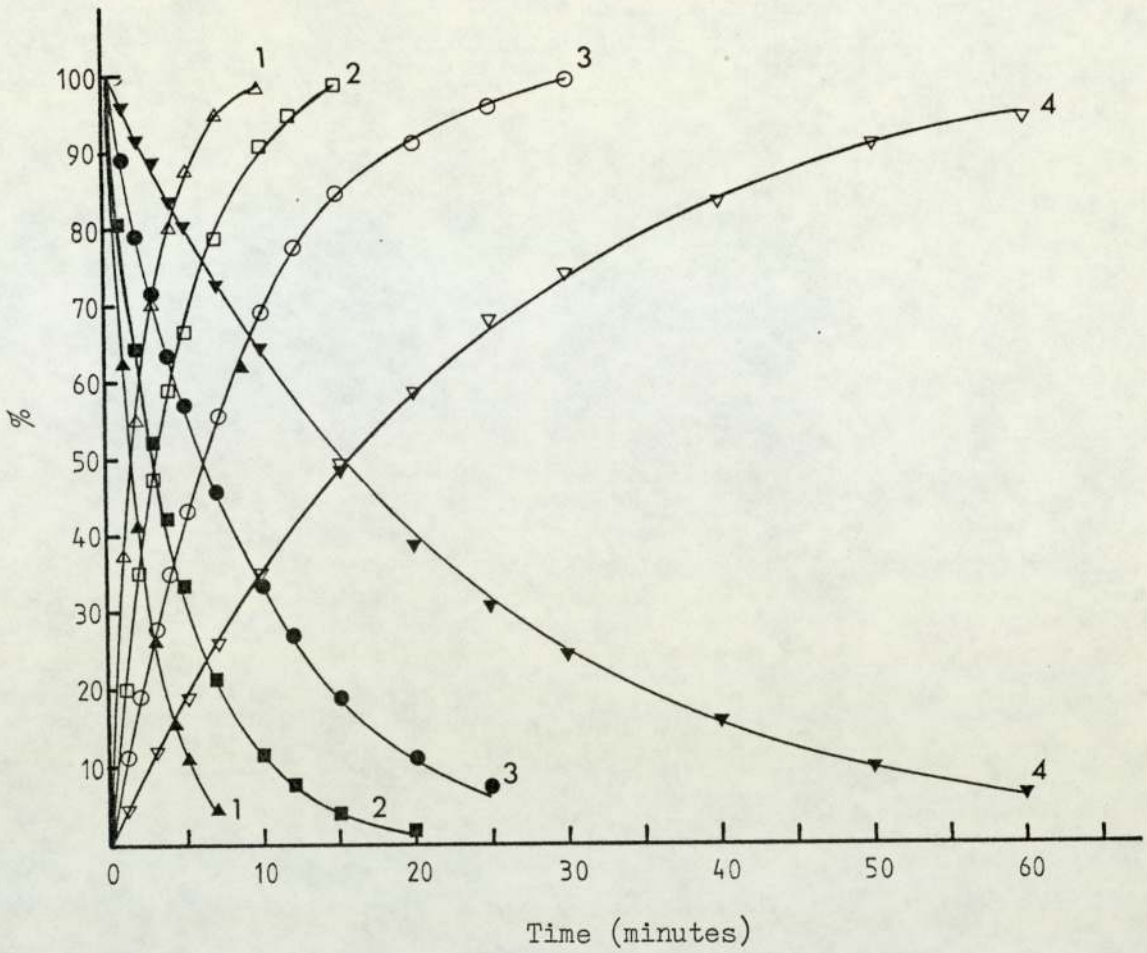


Figure 47. Enzymic hydrolysis of betamethasone-21-valerate 0.02 mg/ml, by esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

Key: ▲, ■, ● and ▼: betamethasone-21-valerate remaining
△, □, ○ and ▽: betamethasone formed
1. with 0.01% esterase
2. with 0.004% esterase
3. with 0.002% esterase
4. with 0.001% esterase

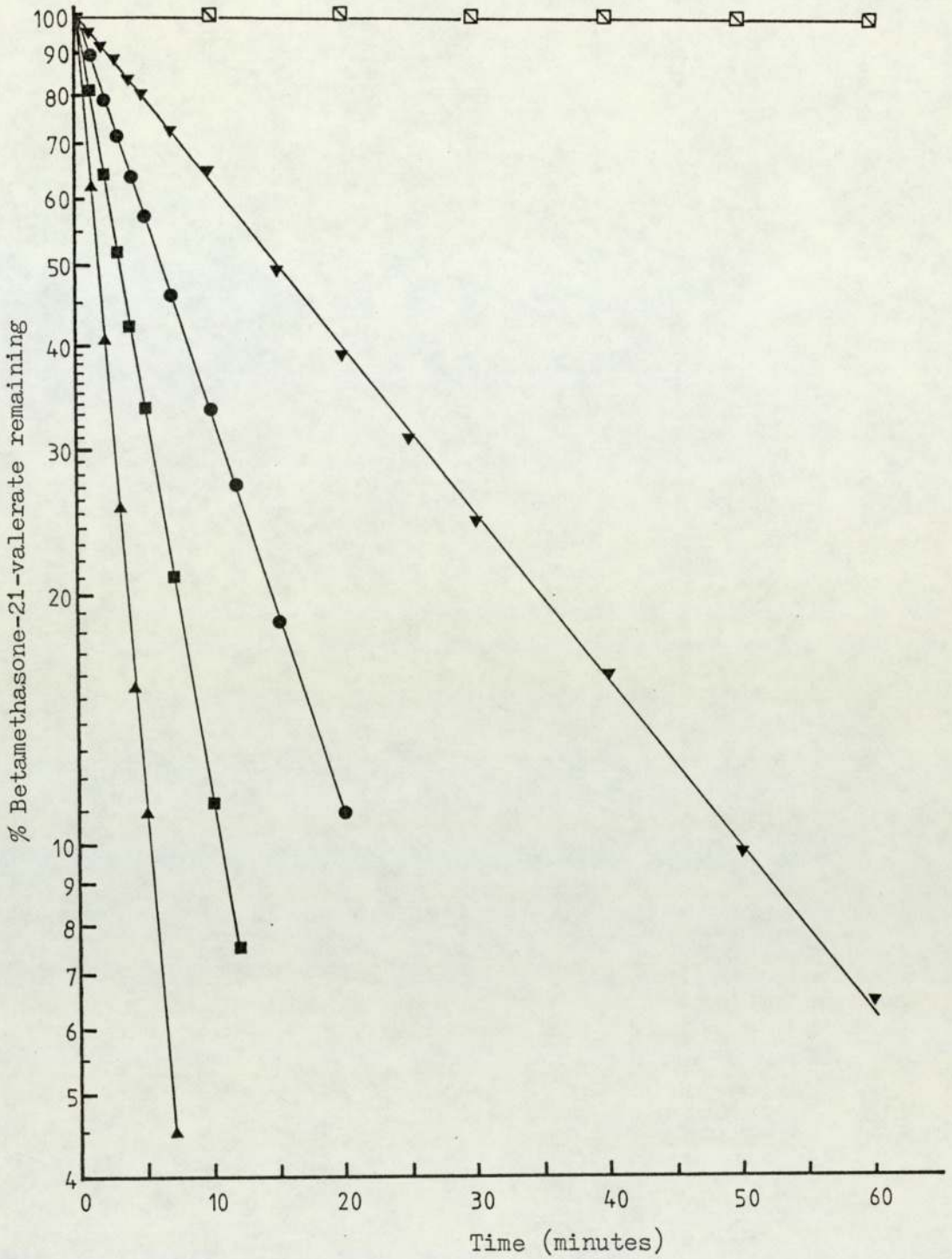


Figure 48. Enzymic hydrolysis of betamethasone-21-valerate 0.02 mg/ml by esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

Key:

- ▲ with 0.01% esterase
- ■ with 0.004% esterase
- ● with 0.002% esterase
- ▼ with 0.001% esterase
- ⧅ control (without estrase)

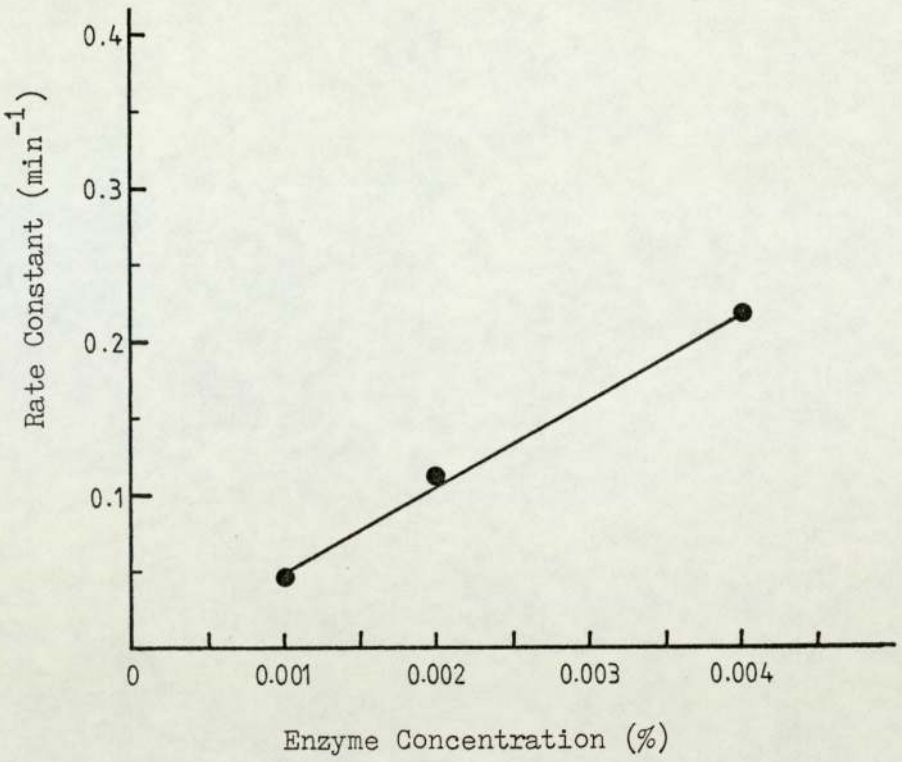


Figure 49. Effect of enzyme concentration on hydrolysis rate of betamethasone-21-valerate 0.02 mg/ml by esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

betamethasone-21-valerate or hydrocortisone-21-butyrate was detected (Figure 50a and 51a respectively). When hydrocortisone-17-butyrate is hydrolysed with 0.01% enzyme, less than 1% of the 21-butyrate was detected (Figure 52a) and in the case of betamethasone-17-valerate hydrolysed with 0.004% enzyme, only up to 3% of betamethasone-21-valerate could be seen (Figure 53a). The sum of the % of the 21-ester and free alcohol formed in the control matched the % of alcohol formed by the enzymic hydrolysis (Figures 51a, 52a and 53a). In the control study of betamethasone-17-valerate 0.1 mg/ml (Figure 50a), the amount of betamethasone-21-valerate formed exceeded its solubility and led to precipitation. Therefore, no reliable data were obtained.

If the assumption that the 17-esters are resistant to esterase is true, the 10% difference between the enzymic hydrolysis rate and the isomerization rate observed in the control needs to be explained. Three factors may contribute to this difference: (i) the presence of trace amount of ammonium sulphate in the test solution, (ii) reversibility in the isomerization, and (iii) experimental error. In a study of non-enzymic degradation of hydrocortisone-17-butyrate, 0.1 mg/ml, a control with the same amount of ammonium sulphate (0.1 ml of 3.2 M) as in the enzyme solution was carried out. Practically, the same rate constant as the plain control was observed (Figure 55): 0.0506 hr^{-1} and 0.0482 hr^{-1} respectively. The main reason for the rate difference between the enzymic and non-enzymic hydrolyses appears to be that the isomerization of the 17-esters to the 21-esters is not completely irreversible. With the presence of enzyme, the formed 21-ester is immediately removed through hydrolysis to the free alcohol. There is very little chance for the reverse conversion to the 17-ester to take place, especially in the presence of high amount of enzyme. But in the control, without the enzyme, the hydrolysis of the 21-esters to the

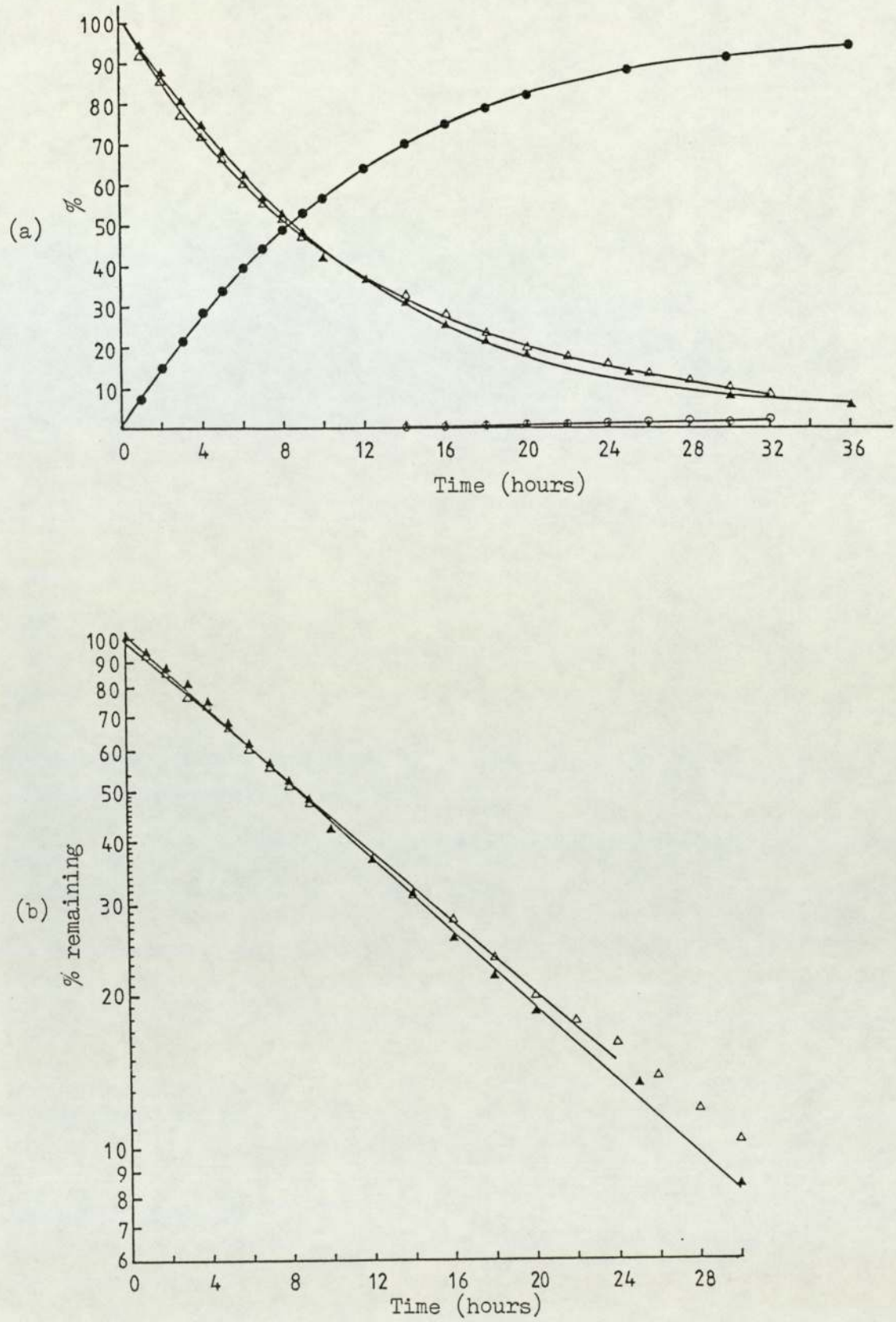
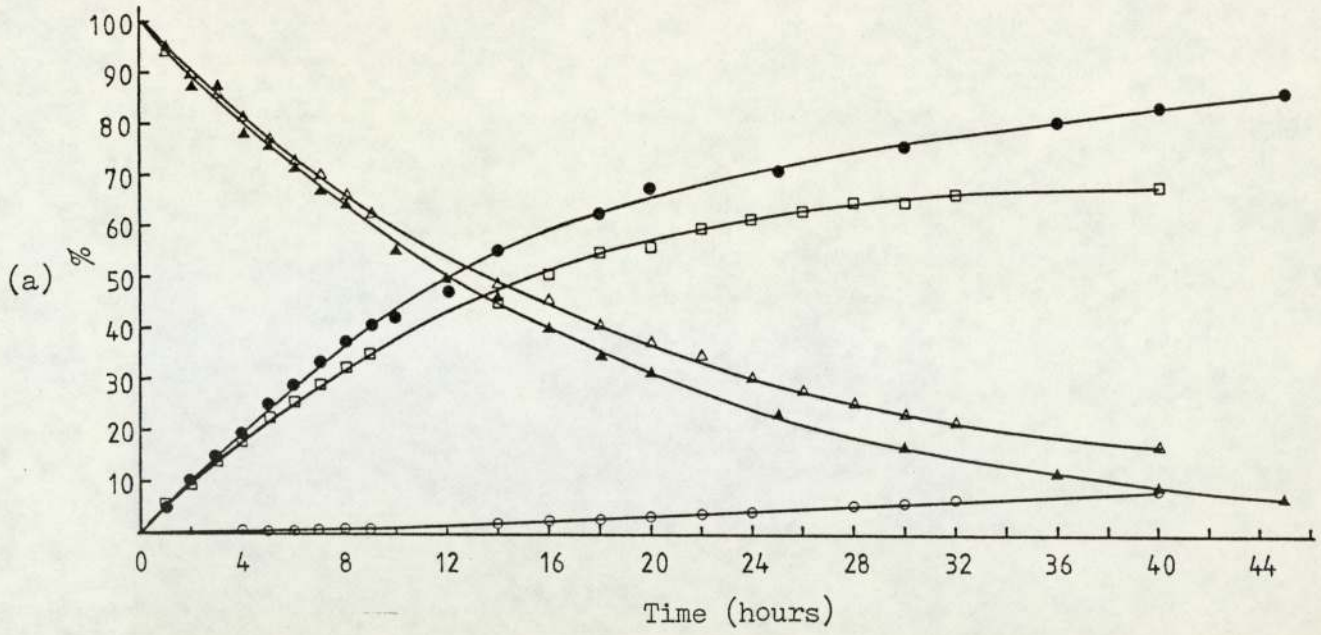


Figure 50. Enzymic and non-enzymic decomposition of betamethasone-17-valerate, 0.1 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

(a) Time-course profiles
(b) Log % betamethasone-17-valerate remaining vs. Time

key: ▲ betamethasone-17-valerate remaining
● betamethasone formed
} with 0.2% esterase

△ betamethasone-17-valerate remaining
○ betamethasone formed
} Control



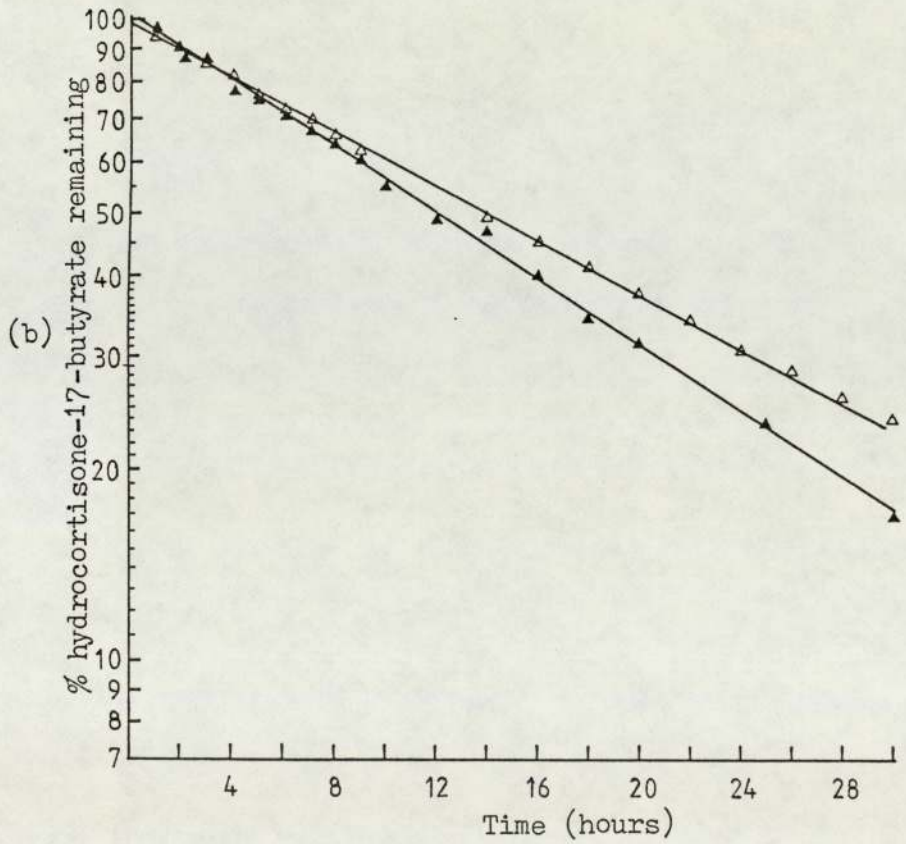


Figure 51. Enzymic and non-enzymic decomposition of hydrocortisone-17-butyrate 0.1 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

(a) Time-course profiles
(b) Log % hydrocortisone-17-butyrate vs. Time

key: ▲ hydrocortisone-17-butyrate remaining
● hydrocortisone formed
} in the presence of 0.2% esterase

