ASPECTS OF THE BIOAVAILABILITY OF TOPICAL CORTICOSTEROID FORMULATIONS.

A Thesis Submitted to

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by

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ABSTRACT

Two possible variables of the McKenzie/Stoughton blanching assay, namely amount applied to the test site and occlusion time have been investigated.

Subsequently, two topical steroid preparations, Synalar cream (0,025% fluocinolone acetonide) and Betnovate cream (0,1% betamethasone 17-valerate) were extemporaneously diluted with five and six placebo bases respectively.

Taking cognizance of the two possible variables, these diluted preparations were assessed *in vivo* using a modified version of the McKenzie/Stoughton blanching assay for blanching activity over a 14 month period. It was found that the base E45, which is slightly alkali, had the greatest effect on both preparations. In the case of betamethasone 17-valerate this base caused the conversion to the less active isomer, betamethasone 21-valerate whereas at the end of the 14 month test period it was found that the Synalar/E45 dilution contained no fluocinolone acetonide.

Quantitative analysis of all the diluted preparations by high performance liquid chromatography using a reverse-phase system was performed.

The data obtained from the systematic studies of the effects of varying concentrations and occlusion times were presented at the Eleventh National Congress of the South African Pharmacological Society.

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PREPARATIONS USED IN THIS STUDY

BETNOVATE[R] cream (0,1% betamethasone 17-valerate) Glaxo-Allenburys (SA) (Pty) Ltd., Manchester Road, Wadeville, Transvaal, South Africa. Glaxo Division.

BETNOVATE HALF STRENGTH[R] cream (0,05% betamethasone 17-valerate) Glaxo-Allenburys (SA)(Pty) Ltd., Manchester Road, Wadeville, Transvaal, South Africa. Glaxo Division.

BETNOVATE[R] cream base, Glaxo-Allenburys (SA)(Pty) Ltd., Manchester Road, Wadeville, Transvaal, South Africa. Glaxo Division.

SYNALAR[R] cream (0,025% fluocinolone acetonide) I.C.I. South Africa (Pharmaceuticals) Ltd., 1 Leyds Street, Braamfontein, Johannesburg, South Africa.

SYNALAR FORTE[R], fluocinolone cream B.P.C. (0,2%), fluocinolone acetonide (0,2%) I.C.I. Limited, Macclesfield, Cheshire, Great Britain.

SYNADONE[R] cream (0,01% fluocinolone acetonide) I.C.I. South Africa (Pharmaceuticals) Ltd., 1 Leyds Street, Braamfontein, Johannesburg, South Africa.

SYNALAR[R] cream base, I.C.I. South Africa (Pharmaceuticals) Ltd., 1 Leyds Street, Braamfontein, Johannesburg, South Africa.

AQUEOUS CREAM B.P., Lennon Limited, 7 Fairclough Road, Port Elizabeth, South Africa.

EMULSIFYING OINTMENT B.P., Lennon Limited, 7 Fairclough Road, Port Elizabeth, South Africa.

CREAM E45, The Boots Company Ltd., Nottingham, England.

ULTRABASE, Berlimed (Pty) Ltd., P.O. Box 10259, Johannesburg, South Africa.

AUTHENTIC SPECIMENS AND INTERNAL STANDARDS USED IN THIS STUDY

FLUOCINOLONE ACETONIDE, British Pharmacopoeia Commission, Authentic Specimen, Batch No. 563.

BETAMETHASONE 17-VALERATE, British Pharmacopoeia Commission, Authentic Specimen, Batch No. 427.

BETAMETHASONE 21-VALERATE, British Pharmacopoeia Commission, Authentic Specimen, Batch No. 339.

MEDROXYPROGESTERONE ACETATE U.S.P., Upjohn (Pty) Ltd., 44 Monteer Road, Isando, Transvaal, South Africa, Lot: 643 CM.

NORETHISTERONE, Ethnor (Pty) Ltd., New Road, Halfway House, Transvaal, South Africa, Mat. No. 2987.

TOLUENE, J.T. Baker Chemical Co., Phillipsburg, New Jersey, 08865, Lot: 512075.

INSTRUMENTATION USED IN THIS STUDY

COMPUTER

Wang 2200, Basic Desk Top Mini-Computer, with Graph Plotter and Print Out Device.

BALANCES

Sartorius Precision 5 Figure Analytical Balance, Type 2474.

Sartorius Precision 4 Figure Analytical Balance, Type 2462.

Sartorius 2 Figure Top Loading Balance, Type 2200.

HIGH PERFORMANCE LIQUID CHROMATOGRAPH

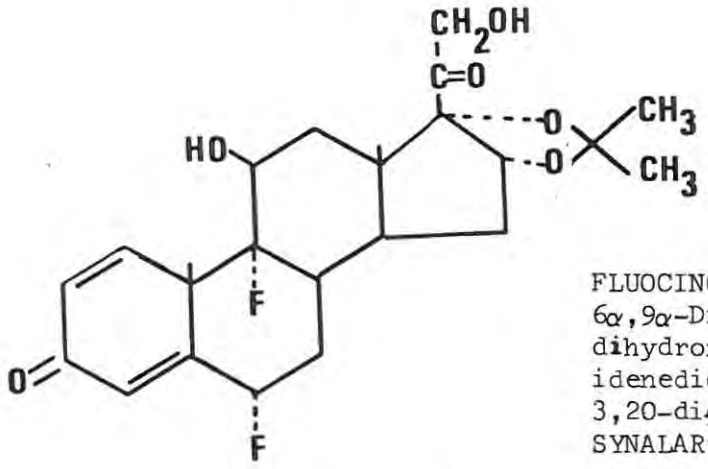
VARIAN 8500 Dual Pump System with Solvent Programmer.

VARIAN Varichrome Detector 200 - 800 nm.

VARIAN Computer Data System (CDS III).

PERKIN ELMER Electric, Self Balancing Recorder, Model 56.

STRUCTURES OF CORTICOSTEROIDS STUDIED IN THIS WORK.



FLUOCINOLONE ACETONIDE
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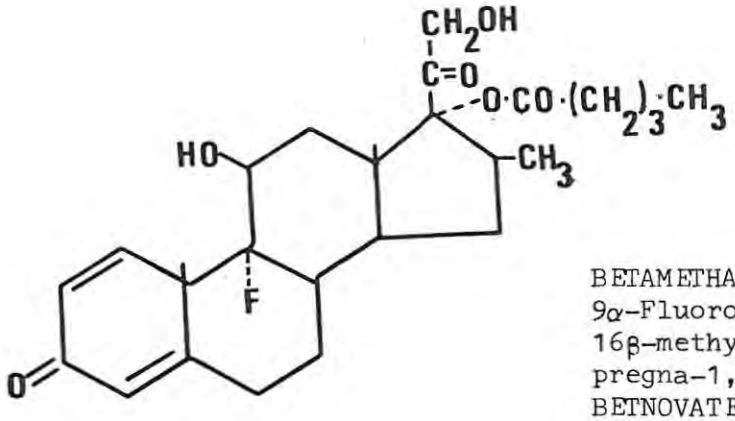
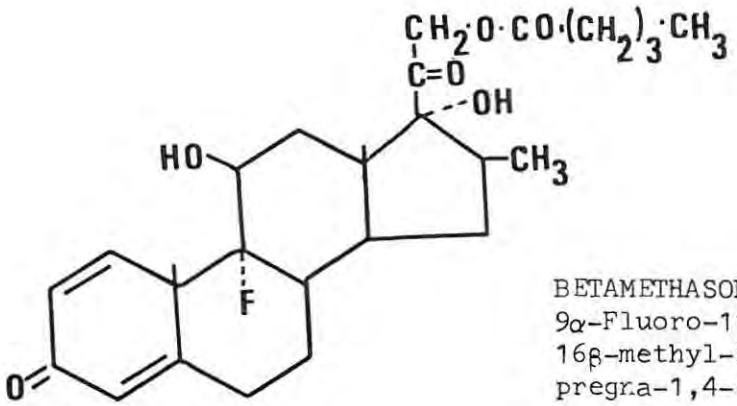
SYNALAR^{R}



BETAMETHASONE 17-VALERATE
9α-Fluoro-11β,21-dihydroxy-16β-methyl-17α valeryloxy-pregna-1,4-diene-3,20-dione
BETNOVATE^{R}



BETAMETHASONE 21-VALERATE
9α-Fluoro-11β,17α-dihydroxy-16β-methyl-21-valeryloxy-pregna-1,4-diene-3,20-dione

1. INTRODUCTION

Topical steroids account for about half of the prescriptions given to patients in the practice of dermatology.[1] There were 9,500,000 prescriptions written for topical corticosteroids in the United States[2] in 1969 and in Britain in the same year 4% of the National Health Services prescription expenditure was for topical corticosteroids.[3] The main reasons for this are that topical corticosteroids are effective and relatively safe.[1]

This widespread use has created a need for a reliable method of *in vivo* testing of these drugs. Clinical trials are laborious and difficult to mount as well as being unsuitable for the screening of large numbers of drugs. Patients suffering from dermatological complaints are likewise not ideal subjects for the testing of topical steroid preparations as it is difficult to obtain standardised lesions which are necessary for comparison of results between patients.[4] Thus various methods have been developed for the screening of topical steroids in animals and human volunteers.

1.1 BIOASSAYS USED TO ASSESS TOPICAL STEROID ACTIVITY

Various workers have attempted to substitute inflammation caused by natural disease with experimentally induced inflammation in healthy volunteers.

A large number of chemically injurious substances have been used to artificially produce inflammation. In 1956 Scott and Kalz[5] investigated results obtained with nitric acid and mustard oil and in 1959 Witkowski and Kligman[6] used croton oil to induce inflammation. Croton oil 100%[7] or a mixture containing pyridine 20%, water 5%, diethyl ether 74% and

croton oil 1%[8] has been used in various modifications of a technique described by Tonelli et al. in 1965[9] which is commonly known as the rat ear assay. Ortega et al.[10] and Burdick[11] adapted this method for use on human skin. Kerosene was used as the inflammatory inducing agent in experiments carried out by Zaynoun and Kurban[12] and Kaidbey and Kligman[13]. The latter established a corticosteroid rank order which correlated well with clinical efficacy. Tetrahydrofurfuryl alcohol was used as the irritant by Schlagel and Northam in 1959[14] and by Brunner and Finkelstein in 1960[15].

Topical steroid activity has also been evaluated by assessing their ability to suppress erythema produced by pyrexal[16] and Rhus dermatitis[17] experimentally induced in humans by patch application of Rhus oleoresins. The ability of corticosteroids to reduce the size of weals induced by histamine on human skin when the histamine was introduced by the pinprick method has also been used as an assay method.[18] The activity of fluocinolone acetonide has been determined by studying its ability to suppress experimentally induced eczema.[19]

All these methods suffer from the same drawbacks in that they are painful and unpleasant for the volunteer and that it is difficult to reproduce a standardised inflammation.[4]

Ultra violet light can be used in the same way as chemical irritants to produce inflammation. Epidermal cell deaths, increased mitosis, hyperplasia, cellular exudation and vasodilation are all a result of injury to the skin by ultra violet light.[20] Application of topical steroids after exposure to ultra violet light resulted in decreased erythema, but Burdick et al.[21] found that it was not only the time of application that was

critical but also the amount of exposure to ultra violet light. It was found that it was necessary to determine each individual's "minimum erythema dose" in order to obtain good discrimination between test compounds.

In 1940 Wolf[22] observed that if adhesive tape was applied to the skin surface and sharply pulled off, local tissue damage could be produced. In 1957 Wells[23] suggested that this particular damage and repair stimulus might be suitable as a steroid assay method. It was found that hydrocortisone applied after stripping caused a decrease in vasodilation, the maximum response occurring after 12 hours. However some investigators[24-26] felt that the skin stripping method had the disadvantage of removing the physiological barrier to absorption. In fact it was demonstrated[26] that damaging the skin by removing its superficial barrier will allow up to 75% of a corticosteroid to pass through it. Such variability in percutaneous absorption will depend on the extent of cutaneous damage.[27] This method also suffers from the disadvantage in that it is both painful and unpleasant for the volunteers.[4] Nevertheless the method has been employed in the development of formulated topical corticosteroids.[28-33]

The Rat Thymolytic Anti-Inflammatory Activity assay[34] and the Anti-Granuloma assay[35] are systemic assays for corticosteroids but can be used as a preliminary indication of possible topical corticoid activity.[8]

In 1971 Fisher and Maibach[36] suggested that the well documented antimitotic effect of corticosteroids might be used to assay new corticoids, as the antimitotic activity seemed to be related to the steroid activity. In 1973 Marks et al.[37] used a method of artifically boosting the mitotic rate of the dorsal skin of the hairless mouse by stripping the skin five or six

times with adhesive tape. Topical steroids were subsequently applied to the "stripped" skin and the area occluded. By this method they were able to show that it was indeed possible to use the antimitotic effect as an assay for topical steroids.

Jarrett and Spearman[38] correlated thinning of the epidermis with a reduction in mitotic activity and subsequently compared five commercially available creams by comparing their thinning action on the mouse tail epidermis.[39]

Certain ointment bases when applied to guinea pig skin produce a measurable and reproducible epidermal thickening.[40] Barnes et al.[41] were able to relate the suppression of these changes to the type and concentration of corticosteroid used and the nature of the base. This method is therefore useful in determining the most suitable base as well as the efficacy of the steroid.

The effects of anti-inflammatory corticosteroids on fibroblasts, which form the matrix and fibrous substance of the dermis, were demonstrated in vivo by Dougherty and Schneebeli.[42] Fibroblasts react to corticosteroids with morphological changes which can be directly correlated with corticosteroid anti-inflammatory activity.[43] This inhibition of fibroblast growth can be used as an in vitro assay for corticosteroid activity.[44]

Scholtz and Dumas[45] in 1972 developed a method of corticosteroid testing, using patients with chronic stabilised psoriasis. Chronic stabilised patients were used because in any given plaque of psoriasis there is a consistency in the degree of activity and severity from one part of the lesion to another.[46] Good correlation was achieved between results obtained from the psoriasis assay and animal assays when determining the

potency of fluocinolone acetonide.[46]

The main advantages of the psoriasis bioassay are that the inhibitory effect upon the epithelial proliferation is tested. The test is carried out on a human dermatosis which is one of the most important indications for the use of corticosteroids, and the patient suffers no additional trauma. The disadvantages of the test are that the appropriate patients must be available, the test is only semi-quantitative, certain corticosteroids which have no antiproliferative action are not suitable for the test,[47] and the patients must be selected by trained personnel.

1.2 THE HUMAN VASOCONSTRICTOR ASSAY AS A MEASURE OF TOPICAL STEROID ACTIVITY

In 1950 Hollander et al. reported that intra-articular steroids produced blanching of the engorged synovial membrane in rheumatoid arthritis[48] and in 1952 Aston and Cook observed vasoconstriction in superficial corneal vascularization treated with subconjunctival steroids.[49]

It was not until 1962 however, that McKenzie and Stoughton,[50] after observing that topical steroids under occlusion,[51] produced pallor of psoriasis lesions and of the surrounding normal skin, realized that this blanching might be used as an index for percutaneous absorption of steroids.

1.2.1 Alcoholic Vasoconstrictor Studies

The McKenzie/Stoughton assay technique involved the application of solutions or suspensions of steroids in 95% ethanol to normal healthy skin both in the occluded and unoccluded mode. By this method they were able to determine, using the degree of skin pallor as the index, that occlusion produced an

increase in penetration by a factor of approximately one hundred.

McKenzie,[52] using the vasoconstrictor effect, tested a number of topical steroids and was able to indicate that acetates were better absorbed than their parent alcohols, which were in turn better absorbed than phosphates. With regard to triamcinolone he found that the acetonide greatly increased penetration. He also found that precise assessment of the potency of the steroid was impracticable using intradermal injections since vasoconstriction lasting several hours may be seen around an intradermal injection of any sort.

In a similar assay to that used by McKenzie, McKenzie and Atkinson[53] assayed betamethasone and 23 of its esters. They concluded that despite its low precision the vasoconstrictor assay is of value for the screening of potentially active topical steroids.

In 1966 after a modification of the technique by Sarkany et al.,[54] McKenzie[55] pointed out that he felt it was impossible to grade accurately the degree of pallor and that the importance of the test was in the use of high dilutions. Zaun[56] in 1966 also concluded that the vasoconstrictor test was not sufficiently sensitive for the determination of quantitative differences between compounds.

Baker and Sattar[57] were unsuccessful in their attempts to grade the pallor that they observed in an assessment of four fluocortolone analogues, but by using the presence or absence of pallor they were able to differentiate between three fold increments in concentration of the steroids. This experiment also took into account that the steroids were deposited on the skin surface by eliminating all air movement that could cause some of the

steroid to be lost.

In 1968 Child et al.[34] examined six commercially available topical steroids for vasoconstrictor activity in man, systemic anti-inflammatory activity in rats and mice and progestinal activity in rabbits. Good agreement was obtained between ranking order of topical activity and ranking order of systemic activity.

Stoughton[58] tested five glucocorticosteroids suspended or dissolved in 95% ethanol for vasoconstrictor potency in vivo and the ability to penetrate skin in vitro. Unfortunately owing to a lack of randomization, which is essential,[51] these results are dubious.

Place et al[59] modified the vasoconstrictor assay and investigated serial dilutions of several corticosteroids. The assay was modified by duplicating applications, randomised differently on both arms of the same volunteer. Test sites on the volunteers arms were outlined with silicone grease. In this assay particular attention was paid to experimental variables.

The method used by Place et al.[59] was modified by Moore-Robinson and Christie[60] and Moore-Robinson.[61] The modification of the Place et al. assay were:-

(1) Occlusion time was reduced.

(2) After removal of the dressing the arms were washed with soap and water.

(3) A scale of from 0 to 3 was used to quantitate the degree of vaso-constriction.

(4) Blanching was "read" at two time intervals, 8 hours and 24 hours after application.

By this method six corticosteroids dissolved in ethanol were investigated and statistical differences demonstrated.

The method of Moore-Robinson and Christie was extended by Granier[62] who observed blanching at three different time intervals, namely 8, 12 and 24 hours after application.

In 1972 Burdick[63] modified the Moore-Robinson and Christie assay technique by increasing occlusion time to 18 hours and in 1973 Weirich and Lutz[64] again modified the assay by using a 0 to 2 scale and observing the responses at 2, 4, 8, 24 and 32 hours after an occlusion time of 16 hours.

Falconi and Rossi[65] considered that the McKenzie/Stoughton method of evaluating vasoconstrictor activity was time consuming and contained a possible source of error in that the alcoholic solutions had a tendency to spread uncontrollably over the skin surface. They modified the technique by incorporating the test compounds into coded, glassine backed 1 cm squares of thin absorbent paper which were applied to the skin surface. However before application of the squares each arm was shaved if necessary, oils removed with a mixture of alcohol and ethyl ether, and a "small" amount of 5% tincture of thurfyl nicotinate applied to the test site. This pre-treatment does not appear to be standardised and could possibly introduce a source of error. The degree of blanching was evaluated on a 0 to 3 scale.

In 1974 Engel *et al.*[66] used an unmodified form of the McKenzie/Atkinson test, but used two parameters to quantify the response, namely spot diameter and blanching. Both responses were graded on a -, +-, + and ++ scale.