△ hydrocortisone-17-butyrate remaining
□ hydrocortisone-21-butyrate formed
○ hydrocortisone formed
} in the control

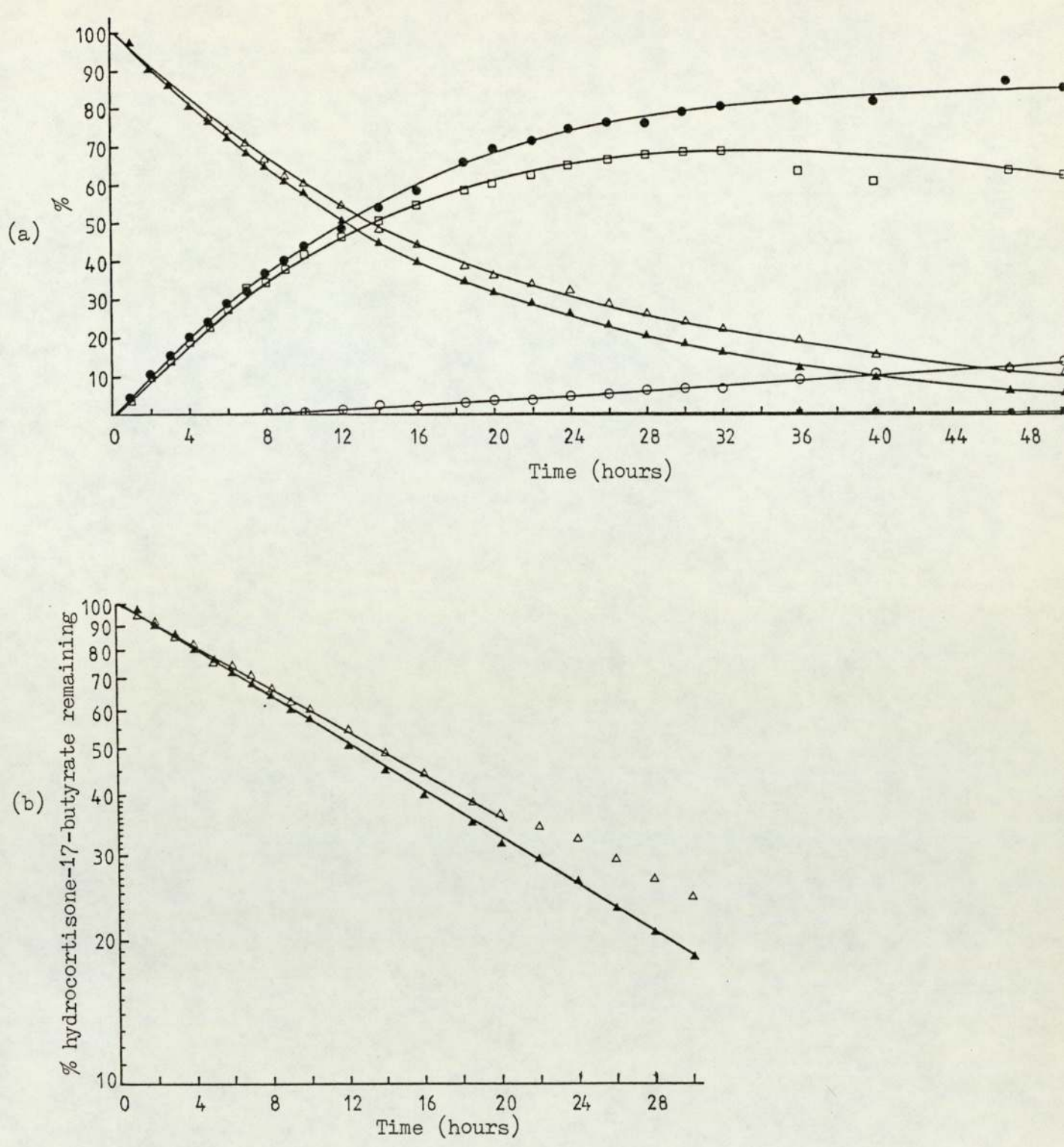


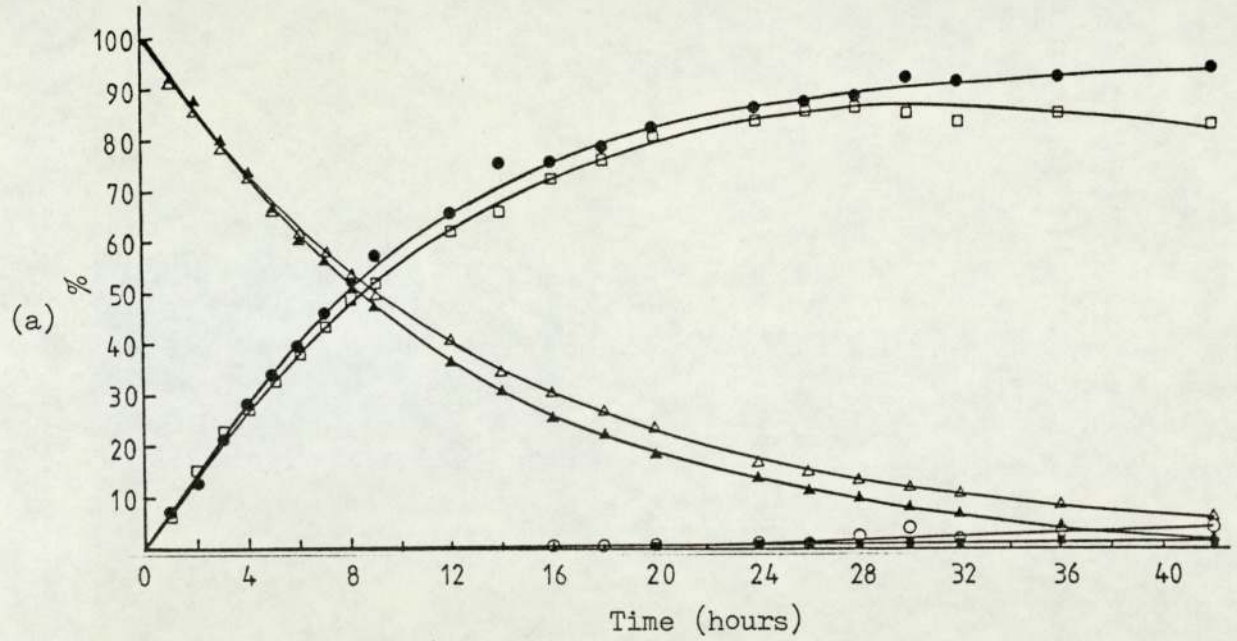
Figure 52. Enzymic and non-enzymic decomposition of hydrocortisone-17-butyrate 0.1 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

(a) Time-course profiles
(b) Log % hydrocortisone-17-butyrate vs. Time

key:-

▲ hydrocortisone-17-butyrate remaining
■ hydrocortisone-21-butyrate formed
● hydrocortisone formed
} in the presence of 0.01% esterase

△ hydrocortisone-17-butyrate remaining
□ hydrocortisone-21-butyrate formed
○ hydrocortisone formed
} in the control



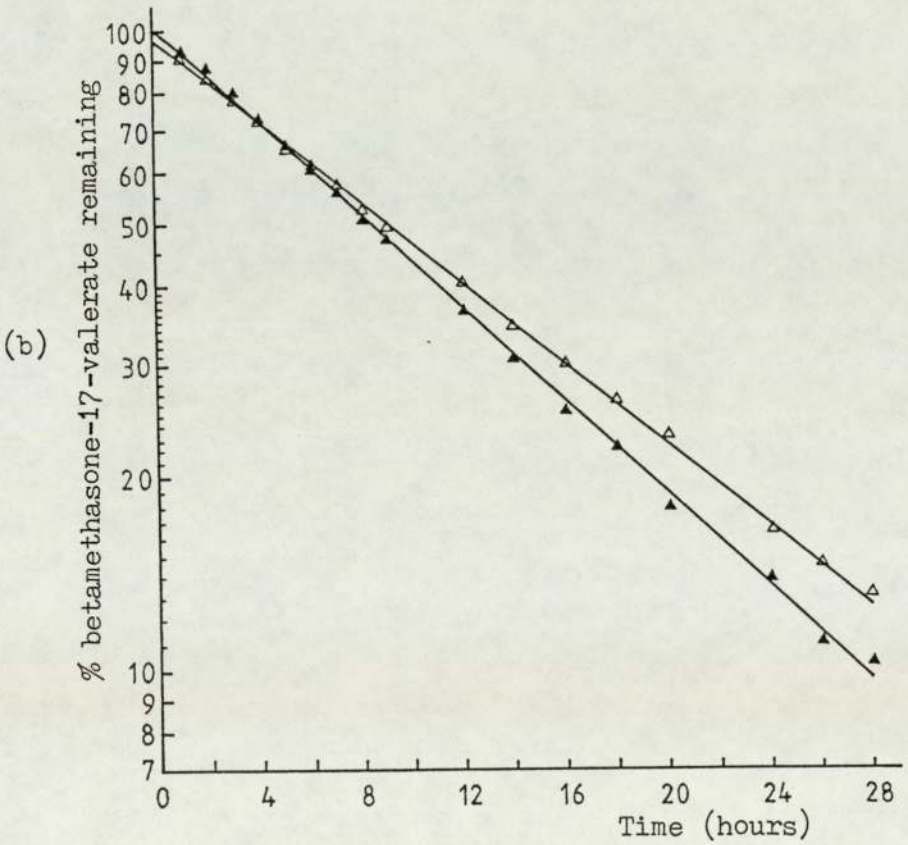


Figure 53. Enzymic and non-enzymic decomposition of betamethasone-17-valerate 0.02 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

(a) Time-course profiles
(b) Log % betamethasone-17-valerate remaining vs. Time

key:	with 0.004% esterase	Control
betamethasone-17-valerate remaining	▲	△
betamethasone-21-valerate formed	■	□
betamethasone formed	●	○

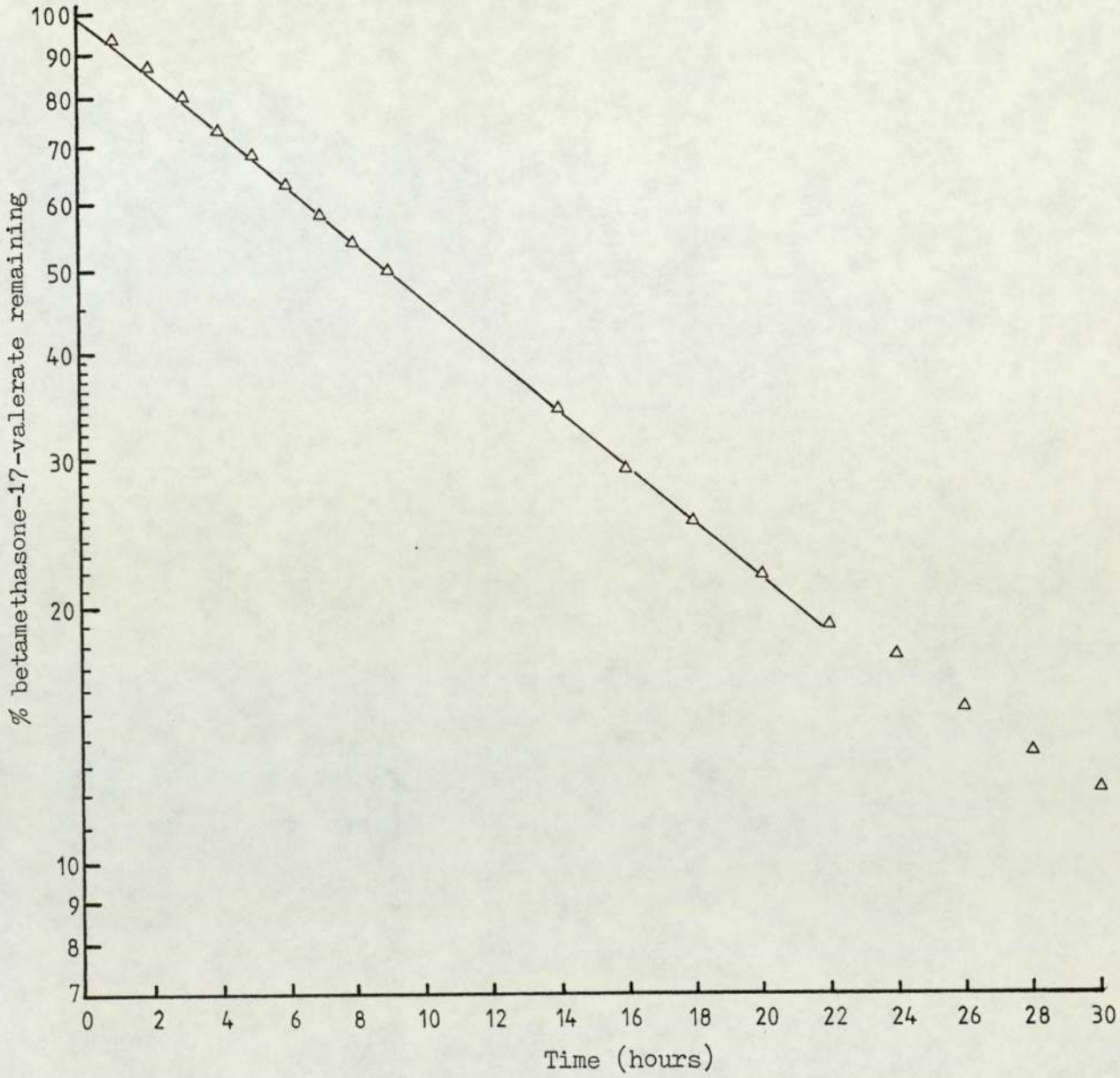


Figure 54. Decomposition of betamethasone-17-valerate 0.02 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

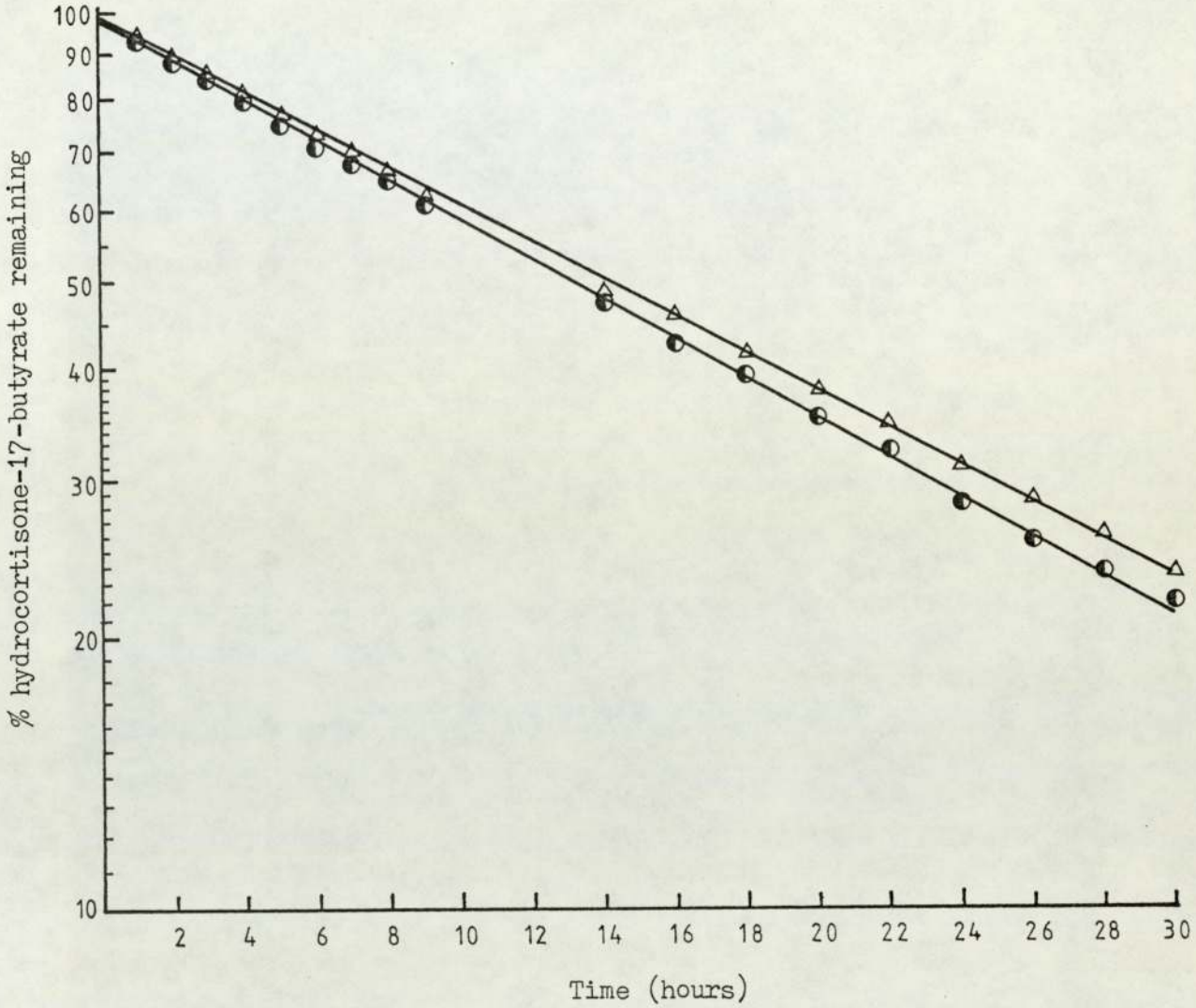


Figure 55. Decomposition of hydrocortisone-17-butyrate 0.1 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.*

Key: ◐ with 0.2% of 3.2 M ammonium sulphate
△ without ammonium sulphate

*data point presented is the mean of duplicates

free alcohol is very slow thus allowing the 21-ester to revert back to the 17-ester to some extent. Therefore, higher concentration of 17-esters could be observed in the control than in the enzymic system. Slower rate constants were therefore obtained based on the disappearance of the 17-esters. In fact, this is supported by stability studies on hydrocortisone-21-butyrate and betamethasone-21-valerate under the same conditions but without enzyme. Initially, there is no 17-ester in the sample solutions; after 30 hours reaction, up to 7% of hydrocortisone-17-butyrate could be detected (Figure 56), and 2% of betamethasone-17-valerate was observed in 5 hours. Therefore, the non-enzymic decomposition rate constant, with the backward reaction taken into account, was calculated by non-linear regression analysis as described in Chapter 3. They were essentially the same as those enzymic rate constants (Table 33). Experimental error also cannot be excluded. Comparing Figure 50b and Figure 53b which show the hydrolysis of betamethasone-17-valerate with different initial drug and enzyme concentrations, the enzymic rate constants in both were the same ($0.084\ hr^{-1}$) while the non-enzymic rate constants varied from 0.072 (Figure 53b) to $0.079\ hr^{-1}$ (Figure 50b). A significant difference between the non-enzymic and the enzymic rate constant is seen in Figure 53b, but those in Figure 50b are essentially the same. Figure 54 shows the non-enzymic decomposition of betamethasone-17-valerate under exactly the same conditions as shown in Figure 53b. If rate constants are calculated on the basis of data from 0 - 22 hours, a rate constant of $0.076\ hr^{-1}$ is obtained. On the other hand, if data points from 0 - 28 hours are used, a value of $0.0726\ hr^{-1}$ is obtained. There is a difference in magnitude of about 4%. Thus, we conclude that the isomerisation is the rate-limiting step in the enzymic hydrolysis of the 17-esters. In other words, the 17-esters are completely

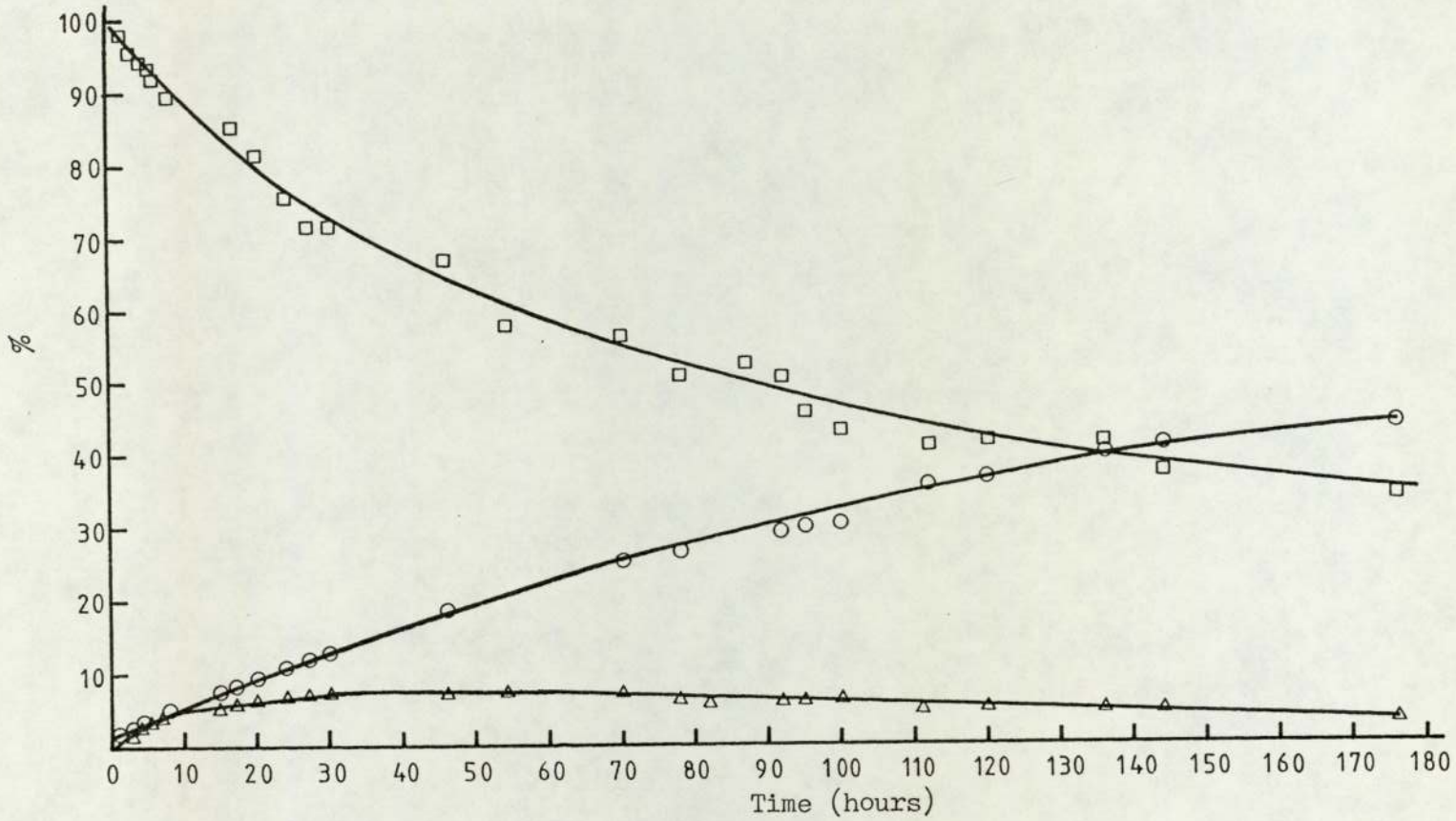


Figure 56. Decomposition of hydrocortisone-21-butyrate 0.1 mg/ml in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

Key: □ Hydrocortisone-21-butyrate remaining

△ Hydrocortisone-17-butyrate formed

○ Hydrocortisone formed

Table 34. Decomposition of hydrocortisone in the presence of 0.01% v/v esterase, in 20% propylene glycol-tris buffer, pH 8.14, at 37°C.