It was shown that this method was sufficiently quantitative to be used in structure activity relationship investigations.

Barry and Brace in 1975[67] assessed the topical activity of eight novel anti-inflammatory steroids using a modified form of the vasoconstrictor assay. After evaporation of the ethanol the sites were occluded for 6 hours and thereafter assessment of the blanching was made using a 0 to 4 scale with half point ratings. Seventeen reading times allowed the determination of complete blanching profiles for each steroid. Three methods of statistical analysis were used to rank the steroids: summed % total possible score, area under the blanching profile and square root transformations of sum scores divided by the number of volunteers.

1.2.2 Studies of Vasoconstriction in Other Solvents

The use of solvents which increase the transport of medicaments through the skin, such as dimethylsulphoxide (DMSO), dimethylformide, dimethylacetamide and tetrahydrofurfuryl alcohol has been widely studied.[68]

Stoughton and Fritsh[69] found that DMSO enhanced the penetration of alcoholic solutions of fluocinolone acetonide and Stoughton[70] found that within a few minutes after application to the surface of human skin, hydrocortisone and fluocinolone acetonide in the presence of DMSO are established in a protected reservoir in the stratum corneum. (See section 1.4)

In 1966 Tissot and Osmunsden[71] using a modification of the McKenzie/ Stoughton vasoconstrictor technique screened a number of different organic solvents for their potential to serve as effective vehicles for the administration of fluorometholone. It was found that the potency

of fluorometholone in the vasoconstriction test was affected by these solvents and that small chemical differences in the configuration of the solvent molecules influenced efficacy.

Triamcinolone acetonide and triamcinolone cumarilate in four solvents were studied by Altmeyer and Zaun in 1974 using a reflex photometric technique.[72]

Several groups of substances have been shown to have penetrant properties; for example, the protein denaturing substances, salicylic acid, latic acid and urea. These penetrants have been studied *in vitro*.[73-74]

1.2.3 Studies of Vasoconstriction in Formulated Products

Solvent studies are useful in the development and establishment of rank order for topical steroids. These studies do not however take into account the influence of the pharmaceutical formulation on the availability of the steroid. It has therefore become necessary to establish bio-availability for the formulated product.

Barrett *et al.*[75] in 1964 observed the vasoconstrictor activity of betamethasone 17-valerate incorporated into four bases: Aqueous Cream B.P., Oily Cream B.P., white soft paraffin and macrogol ointment. No significant differences in the degree of blanching were observed, although a larger area of vasoconstriction was obtained for the macrogol ointment formulation. Sarkany *et al.*[76] extended this work using the four vehicles but incorporating betamethasone 17-valerate, fluocinolone acetonide, hydrocortisone acetate and hydrocortisone alcohol. Similar results to those of Barrett *et al.*[75] were obtained for the betamethasone 17-valerate

but the fluocinolone acetonide failed to produce any consistent response with the different bases. Hydrocortisone acetate and hydrocortisone alcohol, when incorporated into the above bases failed to produce any activity, but when formulated into dimethylacetamide and tetrahydrofurfuryl alcohol bases blanching was obtained.

In a subsequent study, Barrett et al.[77] noted that micronization of the fluocinolone acetonide improved the activity which was further improved by the addition of 5% propylene glycol to the base.

Caldwell et al.[78] dissolved 0,5% beclomethasone dipropionate in propylene glycol and incorporated 5% of this solution into white soft paraffin. The resulting ointment was more active in the vasoconstrictor test than an ointment containing the same concentration of active ingredient in a microcrystalline suspension.

To compare the relative vasoconstrictor potentials of three steroids, fluocinolone acetonide, triamcinolone acetonide and hydrocortisone alcohol, Reid and Brooke[79] selected a common base that, from preliminary work, had been shown to permit the maximum percutaneous absorption of the three steroids. A dimethylacetamide/macrogol formulation was choosen as the "universal base". The activities of the three steroids were compared using a 1 to 4 grading based on the area and degree of pallor. Little difference was noted between the vasoconstrictor potentials of fluocinolone acetonide and triamcinolone acetonide but both of these were about twice as effective as hydrocortisone alcohol.

In 1969 Busse et al.[80] studied the release of betamethasone 17-valerate and 21-desoxybetamethasone 17-valerate from various ointment bases. As

in previous studies,[75-76] high relative potencies were obtained when propylene glycol was incorporated into the ointment.

Christie and Moore-Robinson in 1970[81] assessed the effects of various bases on the vasoconstrictor response. A propylene glycol ointment base was considerably superior to the base that did not contain this ingredient. Ostrenga et al.[82] were also able to demonstrate that ointment delivery systems were greatly improved when the steroid, in this case fluocinonide, was completely dissolved. The effects of three different bases on the absorption of fluocinolone acetonide were studied by Granier.[62] A non-emulsified, anhydrous fatty alcohol/propylene glycol cream base, FAPG, was shown to have better absorption characteristics than the more conventional creams. The effect of ointment base composition on the release of betamethasone 17-benzoate was investigated by Pepler et al.,[83] Soft Paraffin/Propylene Glycol and Soft Paraffin/Isopropyl Myristate gave significantly higher results than conventional bases.

Coldman et al.[84] evaluated several commercially available topical steroid preparations in the unoccluded mode with a short duration of application. Two ointments, both containing the same steroid in the same concentration but compounded by different manufacturers were included in this assessment. One ointment was superior and on examination it was found that the steroid was in solution or fine particles whereas, in the less active preparation the steroid was in a crystalline state. Thus correct formulation of the topical vehicle is important in order to allow the steroid to exert the maximum effect. It was also noted in this test that a placebo preparation had a vasoconstrictor activity which could account for as much as 50% of the response as in the case of White Soft Paraffin B.P.

Subsequently Coldman et al.[85] compared fluocinonide in FAPG base with, and found it to be superior to, commercially available preparations of betamethasone 17-valerate, triamcinolone acetonide and hydrocortisone.

In 1972 Brudick,[11] using a short application time of 6 hours investigated the effects of two different steroids in two different bases and was able to emphasise the importance of the correct base. The shortening of the application time was shown to allow better discrimination between the release characteristics of the bases. As an extension of the work done by Ostrenga et al.[82], Burdick et al.[21] compared several commercially available preparations with a fluocinolone acetonide ointment in which the steroid had been dissolved in a mixture of propylene glycol and propylene carbonate. The short application time was again used. It was concluded from this trial that the most logical basis for comparison is to calculate the area under the curve for each formulation. (See section 2.4)

In 1974 Poulsen et al.[86] compared three different methods of visually assessing blanching. The three techniques were, the presence or absence of pallor, grading the degree of pallor on a 0 to 3 scale and a technique involving paired comparisons at adjacent sites. This latter technique was found to improve the assessment.

In 1972 Stoughton[87], using the unoccluded mode, compared a number of commercially available preparations. However, unlike Coldman et al.,[84] a long (20 hours) application time was used. Ointment vehicles were generally found to be superior to creams and lotions. This could be due to the occlusive nature of the ointment vehicle or as a result of the high placebo effect of white soft paraffin.[84]

Barry and Woodford[88] evaluated thirty proprietary creams and gels using excellent modifications to the vasoconstrictor test. The test sites were occluded for 6 hours which allowed better discrimination between the products. Grading was done on a 0 to 4 scale with half point ratings. Eleven readings were taken from 6 to 96 hours after application which allowed the determination of complete blanching profiles. The retention of the steroid was also assessed in selected volunteers by re-occluding the sites for 12 hours, 8, 12 and 14 days after application. (See section 1.4)

All readings were taken by the investigators except the 9 and 12 hour readings. These were "read by the volunteers" who "were instructed to use similar conditions".[89] This change in observers is not entirely satisfactory, especially as the readings taken by the volunteers are approximately at the time when the maximum intensity of blanching has been reached. However, as this is a comparative technique any small differences will be relative and should not alter the results significantly.

Woodford and Barry[90] observed the bioavailability and activity of betamethasone 17-benzoate in a gel and cream and compared these formulations with several proprietary formulations. In this test it was found that the betamethasone 17-benzoate was significantly less active than a betamethasone 17-valerate preparation. However in a previous study it had been shown that these two steroids were equivalent when dissolved in alcohol. It could be possible that the betamethasone 17-benzoate preparation had not been optimally formulated.

Extending their work on creams and gels Barry and Woodford[91] compared the bioavailabilities of thirty one ointments, and subsequently compared

six proprietary hydrocortisone creams.[92] Barry and Woodford have therefore evaluated, for vasoconstriction, and hence bioavailability, a large proportion of topical steroid products on the United Kingdom market.

In 1977 these two investigators again evaluated nine formulations for blanching activity.[93] Two of these formulations were of an experimental nature whereas the others were proprietary formulations. "Two of the proprietary formulations were marketed in the U.S.A. but not in the U.K.". The investigators made no mention of the manner in which the "U.S.A. formulations" were imported. It is felt that the method of importation is important as it may be possible that the physicochemical nature of the base may change when subjected to extreme conditions which could have resulted during importation.

Barry and Woodford[94] used a non-occluded multiple dose regimen in an attempt to mimic a therapeutic regimen. It was found that the blanching response first increased but then diminished with continued application over a five day period.[95] A "resting period" of two days was allowed before subsequent administration. After the resting period, a response was again apparent, but this also diminished with continual application. The maximum response and the area under the blanching curve for the second week was less than those of the first week. This work may have significant importance in patients who become resistant to topical corticosteroid preparations, in that the vasoconstrictor test could be used to determine the optimal dosage regimen.

The blanching assay has been used specifically to optimise the propylene glycol concentration in formulation development. In 1976, Bluefarb et al.[96] used the test to optimise the propylene glycol concentration for a base

containing 0,05% diflorasone diacetate. In this test a 0 to 3 scale with half point ratings was used and the sites were left unoccluded for 6 hours or occluded for 19 hours. The 6 hour unoccluded study demonstrated that the formulation containing 15% propylene glycol was significantly better than other formulations, however a 6 hour occluded test did not show any marked differences between formulations. Significant differences were shown under 19 hours occlusion where the 15% formulation again outperformed all but one of the other formulations.

1.3 THE MECHANISM OF BLANCHING

The exact mechanism of blanching produced by topical steroids is not clear. Several investigators have implied that a relationship exists between noradrenalin (NA) and corticosteroids, but others have demonstrated that NA is not the only factor involved. Fritz and Levine[97] have shown that vasoconstriction by NA in the mesoappendix of adrenalectomised rats does not occur unless the cortical extract is applied topically. It was thus suggested that corticosteroids may support vascular tone by potentiating the pressor action of NA. Using the rabbit ear[49] and the mesoappendix of the adrenalectomised rat[98] it has been suggested that steroids increase the sensitivity of blood vessels to NA. Reis[99] demonstrated that adrenocortical steroids have a local and direct effect on normal human bulbar conjunctival vessels as well as potentiating NA. Juhlin[100], however, found no evidence of such a potentiation in the vascular reaction of normal skin treated with fluocinolone acetonide. It was therefore assumed that blanching was due to a vasoconstrictor effect of the steroid *per se*.

Frank *et al.*[101] and later Altura[102] showed that steroids did not act

directly by constricting any of the muscular components of the capillary bed but enhanced vascular reactivity to various constrictors while suppressing histamine, bradykinin and alcohol induced vasodilation as well as potentiating NA. Solomon et al.[103] suggested that corticosteroids may be capable of releasing NA from cutaneous stores which had been shown to be present by Moller,[104] but the precise location is unknown.[105,106] Solomon et al.[103] studied the effect of the NA blocking agent guanethidine on vasoconstriction and were able to demonstrate that guanethidine did inhibit vasodilation in normotensive subjects and thus suggested that steroid vasoconstriction was mediated by NA released from these cutaneous stores.

Wolf et al.[107] were able to demonstrate that topically applied corticosteroids potentiated catecholamine induced vasoconstriction. This effect was blocked by phentolamine, but not by propranolol and hence it was suggested that this action is mediated through the increased sensitivity of alpha adrenergic receptors.

Du Vivier and Stoughton[95] demonstrated that it was possible to induce tachyphylaxis to topically applied steroids. As a result of this they suggested that the NA "stores" might be situated in nerve vesicles and that topically applied steroids might act indirectly by releasing this endogenous NA, or influencing its metabolism or re-uptake. Alternatively it was suggested that the steroid may attach itself to a receptor site, which might be specific for each different steroid or a general receptor for all steroids, causing the release of the intracellular mediator adenosine monophosphate or more likely guanosine monophospate. Guanosine monophosphate is a vasoconstrictor and can be stimulated by alpha receptor activity.[108]

1.4 THE SKIN AS A CORTICOSTEROID RESERVOIR

The existence in the skin of a depot or reservoir for topical corticosteroids was suggested by Malkinson and Ferguson.[109] Vickers[110] demonstrated the existence of this reservoir and found it to be situated in the stratum corneum and of considerable capacity. The technique used by Vickers to demonstrate this depot was the same as that used by McKenzie and Stoughton.[50] A stream of warm air was used to promote rapid drying of the test sites. This did not take into account that the steroid is deposited on the skin surface and could have lead to variation by loss of the steroid. The test areas were occluded for 16 hours. On removal of the occlusion blanching was visible but faded after 10 to 16 hours. The test sites were subsequently re-occluded every "two to three days" and on removal of the occlusion blanching was again visible. In some volunteers it was possible to demonstrate blanching up to 15 days after the initial application. The existence of this resevoir has been confirmed by a number of workers.[70,90,111]

Through a series of stripping experiments as described by Wolf[22] and by the use of intradermal injections, Vickers was able to locate the reservoir in the stratum corneum. However Feldman and Maibach[127] suggested that the deeper layers were also implicated. Subcutaneous fat has also been suggested as the possible site for the depot.[27]

The retention of a corticosteroid in the skin should also be considered as a factor affecting its relative potency[27] as the existence of a reservoir allows the steroid to be released slowly over a long period of time. Barry and Woodford[88,90,91] determined that the degree of blanching following re-occlusion of proprietary formulations was

proportional to the ranking order of the preparations. However this was only in the case of the more potent steroids as they were unable to demonstrate the existence of a depot for hydrocortisone formulations.[92] Barry and Woodford[94] in their assessment of vasoconstriction using a multiple dosage regimen, found that the reservoir for the more potent steroids was erratic and it was again not possible to demonstrate a reservoir produced by hydrocortisone preparations.

1.5 THE CORRELATION OF VASOCONSTRICTION WITH CLINICAL EFFICACY

The McKenzie/Stoughton blanching test is based on the property of corticosteroids to produce measurable pallor of the human skin. However according to Marks et al.[37] the disadvantage of this test lies in the fact that although the vasoconstriction is a component part of the therapeutic effect it does not play the leading role. It has therefore become necessary to compare the vasoconstrictor activity of a topical corticosteroid with the clinical efficacy of that steroid.

The strong degree of parallelism between blanching activity and clinical efficacy has been the subject of a number of papers.[11,46,47,77,78,88]

Reid and Brookes[79] correlated the blanching activity of three corticosteroid ointments, hydrocortisone (1%), fluocinolone acetonide (0,025%) and triamcinolone acetonide (0,025%) with their clinical efficacy in a group of patients with eczema. The results of both assessments were the same, with fluocinolone acetonide being slightly superior to triamcinolone acetonide which in turn was better than hydrocortisone.

Moore-Robinson[46] found good correlation between the blanching activity of fluocinolone acetonide and its clinical efficacy.

Whitfield and McKenzie[112] using the blanching assay compared a hydrocortisone 0,1% two phase cream with a 1% hydrocortisone B.P.C. cream. The two phase formulation produced blanching in 9 out of 10 volunteers, whilst the B.P.C. formulation produced a response in only 1 volunteer. The clinical efficacy of both these creams correlated well with these results.

The blanching assay is reliable and quick whereas other screening tests have been shown to be inconsistent with clinical efficacy.[113] Thus the human vasoconstrictor test is an excellent method for screening new corticosteroid derivatives for activity, and for comparing existing formulations. It also has the advantages that healthy skin is used, it is not painful to the volunteers and a number of preparations can be evaluated simultaneously.

1.6 THE EFFECT OF EXTEMPORANEOUS DILUTION OF TOPICAL CORTICOSTEROID FORMULATIONS

There is a wide range of topical steroid preparations on the market which can broadly be divided into three categories; highly active, moderately active and mildly active. If a practitioner wishes to avoid a potent steroid, the rational approach would be to prescribe a milder preparation.[114] However despite this, practitioners continue to prescribe extemporaneously diluted topical steroid preparations. Keipert[115] felt that most commercial preparations were unnecessarily strong and by dilution the preparation would be cheaper and lessen the risk of significant absorption.

A number of surmises have appeared in the literature concerning extemporaneous dilution of topical steroids.[116,117] These have

generally arisen as a result of theoretical considerations with very little, if any, experimental back up. These theoretical considerations can be divided into three main categories; pharmaceutical, bacteriological and biopharmaceutical.

1.6.1 Pharmaceutical Considerations

Manufacturers take a great amount of trouble to produce sophisticated vehicles in which to incorporate topical corticosteroids. The components of these vehicles have been shown to be compatible with each other and the efficiency of the vehicle as a corticosteroid donor has been demonstrated. It is therefore reasonable to suggest that extemporaneous dilution of these vehicles would have a deleterious effect, and as such manufacturers do not recommend dilution of their topical corticosteroid formulations. The highly sophisticated vehicle, FAPG base, will crack if diluted with many creams or ointments.[117]

It would also be possible to inadvertently choose a diluting medium of opposite ionic type to the proprietary product resulting in a breakdown of the emulsion.[118] The British Pharmaceutical Codex[119] and Martindale Extra Pharmacopoeia[120] recommend certain bases with which to dilute proprietary topical corticosteroid preparations should a dilution be necessary. It is also stated in the monograph[119] that these extemporaneous preparations must be used within one month of manufacture and that Betamethasone Cream diluted with a base of alkaline pH will accelerate the conversion of the betamethasone 17-valerate to the less active betamethasone 21-valerate. However, nothing has appeared in the literature in support of these statements.

In an article containing no experimental evidence, Mooney and Pearce[116] found that in an alkaline vehicle, such as E45 cream, the fluocinolone acetonide in Synalar and the fluocinonide in Metosyn underwent oxidative degradation at the 11 and 21 positions. "Thus a dilution of 20% Metosyn in E45 cream loses 50% of its steroid concentration after one month's storage at 23°C."

Topical steroids in ointment vehicles are generally diluted with white soft paraffin.[121] This was thought to be satisfactory[122] but now with the more sophisticated ointment bases, that is, ointments containing a fine dispersion of steroid dissolved in propylene glycol, it has been suggested that it would be difficult to maintain this dispersion on extemporaneous dilution and that trituration might cause phase separation.[117,123]

1.6.2 Bacteriological Considerations

During dilution of topical corticosteroid preparations pathogenic bacteria may be introduced into the vehicle. Strains of Pseudomonas have been isolated from extemporaneously diluted topical steroid creams.[124] However, these surveys of bacteriological content of diluted topical steroid have generally been carried out in hospitals where there is usually a higher bacterial count.

Proprietary topical corticosteroid preparations are manufactured under ideal, carefully controlled conditions and preservatives are added to those preparations in which microorganisms can be sustained.[116,122] It is possible that on dilution, the preservatives may be inactivated due to incompatibility or excessive dilution,[118] but there is no experimental evidence to support this and an investigation is required to determine

the actual extent of bacteriological contamination and what effect dilution has on the preservative.

1.6.3 Biopharmaceutical Considerations

The release of topical corticosteroids is dependent on the nature of the vehicle in which it is incorporated. In the development of a new topical steroid formulation, manufactures go to a great deal of trouble to ensure the optimum release from the vehicle, as incorrect formulation will inhibit the release, and consequently, the activity of the corticosteroid. Dilution of proprietary formulations, may produce a preparation which appears to be physically acceptable, but which has poor release characteristics.[125]

Topical corticosteroid vehicles generally contain a critical amount of propylene glycol in which the corticosteroid is dissolved.[96] Over-solubilization of the steroid with propylene glycol will result in the steroid preferentially remaining in the vehicle, whereas under-solubilization will cause the steroid to revert to the crystalline form, which is less active. It is possible that on dilution the corticosteroid becomes either over- or under-solubilized, which would result in a poor release and consequently a poor activity. Alternatively it is possible that the corticosteroid could be precipitated if the diluting base contained an large proportion of water in which the steroid is not readily soluble.[117]

It is also possible that on dilution the resultant base could be superior to the original base either due to a better release ability or to the occlusive nature of the base being increased. The latter would be expected if a cream formulation were diluted with an ointment base.

Woodford[89] noted that extemporaneous dilutions of Betnovate cream stored for short periods of time before being examined by the blanching assay were little affected by the diluent and suggested a more thorough investigation.

2. METHODS

2.1 THE BLANCHING ASSAY

Proprietary formulations and placebo bases were purchased from a local pharmacy or manufactured according to B.P.C. procedures. No manufacturer's samples were used.

2.1.1 Volunteers

Healthy male and female Caucasians were screened to determine their response to a standard preparation, Betnovate cream (betamethasone 17-valerate 0,1%), applied to the flexor aspects of either forearm and occluded for 6 hours. Only those volunteers who demonstrated a positive response to the standard and who had not received either topical or systemic corticosteroids for at least four weeks were included in the investigations.[94,95] Where possible, the same volunteers were used when preparations were to be investigated routinely.

2.1.2 Mode of Application

Adhesive labels ("Self Seal" Ref. 2438, Gateway Fine Stationery) from which two independent 7 mm squares had been punched were used to mark the flexor aspects of both forearms producing 12 application sites. One set of applications was occluded, the other left unoccluded. The occluded experiments were performed using a nonporous plastic film, (Blenderm Surgical Tape No. 1525[12] 25 mm x 4,5 mm). Six separate strips of sufficient length to prevent separation of the tape from the arm[89] was found to reduce tunnelling.[81] In the unoccluded experiments the sites were protected with a cardboard frame to prevent accidental removal of

the test preparations. In both modes of application the adhesive labels were left in place for the duration of the application and the arms were not bandaged.

The preparations were coded by a person not involved directly with the investigation as were the application charts. Four 7 mm stripes of each preparation, approximating to 3,03 mg (see section 3.1) were applied to each discrete application site. The standard mass was extruded from 1 ml disposable tuberculin syringes, the needles of which had been cut to 5 mm in order to facilitate the extrusion of the formulated product. The syringes were filled immediately prior to use to prevent and minimise any possible interaction between the preparation and the plastic syringe. The syringes were discarded after use.

The extruded formulations were spread on the 7 mm square application sites using a different glass rod for each preparation.

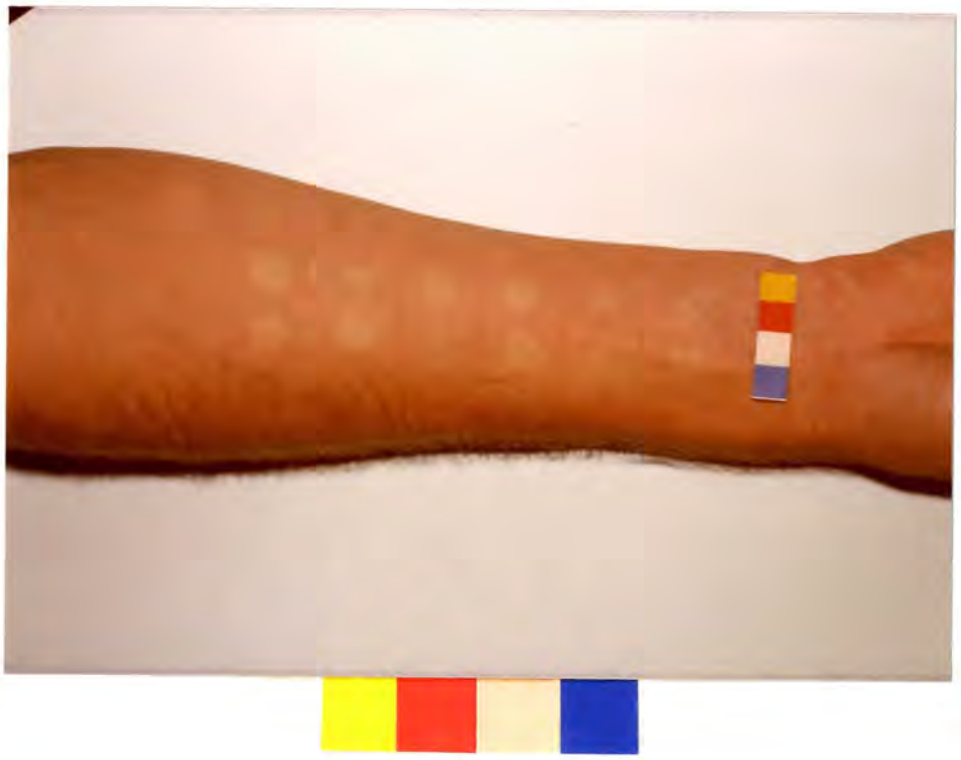
The formulations were allowed to remain in contact with the application sites for exactly 6 hours after which time the occlusions and protections were removed. The adhesive labels and plastic occlusions were removed slowly so as to reduce erythema and to prevent possible stripping[23] of the epidermis. The sites were washed by the volunteer using soap and warm water to remove any residual formulation after which they were patted dry with a towel. The sites often showed puckering but this reverted to normal within half an hour. Occasionally erythema was present around the application but this too disappeared within half an hour.

2.1.3 Reading of Results:

Three independent observers were used to evaluate results in a double blind technique. Blanching was determined at 7, 8, 10, 12, 14, 16, 18, 24 and 28 hours after application which allowed the establishment of a blanching profile.

Standard lighting by overhead fluorescent lamps was used throughout the investigation. The arms were placed horizontally on a desk directly in front of the investigator.

The averaged readings of all three observers were used to analyse the data.



A typical blanching response ten hours after application utilizing a six hour occlusion time.

2.2 STATISTICAL EVALUATION

Three methods were used to evaluate the results.[86]

Method 1

Number of Sites Exhibiting Blanching:

This method involved a simple yes-no determination of whether or not vasoconstriction was present at each application site. These data were reported in terms of total number of sites responding to a given formulation.

Method 2

Intensity of Vasoconstriction:

A visual determination of the degree of vasoconstriction produced at each site was made with this method. An arbitrary response scale was defined as follows:

0 No blanching. Application site appeared identical to surrounding area of skin.

1 Faint pallor observed, clear outline of application square was not seen.

2 Moderate blanching, square outline of application site clearly visible.

3 Intense pallor was observed over entire square of application site.

Method 3

Paired Comparison of Adjacent Application Sites:

This method involved the direct comparison of different formulations applied as pairs. For each pair the following decision was required:

(i) one site exhibited a greater degree of blanching;

(ii) both sites exhibited equal blanching;

(iii) blanching was not observed at either site.

Blanching readings were terminated at 28 hours after application. However, in most cases a response was still evident at this terminal reading and hence it was necessary to calculate a correction factor.

$$(AUC)_{0-\infty} = (AUC)_{0-28hr} + (AUC)_{28hr-\infty}$$

and

$$(AUC)_{28hr-\infty} = \frac{(R)_{28hr}}{k_e}$$

Where $(R)_{28hr}$ is the response at the 28 hour reading time

k_e is the elimination constant

The elimination constant is determined from the slope of the terminal 24 hour - 28 hour portion of the semi-log plot of % TPS vs. time.[128,129]

The area from the correction factor was added to the area obtained from the trapezoidal rule to yield a corrected AUC value.

The disadvantage of calculating the correction factor in this manner is that the elimination constant is determined from the slope between the 24 hour and 28 hour readings. Thus only two points are used to determine this slope and any minor error will significantly affect the correction factor.

As an example of this, if the 24 hour % TPS reading remains constant and the 28 hour % TPS reading decreases successively by 0,1 units then the change in area under the curve and corrected area under the curve, determined by this change in slope, will be as listed below.

% TPS 24 hour	% TPS 28 hour	Area under the curve	Corrected area under the curve
1	0,9	3,8	37,97
1	0,8	3,6	17,94
1	0,7	3,4	11,25
1	0,6	3,2	7,90
1	0,5	3,0	5,89
1	0,4	2,8	4,55
1	0,3	2,6	3,60
1	0,2	2,4	2,90
1	0,1	2,2	2,37

It can be seen from the above Table that the possible error in the corrected area under the curve value is greatest when the difference between the 24 hour % TPS reading and the 28 hour % TPS reading is small.

It would be statistically more correct to extend the observation times to include at least one more reading after the 28 hour reading time, and to determine the slope of the extended portion by linear regression. This, however, is not practical as it becomes extremely difficult to assess the blanching response after the 28 hour period.

3. DISCUSSION

3.1 THE ASSESSMENT OF VARIABLES AFFECTING THE BLANCHING ABILITY OF FORMULATED PRODUCTS

Since its inception in 1962, the McKenzie/Stoughton blanching assay[48] has undergone many modifications in an attempt to make it a precise, reproducible and quantitative method of analysis for topical corticosteroids and formulated products. The parameters which are subject to most variation are:

(i) Measurement of the blanching response.

(ii) The mass of formulated product applied to each test site.

(iii) The length of occlusion time.

Of these parameters, the assessment of the blanching response has been subjected to most modifications. This has been discussed elsewhere. (See sections 1.2.1 and 1.2.3.)

The application of reproducible amounts of formulated product to the test site is extremely difficult. Woodford[88] noted that for Betnovate cream and ointment a variation in mass of 3 to 8 mg did not significantly affect the degree of pallor produced. Polano *et al.*[130] found that corticosteroids in amounts as different as 8 to 55 mg in 0,25% hydrocortisone butyrate cream did not affect the rate of penetration through cadaver epidermis. Baker and Sattar[57] using alcoholic dilutions of four new derivatives of corticosterone demonstrated that different concentrations produced differing degrees of blanching. It is possible that a saturated state could arise, *i.e.*, the amount of steroid diffusing from the base could be greater than the amount which can passively diffuse through the skin.

McKenzie[50] demonstrated that for a number of different steroids dissolved in 95% alcohol an occlusion time of 16 hours increased the absorption of the steroids by one hundred fold over the unoccluded mode. It is not known whether the blanching response will increase proportionally with an increase in occlusion time or whether there is an optimum time above which no further increase in blanching response will be produced.

It was therefore decided to conduct an in depth study on the effect of the amount of formulated product applied to the test site and the effect of occlusion time on the degree of blanching observed.

The formulation chosen for these experiments was Betnovate cream (0,1% betamethasone 17-valerate). Although the results obtained and conclusions drawn can only apply to this preparation it is reasonable to suggest that similar trends would be obtained with other similar topical corticosteroid preparations.

3.1.1 The Effect of the Amount of Formulated Product Applied to the Test Site.

For this trial 11 volunteers were used. Both forearms were masked to produce 12 application sites per arm. Betnovate cream was purchased from a local pharmacy and the first gram of cream was discarded.[90] To each discrete site, 2, 3, 4 or 5 seven mm stripes of cream were applied in a random manner. Both arms were occluded for 6 hours.

Using differential weighings it was found that the mass of cream applied to each application site varied. Four stripes of Betnovate cream have an average mass of 3,03 mg. Forty weighings were performed and

the standard deviation was calculated to be 0,71 mg. This is high and for this reason a large number of sites (66) were used for each different mass applied.

Fig. 1 depicts the blanching profiles obtained for each different mass applied and Table 1 lists the results of the statistical analyses. Statistically significant differences were obtained between 2 and 3 stripes and 3 and 4 stripes, but very few significant differences were observed between 4 and 5 stripes. There is an increase in blanching with an increase in the amount of formulation applied to the skin. As can be seen from the AUC values there is a decrease in the difference between successive values with an increase in concentration. This suggests that it could be possible to determine the concentration limit in terms of blanching response. Consequently an additional trial was mounted.

In the second trial 11 volunteers were also used. To each discrete site, 5, 6, 7 and 8 seven mm stripes of cream were applied in a random manner. The use of 5 stripes allowed for intercomparison between this trial and the previous one. Fig. 2 depicts the blanching profiles obtained for each different mass applied.

A few statistically significant differences were noted between 5 and 6 stripes but no differences were observed between 6 and 7 and 8 stripes.

From the results of these two trials it can be seen that an increase in the amount of preparation applied to the skin causes an increase in the blanching response, but there appears to be a maximum amount of cream

FIG.1

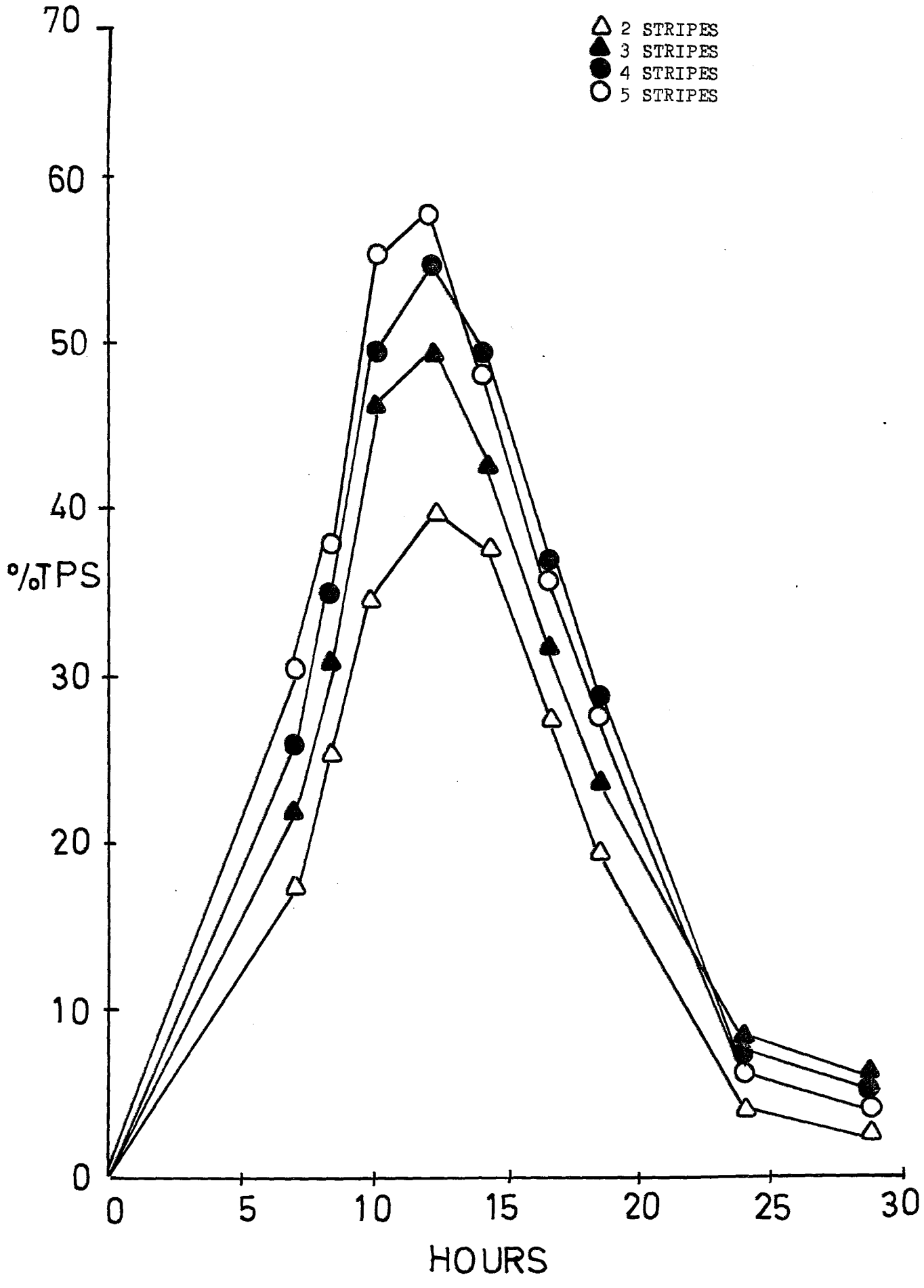

2 STRIPES
3 STRIPES
4 STRIPES
5 STRIPES
70
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG. 2

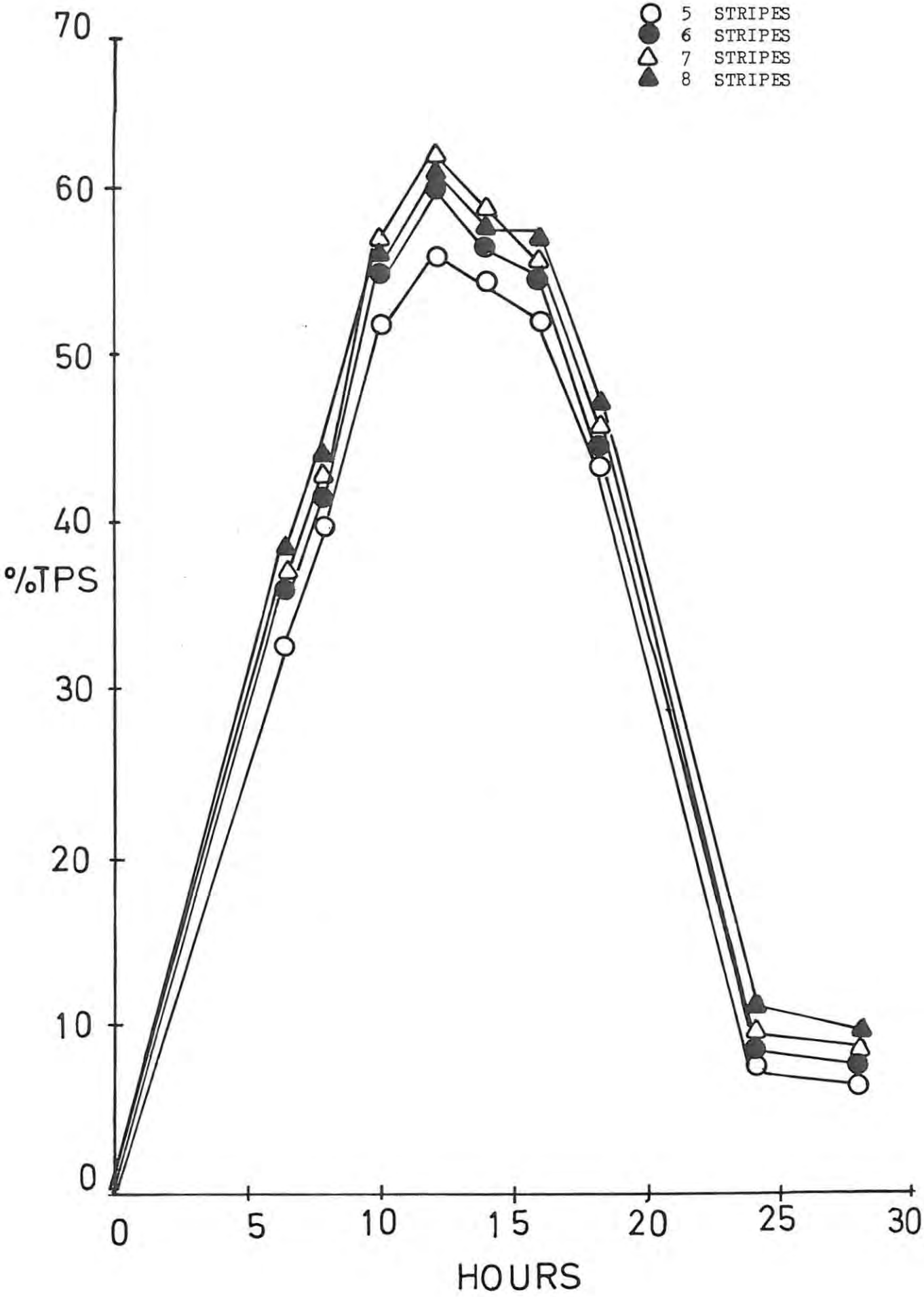

5 STRIPES
6 STRIPES
7 STRIPES
8 STRIPES
70
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

TABLE 1

TWO STRIPES

TIME(HOURS)