Time (hours)	% Hydrocortisone Remaining	
	with esterase	control
0	100	100
2	95.05	98.76
4	98.57	99.46
6	98.29	100.70
8	98.57	99.43
10	99.43	100.96
12	98.92	|
14	97.04	|
16	99.19	N.D.
18.5	101.87	|
20	99.01	|
22	100.69	|
24	97.05	98.03
30	97.00	101.44
36	97.2	|
40	99.7	N.D.
43	100.24	|

N.D. = not determined

Table 35. Decomposition of betamethasone, 0.02 mg/ml in the presence of 0.004% v/v esterase, in 20% propylene glycol-tris buffer, pH 8.14, 37°C.

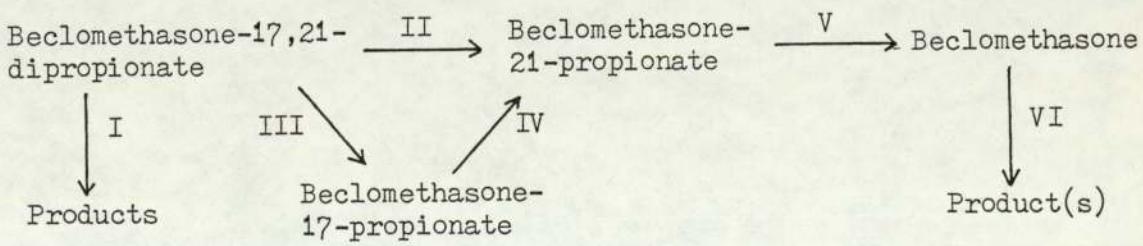
	% Betamethasone Remaining	
Time (hours)	with esterase	control
0	100	100
4	102.9	96.04
6	99.5	98.97
8	101.24	97.76
15	103.65	96.37
18	107.5	98.8
20	105.7	99
23	100.2	93.85
25	100.57	96.1
30	101.3	96.7
40	105.2	102.5
47	99.5	92.5
50	98.81	93.7

resistant to esterase. O'Neill and Carless's (214) observation reported that about 7% of hydrocortisone-17-butyrate and valerate were metabolically hydrolysed after 30 minutes incubation. Rawlins et al (196) also reported that betamethasone-17-valerate is susceptible to enzymic hydrolysis. However, it appears that in these reports, the isomerisation of the 17-ester to the 21-ester was not considered. Under the incubation condition used (pH 7.5), it is quite likely that hydrocortisone-17-butyrate decomposed to the 21-butyrate which was then immediately hydrolysed by the enzyme. The study was therefore inadequate in omitting to follow the kinetics of formation of the 21-ester. Hydrocortisone and betamethasone are resistant to esterase as expected (Table 34 and Table 35 respectively).

To investigate the effect of steric hindrance on the resistance of 17-ester steroids to esterase, the enzymic hydrolysis of a 17,21-diester steroid, beclomethasone-17,21-dipropionate was studied. Its enzymic degradation profile is shown in Figure 57. The same profile was obtained in the absence of the enzyme, although the concentration for the alcohol was marginally higher than that in the control (Figure 57). The rate constants of the enzymic and non-enzymic degradation were 0.00446 hr^{-1} and 0.00390 hr^{-1} respectively (Figure 58). The enzymic and non-enzymic degradation of its monoester, the 17-propionate and 21-propionate were also studied, for comparison of their sensitivities to esterase. The degradation of beclomethasone-17-propionate was first order with respect to the parent compound (Figure 58) and the rate constants were 0.0247 hr^{-1} for the enzymic and 0.0216 hr^{-1} for the non-enzymic transformation. By comparing their enzymic and non-enzymic degradation profiles (Figure 59), the sum of the decomposition products, beclomethasone and the 21-propionate, in both were equal. It appears that isomerisation of beclomethasone-17-propionate to its 21-propionate is a prerequisite for enzymic hydrolysis by esterase.

This is further supported by studying the enzymic hydrolysis of beclomethasone-21-propionate with different concentrations of esterase (Figure 60). It shows that the enzymic hydrolysis rate is much faster than that in the control, even when the concentration of esterase was reduced to 0.1%. The drop in reaction rate after a certain period of time may be due to the inhibition by propylene glycol, since 50% of propylene glycol was used in the medium. It is clear that beclomethasone-21-propionate is much more sensitive to esterase than the 17-propionate. The findings that the hydrolyses of beclomethasone-17,21-dipropionate and its 17-monopropionate by both the enzymic and non-enzymic pathways proceeded at about the same rate suggests that in order for steroids to be susceptible to enzymic degradation by esterase, the 17-OH group must remain free. This is also true of metabolic degradation by epidermal oxidases and may explain why synthetic steroids with the 17-ester functions are generally potent steroids (264).

The decomposition kinetics of beclomethasone-17,21-dipropionate is very complicated. In a study of ethanolamine-catalysed decomposition of this steroid (Figure 61), it was found to degrade very quickly. Apart from beclomethasone, the 17-propionate and the 21-propionate, at least three unknown decomposition products are formed as shown in the HPLC chromatogram (Figure 62). Beclomethasone-21-propionate was only observed for a short time, while the 17-propionate was present at low levels throughout the experiment. From the profiles shown in Figures 57, 59 and 61, a possible Scheme is as follows:



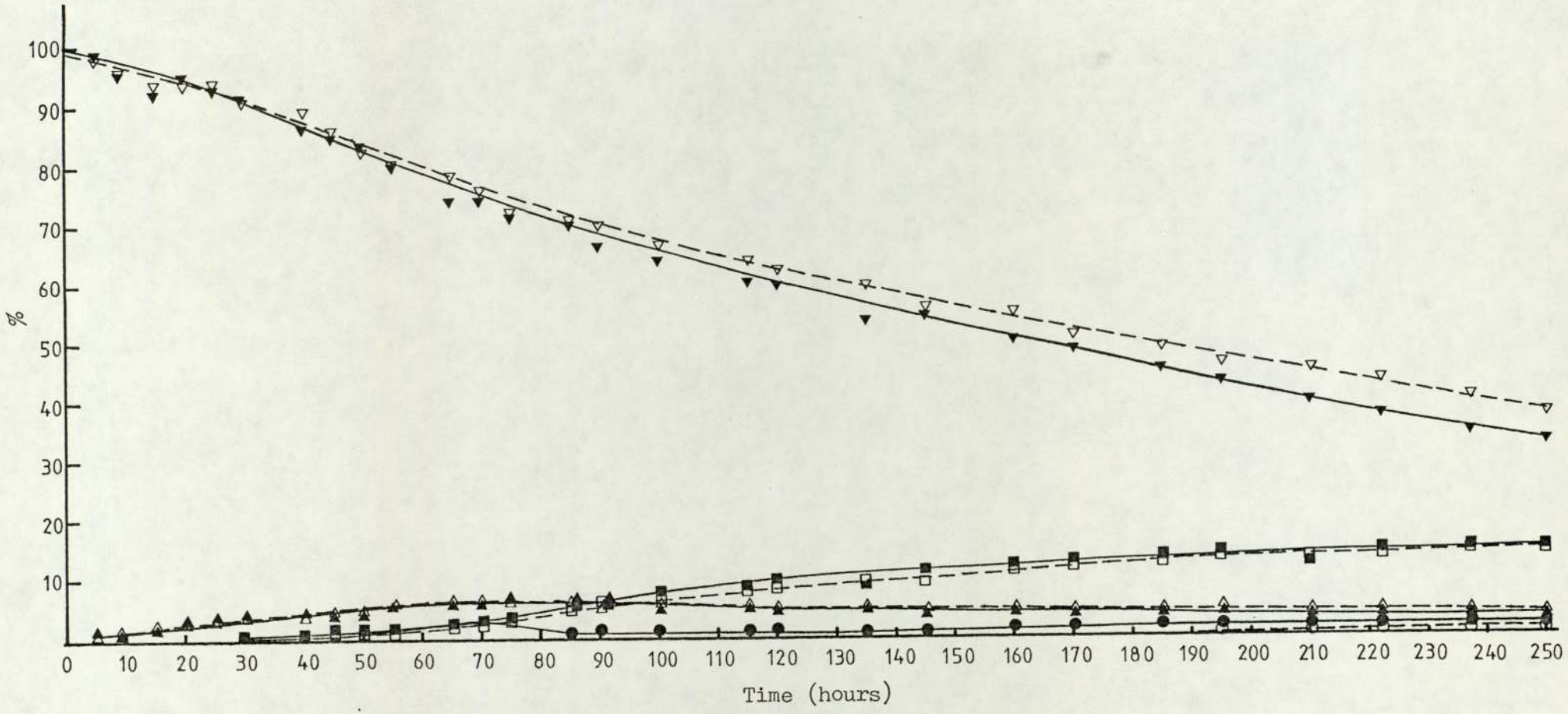


Figure 57. Profiles of enzymic and non-enzymic decomposition of beclomethasone-17,21-dipropionate, 0.1 mg/ml, in 50% propylene glycol-tris buffer, pH 8.04, 37°C.

key: with 0.4% esterase:-

- ▼ Beclomethasone-17,21-dipropionate
- ▲ Beclomethasone-17-propionate
- ■ Beclomethasone-21-propionate
- ● Beclomethasone

Control:-

- ▽ Beclomethasone-17,21-dipropionate
- △ Beclomethasone-17-propionate
- □ Beclomethasone-21-propionate
- ○ Beclomethasone

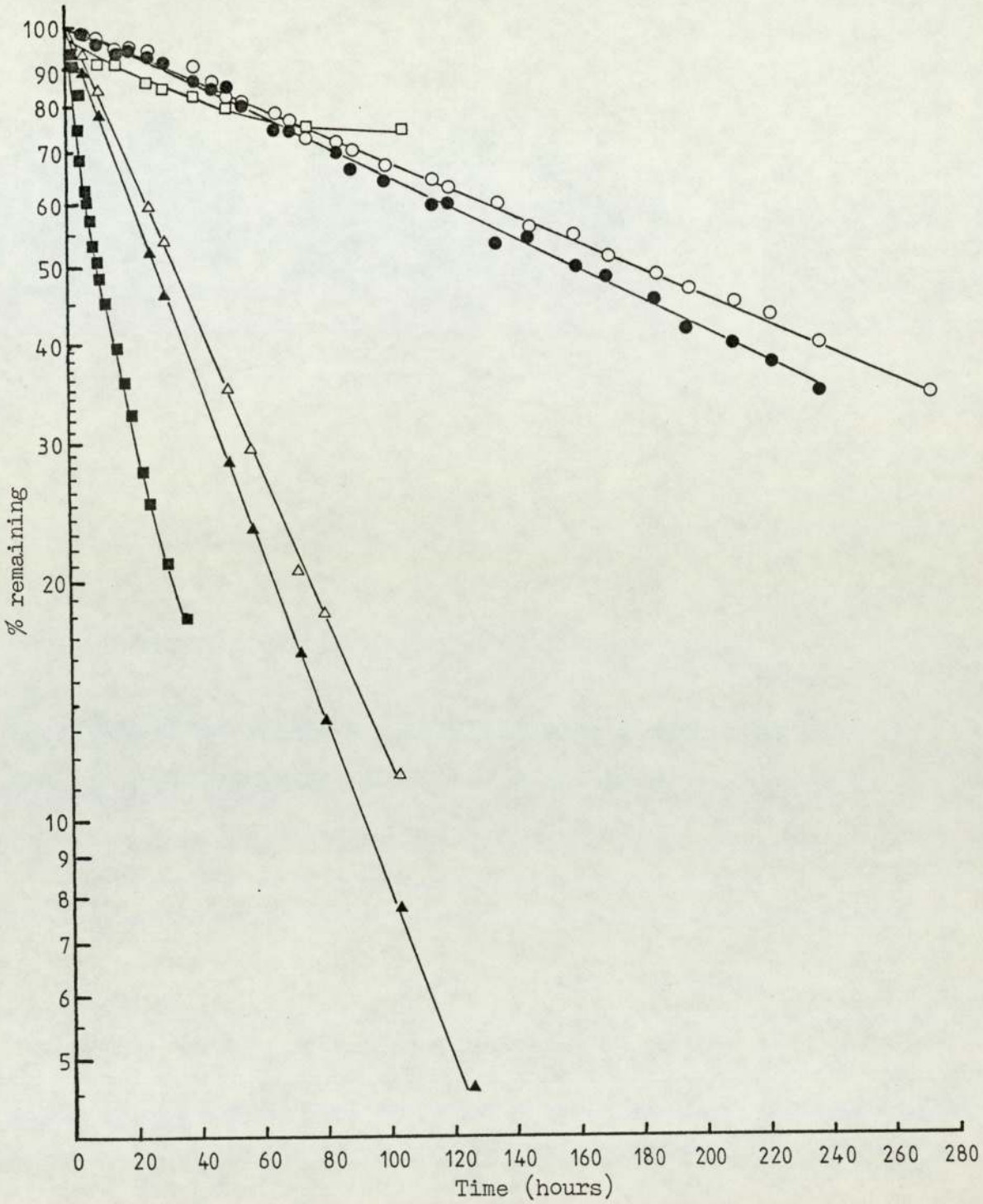


Figure 58. Hydrolysis of beclomethasone esters 0.1 mg/ml by esterase, in 50% propylene glycol-tris buffer, pH 8.04, 37°C.

key:

- ● beclomethasone-17,21-dipropionate
- ▲ beclomethasone-17-propionate
- ■ beclomethasone-21-propionate

} with 0.4% esterase

- ○ beclomethasone-17,21-dipropionate
- △ beclomethasone-17-propionate
- □ beclomethasone-21-propionate

} Control

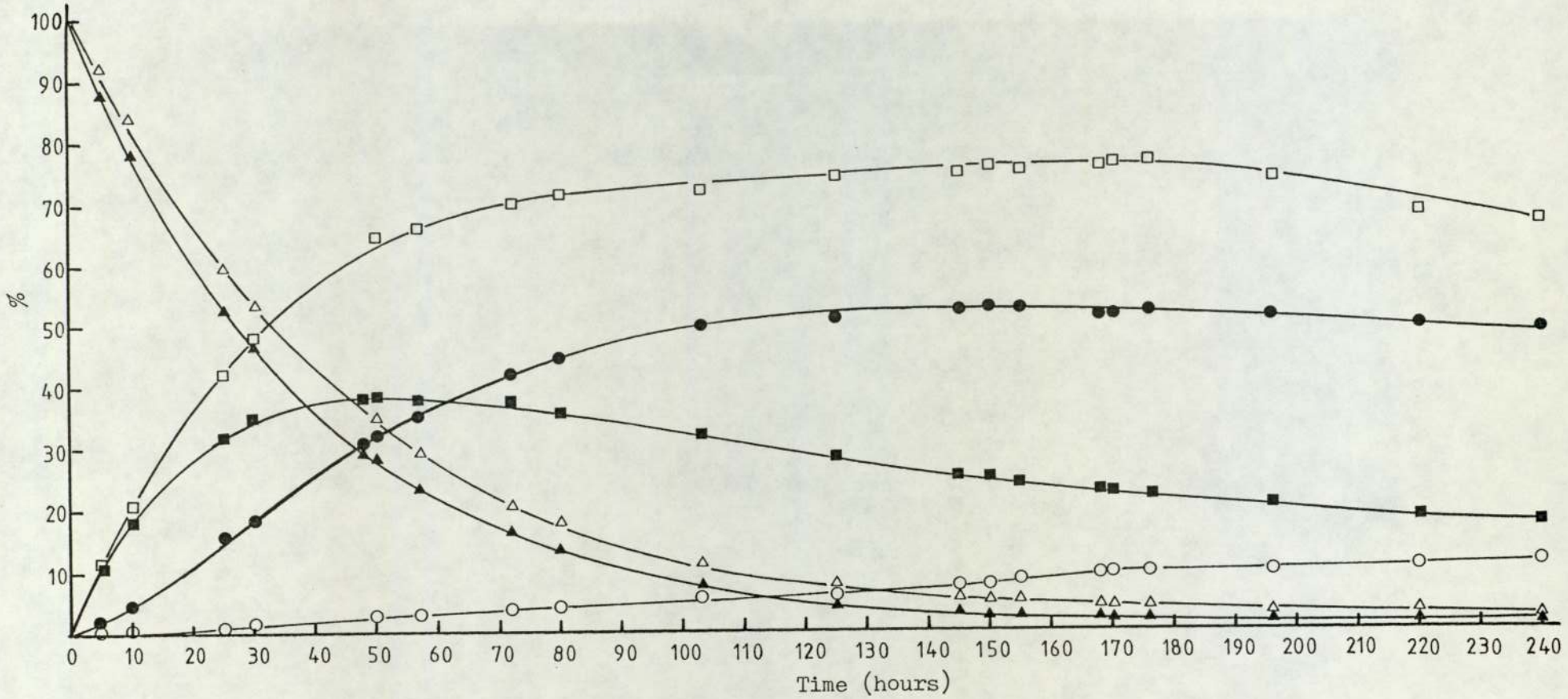


Figure 59. Enzymic and non-enzymic decomposition of beclomethasone-17-propionate, 0.1 mg/ml, in 50% propylene glycol-tris buffer, pH 8.04, 37°C.

key:	with 0.4 % esterase	control
Beclomethasone-17-propionate remaining	▲	△
Beclomethasone-21-propionate formed	■	□
Beclomethasone formed	●	○

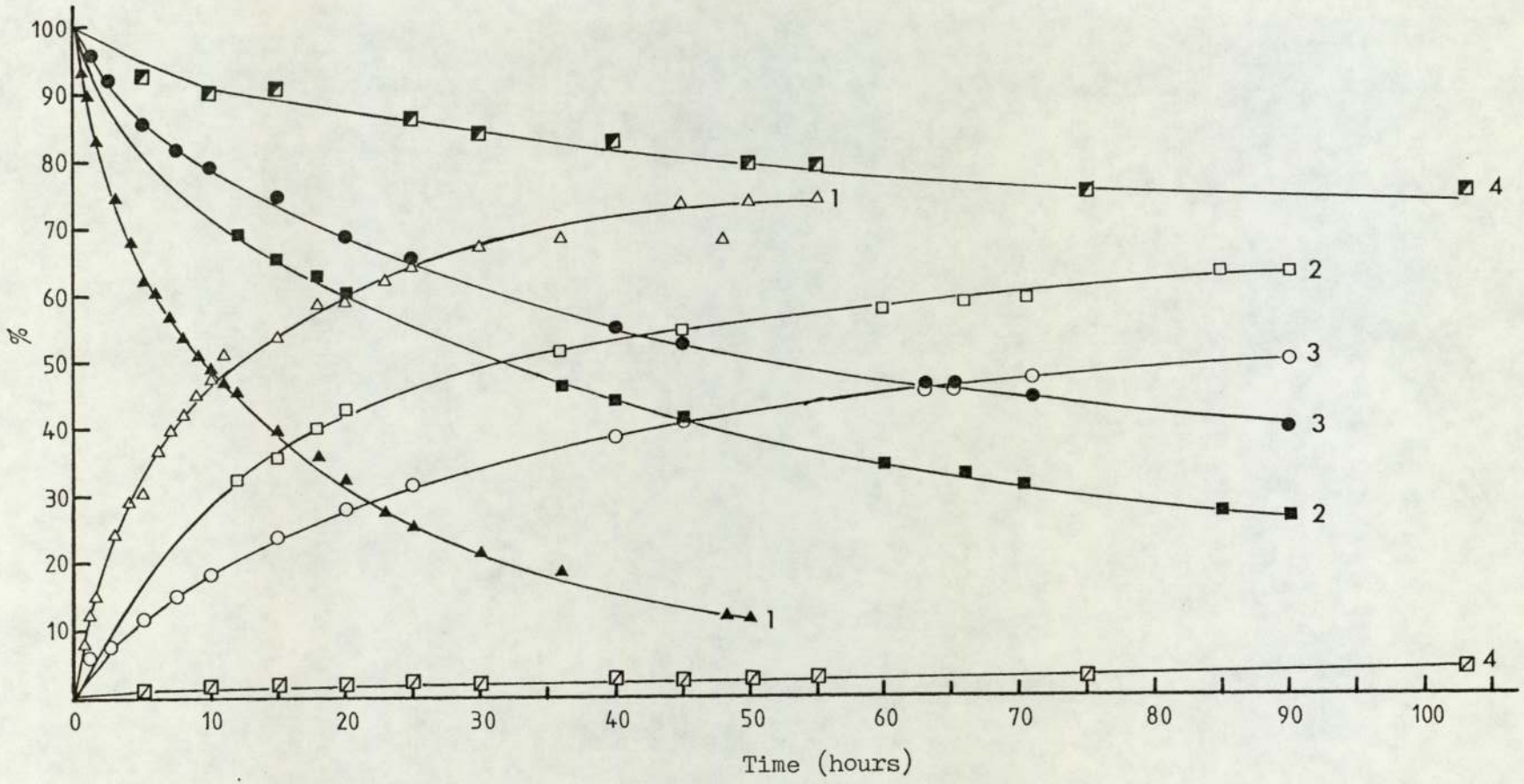


Figure 60. Biotransformation of beclomethasone-21-propionate, 0.1 mg/ml, by esterase, in 50% propylene glycol-tris buffer, pH 8.04, 37°C.