RESPONSE	7	8	10	12	14	16	18	24	28
0	103	70	36	30	40	53	82	160	168
1	63	91	98	89	78	103	92	20	12
2	11	13	42	56	61	22	6	0	0
3	3	6	4	5	1	2	0	0	0
TOTAL	94	135	194	216	203	153	104	20	12
TPS	540	540	540	540	540	540	540	540	540
% TPS	17,4	25,0	35,9	40,0	37,6	28,3	19,3	3,7	2,2

THREE STRIPES

RESPONSE	7	8	10	12	14	16	18	24	28
0	84	49	20	10	16	37	64	137	152
1	76	107	80	80	103	111	107	43	28
2	18	20	71	83	56	30	9	0	0
3	2	4	9	7	5	2	0	0	0
TOTAL	118	159	249	267	230	177	125	43	28
TPS	540	540	540	540	540	540	540	540	540
% TPS	21,9	29,4	46,1	49,4	42,6	32,8	23,2	8,0	5,2

FOUR STRIPES

RESPONSE	7	8	10	12	14	16	18	24	28
0	69	47	20	9	16	27	45	144	156
1	86	96	59	60	71	106	118	36	24
2	21	29	92	96	85	45	17	0	0
3	4	8	9	15	8	2	0	0	0
TOTAL	140	178	270	297	265	202	152	36	24
TPS	540	540	540	540	540	540	540	540	540
% TPS	25,9	33,0	50,0	55,0	49,1	37,4	28,2	6,7	4,4

..... Table 1 continued/

TABLE 1 continued/

FIVE STRIPES

	TIME(HOURS)								
RESPONSE	7	8	10	12	14	16	18	24	28
0	56	38	10	9	14	30	46	147	158
1	91	99	59	48	75	98	114	32	22
2	28	35	94	104	82	48	20	1	0
3	5	8	17	19	9	4	0	0	0
TOTAL TPS	162 540	193 540	298 540	313 540	266 540	206 540	154 540	34 540	22 540
% TPS	30,0	35,7	55,2	58,0	49,3	38,2	28,5	6,3	4,1

AREA UNDER THE CURVE VALUES

	UNCORRECTED AREA	CORRECTED
TWO STRIPES	490,80	507,7
THREE STRIPES	616,70	664,98
FOUR STRIPES	691,20	733,06
FIVE STRIPES	728,65	766,83

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR INTENSITY OF BLANCHING STATISTICAL METHOD.

TWO STRIPES VS. THREE STRIPES

TIME(HOURS)

	7		8		10		12		14		16		18		24		28	
0	103	84	70	49	36	20	30	10	40	16	53	37	82	64	160	137	168	152
1	63	76	91	107	98	80	89	80	78	103	103	111	92	107	20	43	12	28
2	11	18	13	20	42	71	56	83	61	56	22	30	6	9	0	0	0	0
3	3	2	6	4	4	9	5	7	1	5	2	2	0	0	0	0	0	0
χ^2 VALUE	5,04		6,88		15,76		16,06		16,62		4,37		3,95		10,18		7,20	

THREE STRIPES VS. FOUR STRIPES

	7		8		10		12		14		16		18		24		28	
0	84	69	49	47	20	20	10	9	16	16	37	27	64	45	137	144	152	156
1	76	86	107	96	80	59	80	60	103	71	111	106	107	118	43	36	28	24
2	18	21	20	29	71	92	83	96	56	85	30	45	9	17	0	0	0	0
3	2	4	4	8	9	9	7	15	5	8	2	2	0	0	0	0	0	0
χ^2 VALUE	2,99		3,62		5,88		6,76		12,54		4,68		6,31		0,79		0,36	

FOUR STRIPES VS. TWO STRIPES

	7		8		10		12		14		16		18		24		28	
0	69	103	47	70	20	36	9	30	16	40	27	53	45	82	144	160	156	168
1	86	63	96	91	59	98	60	89	71	78	106	103	118	92	36	20	24	12
2	21	11	29	13	92	42	96	56	85	61	45	22	17	6	0	0	0	0
3	4	3	8	6	9	4	15	5	8	1	2	2	0	0	0	0	0	0
χ^2 VALUE	13,54		11,04		34,84		32,48		20,00		16,39		19,26		5,41		4,44	

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR INTENSITY OF BLANCHING STATISTICAL METHOD (continued).

FOUR STRIPES VS. FIVE STRIPES

TIME(HOURS)

	7	8	10	12	14	16	18	24	28
0	69 56	47 38	20 10	9 9	16 14	27 30	45 46	144 147	156 15
1	86 91	96 99	59 59	60 48	71 75	106 98	118 114	36 32	24 2
2	21 28	29 35	92 94	96 104	85 82	45 48	17 20	0 1	0 (
3	4 5	8 8	9 17	15 19	8 9	2 4	0 0	0 0	0 (
χ^2 VALUE	2,60	1,56	5,81	2,12	0,36	1,24	0,32	1,27	0,1(

FIVE STRIPES VS. TWO STRIPES

	7	8	10	12	14	16	18	24	28
0	56 103	38 70	10 36	9 39	14 40	30 53	46 82	147 160	158 168
1	91 63	99 91	59 98	48 89	75 78	98 103	114 92	32 20	22 12
2	28 11	35 13	94 42	104 56	82 61	48 22	20 6	1 0	0 0
3	5 3	8 6	17 4	19 5	9 1	4 2	0 0	0 0	0 0
χ^2 VALUE	26,89	20,19	52,31	46,14	22,06	16,82	20,01	4,32	3,25

FIVE STRIPES VS. THREE STRIPES

	7	8	10	12	14	16	18	24	28
0	56 84	38 49	10 20	9 10	14 16	30 37	46 64	147 137	158 152
1	91 76	99 107	59 80	48 80	75 103	98 111	114 107	32 43	22 28
2	28 18	35 20	94 71	104 83	82 56	48 30	20 9	1 0	0 0
3	5 2	8 4	17 9	19 7	9 5	4 2	0 0	0 0	0 0
χ^2 VALUE	10,41	7,13	12,17	15,95	10,58	6,36	7,34	2,97	0,84

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR YES/NO STATISTICAL METHOD.

TWO STRIPES VS. THREE STRIPES

TIME(HOURS)

	7	8	10	12	14	16	18	24	28
YES	77 96	110 131	144 160	150 170	140 164	127 143	98 116	20 43	12 28
NO	103 84	70 49	36 20	30 10	40 16	53 37	82 64	160 137	168 152
χ^2 VALUE	3,61	5,02	4,76	10,15	11,19	3,33	0,37	9,31	6,33

THREE STRIPES VS. FOUR STRIPES

	7	8	10	12	14	16	18	24	28
YES	96 111	131 133	160 160	170 171	164 164	143 153	116 135	43 36	28 24
NO	84 69	49 47	20 20	10 9	16 16	37 27	64 45	137 144	152 156
χ^2 VALUE	2,23	0,01	0,03	0,00	0,03	1,54	4,26	0,58	0,20

FOUR STRIPES VS. TWO STRIPES

	7	8	10	12	14	16	18	24	28
YES	111 77	133 110	160 144	171 150	164 140	153 127	135 98	36 20	24 12
NO	69 103	47 70	20 36	9 30	16 40	27 53	45 82	144 160	156 168
χ^2 VALUE	12,12	6,13	4,76	11,50	11,19	10,04	15,77	4,76	3,74

FOUR STRIPES VS. FIVE STRIPES

	7	8	10	12	14	16	18	24	28
YES	111 124	133 142	160 170	171 171	164 166	153 150	135 134	36 33	24 32
NO	69 56	47 38	20 10	9 9	16 14	27 30	45 46	144 147	156 158
χ^2 VALUE	1,77	0,99	2,95	0,06	0,04	0,08	0,00	0,07	0,63

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR YES/NO STATISTICAL METHOD (continued).

FIVE STRIPES VS. TWO STRIPES

TIME(HOURS)

	7	8	10	12	14	16	18	24	28
YES	124 77	142 110	170 144	171 150	166 140	150 127	134 98	33 20	32 12
NO	56 103	38 70	10 36	9 30	14 40	30 53	46 82	147 160	158 168
χ^2 VALUE	23,84	12,71	15,58	11,50	13,62	7,58	14,85	3,19	8,19

FIVE STRIPES VS. THREE STRIPES

	7	8	10	12	14	16	18	24	28
YES	124 96	142 131	170 160	171 170	166 164	150 143	134 116	33 43	32 28
NO	56 84	38 49	10 20	9 10	14 16	30 37	46 64	147 137	158 152
χ^2 VALUE	8,42	1,52	2,95	0,00	0,04	0,66	3,78	1,35	0,04

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR PAIRED COMPARISON STATISTICAL METHOD.

TWO STRIPES VS. THREE STRIPES

TIME(HOURS)

	7	8	10	12	14	16	18	24	28
0	45	17	7	1	5	14	25	89	96
=	8	3	2	4	4	10	5	1	0
<	45	65	85	76	74	59	50	23	17
>	22	35	26	39	37	37	40	7	7
χ^2 VALUE	7,22	8,41	30,31	11,27	11,68	4,59	0,90	7,50	3,38

THREE STRIPES VS. FOUR STRIPES

	7	8	10	12	14	16	18	24	28
0	29	14	6	3	4	7	11	81	96
=	7	7	3	5	7	11	10	2	2
<	46	49	59	54	57	56	55	17	8
>	38	50	52	58	52	46	44	20	14
χ^2 VALUE	0,58	0,00	0,32	0,08	0,15	0,79	1,01	0,11	1,14

FOUR STRIPES VS. TWO STRIPES

	7	8	10	12	14	16	18	24	28
0	33	16	6	2	5	10	22	93	104
=	6	5	8	7	6	7	11	1	0
<	23	31	29	42	33	30	32	8	9
>	58	68	77	69	76	73	55	18	7
χ^2 VALUE	14,27	13,09	20,84	6,09	16,18	17,13	5,56	3,11	0,06

..... Table 1 continued/

Table 1 continued/

TABLE USED IN CALCULATING χ^2 VALUES FOR PAIRED COMPARISON STATISTICAL METHOD (continued).

FOUR STRIPES VS. FIVE STRIPES

TIME(HOURS)

	7	8	10	12	14	16	18	24	28
0	22	10	4	0	1	8	18	91	108
=	10	10	7	7	11	7	9	3	1
<	45	57	67	63	55	53	37	11	4
>	43	43	42	50	53	52	56	15	7
χ^2 VALUE	0,01	1,69	5,28	1,27	0,09	0,00	3,48	0,35	0,36

FIVE STRIPES VS. TWO STRIPES

	7	8	10	12	14	16	18	24	28
0	27	15	3	1	3	13	19	88	102
=	7	5	5	3	13	13	7	1	0
<	14	17	16	23	29	24	32	10	9
>	72	83	96	93	75	70	62	21	9
χ^2 VALUE	37,78	42,25	55,72	41,04	19,47	21,54	8,95	3,23	0,05

FIVE STRIPES VS. THREE STRIPES

	7	8	10	12	14	16	18	24	28
0	23	11	1	1	2	10	14	80	95
=	6	9	5	7	5	7	13	3	1
<	26	35	35	38	46	40	37	18	9
>	65	65	79	4	67	63	56	19	15
χ^2 VALUE	15,87	8,41	16,22	25,93	3,54	4,70	3,48	0,00	1,04

above which no further blanching response is observed. There are a number of reasons which could account for this observation.

Reference to figs. 1 and 2 indicates that the preparation being tested has not caused the maximum possible blanching. It may be that the response observed for 6, 7 and 8 stripes is a maximum response for betamethasone 17-valerate and no matter how much more of the steroid is applied to the skin no further increase in blanching would be produced. It is presumed that only a certain amount of betamethasone 17-valerate can passively diffuse through the skin, no matter how much steroid is being released from the cream base. Alternatively it may be possible that more steroid penetrates through the skin than there are receptor sites available at the test site.

The maximum response observed may also be due to the thickness of the layer of cream applied to the test site. All the sites are the same area, 7 mm^2. The more cream applied, the thicker the layer but the area in contact with the skin remains the same. The steroid will only be able to diffuse through a certain amount of cream and it may be that once the thickness of the layer reaches a certain limit that the steroid is physically too far removed from the skin for it to be absorbed at all. No log dose response curve has been drawn as, although the amount applied is related to the dose, the actual concentration at the receptor site is not known.

3.1.2 The Effect of Occlusion Time on the Blanching Ability of Formulated Products

Occlusion of topical steroid preparations with a non-porous plastic film

has been used for a number of years as a means of increasing the absorption of topical corticosteroids through the skin.

No in-depth study has been reported, however, to investigate the effect of occlusion time on response, or to determine how critical the period of occlusion may be. For this trial 17 volunteers were used. Both forearms were masked to produce 12 application sites per arm. The standard mass of Betnovate cream was applied to each site after which all sites were occluded. The occluding films were removed in a random manner 2, 4, 6 and 8 hours after application.

The results of this trial are depicted graphically in fig. 3. χ^2 analyses of the result demonstrated significant differences between all occlusion times. The AUC values tended to suggest that, as in the case of increasing the concentration with respect to response, increasing the occlusion further may result in the achievement of a plateau.

In order to confirm this, a further trial was undertaken. In this trial 16 volunteers were used. The 12 demarcated application sites were occluded for 8, 10 and 12 hours, the order of which had previously been randomised. The 8 hour occlusion time allowed intercomparison between this and the previous trial.

Graphical representation of the results are shown in fig. 4. χ^2 analyses showed a few statistically significant differences between the 8 and 10 hour occlusion times but there were no differences between the 10 and 12 hour occlusion times. The differences between successive occlusion times decrease with increase in occlusion time, which suggests that a plateau was being attained, but the actual AUC values increased with

FIG.3

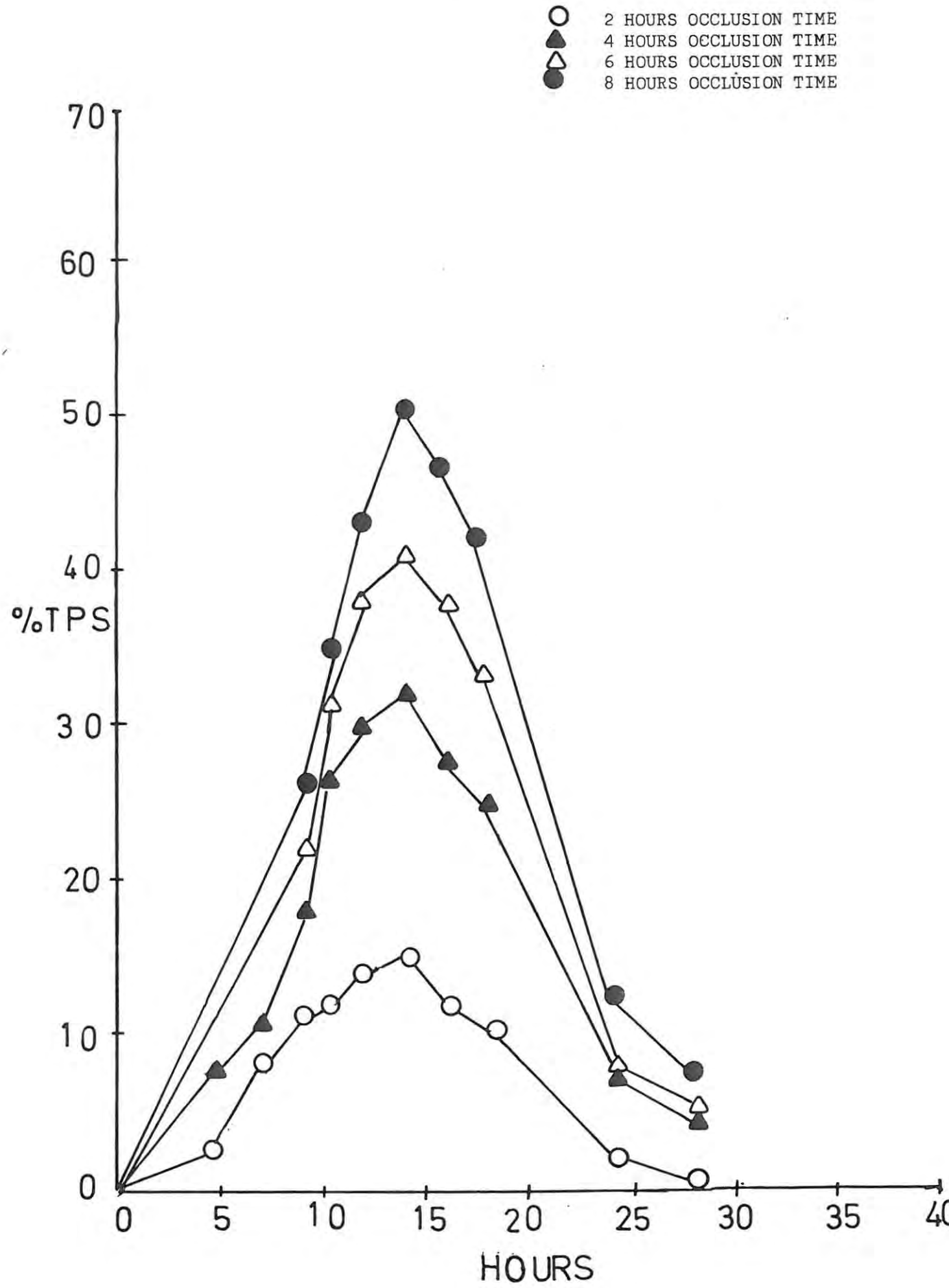
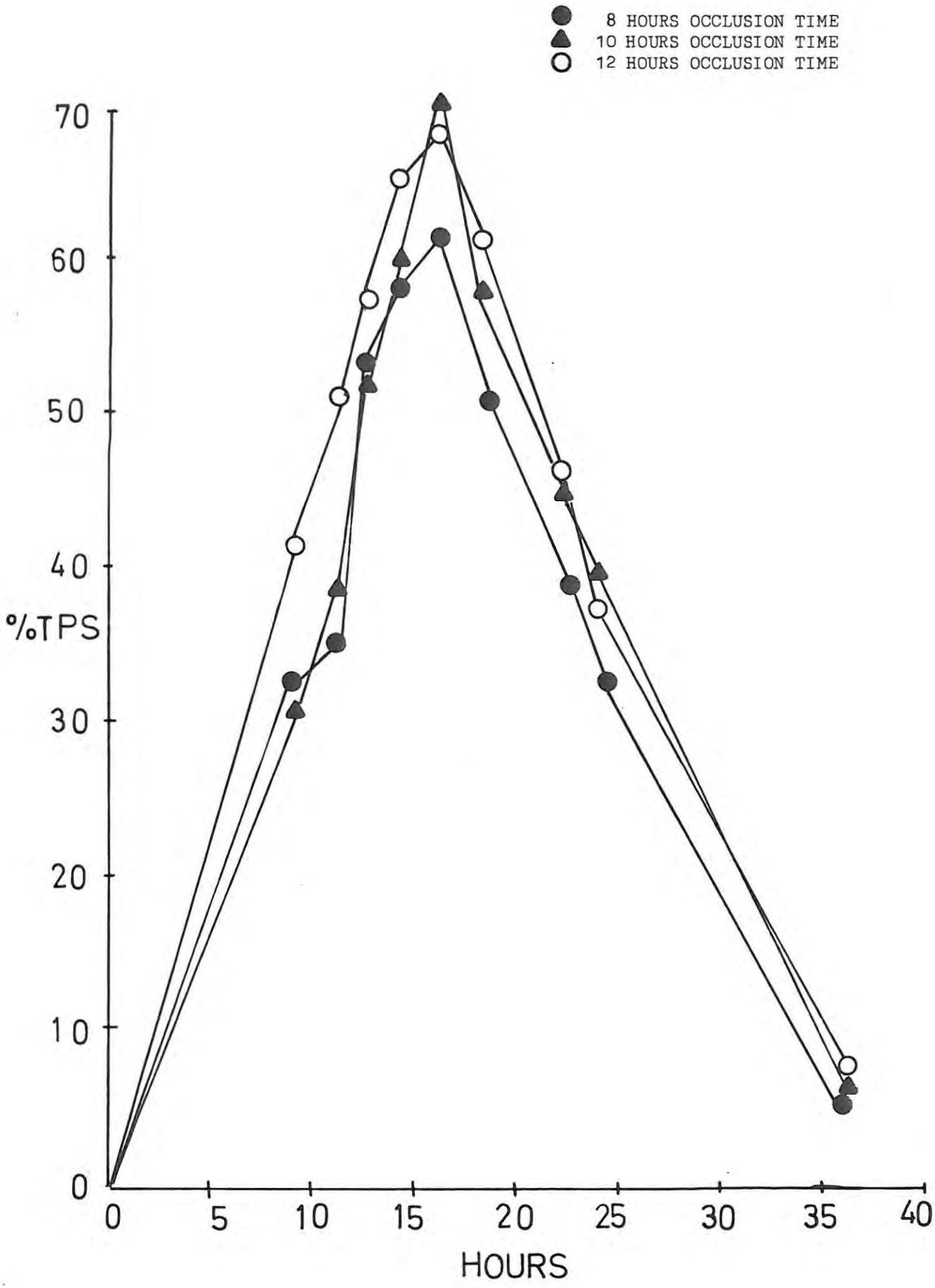

2 HOURS OCCLUSION TIME
4 HOURS OCCLUSION TIME
6 HOURS OCCLUSION TIME
8 HOURS OCCLUSION TIME
70
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
35
40
HOURS

FIG.4


8 HOURS OCCLUSION TIME
10 HOURS OCCLUSION TIME
12 HOURS OCCLUSION TIME
70
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
35
40
HOURS

increase in occlusion time. This corresponding increase, which was not proportional to increased occlusion time suggests that an absolute maximum response had not been attained with the 12 hour occlusion time.

From figs. 3 and 4 it can be seen that the response increases with an increase in occlusion time, but above a certain period of occlusion no further increase in response is obtained.

This plateau effect could be due to maximum absorption of the steroid present, i.e., unless the concentration of steroid per site is increased there can be no further increase in response. If this plateau is due to, too low a concentration of steroid, it would require additional trials to determine the period of occlusion above which no further response is observed. An increased mass of preparation would have to be applied to the site and occlusion times of 10 and 12 hours used. If an increased response is obtained using these larger masses then it can be assumed that the plateau depicted in fig. 4 is due to the concentration of steroid. If on the other hand no increase in response is observed, then it can be assumed that the plateau is due to other parameters.

One explanation for attaining a plateau effect is that the steroid present has reached a maximum blanching effect and that no matter how much the concentration is increased no greater response will result.

Another possible explanation for the plateau effect is that above a certain occlusion time there is maximum hydration of the stratum corneum and thus, maximum absorption of the steroid.

Because of the results obtained from these two trials it was decided to use a 6 hour occlusion time in all subsequent trials. The reasons for this are that this period allows excellent discrimination between different preparations. Also, since most experimental work in this field makes use of a 6 hour occlusion time, it was felt that standardisation would be an advantage. Six hour occlusion time was found to be convenient and did not subject the volunteers to undue discomfort. Because of the increase in response with longer occlusion times in all subsequent trials a 6 hour period of occlusion was rigidly adhered to.

3.2 THE IN VIVO ASSESSMENT OF EXTEMPORANEOUS DILUTIONS OF TOPICAL CORTICOSTEROID PREPARATIONS

3.2.1 Introduction

Considerable time, effort and money is spent in the development of vehicles for topical steroid preparations. These should provide the optimum release characteristics for any particular steroid. The vehicles are extremely sophisticated and any extemporaneous dilution of these may therefore alter their release characteristics. Manufacturers therefore continually warn about the possible dangers of extemporaneous dilution of their commercially available topical steroid formulations. Despite these warnings, topical steroid preparations are frequently diluted with a variety of bases in an attempt to either decrease the potency of the formulation or to alter the release characteristics of the vehicle. Very little work has, however, been carried out on these diluted formulations and thus it is not known to what extent the potency is affected or to what extent the release characteristics of the original base is affected.

It was therefore decided, to investigate the bioavailability of extemporaneously diluted topical steroid preparations at regular intervals over a prolonged period of time.

For this study to be of practical use it was necessary to determine which commercially available topical steroid preparations were most often diluted, which bases were most commonly used as the diluting media and to what extent the commercial products were generally diluted. In order to ascertain the above information it was necessary to conduct a small survey at the local pharmacies. The results of the survey showed that both Betnovate and Synalar creams were those preparations most commonly diluted, and the bases which were used to dilute these preparations were, Emulsifying Ointment B.P.; Aqueous Cream B.P.; Buffered Cream B.P.C.; E45 cream and Ultrabase. These bases were most commonly used to dilute Betnovate and Synalar creams to 50% of their original strength. As a half strength Betnovate cream was commercially available and as we had the placebo Betnovate base it was also decided to include the commercially available preparation and an extemporaneously prepared 50% dilution of Betnovate cream with the placebo base.

Unfortunately it was not possible to obtain a commercially prepared half strength Synalar cream, but as Synadone (0,01% fluocinolone acetonide) and a small quantity of Synalar placebo cream base was available, from which a 50% extemporaneous formulation could be prepared, a single assessment of each was carried out.

3.2.2 Preparation of Extemporaneous Dilutions

Conditions in a pharmacy were simulated so that the study would have

practical application. Therefore the Betnovate and Synalar creams as well as all the bases apart from the Buffered Cream B.P.C. were purchased from a local pharmacy. No manufacturer's samples were used. Buffered Cream B.P.C. has a shelf life of 1 month, thus manufacturers do not find it a viable proposition to produce the cream. The cream was therefore prepared the day prior to use.

Exactly equal amounts (150 g) of the corticosteroid formulation and the diluting bases were weighed out on a Sartorious 2 figure top loading balance. The vehicles were then thoroughly mixed on an ointment slab after which the resultant preparations were placed in clear glass ointment jars and tightly sealed. Clear glass jars were used as extemporaneously prepared corticosteroid preparations are generally dispensed from pharmacies in these containers.

Within 12 hours of manufacture these freshly prepared formulations were assessed *in vivo* for biological availability.

3.2.3 50% Preparations Studied Routinely

Preparation	Abbreviation
Betnovate / Aqueous Cream B.P.	a
Betnovate / Buffered Cream B.P.C.	b
Betnovate / E45 cream	c
Betnovate / Ultrabase	d
Betnovate / Emulsifying Ointment B.P.	e
Betnovate / Betnovate placebo base	f
Betnovate Half Strength	g

Preparation	Abbreviation
Synalar / Aqueous Cream B.P.	A
Synalar / Buffered Cream B.P.C.	B
Synalar / E45 cream	C
Synalar / Ultrabase	D
Synalar / Emulsifying Ointment B.P.	E

3.2.4 Preliminary Investigation

In each assessment either Betnovate or Synalar creams were used as well as the extemporaneously prepared creams to allow intercomparison between successive trials.

In the first assessment, Betnovate cream and 4 dilutions of Betnovate (a, d, f, g) were tested. Twelve volunteers with both forearms masked to produce 12 test sites per arm were used. The standard mass of preparation was applied to each test site in a random manner after which one arm was occluded, the other left unoccluded, but protected with a cardboard frame to prevent spreading.

The results are depicted graphically in figs. 5 and 6. It can be seen from the blanching profiles of the unoccluded study, fig. 5, that the results are totally unsuitable for statistical analyses as it is not possible to visually discriminate between the test preparations due to the poor blanching response observed.

It was therefore decided that in all subsequent *in vivo* assessments of 50% extemporaneous dilutions of topical steroid preparations only the occluded mode would be used.

FIG.5

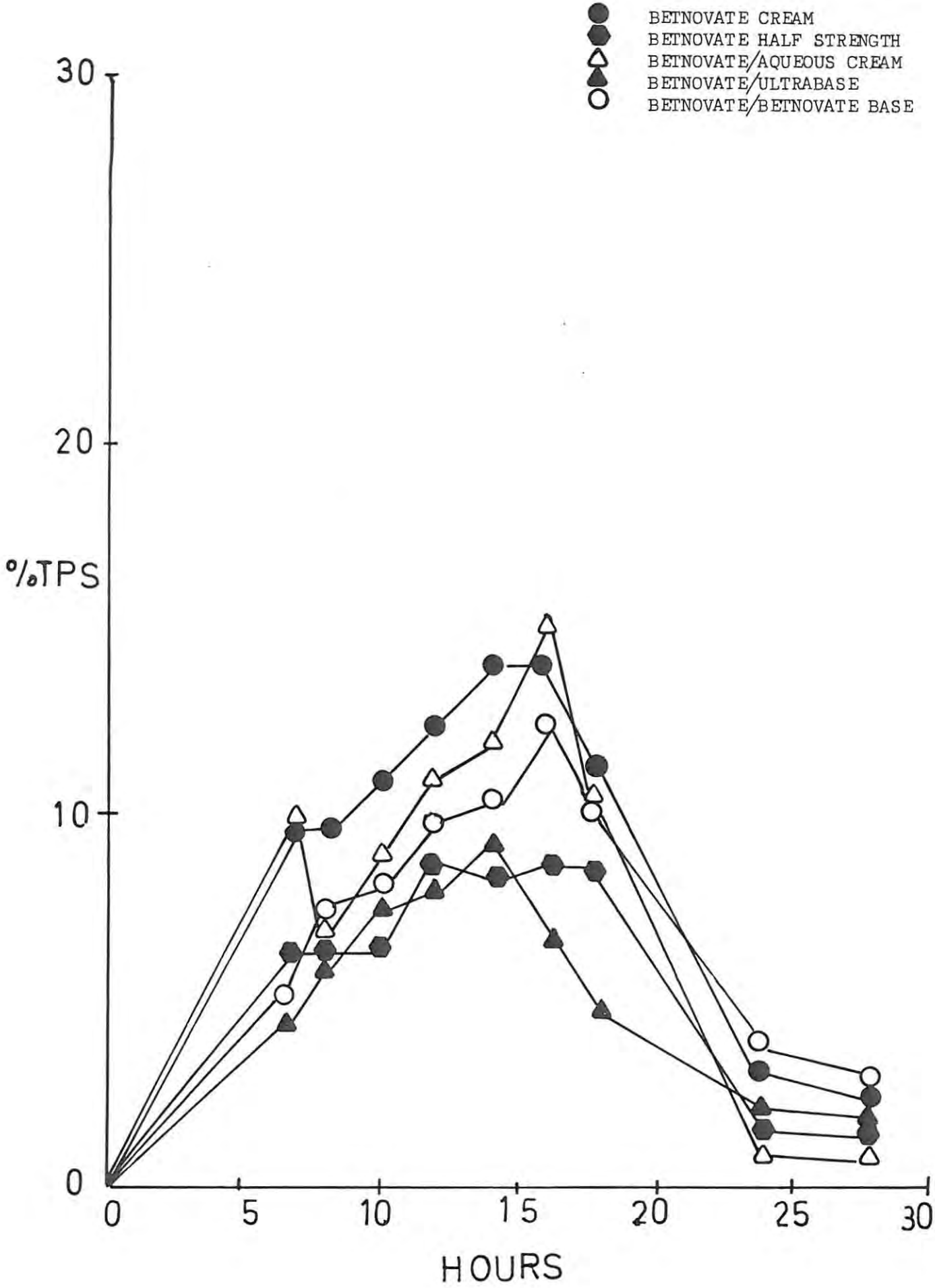
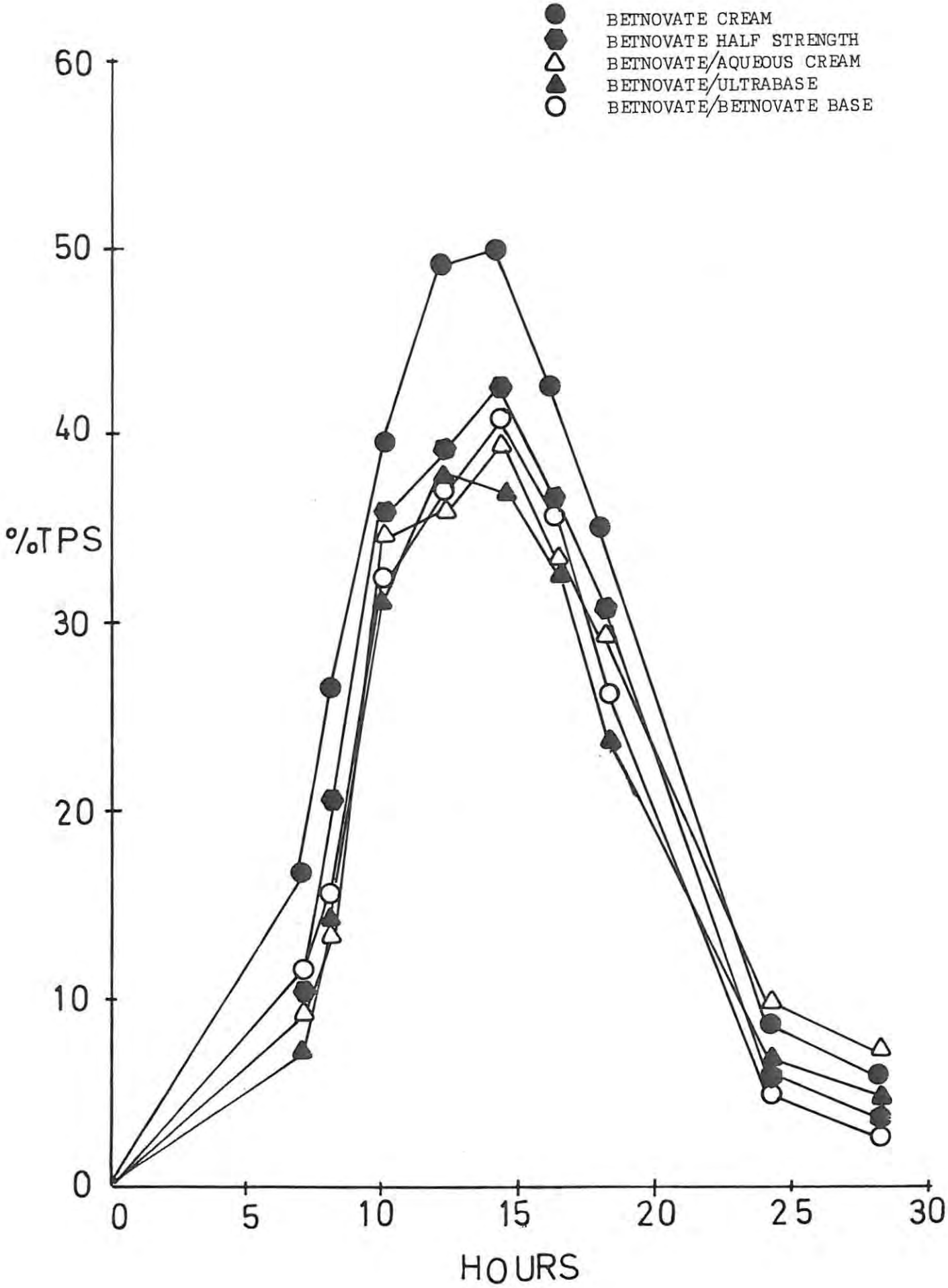

BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG.6


BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

3.2.5 The *in vivo* Assessment of Betnovate and Betnovate/Dilutions

The above trial was repeated using freshly prepared dilutions (a, d, f, g). The results of this trial as well as the remainder of the freshly prepared dilutions (b, c, e) are depicted in figs. 7 and 8.

In all of the above mentioned trials, between 10 and 12 volunteers were used, 12 discrete sites were demarcated on both forearms and both arms were occluded for a period of exactly 6 hours.

The results of the Betnovate dilution studies demonstrated no statistically significant differences between any of the half strength preparations. In one trial (fig. 7) there are statistically significant differences between the half strength preparations and Betnovate cream. In the other trial (fig. 8) no statistically significant differences were demonstrated. It is interesting to note that the response obtained from the half strength preparations as measured by AUC values are not very much less than Betnovate in fig. 7, but significantly different in fig. 8. This difference, however, is not linearly related to the concentration of corticosteroid present.

The diluted preparations were stored at room temperature on open shelves that were neither exposed to, nor protected from, excess light. Conditions were thus similar to those found in most pharmacies.

Approximately one month after dilution, the preparations were thoroughly mixed and then tested *in vivo* again for blanching activity. Where possible, the same volunteers as those used to assess the freshly prepared dilutions were used to assess the same preparations.