key: ▲, ■, ● and ◪: beclomethasone-21-propionate remaining △, □, ○ and ⊠: beclomethasone formed

1 with 0.4% esterase
2 with 0.2% esterase
3 with 0.1% esterase
4 control

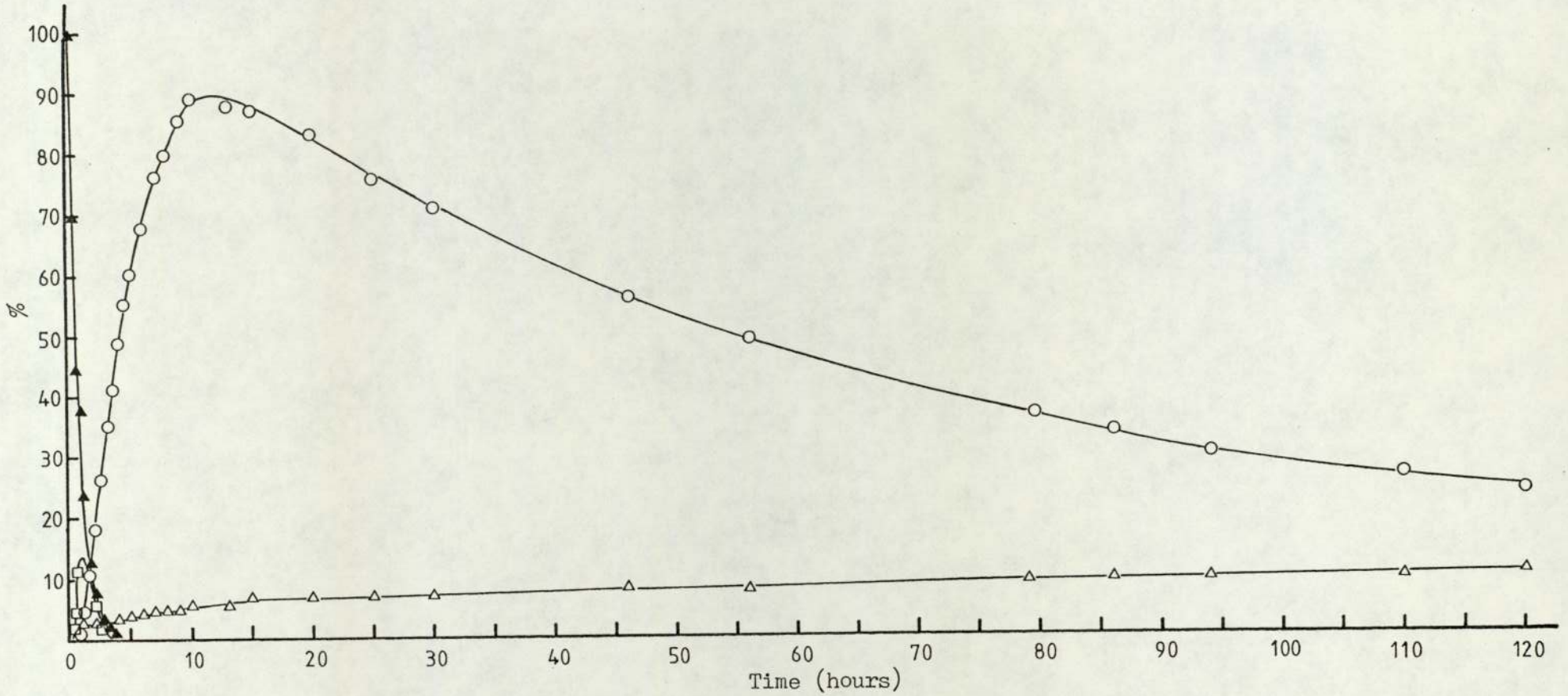


Figure 61. Profiles of ethanolamine-catalysed decomposition of beclomethasone-17,21-dipropionate in 50% propylene glycol-water, pH 10.113, 37°C.

key: ▲ beclomethasone-17,21-dipropionate
△ becolmethasone-17-monopropionate
□ beclomethasone-21-monopropionate
○ beclomethasone

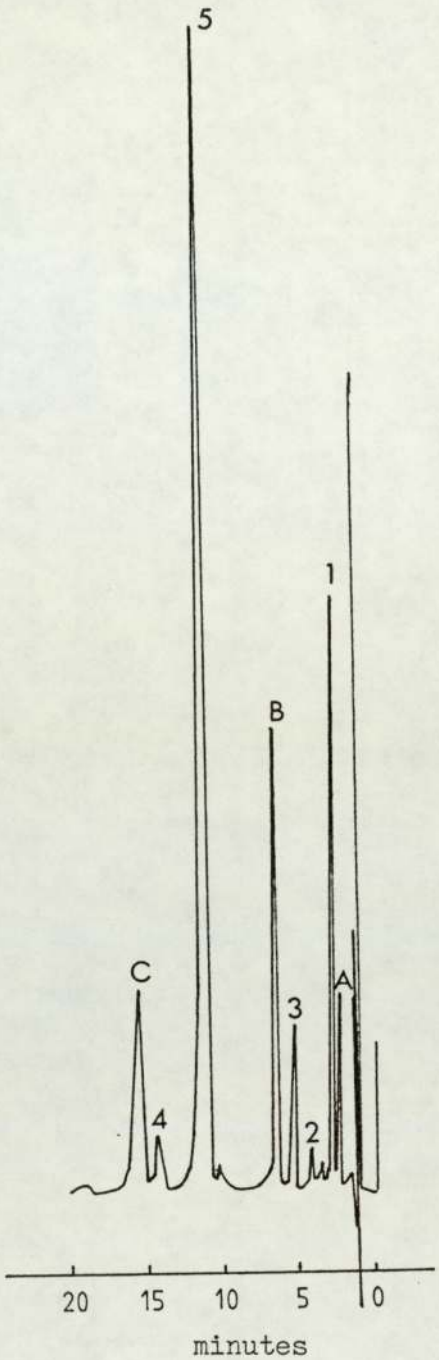


Figure 62. Chromatogram of beclomethasone-17,21-dipropionate and its decomposition products (ethanolamine-catalysed decomposition after 2 hours, in 50% propylene glycol-water, pH 10.113, 37°C).

key: 1 : beclomethasone
2 : beclomethasone-17-propionate
3 : beclomethasone-21-propionate
4 : beclomethasone-17,21-dipropionate
5 : betamethasone-17,21-dipropionate (internal standard)
A,B,C: decomposition products

Pathway I appears to be an important route. The C-17 and C-21 esters are both subject to base-catalysed decomposition and from the pH 8.09 data (Figures 59 and 60), it appears that the isomerisation (pathway IV) is very fast compared to the hydrolyses. The hydrolysis from 17-propionate to the free alcohol is probably insignificant. However, the true decomposition pathway has yet to be determined.

Hitherto the studies described were done using the pure esterase in vitro. The enzymes of the skin may behave in a different way and the existence of similar enzymes has to be confirmed. Mouse skin was therefore used. The whole skin was used, no attempt was made to separate the dermis and epidermis at this stage. The skin was homogenized and the fatty tissue was separated by centrifuge at $4^{o}C$. Preliminary studies confirmed that carboxylic ester hydrolase does exist in mouse skin.

Hsia et al (212) reported that the coenzyme, NADPH, is necessary for restoring the ability of cadaverous skin to metabolize hydrocortisone. In our study, it was experimentally found that the coenzyme was not necessary when using freshly prepared skin homogenates for hydrolysing betamethasone-21-valerate. The same hydrolysis rates were obtained with or without NADPH. The NADPH may have a small stabilizing effect on the enzyme activity. As Figure 63 shows the hydrolysis profiles of betamethasone-21-valerate by a skin homogenates with and without NADPH added prior to storage. The latter has a slower rate although the same amount of NADPH was added before reacting with the steroid. However, as far as the initial rate is concerned, the difference is insignificant (Table 36). In the determination of the initial velocity in enzymic reactions, it is only necessary to draw a tangent at the origin to the first part of the progress curve with the amount of change not exceeding 20% of the total (214a). The addition

of NADPH before initiation of the reaction did not affect the rate ((2) cf (3), Table 36). It has been shown that the activity of the enzyme can be preserved for at least 3 days by freezing, although the skin homogenates was used within 24 hours ((5), Table 36).

In the study of the metabolic transformation of betamethasone-17-valerate by skin homogenates, a faster rate of disappearance of the parent compound was observed in the control (Figure 64). Such a phenomenon was confirmed by repeating the same experiment. The possible reason is that the corticosteroid is bound to the protein; consequently less free betamethasone-17-valerate was available for base-catalysed degradation. This further indicates that betamethasone-17-valerate is essentially resistant to the enzyme. It is the decomposition product, the 21-valerate, which is subject to rapid enzymic hydrolysis, since lower concentration of betamethasone-21-valerate and higher concentration of betamethasone were observed in the enzymic reaction than those in the control, and the sum is equal to that in the control (Figure 64). The possibility of protein binding is further supported by the fact that lower concentration was obtained if the sample aliquots were filtered before dilution with the internal standard and acidified acetonitrile solution (Table 37). When the sample aliquots were diluted before filtration, the steroid was released from the protein, then extracted in the solvent which passed through the filter paper and gave the real value.

Figure 65 shows an enzymic hydrolysis profile of betamethasone-21-valerate by the mouse skin homogenates, in which the rate falls with time, and after 15 hours, the enzymic rate is equal to the non-enzymic rate. It seems that after 15 hours, the enzyme activity is lost. Various causes may contribute to this falling off in activity: the products of the reaction may inhibit the enzyme, the degree of

saturation of the enzyme with substrate may fall because of the fall in substrate concentration, or the enzyme may undergo some inactivation by factors such as propylene glycol. The metabolites of betamethasone-21-valerate are betamethasone and valeric acid. Either one or both may inhibit the enzyme or compete with betamethasone-21-valerate for the enzyme. The activity or amount of the enzyme may vary among different mice, so whenever different batches of skin homogenates were used, a hydrolysis study of betamethasone-21-valerate using the same batch of skin was used as standard, the parameter to be considered is compared to this standard.

Figure 66 shows the lack of effect of betamethasone on the activity of the enzyme. A very high concentration (0.06 mg/ml, same concentration as betamethasone-21-valerate) of betamethasone was used and the reaction rate compared with a standard with no added betamethasone. Although the profiles look slightly different, the initial rates are essentially the same (Table 38). Further data with four different betamethasone concentrations between 0 - 0.06 mg/ml are shown in Figures 66b and 66c). It is concluded that betamethasone does not affect the enzyme activity. Similarly, no inhibition was observed in the presence of valeric acid (Figure 67a) or the combination of valeric acid and betamethasone (Figure 67b). All the rates were equal (Table 39).

It is clear that propylene glycol inactivates the enzymes (Table 40), but this can be inhibited by freezing (Figure 68a). When the skin homogenates was stored in the presence of propylene glycol at 37°C for 12 hours, the enzyme became less active (Figure 68b). However, if the sample was stored under the same conditions for 5 hours only, the enzyme activity was not changed (Figure 68b). Reducing the propylene glycol concentration to 20%, eliminates the inhibition and a zero order enzymic hydrolysis is observed (Figure 69).

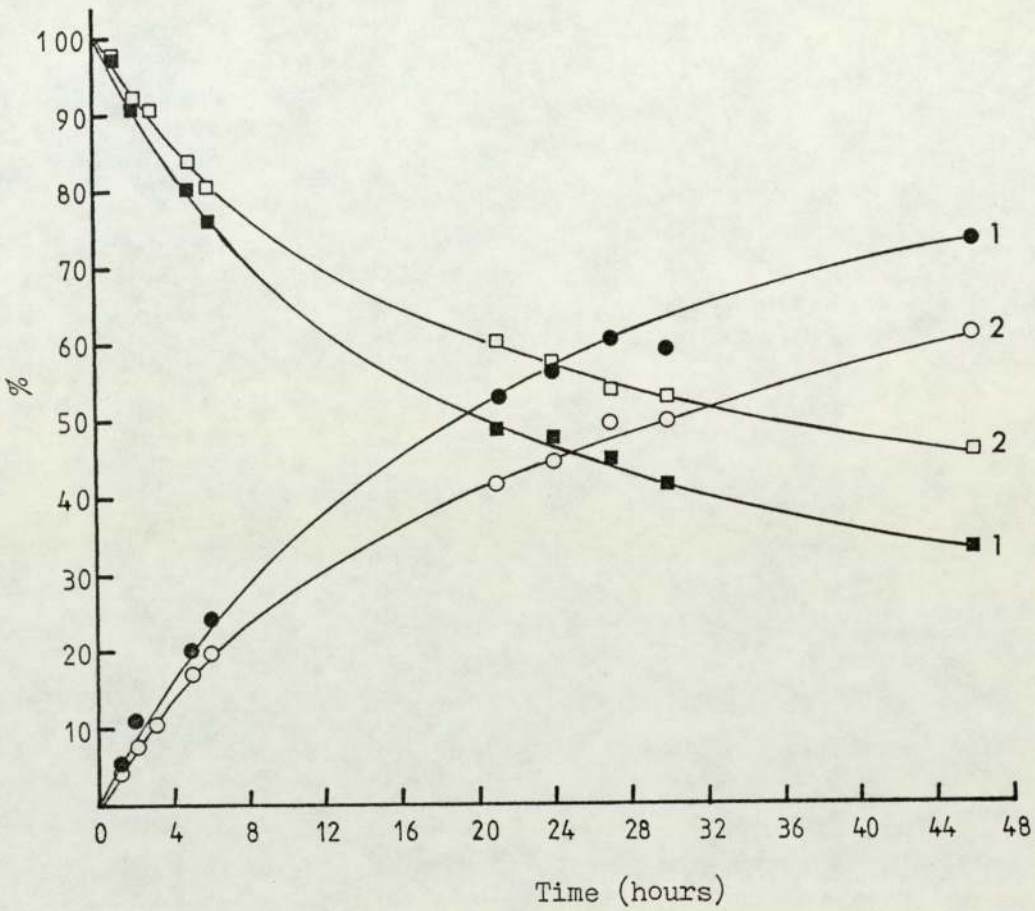


Figure 63. Effect of coenzyme on enzymic activity of mouse skin homogenates.

key:

1:NADPH was added to the skin homogenates, stored at -15°C for one day:
- ■ betamethasone-21-valerate remaining
- ● betamethasone formed

2:Skin homogenates was stored at -15°C for one day, then NADPH was added:-
- □ betamethasone-21-valerate remaining
- ○ betamethasone formed

Table 36. Biotransformation of betamethasone-21-valerate 0.06 mg/ml by mouse skin homogenates in the presence and absence of NADPH, 37°C.

Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.5

sample No. / composition	(1)		(2)		(3)		(4)		(5)	
Time (hours)	% B-21-val	% B	% B-21-val	% B	% B-21-val	% B	% B-21-val	% B	% B-21-val	% B
0	100.0	0	100.0	0	100.0	0	100.0	0	100.0	0
1	98.6	5.5	98.0	4.8	96.3	4.8	93.7	5.2	94.1	5.5
2	91.0	11.1	92.3	7.8	92.5	8.7	88.7	8.8	89.4	10.2
3	N.D.	N.D.	90.7	10.5	91.0	11.3	89.2	11.8	87.7	13.5
4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	84.8	14.6	N.D.	N.D.
5	80.4	20.1	84.1	17.2	N.D.	N.D.	78.7	17.6	81.3	19.7
6	76.0	24.5	80.8	19.9	N.D.	N.D.	N.D.	N.D.	76.1	22.3
7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	76.8	23.1	75.9	24.7
18	N.D.	N.D.	N.D.	N.D.	64.2	38.0	N.D.	N.D.	N.D.	N.D.
21	48.9	52.9	60.3	41.6	61.1	39.5	N.D.	N.D.	N.D.	N.D.
23	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	59.2	42.5	N.D.	N.D.
24	47.9	56.2	57.7	44.4	59.6	43.0	N.D.	N.D.	55.6	45.6
27	45.0	60.2	54.2	49.7	56.0	43.9	N.D.	N.D.	N.D.	N.D.
28	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	53.8	48.1
30	41.4	59.0	53.3	49.7	N.D.	N.D.	54.8	47.3	N.D.	N.D.
Initial Rate Constant (hr^{-1})	Disappearance of B-21-val: 4.1	Formation of B: 3.9	Disappearance of B-21-val: 3.2	Formation of B: 3.2	Disappearance of B-21-val: 3.4	Formation of B: 3.7	Disappearance of B-21-val: 3.5	Formation of B: 3.4	Disappearance of B-21-val: 3.6	Formation of B: 3.9

(1) NADPH was added to mouse skin homogenates and stored at -15°C for 1 day

(2) Mouse skin homogenates was stored at -15°C for one day, and freshly prepared NADPH was then added

(3) Mouse skin homogenates was stored at -15°C for one day, without NADPH

(4) Mouse skin homogenates was stored at -15°C for two days, without NADPH

(5) Mouse skin homogenates was stored at -15°C for three days, without NADPH

N.D. = not determined

B-21-val = betamethasone-21-valerate

B = betamethasone

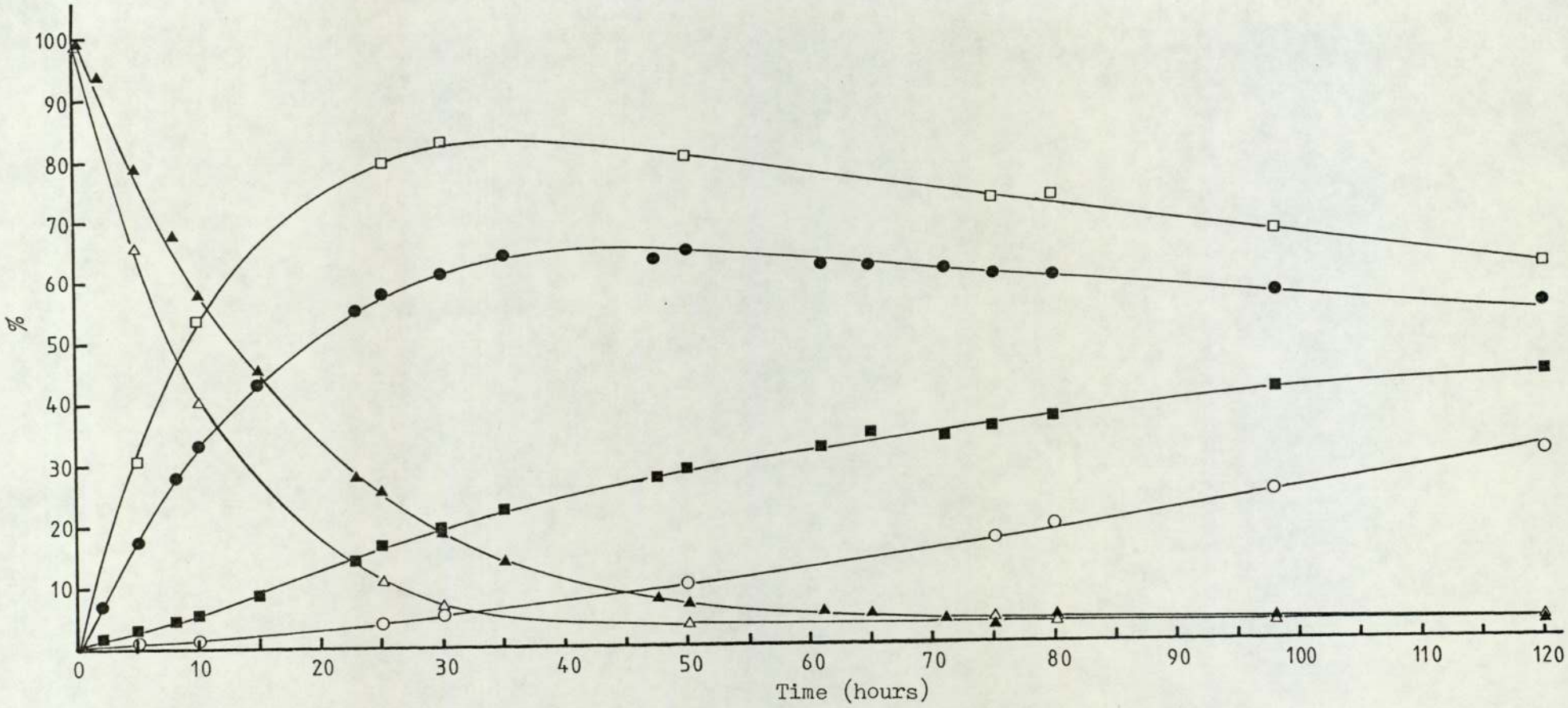


Figure 64. Biotransformation of betamethasone-17-valerate 0.06 mg/ml by mouse skin homogenates, in 50% propylene glycol-Krebs buffer, pH 7.54, 37°C.

key:

with mouse skin homogenates:-
- ▲ betamethasone-17-valerate remaining
- ■ betamethasone-21-valerate formed
- ● betamethasone formed

control:-
- △ betamethasone-17-valerate remaining
- □ betamethasone-21-valerate formed
- ○ betamethasone formed

Table 37. Effect of the order of filtration and dilution on assay of steroids in the presence of mouse skin homogenates.

	% Composition					
	Sample solutions were filtered before dilution			Sample solutions were diluted before filtration		
Sample No.	% B-21-val	% B	% B-17-val	% B-21-val	% B	% B-17-val
1	51.7	5.7	37.6	54.7	6	39.7
2	40.3	40.7		51.3	50.6	
3	43.3	54.3		44.9	58.3	
4	34.6	22.1		43.2	57.3	

B = betamethasone
B-17-val = betamethasone-17-valerate
B-21-val = betamethasone-21-valerate

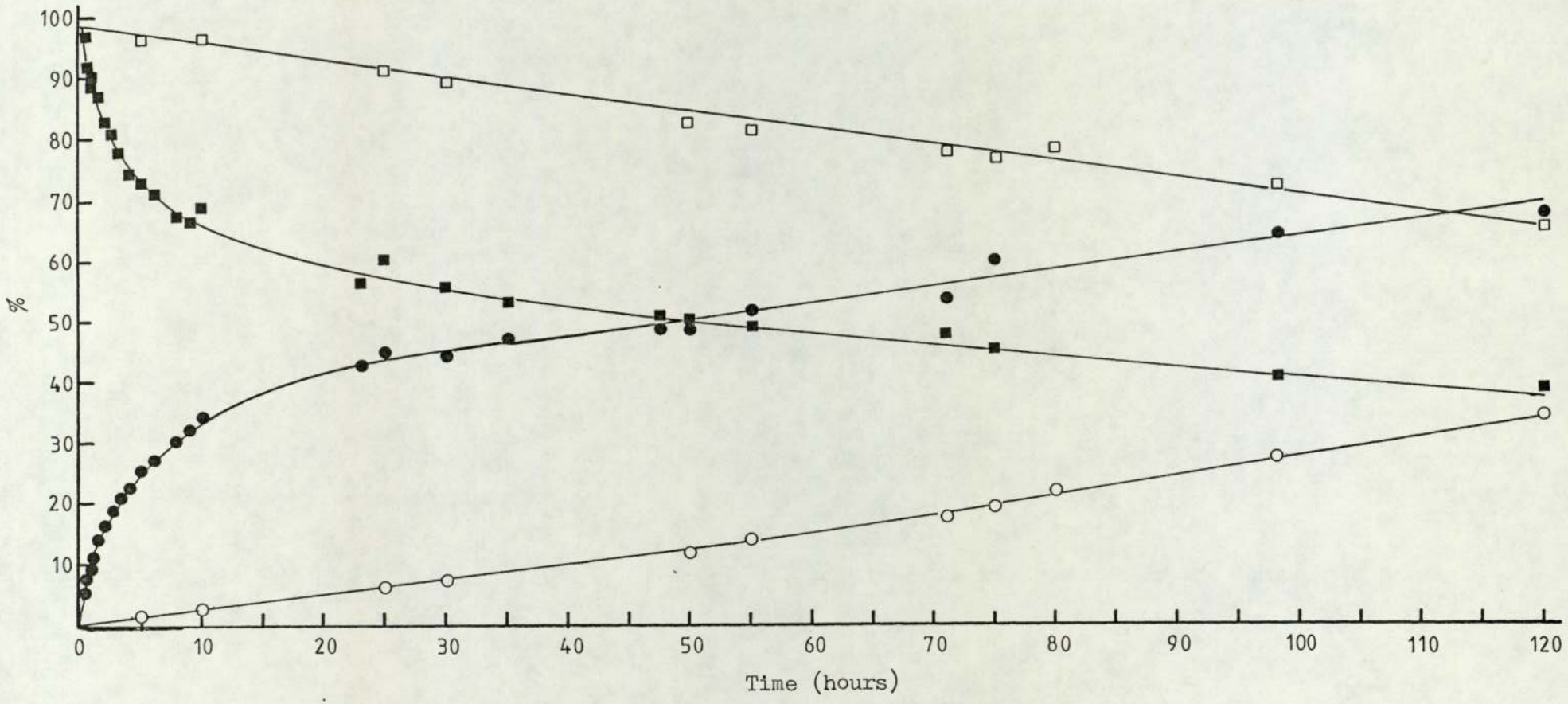
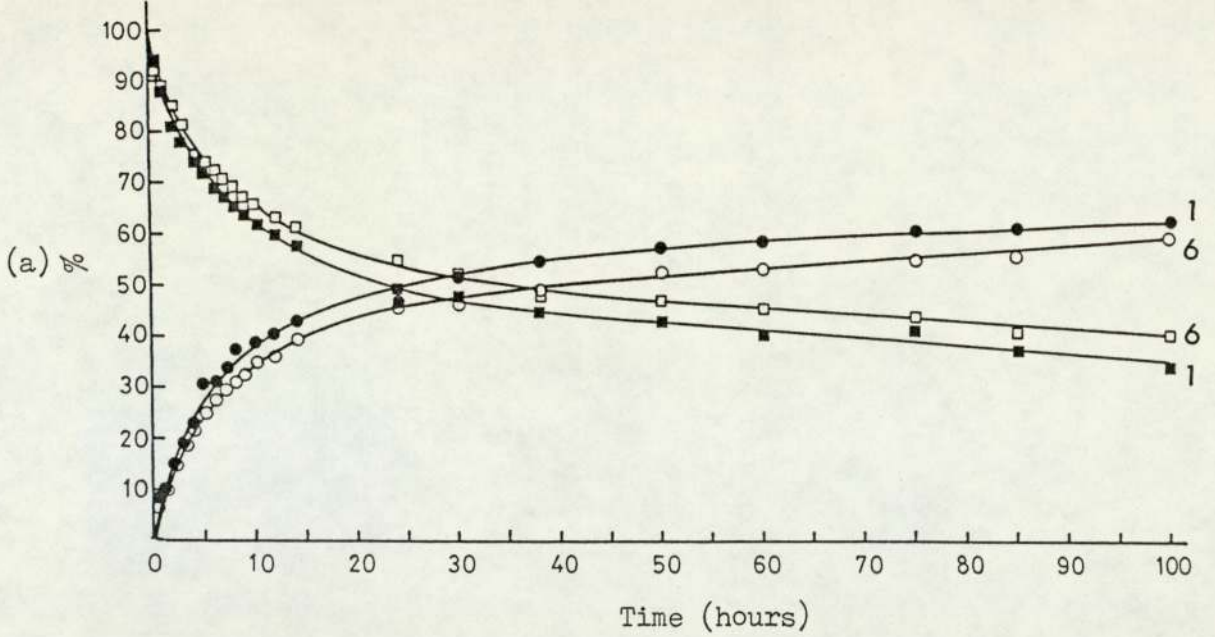


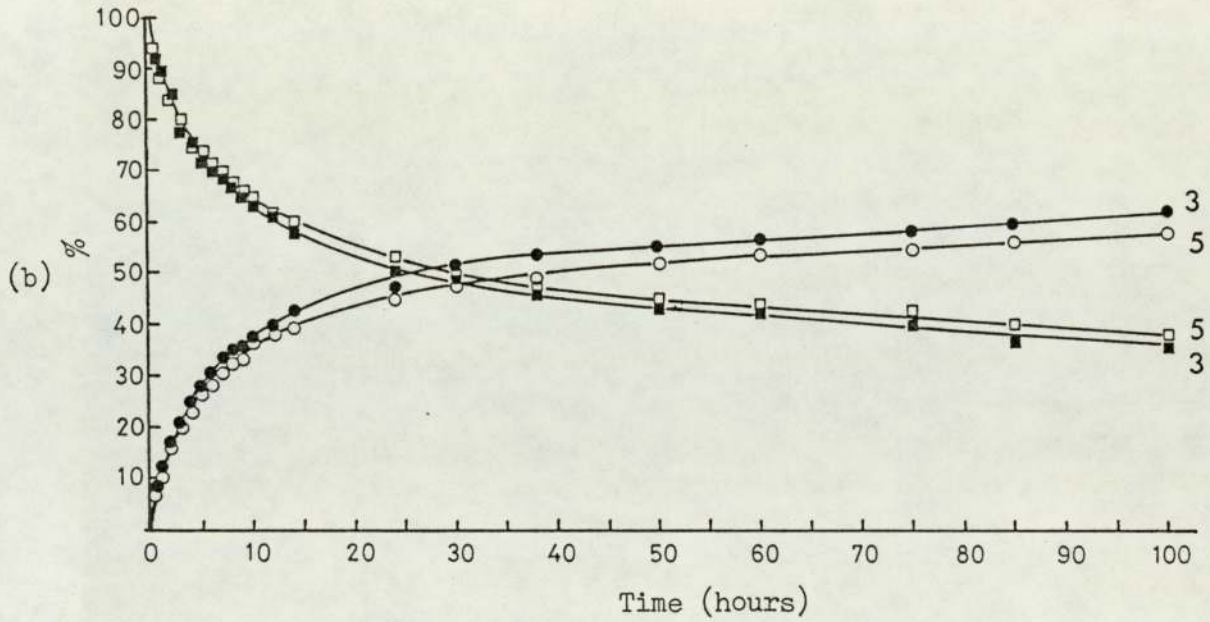
Figure 65. Biotransformation of betamethasone-21-valerate 0.06 mg/ml by mouse skin homogenates, in 50% propylene glycol-Krebs buffer, pH 7.5, 37°C.

key:

with mouse skin homogenates:-
■ betamethasone-21-valerate remaining
● betamethasone formed

control:-
□ betamethasone-21-valerate remaining
○ betamethasone formed


(a) %
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
1
6
6
1


(b) %
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
3
5
5
3

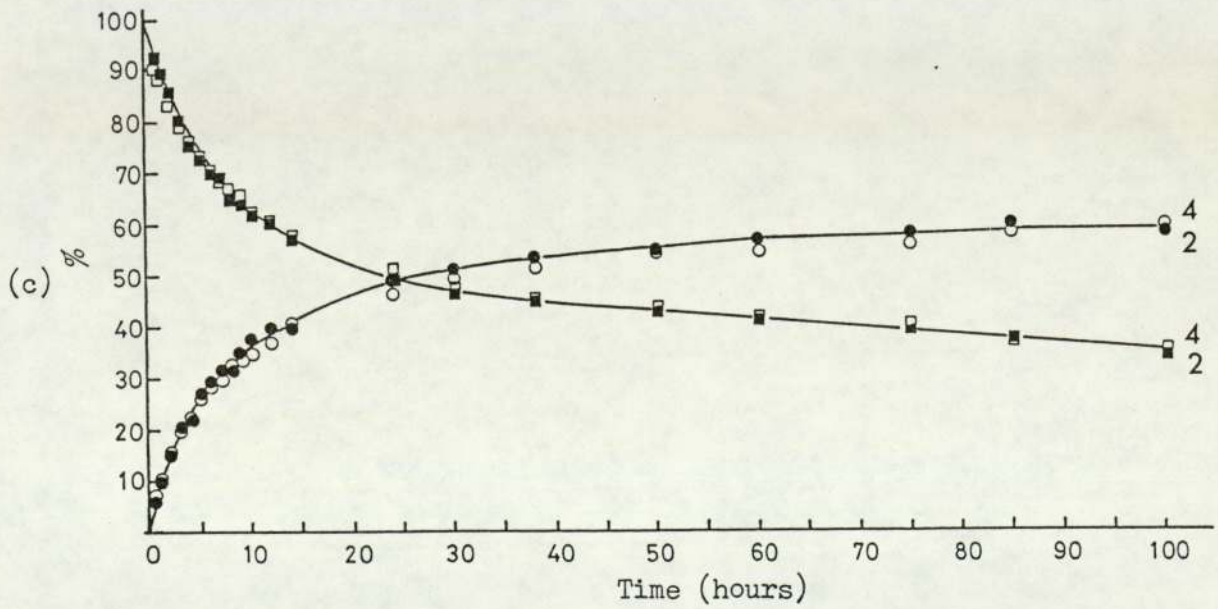

(c) %
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
4
2
4
2

Table 38. Initial rates of enzymic hydrolysis of betamethasone-21-valerate, 0.06 mg/ml, by mouse skin homogenates in the presence of betamethasone, at 37°C.
Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.54

Concentration of betamethasone (mg/ml)	Initial Rate Constant (hr^{-1})	
	Disappearance of betamethasone-21-valerate	Formation of betamethasone
0.06	8.54	6.64
0.02	5.6	7.4
0.015	7.26	6.4
0.010	7.7	6.7
0.005	7.28	6.65
0	8.6	6.63

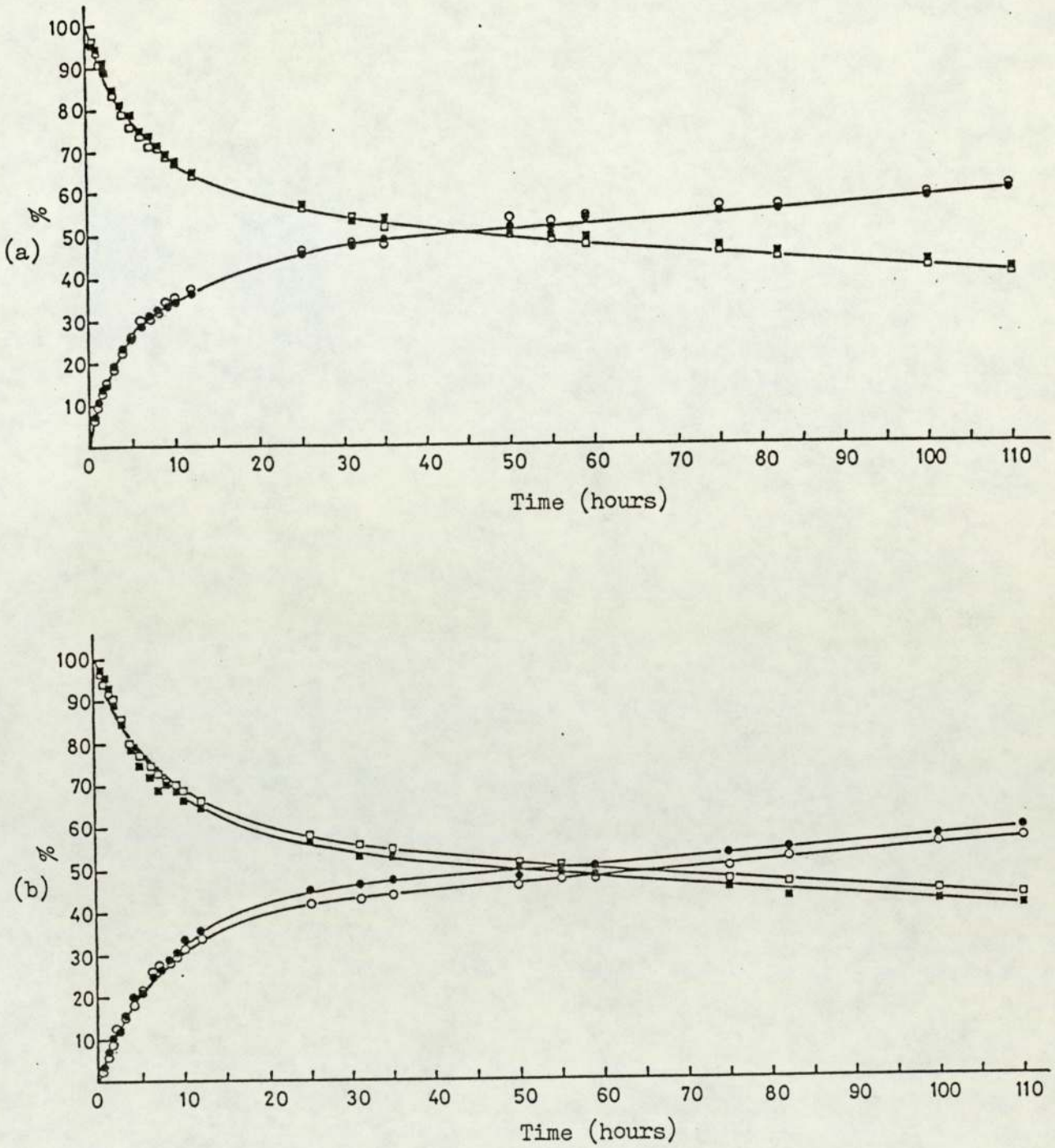


Figure 67. Enzymic hydrolysis of betamethasone-21-valerate 0.06 mg/ml by mouse skin homogenates in the presence of betamethasone and valeric acid, at 37°C.
Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.54

key: ■ and □ : betamethasone-21-valerate remaining
● and ○ : betamethasone formed
(a) □ , ○ without betamethasone and valeric acid (control standard)
(a) ■ , ● with 0.02 mg/ml valeric acid
(b) with 0.02 mg/ml betamethasone and valeric acid

Table 39. Initial rates of enzymic hydrolysis of betamethasone-21-valerate, 0.06 mg/ml, by mouse skin homogenates in the presence of betamethasone and valeric acid, at 37°C. Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.54

Concentration of betamethasone (mg/ml)	Concentration of Valeric acid (mg/ml)	Initial Rate Constant (hr^{-1})	
		Disappearance of betamethasone-21-valerate	Formation of betamethasone
0	0	4.7	5.4
0	0.02	5.4	5.9
0.02	0.02	4.93	5.1
0.02	0.02	4.75	4.73

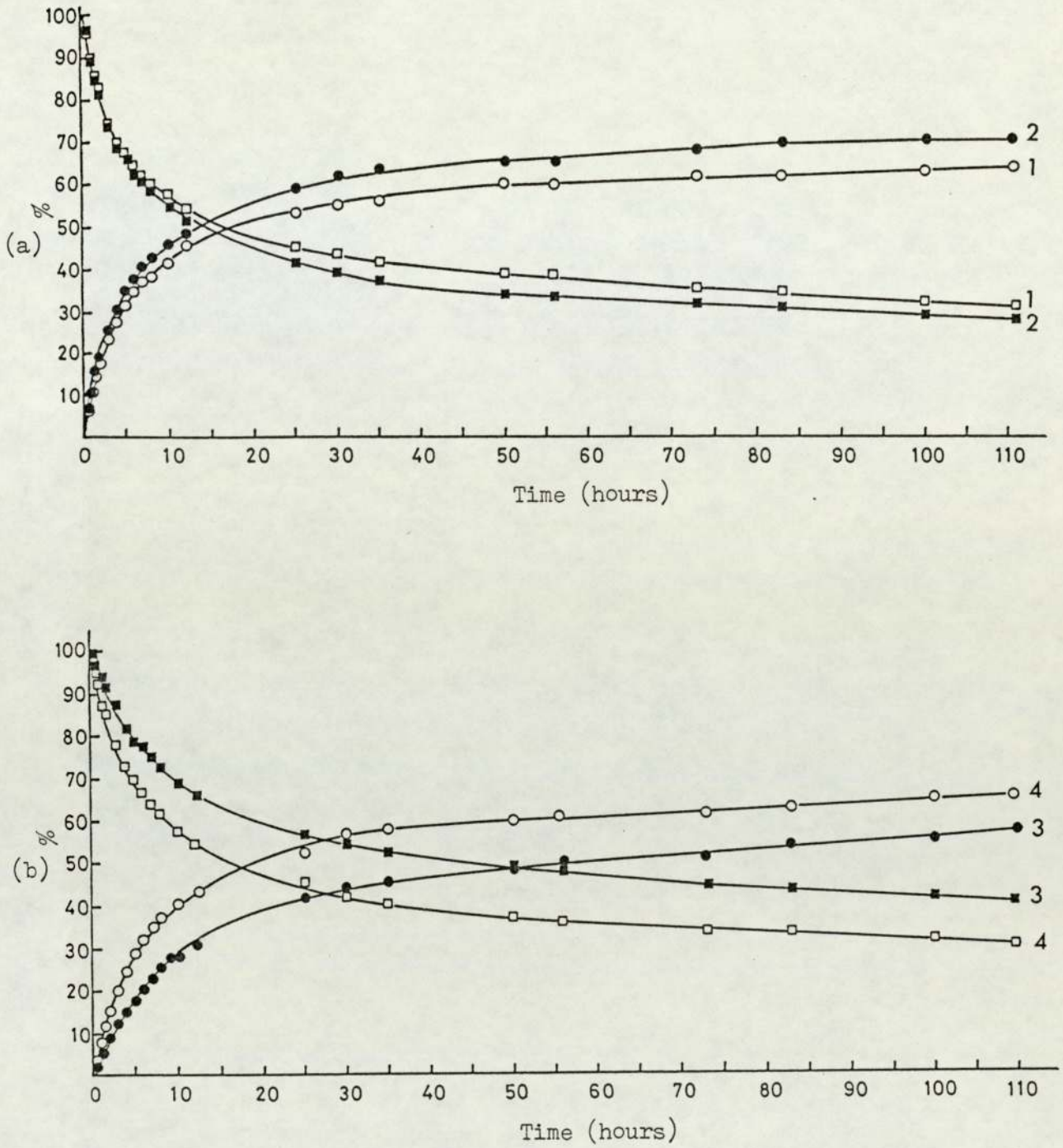


Figure 68. Effect of propylene glycol on mouse skin enzymic activity, 37°C.
Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.54
Substrate: 0.06 mg/ml betamethasone-21-valerate

key: ■ and □ : betamethasone-21-valerate remaining

● and ○ : betamethasone formed

1 mouse skin homogenates stored at -15°C for 12 hours (control standard)