FIG. 7

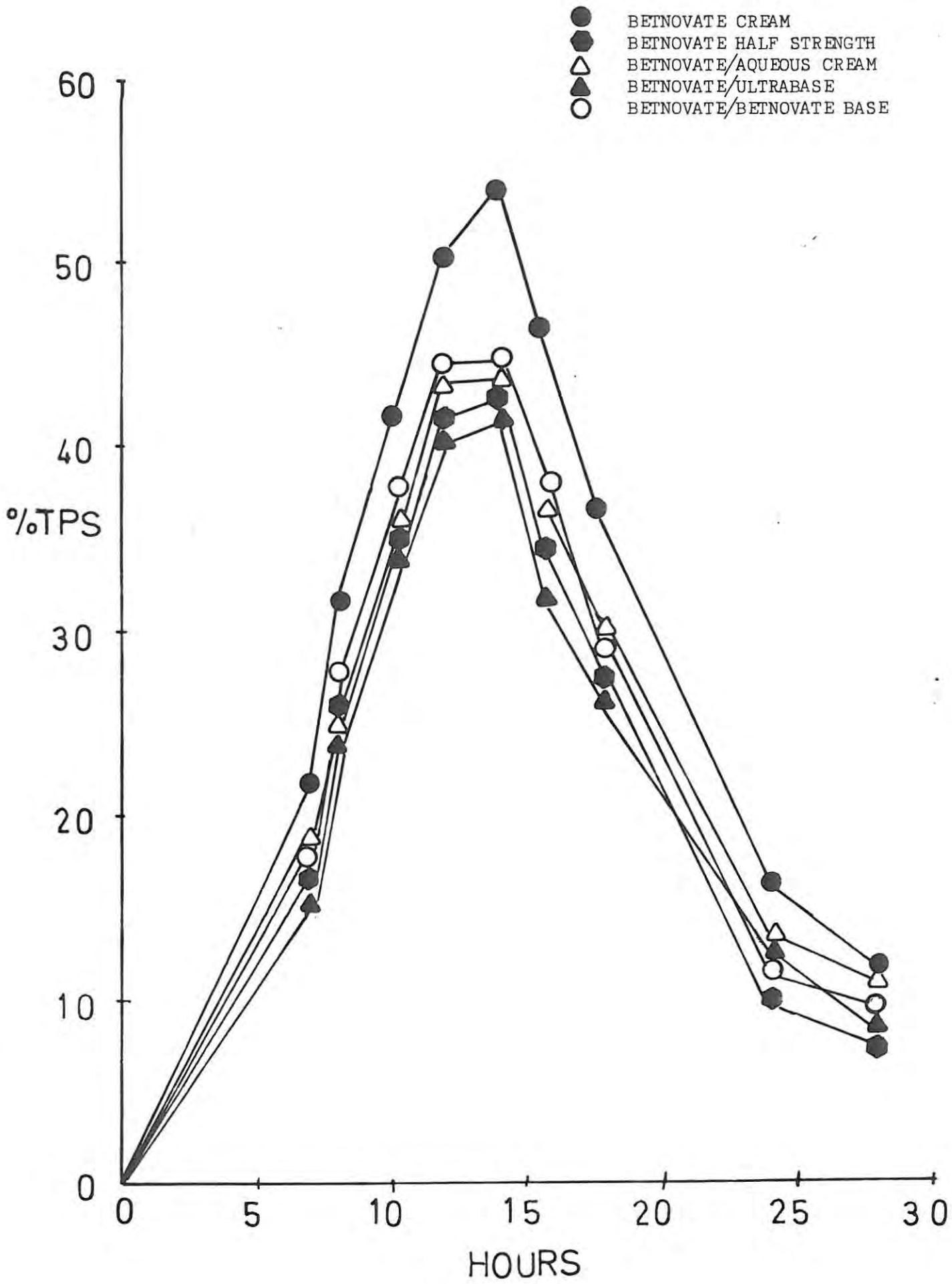
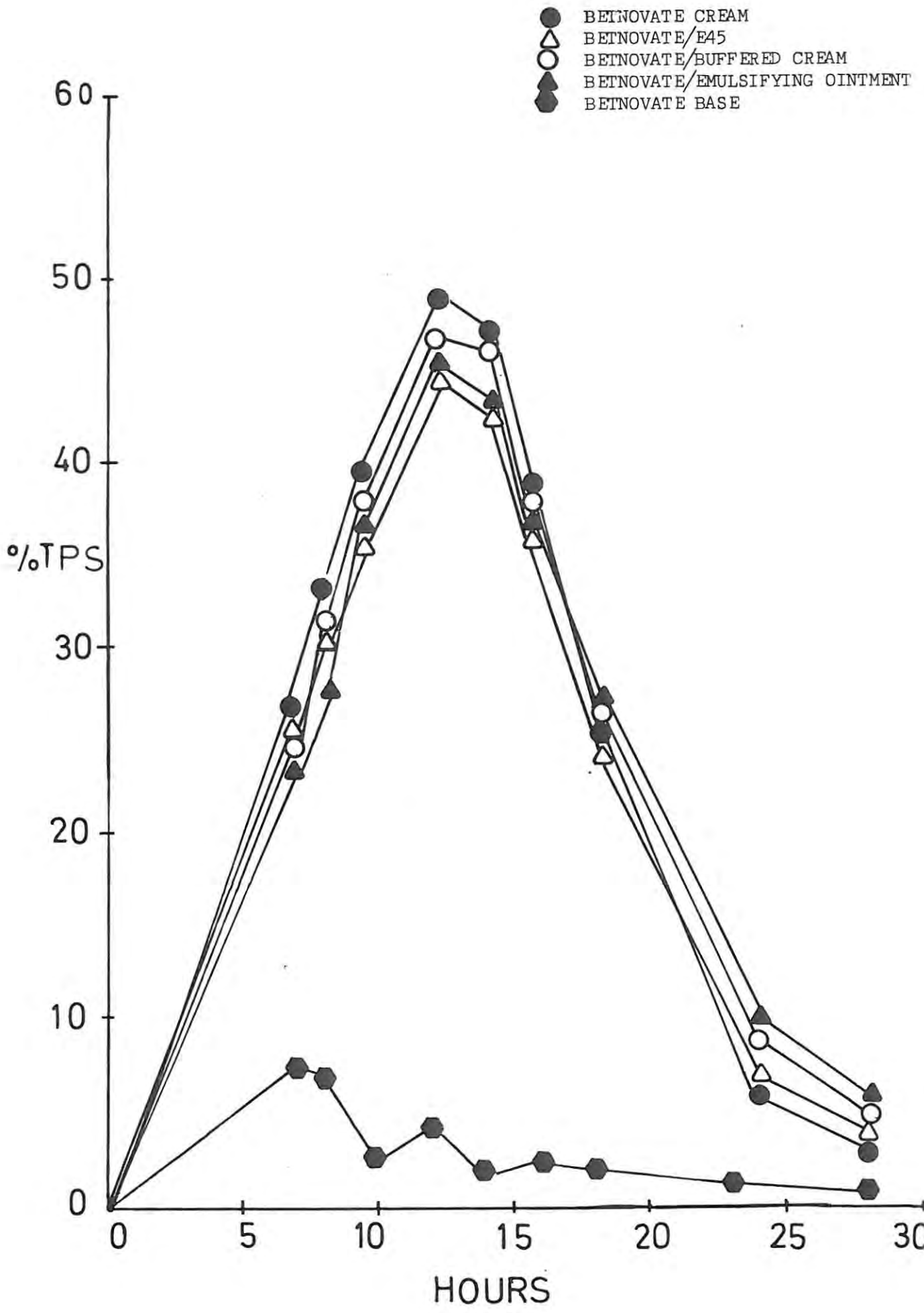

BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

FIG.8


BETNOVATE CREAM
BETNOVATE/E45
BETNOVATE/BUFFERED CREAM
BETNOVATE/EMULSIFYING OINTMENT
BETNOVATE BASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

The results for the two trials are depicted graphically in figs. 9 and 10.

χ^2 analyses of the results depicted in fig. 9 generally show very few statistically significant differences between the various preparations. The intensity of blanching of the Betnovate/Aqueous Cream formulation is greater than that of Betnovate. Betnovate, however, has a slightly longer response, giving it an AUC value marginally greater than Betnovate/Aqueous Cream, which in turn is superior to the other half strength formulations (d, f, g). It could be suggested that this superior response is a concentration effect but this seems unlikely as a large number of sites (58) were used for each preparation.

χ^2 analyses of the results depicted in fig. 10 show no statistically significant differences between Betnovate/Buffered Cream and Betnovate/Emulsifying Ointment which may be expected because of the similarity between Buffered Cream B.P.C. and Emulsifying Ointment B.P. There are a few statistical differences between the various blanching profiles, but these differences are due mainly to a shift to the left of the Betnovate/E45 blanching profile. Despite this shift to the left, AUC values for the 3 half strength preparations (b, c, e) are very similar although less than the AUC value for Betnovate. This shift to the left suggests that Betnovate/E45 produces a slightly greater initial response with a faster onset of blanching activity, but once the maximum intensity of blanching has been reached the amount of blanching produced by Betnovate, Betnovate/E45 decreases at a greater rate than the blanching effect of Betnovate/Emulsifying Ointment and Betnovate/Buffered Cream. It therefore appears that the Betnovate/E45 formulation allows a faster rate of release and subsequent removal of betamethasone 17-valerate from the site of action.

FIG.9

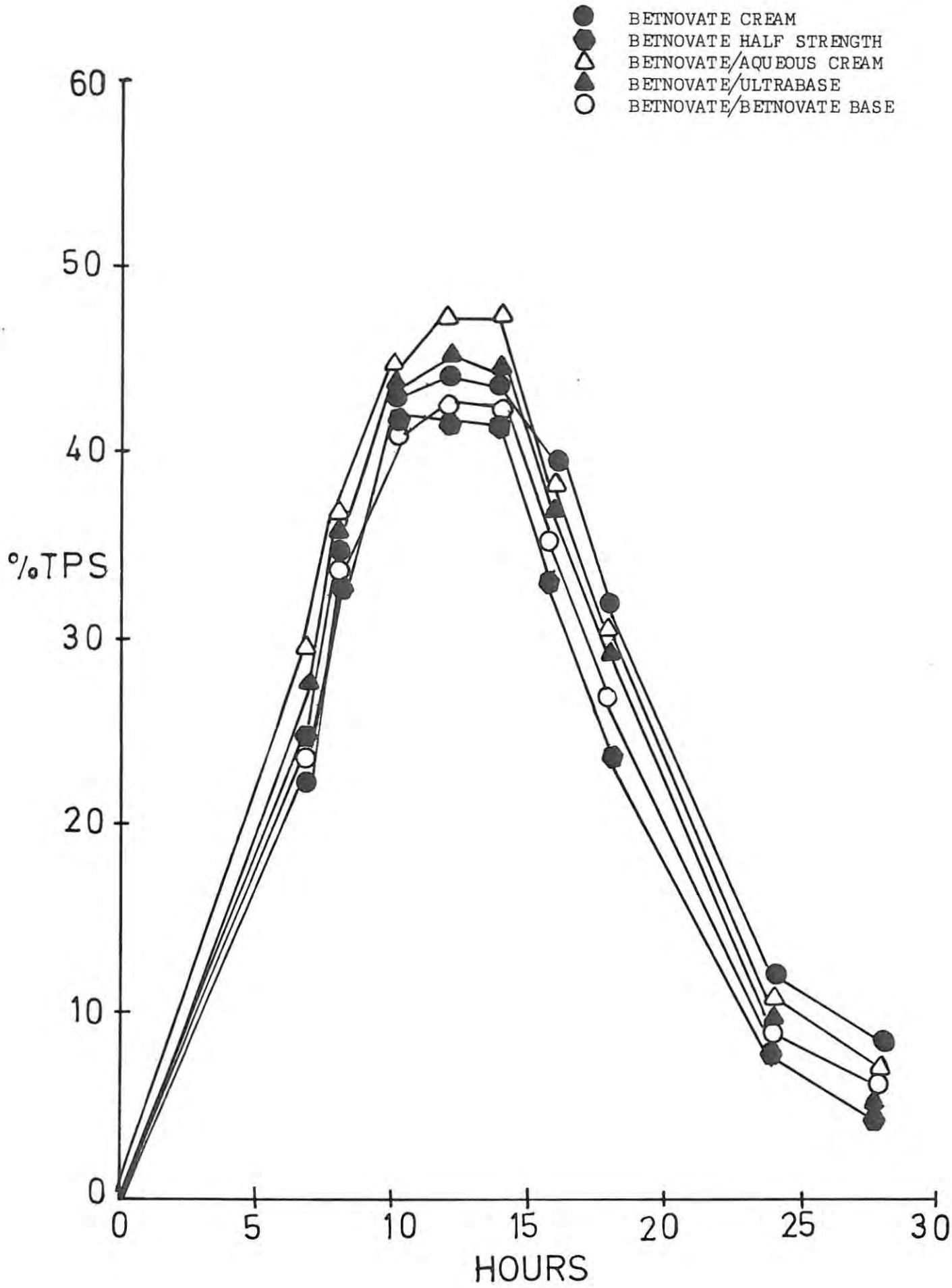
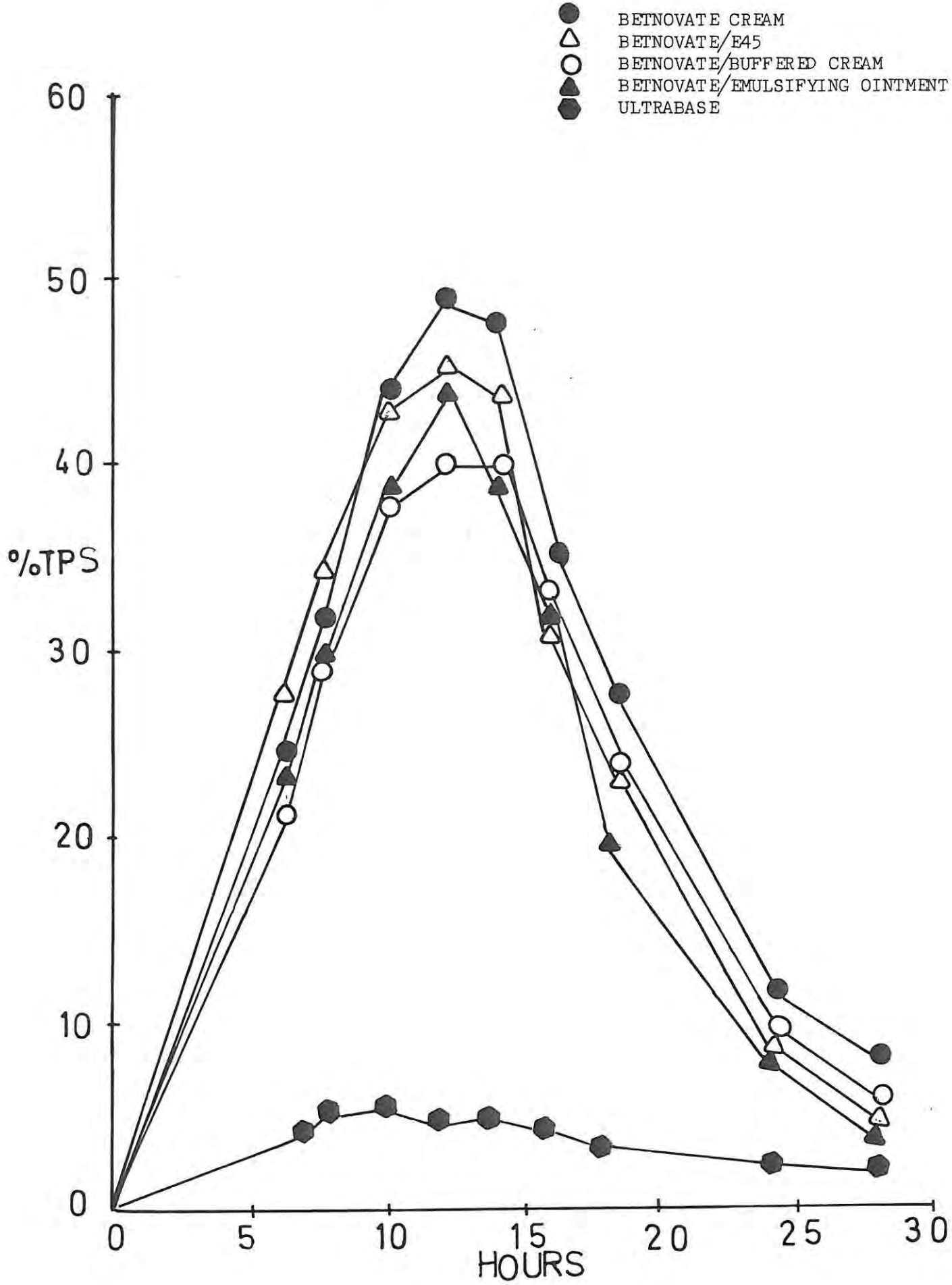

BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG.10


BETNOVATE CREAM
BETNOVATE/E45
BETNOVATE/BUFFERED CREAM
BETNOVATE/EMULSIFYING OINTMENT
ULTRABASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

Comparing the results of these two trials with the results obtained from the previously described data (figs. 7 and 8) there appears to be very little change in the potency and release characteristics of the preparations. No physical tests or assays were conducted on the preparations after one month, but a visual inspection of them showed no physical change.

The Betnovate dilutions were tested in vivo for blanching activity again 3 months after manufacture. The results are depicted graphically in figs. 11 and 12. χ^2 analyses of the results depicted in fig. 11 show virtually no statistically significant differences between any of the formulations (a, d, f, g). Area under the curve values were likewise very similar.

χ^2 analyses of the results depicted in fig. 12 show statistically significant differences between Betnovate and Betnovate/Emulsifying Ointment at most reading times in favour of the former and a few significant differences between Betnovate/E45 and Betnovate in favour of the latter.

It is interesting to note the similarity between the relative AUC values of Betnovate/Aqueous Cream and Betnovate/Buffered Cream, and their superiority compared to Betnovate/Emulsifying Ointment. Aqueous Cream B.P. and Buffered Cream B.P.C. both contain the same amount of water, whereas Emulsifying Ointment B.P. is formulated without water. It therefore seems possible that the amount of water present may effect the blanching response, possibly by increasing the hydration of the stratum corneum which in turn allows better penetration of the steroid.

FIG.11

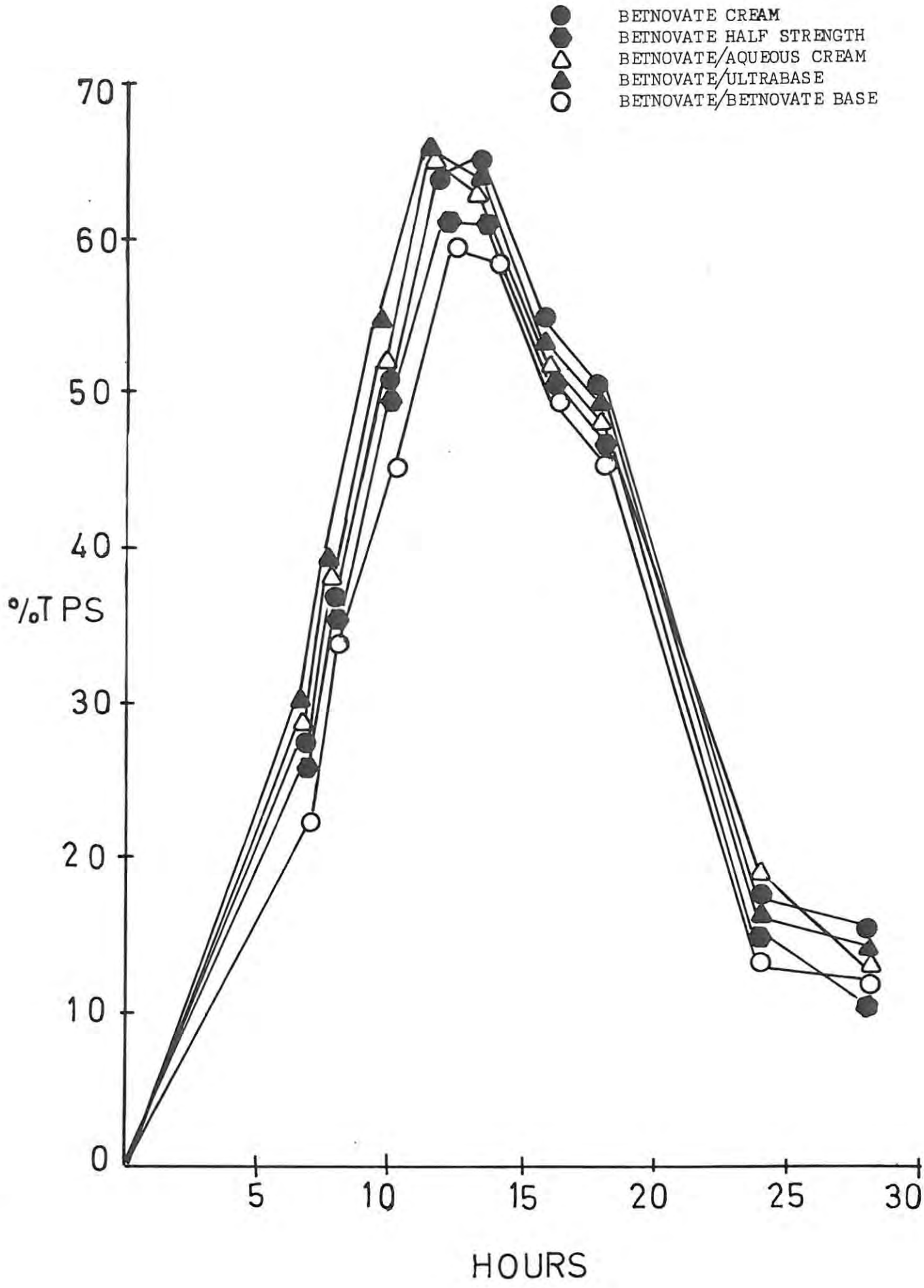
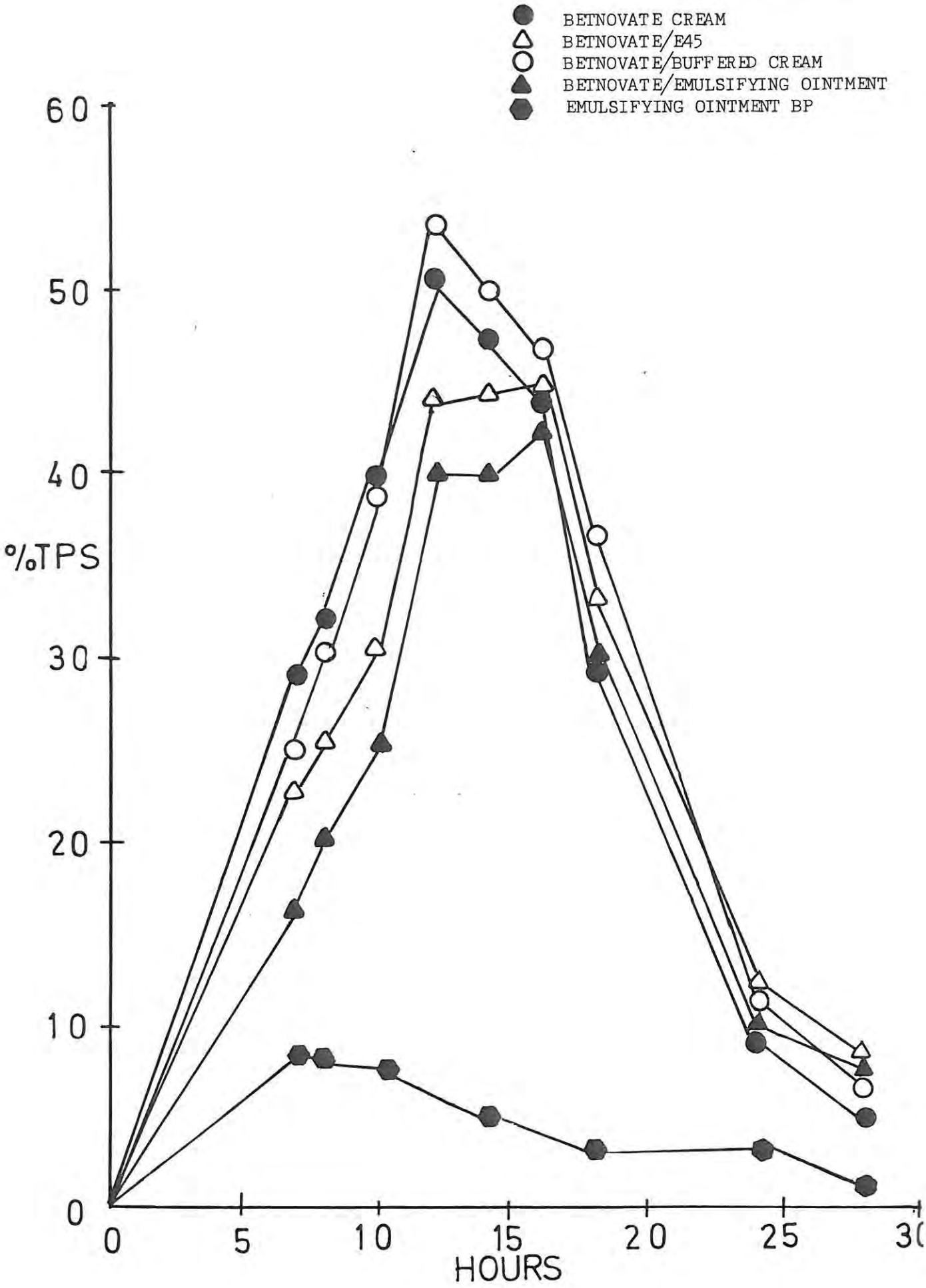

BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
70
60
50
40
30
20
10
0
%TPS
5
10
15
20
25
30
HOURS

FIG.12


BETNOVATE CREAM
BETNOVATE/E45
BETNOVATE/BUFFERED CREAM
BETNOVATE/EMULSIFYING OINTMENT
EMULSIFYING OINTMENT BP
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

The Betnovate dilutions were again tested in vivo for blanching activity 9 months after manufacture. The results are depicted graphically in figs. 13 and 14. χ^2 analyses of the results depicted in fig. 13 show statistically significant differences between the extemporaneously prepared Betnovate/Betnovate Base and Betnovate. Area under the curve values for all half strength preparations (a, d, f, g) are larger than the corresponding AUC value for Betnovate cream.

χ^2 analyses of the results depicted in fig. 14 show very few statistically significant differences between any of the preparations (b, c, e).

Comparison of the results of all the in vivo assessments of Betnovate dilutions over a period of 9 months show very little change in the potency and release characteristics of the preparations. Visually there appears to be no change in the physical properties of the preparations.

Six months after manufacture all the Betnovate dilutions were assayed by HPLC (See section 4) and were found to be within B.P. limits. However, when these dilutions were again analysed 14 months after manufacture, it seemed that some of the betamethasone 17-valerate had been converted to the 21-valerate isomer. (See section 4.5).

Because of this conversion it was decided to again assess the blanching ability of the Betnovate/E45 dilution. The results of this trial are depicted in fig. 15.

χ^2 analyses of the blanching profiles depicted in fig. 15 demonstrated statistically significant differences between Betnovate and Betnovate/E45

FIG.13

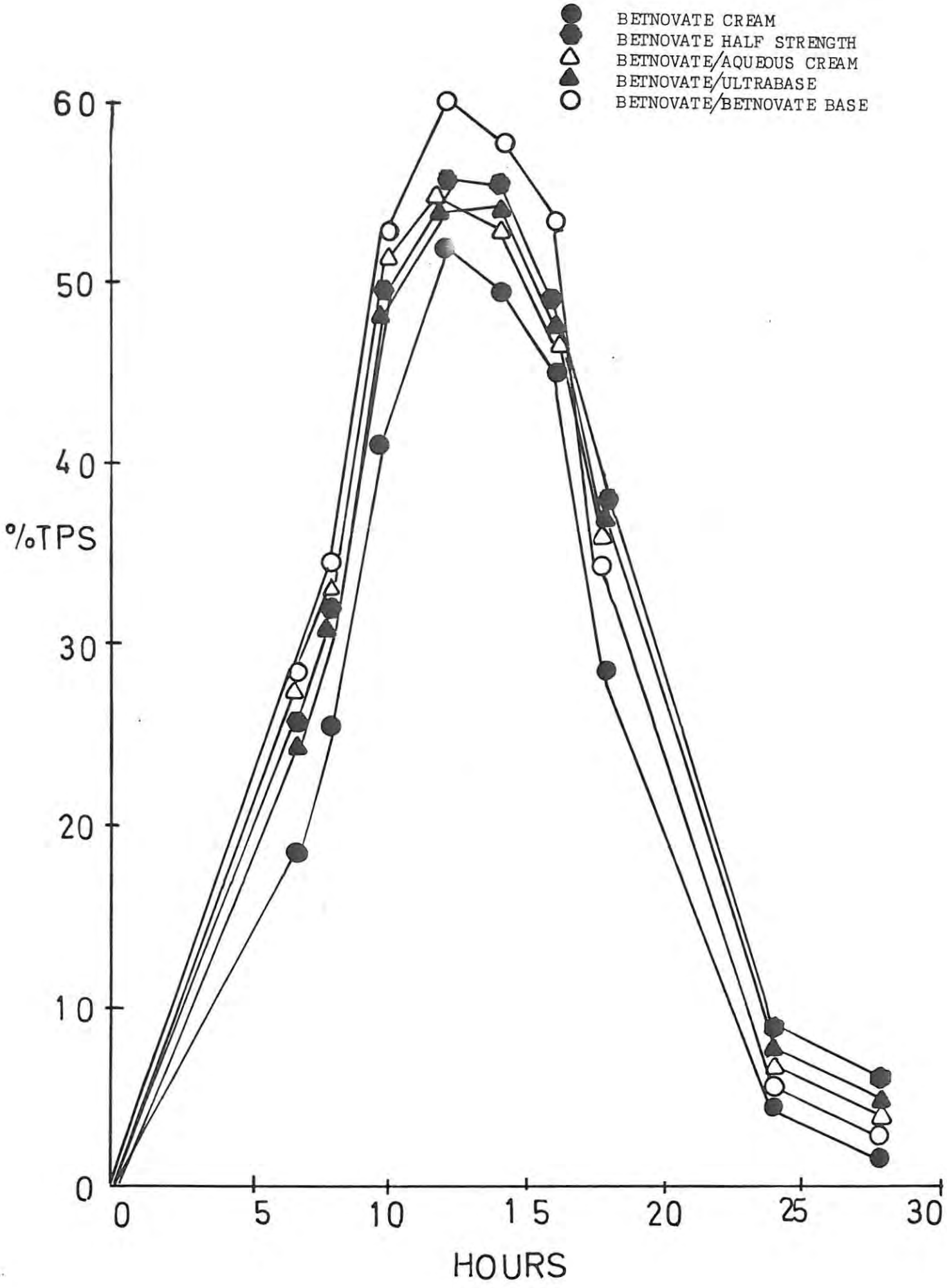

BETNOVATE CREAM
BETNOVATE HALF STRENGTH
BETNOVATE/AQUEOUS CREAM
BETNOVATE/ULTRABASE
BETNOVATE/BETNOVATE BASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG.14

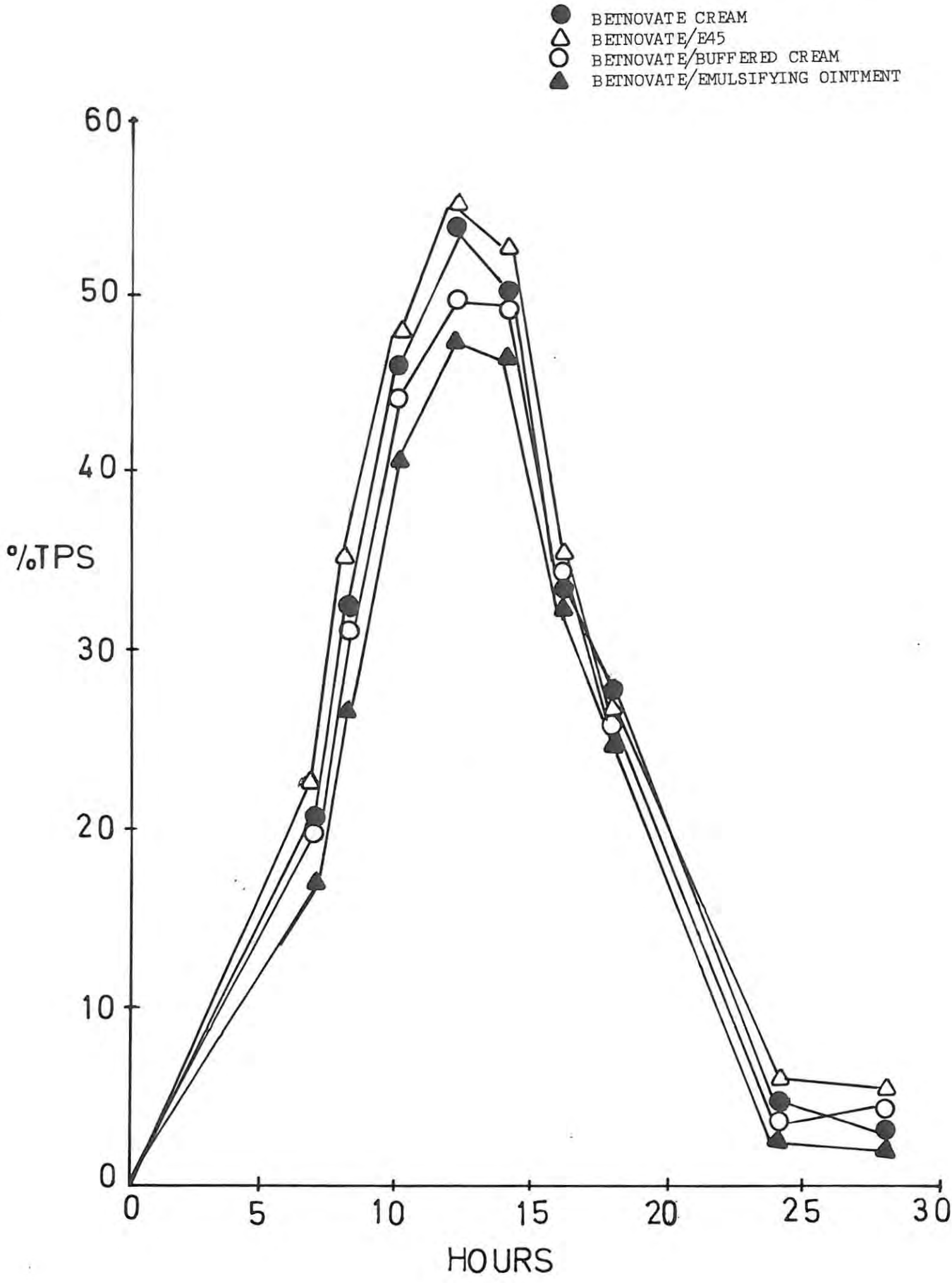
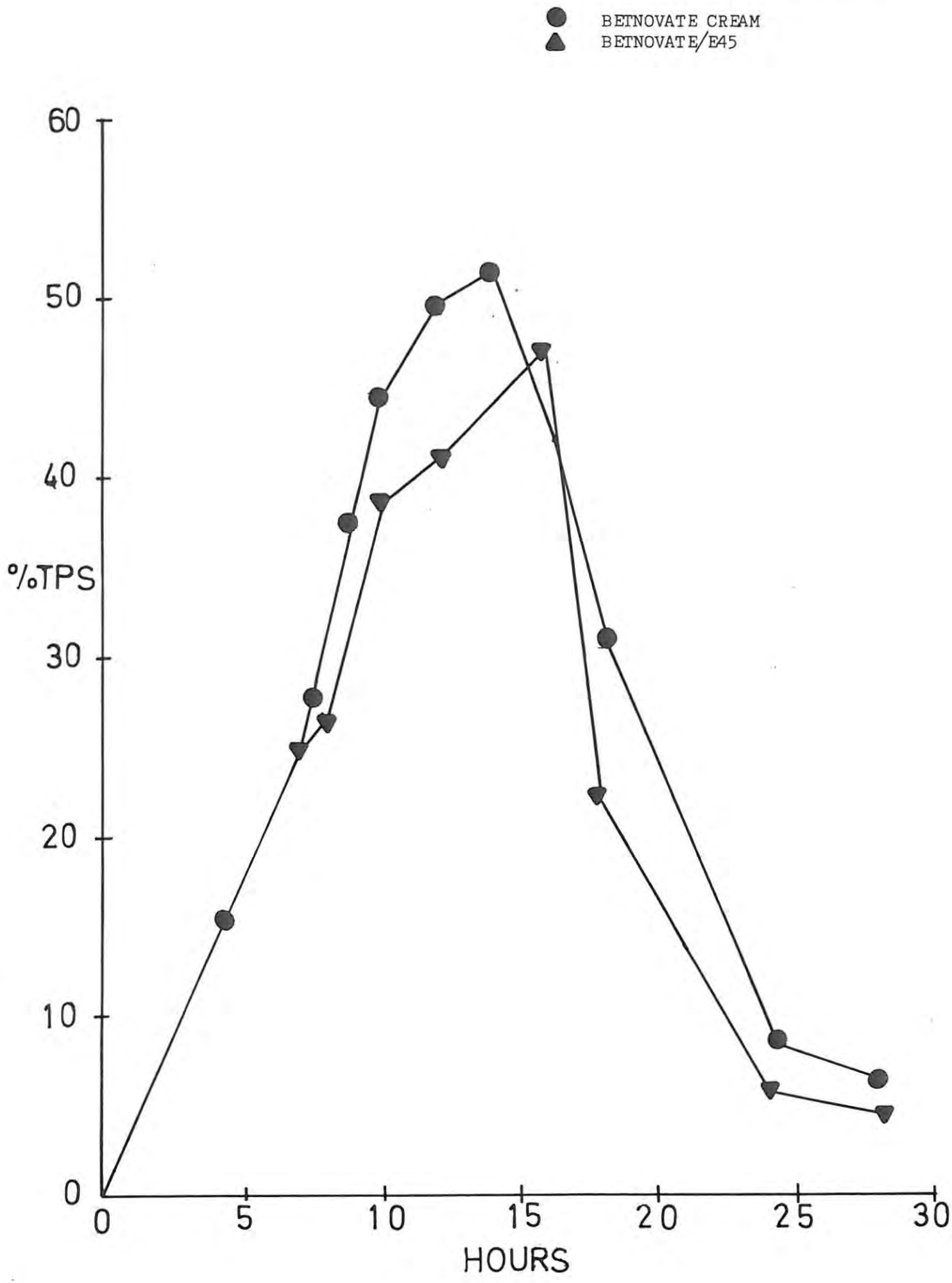

BETNOVATE CREAM
BETNOVATE/E45
BETNOVATE/BUFFERED CREAM
BETNOVATE/EMULSIFYING OINTMENT
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG.15


BETNOVATE CREAM
BETNOVATE/E45
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

at most reading times. These differences are due mainly to the lower intensity of blanching and subsequent faster decrease in the blanching effect of Betnovate/E45 compared to Betnovate. The AUC values of Betnovate/E45 is however, lower than that of Betnovate, but the difference between the 2 AUC values is not as large as one would expect considering that the concentration of betamethasone 17-valerate in Betnovate/E45 is approximately one quarter (see Section 4.5) of that of Betnovate and that the blanching ability of betamethasone 21-valerate is lower than that of betamethasone 17-valerate.[53]

3.2.6 The in vivo Assessment of Synalar Cream and Synalar Dilutions

Graphical representation of the in vivo assessment of freshly prepared Synalar dilutions are shown in figs. 16 and 17. χ^2 analyses of the results depicted in fig. 16 show no statistically significant differences amongst the half strength formulations (A, B, E) but statistically significant differences between the half strength formulations and Synalar cream in favour of Synalar cream at the 10, 12 and 14 hour readings.