2 mouse skin homogenates with propylene glycol, stored at -15°C for 12 hours

3 mouse skin homogenates with propylene glycol, stored at 37°C for 12 hours

4 mouse skin homogenates with propylene glycol, stored at 37°C for 5 hours

Table 40. Effect of propylene glycol on mouse skin enzymic activity, at 37°C.
Incubation medium: 50% propylene glycol-Krebs buffer, pH 7.54
Substrate: betamethasone-21-valerate 0.06 mg/ml

	Initial Rate Constant (hr^{-1})	
	Disappearance of betamethasone-21-valerate	Formation of betamethasone
1. Mouse skin homogenates stored at -15°C for 12 hours (control standard)	8.58	8.78
2. Mouse skin homogenates with propylene glycol, stored at -15°C for 12 hours	9.54	9.2
3. Mouse skin homogenates with propylene glycol, stored at 37°C for 12 hours	4.4	3.84
4. Mouse skin homogenates with propylene glycol, stored at 37°C for 5 hours	7.98	7.74

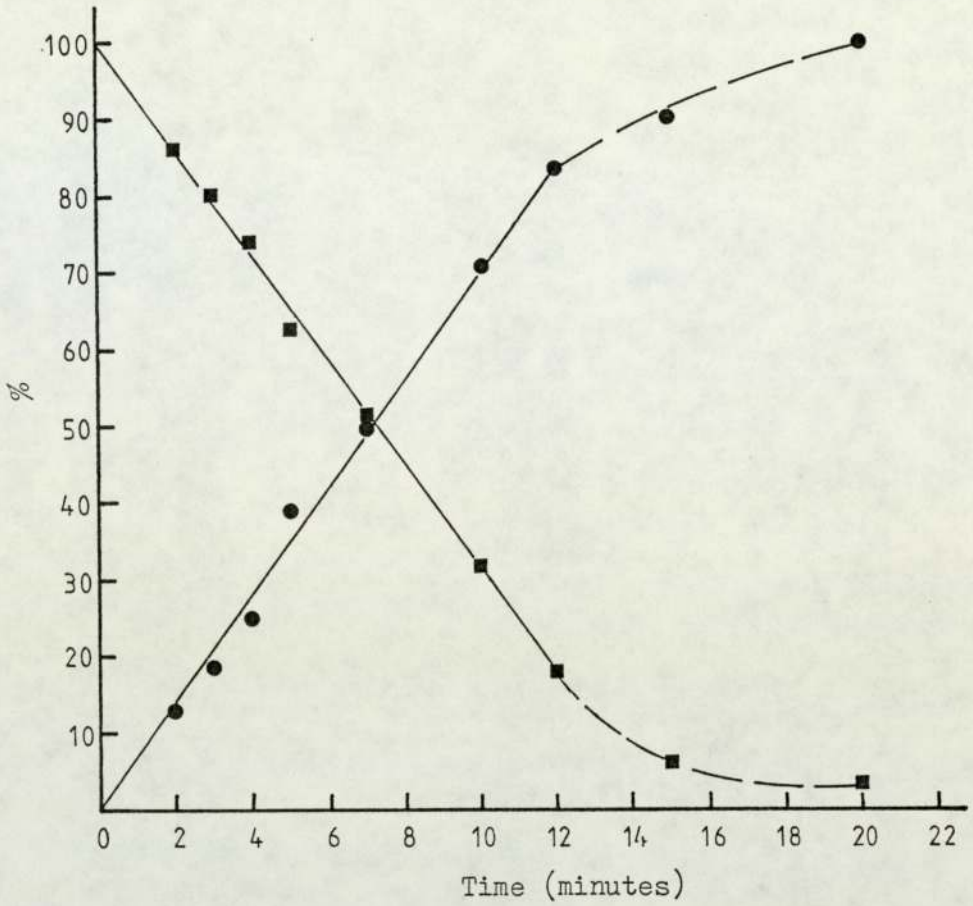


Figure 69. Enzymic hydrolysis of betamethasone-21-valerate 0.02 mg/ml by mouse skin homogenates, in 20% propylene glycol-Krebs buffer, pH 7.5, 37°C.

key: ■ betamethasone-21-valerate remaining
● betamethasone formed

Results from the in vivo biotransformation of betamethasone-17- and 21-valerates and hydrocortisone-17- and 21-butyrates, using mouse skin homogenates, parallel the results obtained using pure esterase. The 21-esters are much more susceptible to the skin enzymes whereas the 17-esters are resistant. As shown in Figures 70 and 71, the enzymic hydrolyses of the 21-esters are very rapid. Hydrocortisone-21-butyrate is relatively resistant to the mouse skin enzymes when compared to betamethasone-21-valerate. The initial rate constants are 1.296 min^{-1} and 2.174 min^{-1} respectively. It appears that the corresponding free alcohol is the only product. No other product(s) were detected by the HPLC system used, and the sum of the 21-ester and the alcohol reached 100%. In the absence of mouse skin, the steroid showed no decomposition within 2 hours. On the other hand, the enzymic decomposition rate constants of hydrocortisone-17-butyrate and betamethasone-17-valerate are 0.0472 hr^{-1} and 0.0635 hr^{-1} respectively, which are identical to those in the control: 0.0479 and 0.0667 hr^{-1} respectively (Table 41). The rate-limiting step is the isomerisation from the 17-esters to the 21-esters which then are hydrolysed rapidly by the skin enzymes. This is reflected in the similar rates of appearance of the 21-esters and of the corresponding alcohol in the control and in the enzymic reaction (Figures 72 and 73).

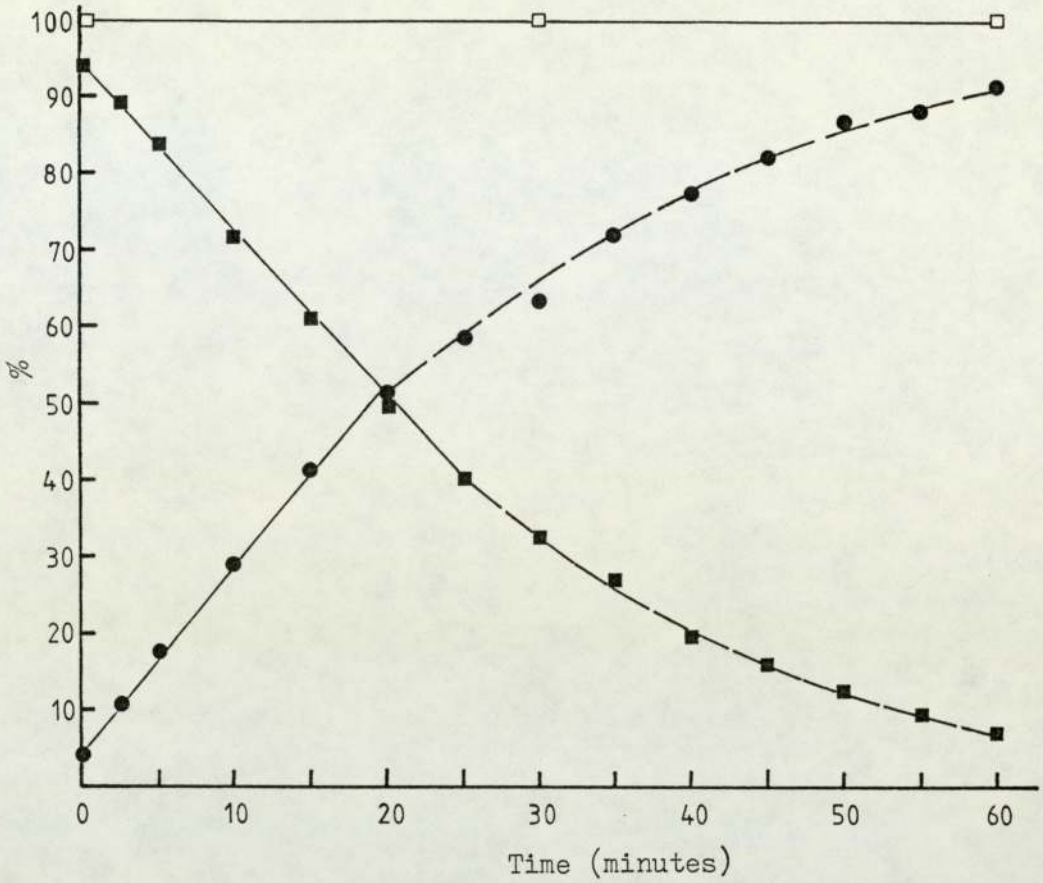


Figure 70. Biotransformation of betamethasone-21-valerate 0.02 mg/ml by mouse skin homogenates, in 20% propylene glycol-tris buffer, pH 7.93, 37°C.

key:

■ betamethasone-21-valerate remaining
● betamethasone formed
} with mouse skin homogenates

□ betamethasone-21-valerate remaining in the control

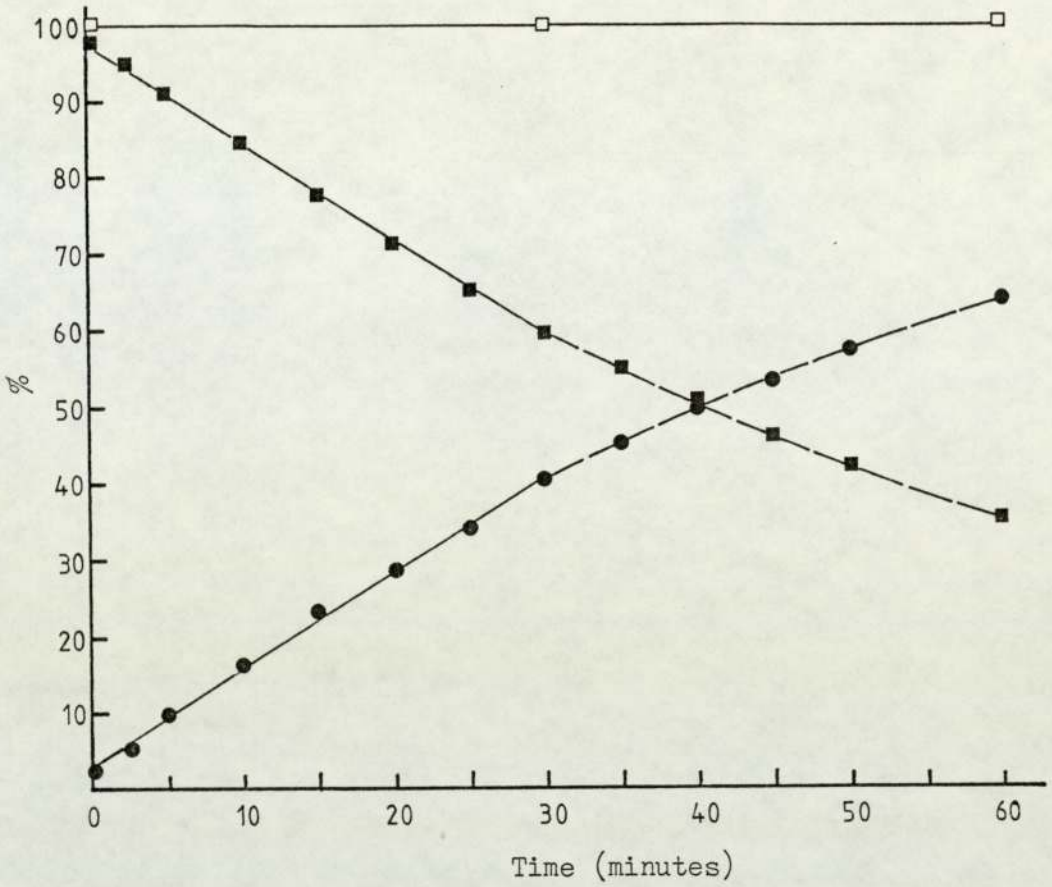


Figure 71. Biotransformation of hydrocortisone-21-butyrate 0.02 mg/ml by mouse skin homogenates, in 20% propylene glycol-tris buffer, pH 7.93, 37°C.

key:

■ hydrocortisone-21-butyrate remaining ┐
● hydrocortisone formed ┘ with mouse skin homogenates

□ hydrocortisone-21-butyrate remaining in the control

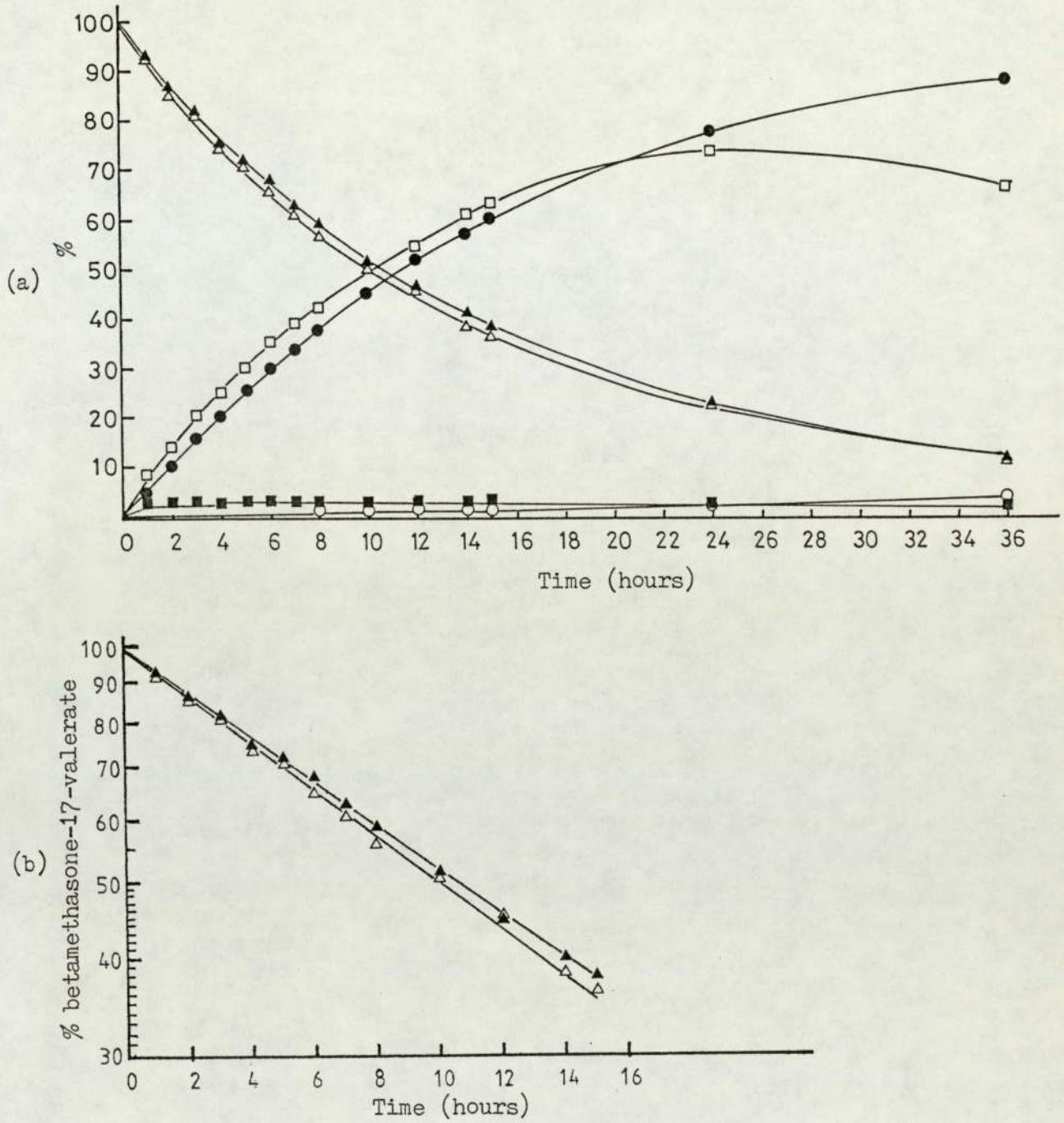


Figure 72. Biotransformation of betamethasone-17-valerate 0.02 mg/ml by mouse skin homogenates, in 20% propylene glycol-tris buffer, pH 7.93, 37°C.

(a) Time-course profiles
(b) Log % betamethasone-17-valerate remaining vs. Time

key:

▲ betamethasone-17-valerate remaining
■ betamethasone-21-valerate formed
● betamethasone formed
} in the presence of mouse skin homogenates

△ betamethasone-17-valerate remaining
□ betamethasone-21-valerate formed
○ betamethasone formed
} in the control

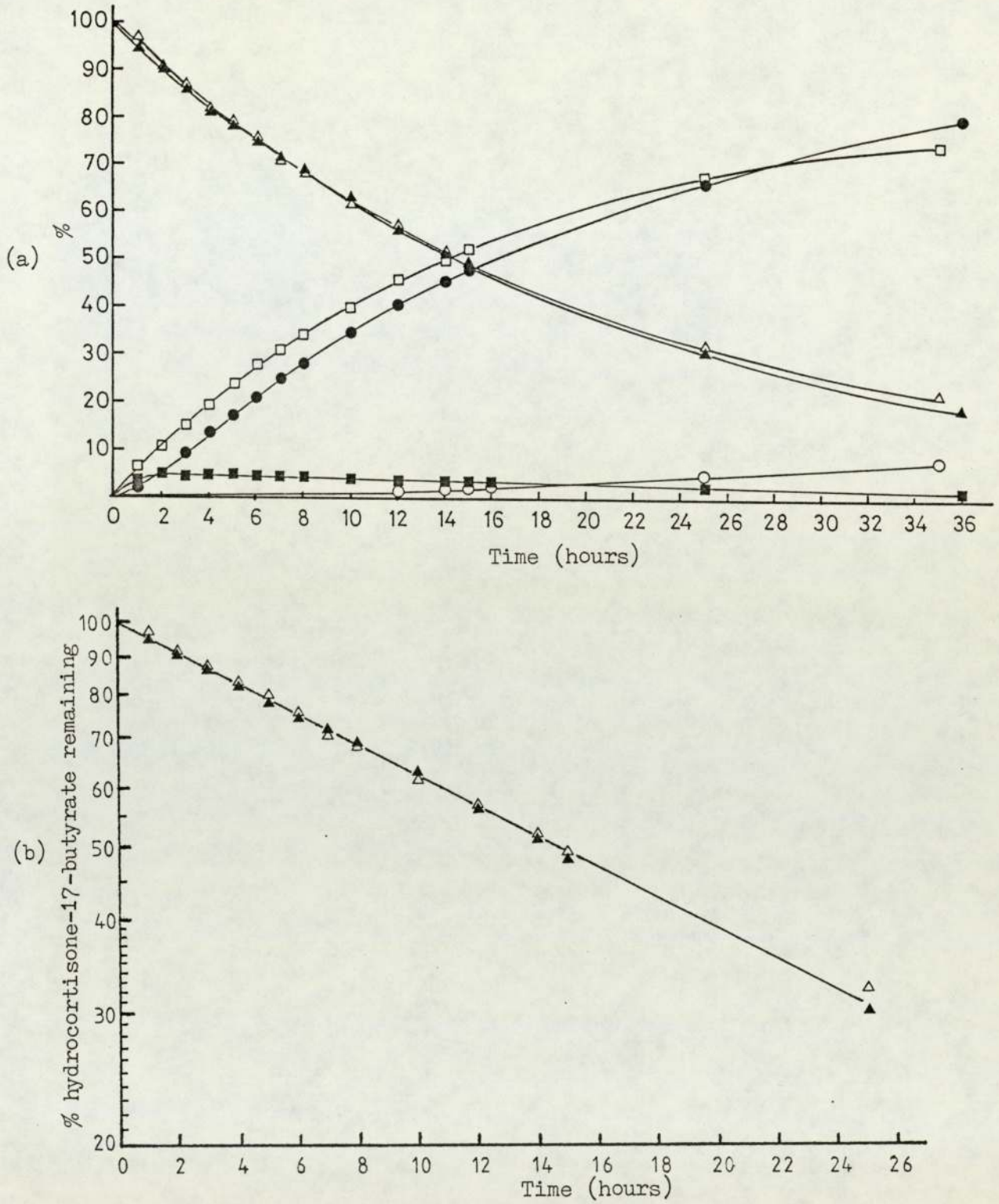


Figure 73. Biotransformation of hydrocortisone-17-butyrate 0.02 mg/ml by mouse skin homogenates, in 20% propylene glycol-tris buffer, pH 7.93, 37°C.
(a) Time-course profiles
(b) Log % hydrocortisone-17-butyrate remaining vs. Time

key: ▲ hydrocortisone-17-butyrate remaining
■ hydrocortisone-21-butyrate formed
● hydrocortisone formed
} in the presence of mouse skin homogenates

△ hydrocortisone-17-butyrate remaining
□ hydrocortisone-21-butyrate formed
○ hydrocortisone formed
} in the control

Table 41. Metabolic transformation of steroid esters, 0.02 mg/ml, by mouse skin homogenates in 20% propylene glycol-tris buffer, pH 7.93, at 37°C.

	Rate Constant	
	Enzymic hydrolysis	Non-enzymic decomposition (Control)
Betamethasone-17-valerate	0.0635 hr^{-1}	0.0667 hr^{-1}
Betamethasone-21-valerate	2.174 min^{-1} (130.44 hr^{-1})	No change after 2 hours
Hydrocortisone-17-butyrate	0.0472 hr^{-1}	0.0479 hr^{-1}
Hydrocortisone-21-butyrate	1.296 min^{-1} (77.76 hr^{-1})	No change after 2 hours

CHAPTER 6. CONCLUSION

New normal phase and reversed phase HPLC systems were developed for analysing the steroids used in this project: hydrocortisone and its 17- and 21-butyrates and 21-acetate; betamethasone and its 17- and 21-valerates; beclomethasone, its 17,21-dipropionate and 17-, 21-monopropionates; prednisolone and cortisone acetate. These assay methods enabled the elucidation of some of the complex decomposition pathways of the steroids used.

It has been shown that under non-ideal conditions, betamethasone-17-valerate undergoes acyl migration to betamethasone-21-valerate. The 21-valerate is then hydrolysed to betamethasone which in turn decomposes to other product(s). The reactions were shown to be sequential first order and base-catalysed. Hydrocortisone-17-butyrate behaves in a similar manner. The 17-butyrate isomerizes to the 21-butyrate which further hydrolyses to hydrocortisone. Hydrocortisone then undergoes further degradation to give glyoxals or 17-oxo steroids (Figure 3, p.23). The last step is known to be metal-catalysed and addition of sodium edetate decreases the decomposition. Sodium edetate as expected had no effect on the isomerization and hydrolysis reactions. The isomerization from the 17-ester to the 21-ester is reversible with the forward rate constant being more than 10 times higher than the reverse constant. The hydrolysis process is shown to be irreversible.

An attempt has been made to formulate a stable 0.1% w/w hydrocortisone-17-butyrate gel. Gelling agents used included cellulose derivatives, methacrylate polymers, carboxypolymethylene resins and

Xanthan Gum. Only the gel formed by carboxypolymethylene resins was found to possess sufficiently good cosmetic appearance. The others produced an unacceptable texture or left films on the skin after application. The solubility profile of the steroid was determined in order to optimise delivery. The aim was to maximise the thermodynamic activity of the steroid by careful choice of solvent. The shelf-life of the gel is about one year. Stability studies at various temperatures revealed that the decomposition followed the Arrhenius equation. Replacing most of the water in the gel base by propylene glycol does not improve its stability and may have a slight adverse effect. Monitoring the stability of hydrocortisone-17-butyrate in gels with various pH values show that the disappearance of the steroid follows first order kinetics and the decomposition pathway parallel those observed in buffered propylene glycol. However, the overall decomposition was not sequential first order, probably due to diffusion being the rate limiting step (265).

For a topical preparation to be effective, the drug has to diffuse out of the vehicle and penetrate through the skin barrier to exert its effect. Either process could be the rate-limiting step. Factors of importance in the percutaneous absorption of hydrocortisone and its 17-, 21-butyrates and 21-acetate were modelled by using three in vitro systems. The first involved release of the steroids from gels into isopropyl myristate, the diffusion from vehicle was the rate-limiting process. The second model involved studying the kinetics of partitioning of the steroids in a Schulman cell. Some of the difficulties, such as the interphase transfer of solvent, encountered in the use of this model have been discussed. The third model used consisted of diffusion cells for monitoring the kinetics of transfer of the steroids

through mouse skin. A novel approach in the use of this system was the simultaneous monitoring of more than one steroid, so that direct comparisons could be made thus overcoming the serious problem of variation between skins.

The release rates of the steroids into isopropyl myristate from gel formulations made up with various concentrations of propylene glycol were highly dependent on the composition of the vehicle. Maximum releases were found as expected with vehicles fully saturated with the steroid studied (Figure 32, p.159). The microviscosity of the vehicle also plays a role. The relative viscosity of aqueous propylene glycol increases from 2 cps for 20% propylene glycol to 20 cps for 80% propylene glycol (at 23°C). The diffusion coefficient decreases with increasing amount of propylene glycol (Table 28, p.164). The rank solubility of the steroids in aqueous propylene glycol (20% - 100%) was hydrocortisone > hydrocortisone-17-butyrate > hydrocortisone-21-butyrate ≃ hydrocortisone-21-acetate (Figure 28, p.153). The rank order of their partition coefficient between isopropyl myristate and aqueous propylene glycol was hydrocortisone-21-butyrate > hydrocortisone-17-butyrate > hydrocortisone-21-acetate > hydrocortisone (Figure 29, p.154). As far as the maximum rates of release from the gels were concerned, hydrocortisone and its 17-butyrate were highest and almost identical. Hydrocortisone-21-acetate from a 100% propylene glycol system penetrated mouse skin faster than hydrocortisone-17-butyrate and hydrocortisone (Figure 43, p.184). However, it showed the slowest penetration when the solvent was changed to 40% propylene glycol (Figure 44, p.185). In this system, hydrocortisone acetate is only 6% solubilised whereas hydrocortisone and the 17-butyrate are 100% and 56% solubilised respectively.

The three-phase partitioning model showed that hydrocortisone-17-butyrate transferred much faster than hydrocortisone. This parallels the results obtained with the other two models. The transfer rate of hydrocortisone acetate was slightly slower than the 17-butyrate while the opposite was true in the mouse skin model with the same solvent composition. Hydrocortisone-21-butyrate which is less active than the 17-butyrate and cortisone acetate which is topically inactive, show significantly faster transfer rates than the 17-butyrate. Relative to the other steroids, the topically active steroid, prednisolone, was only very slowly transferred.

All these results suggest that the skin permeability of a steroid does not necessarily parallel its topical activity. Other workers have produced similar evidence (122, 171). For example, the higher potency of betamethasone-17-valerate over betamethasone, and of fluocinolone acetonide acetate over fluocinolone acetonide, cannot be explained by differences in skin penetration, since their rates of topical absorption are about the same (171). If properly formulated, hydrocortisone can be released as well as hydrocortisone-17-butyrate; hydrocortisone acetate and hydrocortisone-21-butyrate can under certain circumstances be superior to hydrocortisone-17-butyrate (Chapter 4). Another report (122) has shown that hydrocortisone penetrated skin faster than the 17-butyrate, when applied in ethanolic solutions. It was once thought that cortisone acetate lacks topical activity because of its poor percutaneous absorption but it is now known that hydrocortisone and cortisone acetate are absorbed to about the same extent (155).

Human skin contains a large number of enzymes which are able to

transform foreign substances. Utilizing pure esterase and mouse skin homogenates, the resistance of the 17-esters and 21-esters to enzymic decomposition were compared. Esterase was identified in mouse skin. High concentrations of propylene glycol were found to inactivate the enzymes. Results indicate that the 21-esters —— hydrocortisone-21-acetate, hydrocortisone-21-butyrate and betamethasone-21-valerate, are very sensitive to both hog esterase and mouse skin enzymes leading to the formation of the corresponding free alcohol. Control samples showed that under identical conditions, the non-enzymic decompositions of the steroids were negligible. Hydrocortisone-21-acetate, with the shortest side chain from among the steroids studied, showed the slowest enzymic decomposition; a result which is in agreement with those of O'Neill and Carless's (214). For hydrocortisone-17-butyrate and betamethasone-17-valerate, based on the disappearance of the starting compound, slightly faster (10%) decompositions were observed in the presence of esterase than in the absence of the enzyme. The corresponding free alcohol was the only major product. Trace amounts of the corresponding 21-ester were also detected at low concentration of esterase. With the available data on the kinetics of decomposition of hydrocortisone-17-butyrate and betamethasone-17-valerate, it is clear that their enzymic and non-enzymic decomposition rates are essentially the same. That is, hydrocortisone-17-butyrate and betamethasone-17-valerate are resistant to the enzyme. But once the 17-esters isomerize to their 21-esters, the latter are immediately hydrolysed by the enzyme. The reversed isomerization is prevented, thus explaining the higher observed rates in the presence of esterase. Such findings are also true when mouse skin homogenates were used as the enzyme source. The total resistance of beclomethasone-17,21-dipropionate and its 17-monopropionate to esterase further support this conclusion. The 21-mono-

propionate is much more sensitive to esterase though the reaction was slower than those for hydrocortisone-21-acetate and 21-butyrate and betamethasone-21-valerate. This suggests that for steroids to be susceptible to enzymic degradation, the 17-hydroxy group must remain free. Hydrocortisone and betamethasone are resistant to esterase but evidence has shown that hydrocortisone is subject to metabolism in human skin (202 - 204).

Psoriasis is characterised by a greatly enhanced proliferation of epidermal cell. Effective antipsoriatic agents such as corticosteroids inhibit glucose-6-phosphate dehydrogenase activity. Betamethasone-17-valerate (266) and hydrocortisone-17-butyrate (267) are much more effective in this respect than betamethasone, hydrocortisone and hydrocortisone acetate. These marked differences indicate that betamethasone-17-valerate and hydrocortisone-17-butyrate are not just esters with better transport properties but exert biochemical actions of their own. Hydrocortisone-21-esters with chain length of about five or six carbons showed high inhibition to glucose-6-phosphate dehydrogenase in vitro, but hydrocortisone and longer chain esters exhibited low activity (268).

It appears that hydrocortisone-17-butyrate and betamethasone-17-valerate owe their high potency to their inherent activity. Perhaps their corresponding 21-esters possess similar activity, but are too vulnerable to cutaneous enzymes and are therefore readily transformed to the less active form, the free alcohol. On the other hand, the 17-esters are resistant to enzymic hydrolysis. Because of this striking difference in cutaneous metabolism, skin absorption does not seem to be a dorminant factor for explaining the activity differences between the 17- and 21-esters.

APPENDIX 1

List of materials and their sources

Chemicals	Source	Grade
acetonitrile	Fisons, U.K.	H.P.L.C.
ammonia solution	Fisons, U.K.	S.L.R.
ammonium sulphate	BDH, U.K.	AnalaR
Aqueous Cream, B.P.	Evans, U.K.	
beclomethasone	Sigma Chem. Co., U.K.	
beclomethasone-17-propionate	British Pharmacopoeia Commission Lab., U.K.	
beclomethasone-21-propionate	"	
beclomethasone-17,21-dipropionate	"	
betamethasone	Glaxo Ltd., U.K.	
betamethasone-17-valerate	Glaxo Ltd., U.K.	
betamethasone-21-valerate	British Pharmacopoeia Commission Lab., U.K.	
Betnovate Cream 0.1% w/w	Glaxo Ltd., U.K.	
Caffeine	Fisons, U.K.	S.L.R.
Carbopol (carboxypolymethylene polymers)	Goodrich Chem. Co.	
Cetomacrogol Cream, B.P.C. (formula A)	Macarthys, U.K.	
chloroform	Fisons, U.K.	A.R.
citric acid	BDH, U.K.	AnalaR
cortisone acetate	Boots, U.K.	
dimethyl sulphoxide	Sigma Chem. Co., U.K.	A.R.
E45 Cream	Boots, U.K.	
esterase	Sigma Chem. Co., U.K.	
ethanol (absolute)	Fisons, U.K.	A.R.
ethanolamine	BDH, U.K.	A.R.

Chemicals	Source	Grade
ethylacetate	Fisons, U.K.	H.P.L.C.
Eudispert hv (methacrylate polymers)	Rohm Pharma, U.K.	
glucose-6-phosphate	Sigma Chem. Co., U.K.	
glucose-6-phosphate dehydrogenase	"	
hexane	Fisons, U.K.	H.P.L.C.
hydrochloric acid (volumetric solution)	BDH, U.K.	AnalaR
hydrocortisone	Sigma Chem. Co., U.K.	
hydrocortisone acetate	"	
hydrocortisone-17-butyrate	Gist-Brocades, Holland	
hydrocortisone-21-butyrate	"	
isopropanol	Fisons, U.K.	A.R.
isopropyl myristate	Croda Ltd., U.K.	A.R.
Klucel HF (hydroxypropyl cellulose	Hercules Ltd., U.K.	
methanol	Fisons, U.K.	H.P.L.C.
magnesium sulphate, hepta-hydrate	BDH, U.K.	AnalaR
Natrosal (hydroxyethylcellulose)	Hercules, Ltd., U.K.	
NADP (nicotinamide-adenine dinucleotide phosphate)	Sigma Chem. Co., U.K.	
phenacetin	BDH, U.K.	L.R.
potassium chloride	BDH, U.K.	AnalaR
potassium biphosphate (KH_2PO_4)	Fisons, U.K.	A.R.
prednisolone	Boots, U.K.	
prednisolone acetate	Boots, U.K.	
propylene glycol	Fisons, U.K.	S.L.R.
sodium bicarbonate	BDH, U.K.	AnalaR

Chemicals	Source	Grade
sodium carboxymethylcellulose	Hercules Ltd. , U.K.	
sodium chloride	BDH, U.K.	L.R.
sodium edetate	BDH, U.K.	L.R.
Disodium hydrogen phosphate, dodecahydrate (Na_2HPO_4 12 H_2O)	Fisons, U.K.	A.R.
triethylamine	Fisons, U.K.	S.L.R.
tris hydroxymethyl aminomethane	BDH, U.K.	L.R.

APPENDIX 2

Publications:

1. A. Li Wan Po, W. J. Irwin and Y. W. Yip (1979). High-performance liquid chromatographic assay of betamethasone-17-valerate and its degradation products. J. Chromatography, 176 399 - 405
2. A. Li Wan Po, W. J. Irwin and Y. W. Yip (1979). Stability of topical steroids. Proceedings of the Analytical Division of the Chemical Society. 16 333 - 335
3. Y.W. Yip, W. J. Irwin and A. Li Wan Po (1981). HPLC separation of topical steroids: application to kinetic studies. Proceedings of the Symposium on the Analysis of Steroids, 21 - 23, May, 1981. Hungarian Chemical Society.
4. Y. W. Yip, A. Li Wan Po and W. J. Irwin (1982). The kinetics of decomposition and formulation of hydrocortisone-17-butyrate in semi-aqueous and gel systems. J. Pharm. Sci. (in press)

REFERENCES

1. W. Martindale, "The Extra Pharmacopoeia", 27th Ed., The Pharmaceutical Press, London, 1977, pp.389
2. W. C. Cutting, "Handbook of Pharmacology, the Actions and Uses of Drugs", 5th Ed., Appleton-Century-Crofts, New York, 1972, pp.334
3. P. S. Hench et al, Proc. Staff Meetings Mayo Clinic, (1949) 24 181
4. P. S. Hench et al, Arch. Intern. Med., (1950) 85 545
5. C. A. Schlagel, J. Pharm. Sci., (1965) 54 (3) 335 - 353
6. G. W. Liddle, Clinical Pharmacology and Therapeutics, (1961) 2 (5) 615 - 635
7. E. W. Boland, Ann. Rheumatic Diseases, (1962) 21 176
8. C. A. Schlagel and J. I. Northan, Proc. Soc. Exptl. Biol. Med., (1959) 101 629
9. S. W. Perlstein, Antibiol. Med. Clin. Therap., (1959) 6 575
10. G. W. Liddle, Metabolism, (1964) 13 37
11. C. F. H. Vickers and S. M. Tighe, Br. J. Derm., (1960) 72 352
12. C. F. H. Vickers, Br. Med. J., (1962) Jan. 156 - 157
13. E. W. Boland, Ann. Rheumatic Diseases, (1958) 17 376
14. H. Coke, Rheumatism, (1961) 17 70
15. M. J. Busse, Scientific Approach to the Design of Topical Delivery Systems, in "Postgraduate School on Optimisation of Drug Delivery", Apr. 1979, School of Pharmacy, University of London, London, pp.23
16. F. W. Crowe, T. B. Fitzpatrick, and S. A. Walker, J. Invest. Dermatol., (1958) 31 297
17. M. M. Chan, and E. J. Levy, J. New Drugs, (1961) 1 262
18. H. C. Fishman, Western Medicine, (1965) 6 270
19. A. W. McKenzie and R. M. Atkinson, Arch. Derm., (1964) 89 741 - 746

20. M. F. Coldman, L. Lockerbie, and E. A. Laws, Br. J. Derm., (1971) 85 573

21. H. Roth and J. McLeary, Clin. Med., (1973) 80 16

22. H. L. Maibach and R. B. Stoughton, Med. Clin. North. Am., (1973) 57 (5) 1253 - 1264

23. A. W. McKenzie, Arch. Derm., (1962) 86 611 - 614

24. P. A. Desaulles, L. Ehmann, and J. Urech, Steroids, (1965) 6 339

25. P. J. Ashurst, Br. J. Clin. Practice, (1972) 26 (6) 263

26. G. H. Philipps, Locally Active Corticosteroids: Structure-Activity Relationships, in "Mechanisms of Topical Corticosteroid Activity", Ed. Lyne Wilson and R. Marks, A Glaxo Symposium, Churchill Livingstone, 1976

27. A. Ercoli, G. Falconi, R. Gardi, and R. Vitali, J. Med. Chem., (1972) 15 783

28. E. J. Raffle, and W. Frain-Bell, Br. J. Derm., (1967) 79 487

29. E. J. Raffle, and W. Frain-Bell, Br. J. Derm., (1968) 80 124

30. C. G. Sparkes, and L. Wilson, Br. J. Derm., (1974) 90 197

31. E. J. Umberger, Anal. Chem., (1955) 27 (5) 768

32. A. E. Allen and V. Das Gupta, J. Pharm. Sci., (1974) 63 (1) 107

33. C. C. Porter and R. H. Silber, J. Biol. Chem., (1950) 185 201

34. L. Chafetz, D. C. Tsilifonis, and C. Moran, J. Pharm. Sci., (1974) 63 (11) 1771

35. W. J. Mader and R. R. Buck, Anal. Chem., (1952) 24 (4) 666

36. The United State Pharmacopoeia, 19th Rev. (1975), Mack Publishing Co., Easton, Pa., pp.622

37. The National Formulary, 14th Ed. (1975), Mack Publishing Co., Easton, Pa., pp. 976

38. British Pharmacopoeia, 1973, The Pharmaceutical Press, London, pp. A95

39. Y. W. Yip and A. Li Wan Po, J. Pharm. Pharmacol., (1979) 31 400

40. E. Heftmann and I. R. Hunter, J. Chrom., (1979) 165 283

41. M. D. Smith and D. J. Hoffmann, J. Chrom., (1979) 168 163

42. D. Dekker and D. J. Buus, Int. J. Pharm., (1980) 5 195

43. R. Ballerini, M. Chinol, and M. Ghelardoni, J. Chrom., (1980) 193 413

44. H. Bundgaard and J. Hansen, Int. J. Pharm., (1981) 7 197

45. P. A. Williams and E. R. Biehl, J. Pharm. Sci., (1981) 70 (5) 530

46. M. C. Petersen, R. L. Nation and J.J. Ashley, J. Chrom., (1980) 183 131

47. B. D. Anderson and V. Taphouse, J. Pharm. Sci., (1981) 70 (2) 181

48. J. Cornejo, M. C. Hermosin, J. L. White, G. E. Peck, and S. L. Hem, J. Pharm. Sci., (1980) 69 (8) 945

49. J. Hansen and H. Bundgaard, Arch. Pharm. Chemi. Sci. Ed., (1980) 8 91

50. S. Siggia and R. A. Dishman, Anal. Chem., (1970) 42 (11) 1223

51. W. C. Landgraf and E. C. Jennings, J. Pharm. Sci., (1973) 62 (2) 278

52. F. Bailey and P. N. Britain, J. Pharm. Pharmacol., (1972) 24 425

53. M. C. Olson, J. Pharm. Sci., (1973) 62 (12) 2001

54. R. E. Huettemann and A. P. Shroff, J. Chrom. Sci., (1975) 13 357

55. L. M. Upton, E. R. Townley, and F. D. Sancilio, J. Pharm. Sci., (1978) 67 (7) 913

56. C. Burgess, J. Chrom., (1978) 149 233

57. P. M. Kabra, L. L. Tsai, and L. J. Marton, Clin. Chem., (1979) 25 1293

58. S. E. Tsuei, J. J. Ashley, and R. G. Moore, J. Chrom., (1978) 145 213

59. J. A. Mollica and R. F. Strusz, J. Pharm. Sci., (1972) 61 444

60. J. C. Touchstone and W, Wortmann, J Chrom., (1973) 76 244

61. A. G. Butterfield, B. A. Lodge, N. J. Pound, and R. W. Sears, J. Pharm. Sci., (1975) 64 441

62. P. Helboe and M. Thomsen, Int. J. Pharm., (1979) 2 317

63. R. Horikawa, T. Tanimura, Z. Tamura, J. Chrom., (1979) 168 526

64. J. C. Boylan, Drug Development Communications, (1976) 2 (4&5) 325

65. A. N. Martin and G. S. Banker, Advances in Pharm. Sci., (1964) 1 Chap. 1

66. I. Eros et al, Acta. Pharm. Hung., (1970) 40 64

67. N. J. Abbe, Pharm. J., (1959) 193 111

68. N. J. Van Abbe, "Pharmaceutical and Cosmetic Products for Topical Administration", William Hinemann Medical Books Ltd., London, 1969, pp.98

69. K. Munzel, Pharm. Acta. Helv., (1971) 46 513

70. T. Higuchi, J. Soc. Cosm. Chem., (1960) 11 85

71. D. E. Wurster, American Perfumer and Cosmetics, (1965) 80 21

72. W. G. Wagner, J. Pharm. Sci., (1961) 50 379

73. M. Katz and B. J. Poulsen, J. Soc. Cosmet. Chem., (1972) 23 565

74. J. Giroux and M. Schrenzel, Pharm. Acta. Helv., (1964) 39 615

75. H. C. Ansell, "Introduction to Pharmaceutical Dosage Forms", 2nd Ed., Lea and Febiger, Philadelphia, 1976, Chapter 5

76. A. N. Martin, J. Swarbrick, and A. Cammarata, "Physical Pharmacy", 2nd Ed., Lea and Febiger, Philadelphia, 1969, Chapter 19

77. I. B. Chang, Cosmetics and Toiletries, (1977) 92 25

78. I. Adams and S. S. Davis, J. Pharm. Pharmacol., (1973) 25 640

79. A. A. El-Sayed and Y. E. Hamza, Formulation and evaluation of merbromine and chloramphenicol methacrylate polymers gels, Pharma International, (1977) 4

80. W. L. Nobles, Drug and Cosmetic Industry, (1955) 77 178 - 179, 280 - 281

81. L. Cohen, Soap Cosmetics Chemical Specialties, (1956) 32 (12) 50

82. G. Levy and T. W. Schwarz, Drug and Cosmetic Industry, (1957) 81 606 - 607, 696 - 699

83. A. A. El-Sayed, Formulation and Stability of Hydrocortisone in Eudispert Bases, Pharma International, (1972) 2

84. V. D. Gupta, J. Pharm. Sci., (1978) 67 (3) 299

85. D. E. Guttman and P. D. Meister, J. Am. Pharm. Assoc. Sci. Ed., (1958) 47 773

86. T. Chulski and A. A. Forist, J. Am. Pharm. Assoc. Sci. Ed., (1958) 47 553

87. R. E. Graham, E. R. Biehl, and C. T. Kenner, J. Pharm. Sci., (1978) 67 (3) 360

88. R. Gardi, "Hormonal Steroids, Biochemistry Pharmacology and Therapeutics", Vol. 2, Edited by M. Pecile, pp. 99 - 109

89. P. S. Adams and A. L. Cripps, J. Pharm. Pharmacol., (1980) 32 suppl. 47p

90. J. Hansen and H. Bundgaard, Int. J. Pharm., (1980) 6 307

91. J. Hansen and H. Bundgaard, Arch. Pharm. Chemi. Sci. Ed., (1979) 7 135

92. B. A. Lodge, H. Watanabe and L. Watts, Can. J. Pharm. Sci., (1975) 10 107

93. M. L. Lewbart and V. R. Mattox, Nature, (1959) 183 820

94. R. J. Scheuplein, J. Invest. Derm., (1966) 45 334

95. C. F. H. Vickers, Arch. Derm., (1963) 88 20

96. R. D. Griesemer, J. Soc. Cosmet. Chem., (1960) 11 79

97. I. H. Blank, J. Invest. Derm., (1965) 45 249

98. I. H. Blank, R. D. Griesemer, and E. Gould, J. Invest. Derm., (1957) 29 299

99. E. Cronin and R. B. Stoughton, Br. J. Derm., (1962) 74 265

100. F. R. Bettley, Br. J. Derm., (1965) 77 98

101. J. A. Keipert, Med. J. Aust., (1971) 1 1021

102. R. L. Nachman and N. B. Esterly, J. Pediatr., (1971) 79 628

103. R. J. Feldmann and H. I. Maibach, J. Invest. Derm., (1967) 48 (2) 181

104. H. I. Maibach, R. J. Feldmann, T. H. Milby and W. F. Serat, Pesticides Arch. Environ. Health, (1971) 23 208

105. F. N. Marzulli, J. Invest. Derm., (1962) 39 387

106. A. H. McGreesh, Toxicol. Appl. Pharmacol., (1965) 7 20

107. P. K. Noonan and R. C. Wuster, J. Pharm. Sci., (1980) 69 365

108. T. Fredriksson, Acta. Dermatol-Venereol., (1961) 41 353

109. W. J. Van Kooten and J. W. H. Mail, Dermatologica, (1966) 132 141

110. G. H. MacKee, M. B. Sulsberger, F. Herrmann and R. L. Baer, J. Invest. Derm., (1945) 6 43

111. J. Grab, Arch. Derm., (1960) 81 606

112. J. R. Scholtz, Arch. Derm., (1961) 84 1029

113. M. B. Sulzberger and V. H. Witten, Arch. Derm., (1961) 84 1027

114. R. J. Feldmann and H. I. Maibach, Arch. Derm., (1965) 91 661

115. I. Shapiro, Current Therapeutic Research, (1963) 5 (8) 426

116. R. B. Stoughton, Arch. Environ. Health, (1965) 11 551

117. A. W. McKenzie and R. B. Stoughton, Arch. Derm., (1962) 86 608

118. S. Rothman, "Physiology and Biochemistry of the Skin", University of Chicago Press, Chicago, 1954

119. R. B. Stoughton, Arch. Derm., (1965) 91 657

120. C. F. H. Vickerst, The Transactions St. John's Dermatological Society, (1973) 59 10

121. R. J. Scheuplein, I.H. Blank, G. J. Brauner, and D. J. MacFarlane, J. Invest. Derm., (1969) 52 (1) 63

122. M. Ponec and M.K. Polano, Arch. Derm. Res., (1979) 265 101

123. H. I. Maibach, Dermatologica, (1976) 152 suppl. 1 11

124. J. Ostrenga, J. Haleblian, B. Poulsen, B. Ferrell, N. Mueller, and S. Shastri, J. Invest. Derm., (1971) 56 (5) 392

125. M. Ponec, Dermatologica, (1976) 152 suppl. 1 37

126. B. J. Poulsen, E. Young, V. Coquilla and M. Katz, J. Pharm. Sci., (1968) 57 (6) 928

127. J. B. Shelmire, Arch. Derm., (1960) 82 24

128. B. W. Barry and R. Woodford, Br. J. Derm., (1974) 91 323

129. B. W. Barry and R. Woodford, Br. J. Derm., (1975) 93 563

130. R. J. Feldmann and H. I. Maibach, Arch. Derm., (1974) 109 58

131. J. Almeyda and B. W. Burt, Br. J. Derm., (1974) 91 579

132. C. W. Barrett, J. W. Hadgraft, G. A. Caron, and I. Sarkany, Br. J. Derm., (1965) 77 576

133. I. W. Caldwell, S.P. Hall-Smith, R. A. Main, P. J. Ashurst, V. Kirton, W. T. Simpson and G. W. Williams, Br. J. Derm., (1968) 80 111

134. L. Wilson, Current Med. Res. and Opinion, (1973) 1 228

135. V. Das Gupta, and A. N. Deleon, Indian J. Hosp. Pharm., (1973) 10 141

136. M. J. Busse, P. Hunt, K. A. Lees, P. N. D. Maggs and T. M. McCarthy, Br. J. Derm., (1968) suppl. 4 103

137. M. Whitefield and A. W. McKenzie, Br. J. Derm., (1975) 92 585

138. J. S. Turi, D. Danielson and J. W. Woltersom, J. Pharm. Sci., (1979) 68 (3) 275

139. R. J. Feldmann and H. I. Maibach, Arch. Derm., (1966) 94 649

140. H. I. Maibach and R. J. Feldmann, Ann. New York Academic Science, (1967) 141 423

141. J. E. Wahlberg and E. Skog, Acta Derm. Venereol (1967) 47 209

142. A. C. Allenby, A.H. Creasey, A. G. Edginton, J. A. Fletcher and C. Schock, Br. J. Derm., (1969) 81 suppl. 4 47

143. R. B. Stoughton and W. Fritsch, Arch. Derm., (1964) 90 512

144. N. G. Sulzberger, T. A. Cortese, L. Fishman, H.S. Wiley and P. S. Peyakowich, Ann. N. Y. Acad. Sci., (1967) 141 437

145. H. Baker, J. Invest. Derm., (1968) 50 283

146. S. G. Elfbaum and K. Laden, J. Soc. Cosmet. Chem., (1968) 19 119, 163, 841

147. L. F. Montes, J. L. Day, C. J. Wand and L. Kennedy, J. Invest. Dermatol., (1967) 48 184

148. S. M. Nagy, C. Golumbic, W. H. Stein, J. S. Fruton and M. Bergmann, J. Gen. Physiol., (1946) 29 441

149. I. H. Blank, J. Soc. Cosmet. Chem., (1960) 11 59

150. D. H. O. Gemmell and J. C. Morrison, J. Pharm. and Pharmacol., (1958) 10 167

151. M. E. Stolar, G. V. Rossi, and M. Barr, J. Pharm. Sci., (1960) 49 144

152. R. J. Feldmann and H. I. Maibach, J. Invest. Dermatol., (1969) 52 (1) 89

153. M. J. Bartek, J. A. LaBudde, H. I. Maibach, J. Invest. Derm., (1972) 58 114

154. A. J. Wickrema Sinha, R. S. Shaw, and D. J. Weber, J. Invest. Derm., (1978) 71 372

155. F. D. Malkinson, E. H. Ferguson, and M. C. Wang, J. Invest. Derm., (1957) 28 211

156. R. D. Carr and W. M. Tarnowski, Acta. Derm. Vener. Stockh., (1968) 48 417

157. M. Feiwel, V. James, and E. Barnett, Lancet, (1969) 1 485

158. A. Kukita, K. Yamada, and Y. Takeda, Dermatologica, (1976) 152 suppl. 1 197

159. K. J. Child, A. F. English, H. G. Gilbert, A. Hewitt, and E. A. Woollett, Arch. Derm., (1968) 97 407

160. G. A. Christie and M. Moore-Robinson, Br. J. Derm., (1970) 82 suppl. 6 93

161. R. B. Stoughton, Arch. Derm., (1972) 106 825

162. G. Falconi, and G. L. Rossi, Arch. Derm., (1972) 105 856

163. A. F. Pepler, R. Woodford and J.C. Morrison, Br. J. Derm., (1971) 85 171

164. B. W. Barry and R. Woodford, Br. J. Derm., (1976) 95 423

165. B. W. Barry and R. Woodford, Br. J. Derm., (1977) 97 555

166. B. W. Barry and R. Woodford, J. Clin. Pharm., (1978) 3 43

167. B. W. Barry, A. R. Brace, A. C. Norris and R. Woodford, J. Pharm. Pharmacol., (1975) 27 75p

168. B. W. Barry, Dermatologica, (1976) 152 suppl. 1 47

169. P. Grasso and A. B. G. Lansdown, J. Soc. Cosmet. Chem., (1972) 23 481

170. R. B. Stoughton, Dermatologica, (1976) 152 suppl. 1 27

171. R. B. Stoughton, Arch. Derm., (1969) 99 753

172. J. Ostrenga, C. Steinmetz, and B. Poulsen, J. Pharm. Sci., (1971) 60 (8) 1175

173. M. F. Coldman, B. J. Poulsen, and T. Higuchi, J. Pharm. Sci., (1969) 58 1098

174. J. Ostrenga, C. Steinmetz, and B. Poulsen, J. Pharm. Sci., (1971) 60 (8) 1180

175. M. F. Coldman, T. Kalinovsky, and B. J. Poulsen, Br. J. Derm., (1971) 85 457

176. M. Ponec and M. K. Polano, Arch. Derm. Forsch., (1972) 245 381

177. V. Shahi and J. L. Zatz, J. Pharm. Sci., (1978) 67 (6) 789

178. H. Durrheim, G. L. Flynn, W.I. Higuchi, and C. R. Behl, J. Pharm. Sci., (1980) 69 (7) 781

179. J. Haleblian, R. Runkel, N. Mueller, J. Christopherson, K. Ng, J. Pharm. Sci., (1971) 60 (4) 541

180. F. N. Marzulli, D. W. C. Brown, and H. I. Maibach, Toxicol. Appl. Pharmacol., suppl. 3 (1969) 76

181. R. T. Tregear, "Physical Function of Skin", Academic Press, New York, 1966

182. R. B. Stoughton, Animal models for in vitro percutaneous absorption, in "Animal Models in Dermatology", edited by H. Maibach, Churchil Livingstone, New York, 1975, pp. 121

183. S. A. Khalil, and A. N. Martin, J. Pharm. Sci., (1967) 56 (10) 1225

184. M. A. Augustine and J. Swarbrick, J. Pharm. Sci., (1970) 59 314

185. D. L. Berliner, Advances in Biology of Skin, (1972) 12 357 - 365

186. A. Pannatier, O. Jenner, B. Testa, and J. C. Etter, Drug Metabolism Reviews, (1978) 8 (2) 319

187. A. Jarrett, The biochemistry of the epidermis, in "The Physiology and Pathophysiology of the Skin", Academic Press, 1976, vol. 1, Chapter 2

188. W. Voigt, E. P. Fernandez, and S. L. Hsia, J. Biol. Chem., (1970) 245 5594

189. B. P. Davis, "17β-Hydroxysteroid Dehydrogenase of Rat Skin and Human Skin", PH.D. dissertation, University of Miami

190. M. J. C. Im, and J. E. Hoopes, J. Invest. Derm., (1970) 55 277

191. W. Montagna, The epidermis, in "The Structure and Function of Skin", Academic Press, New York and London, 1962, Chapter 2

192. M. B. Hodgins and J. B. Hay, Biochemical Society Transactions, (1976) 4 (4) 605

193. S. L. Hsia, Essays in Biochemistry, (1971) 7 1

194. J. B. Hay and M. B. Hodgins, J. Endocrinol., (1973) 59 475

195. J. B. Hay, J. Endocrinol., (1974) 64 29

196. M. D. Rawlins, V. Shaw, and S. Shuster, Br. J. Pharmacol., (1979) 66 (3) 441p

197. V. V. Täuber and T. Toda, Arzeimittel Forschung (Drug Research), (1976) 26 1484

198. M. I. Dubovie, Ukrain. Biodhem. Lhur., (1954) 26 3

199. M. I. Dubovie, Vestnik. Dermatolog. Venerol., (1960) 34 10

200. M. Julesz, I. Faredin, and I. Tóth, Acta Medica Academiae Scientiarum Hungaricae Tomus, (1966) 22 (1) 25

201. M. Julesz, I. Faredin, and I. Tóth, Acta Media Academiae Scientiarum Hungaricae Tomus, (1966) 22 (1) 49

202. F. Malkinson, M. W. Lee, and I. Cutukovic, J. Invest. Derm., (1959) 32 101

203. S. L. Hsia and Y. L. Hao, Biochemistry, (1966) 5 (5) 1469

204. S. L. Hsia and Y. L. Hao, Steroids, (1967) 10 489

205. D. L. Berliner, J.R. Pasqualini, and A. J. Gallegos, J. Invest. Derm., (1968) 50 (3) 220

206. E. C. Gomez and S. L. Hsia, Biochemistry, (1968) 7 24

207. G. D. Weinstein, P. Frost, and S. L. Hsia, J. Invest. Derm., (1968) 51 4

208. J. K. Grant, Br. J. Derm., (1968) 81 suppl. 2 18

209. V. H. Price, Arch. Derm., (1975) 111 1496

210. G. Sansone and R. M. Reisner, J. Invest. Derm., (1971) 56 (5) 366

211. S. L. Hsia, V. H. Witten, and Y. L. Hao, J. Invest. Derm., (1964) 43 407

212. S. L. Hsia, A. J. Mussallem, and V. H. Witten, J. Invest. Derm., (1965) 45 (5) 384

213. M. S. Greaces, J. Invest. Derm., (1971) 57 (2) 100

214. R. C. O'Neill and J. E. Carless, J. Pharm. Pharmacol., (1980) 32 10p

214a. M. Dixon and E. C. Webb, "Enzymes" 3rd Ed., Longman, 1979, pp.10

215. "Documenta Geigy Scientific Tables", 6th Ed., Edited by Konrad Diem, Ciba Geigy, 1968, pp.382

216. R. Gardi, R. Vitali and A. Ercoli, Gazz. Chim. Ital., (1963) 93 431

217. A. Li Wan Po, W. J. Irwin, and Y. W. Yip, J. Chrom., (1979) 176 399

218. H. Engelhardt, J. Chrom. Sci., (1977) 15 380

219. W. Boehme and H. Engelhardt, J. Chrom., (1977) 133 67

220. L. R. Snyder, J. Chrom. Sci., (1969) 7 595

221. L. R. Snyder and J. J. Kirkland, "An Introduction to Modern Liquid Chromatography", Wiley-Interscience, New York, 1979, Chapter 6 and 7.

222. K. J. Williams, A. Li Wan Po, and W. J. Irwin, J. Chrom., (1980) 194 217

223. J. E. Fairbrother and A. Siam, Proc. Analyst. Div. Chem. Soc., (1978) 15 253

224. B. L. Karger, K. R. Gant, A. Hartkope, and P. H. Weiner, J. Chrom., (1976) 128 65

225. C. Horvath, W. Melander, and I. Molnar, J. Chrom., (1976) 125 129

226. D. C. Locke, J. Chrom. Sci., (1974) 12 433

227. K. Tanaka, H. Goodell, and B. L. Karger, J. Chrom., (1978) 158 233

228. J. W. Munson and T. D. Wilson, J. Pharm. Sci., (1981) 70 (2) 177

229. M. J. Busse, J. Pharm., (1978) 220 25

230. P. J. Elving, J. M. Markowitz, and I. Rosenthal, Anal. Chem. (1956) 28 (7) 1179

231. A. F. Mooney, Br. J. Derm., (1974) 90 109

232. Glaxo Information Sheet No. 11 and suppl., Medical Information Unit, Glaxo Lab. Ltd., U.K., 1978

233. C. M. Metzler, G. L. Elfring, and A. J. McEwen, "A User's Manual for Non-Linear and Associated Programs", Upjohn Co., Kalamazoo, Michigan, 1974

234. W. H. McNeely and K. S. Kang, "Industrial Gum", 1973, Chapter XXI pp.473 - 497

235. P. Calvet, Nature, (1980) 287 (2, Oct.) 388

236. M. Barr and L. F. Tice, J. Am. Pharm. Assoc. Sci. Ed., (1957) 46 217

237. T. O. Oesterling and D. E. Guttman, J. Pharm. Sci., (1964) 53 (10) 1189

238. H. Bundgaard, Arch. Pharm. Chemi. Sci. Ed., (1980) 8 83

239. R. N. Brogden, R. M. Pinder, P. R. Sawyer, T. M. Speight and G. S. Avery, Drugs, (1976) 12 249

240. R. J. Scheuplein and I. H. Blank, Physiol. Rev., (1971) 51 702

241. S. H. Yalkowsky, G. L. Flynn, and G. L. Amidon, J. Pharm. Sci., (1972) 61 (6) 983

242. S. H. Yalkowsky and G. L. Flynn, J. Pharm. Sci., (1974) 63 1276

243. T. Higuchi, J. Pharm. Sci., (1961) 50 (10) 874

244. W. I. Higuchi, J. Pharm. Sci., (1962) 51 (8) 802

245. R. M. Barrer, "Diffusion in and through Solids", Cambridge University Press, London, 1951

246. R. H. Gug and J. Hadgraft, J. Pharm. Sci., (1981) 70 (11) 1243

247. C. Capellos and B. H. J. Bielski, "Kinetic Systems. Methematical Description of Chemical Kinetics in solution", Wiley-Interscience, 1972, Chapter 2

248. B. J. Poulsen, Z. T. Chowhan, R. Pritchard, and M. Katz, Curr. Probl. Dermatol., (1978) 7 107

249. A. L. Weiss and B. J. Sciarrone, J. Pharm. Sci., (1969) 58 (8) 980

250. K. Chandra, T. Chakrabarti, and G. P. Srivastava, Pharmaceutisch Weekblad, (1977) 112 85

251. R. C. Wuster and P. K. Noonan, Int. J. Pharm., (1980) 7 99

252. G. L. Flynn and R. W. Smith, J. Pharm. Sci., (1972) 61 61

253. M. H. Kim and W. L. Herrmann, J. Clin. Endoc. Metabl., (1968) 28 187

254. G. Sansone-Bazzano, A. K. Seeler, B. Cummings, and R. M. Reisner, J. Invest. Dermatol., (1979) 73 (1) 118

255. M. Giacomini and F. Wright, J. Steroid Biochem., (1980) 13 (6) 645

256. J. L. Gaylor, J. Biol. Chem., (1963) 238 1643

257. I. E. Bush, Experienta, (1956) 12 325

258. N. I. Gold, L. L. Smith and F. D. Moore, J. Clin. Invest., (1959) 38 2238

259. R. B. Burton, E. H. Keutman, and C. Waterhouse, J. Clin. Endocrinol. Metab., (1953) 13 48

260. D. J. C. Engel, A. F. Marx, R. F. Rekker, and L. V. Wijk, Arch. Derm., (1974) 109 863

261. E. H. Epstein and N. H. Munderloh, Endocrinology, (1981) 108 (2) 703

262. E. H. Epstein and J. M. Bonifas, J. Invest. Derm., (1982) 78 144

263. A. Hughes and H. J. Yardley, Br. J. Derm., (1982) 106 299

264. M. Whitefield, Lancet, (1977) 2 925

265. J. H. Wood, Drug Development Communications, (1976) 2 (1) 33

266. W. P. Raab and B. M. Gmeiner, Arch. Derm. Res., (1975) 113

267. W. P. Raab and B. M. Gmeiner, Arch. Derm. Res., (1976) 255 265

268. R. C. O'Neill and J. E. Carless, J. Pharm. Pharmacol., (1980) 32 11p