χ^2 analyses of the results depicted in fig. 17 demonstrated equivalence between Synadone (0,01% fluocinolone acetonide) and Synalar/Ultrabase (0,0125% fluocinolone acetonide), but statistically significant differences were observed between Synadone and Synalar/E45 in favour of the latter and between Synalar and Synalar/E45 in favour of the former.

The results of the in vivo assessment of the one month Synalar dilution study are depicted in figs. 18 and 19.

FIG.16

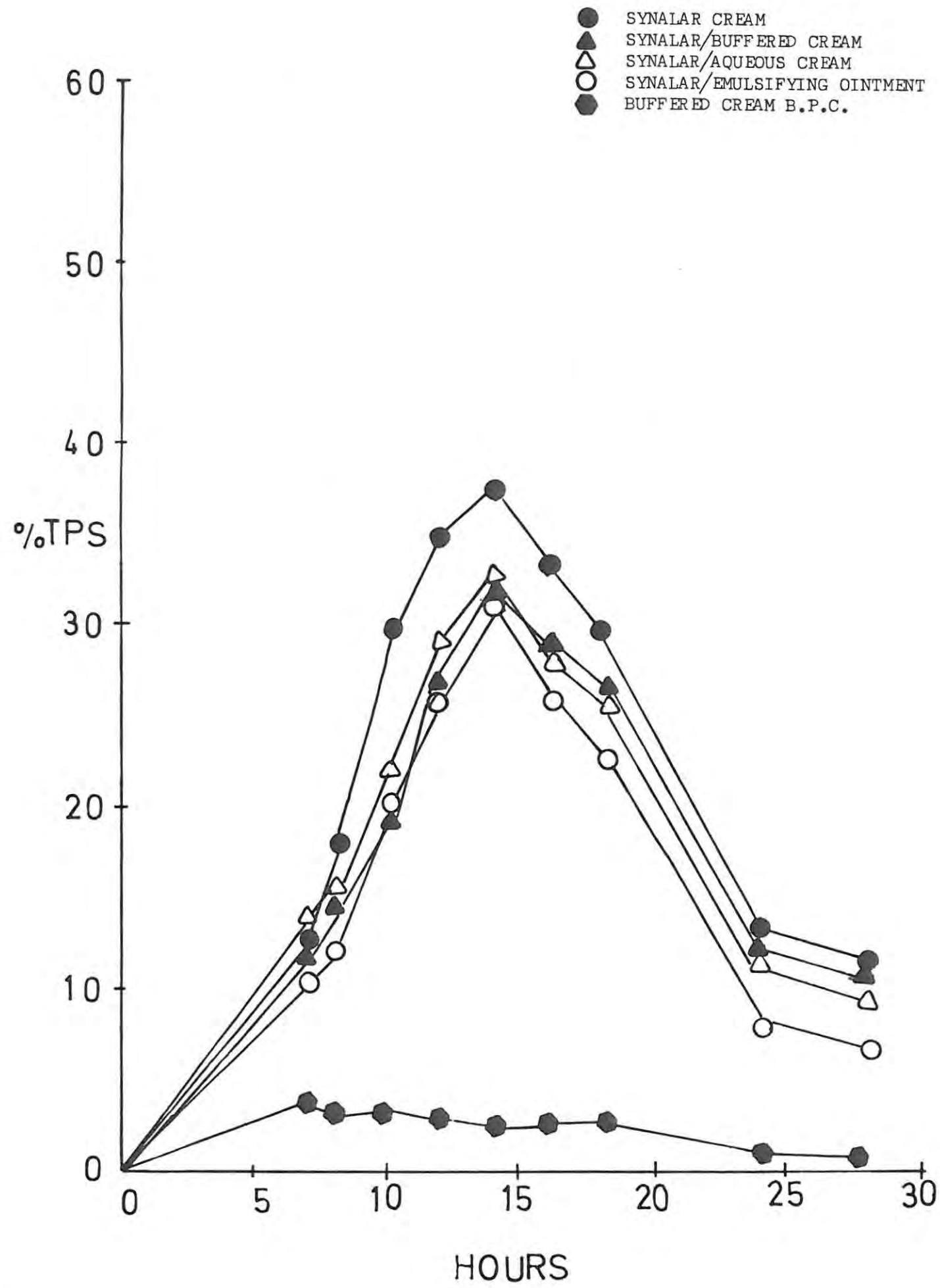

SYNALAR CREAM
SYNALAR/BUFFERED CREAM
SYNALAR/AQUEOUS CREAM
SYNALAR/EMULSIFYING OINTMENT
BUFFERED CREAM B.P.C.
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

FIG.17

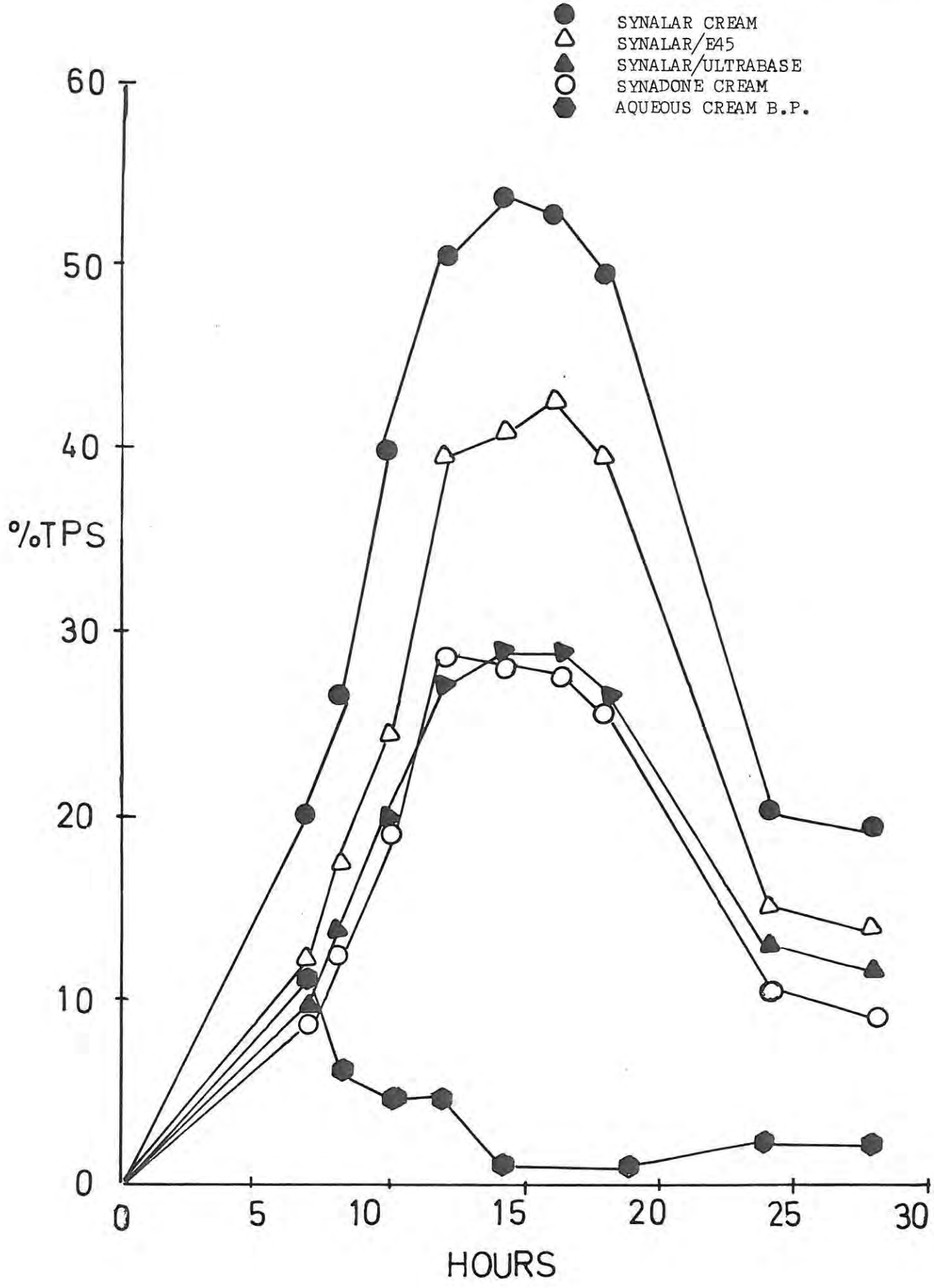

SYNALAR CREAM
SYNALAR/E45
SYNALAR/ULTRABASE
SYNADONE CREAM
AQUEOUS CREAM B.P.
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

FIG.18

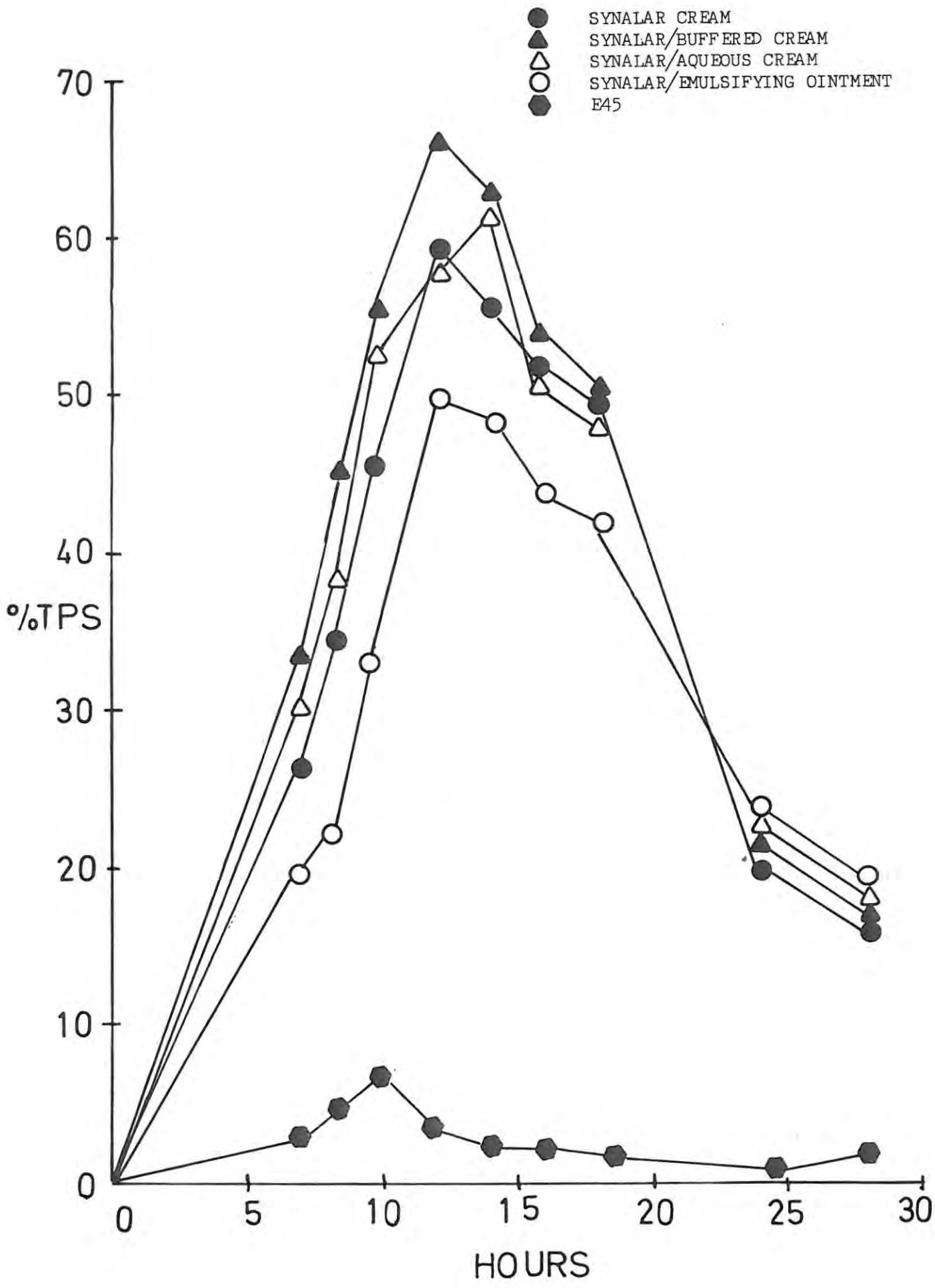
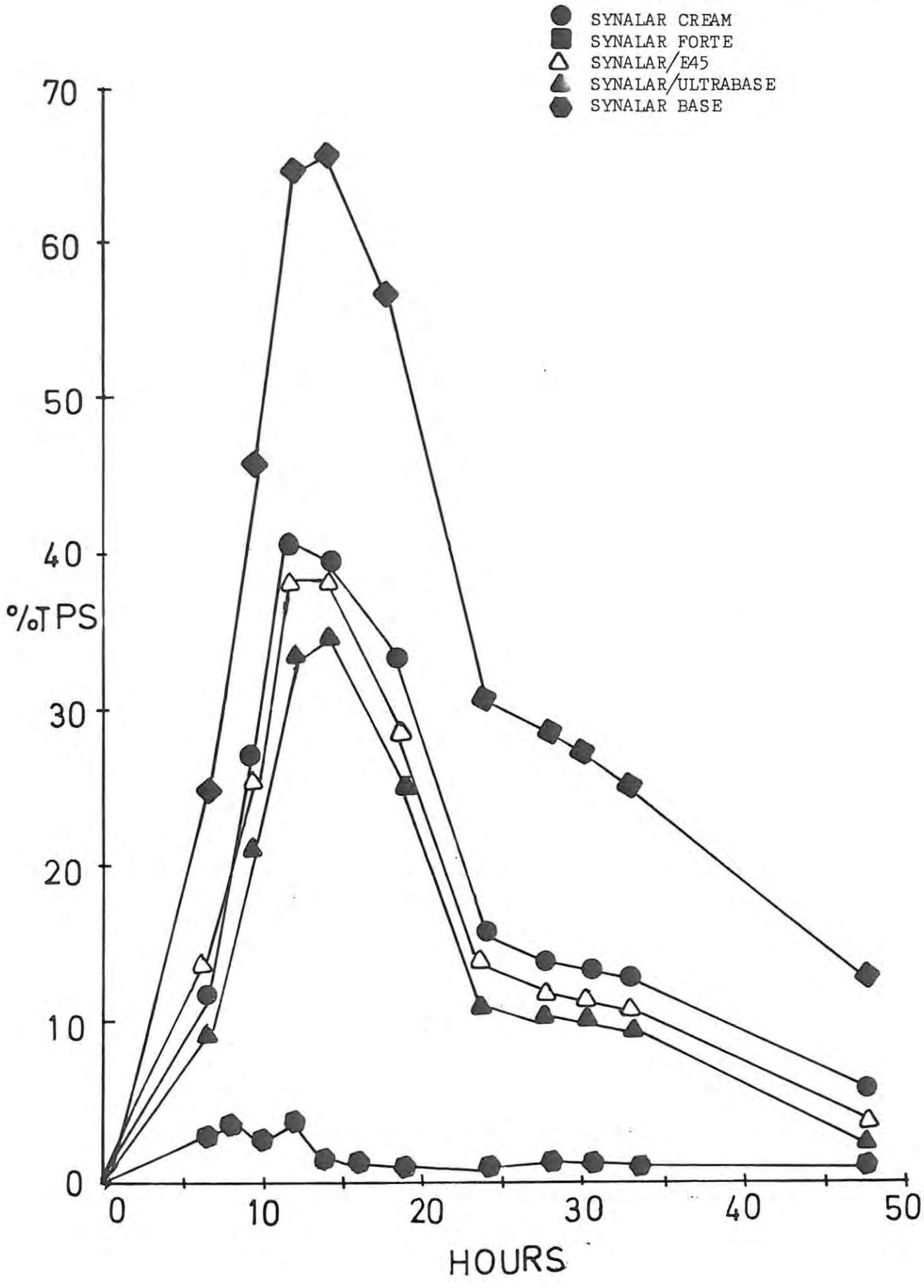

SYNALAR CREAM
SYNALAR/BUFFERED CREAM
SYNALAR/AQUEOUS CREAM
SYNALAR/EMULSIFYING OINTMENT
E45
70
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

FIG.19


SYNALAR CREAM
SYNALAR FORTE
SYNALAR/E45
SYNALAR/ULTRABASE
SYNALAR BASE
70
60
50
40
30
20
10
0
%TPS
0
10
20
30
40
50
HOURS

χ^2 analyses showed no statistically significant differences between the blanching profiles of Synalar and Synalar/Aqueous Cream, but a few differences were noted between Synalar and Synalar/Buffered Cream in favour of the latter and Synalar and Synalar/Emulsifying Ointment in favour of the former.

The largest AUC value was obtained for Synalar/Buffered Cream. The value was considerably larger than the 2 relatively equal AUC values obtained from Synalar and Synalar/Aqueous Cream and suggest a better release of fluocinolone acetonide from Synalar/Buffered Cream than Synalar. This is somewhat alarming in that the data implies that the fluocinolone acetonide is not efficiently released from the commercially available Synalar preparation.

The smallest AUC value was obtained for Synalar/Emulsifying Ointment. As in the case of the Betnovate dilution study it is suggested that the increased amount of water in both Aqueous Cream B.P. and Buffered Cream B.P.C. compared to Emulsifying Ointment B.P. allows for a greater release rate and subsequent penetration of the corticosteroid from the cream preparations.

During the investigation of the one month old dilutions, Synalar Forte (0,2% fluocinolone acetonide) was included in a trial to observe the effect of an increased concentration on the blanching response of Synalar. χ^2 analyses of the results depicted in fig. 19 show statistically significant differences between Synalar and Synalar Forte at all reading times in favour of the latter. There were also statistically significant differences between Synalar and Synalar/ Ultrabase and a few significant differences between Synalar and Synalar/E45.

Comparing the blanching profiles of Synadone, Synalar and Synalar Forte, it appears that Synalar Forte not only produces an increase in intensity of blanching, but also a much longer blanching response than both Synadone and Synalar. From this comparison it also appears that the concentration of fluocinolone acetonide incorporated in Synalar cream may be lower than the concentration required to produce the maximum response. It is interesting to note that Synalar Forte, which is 8 times more potent than Synalar, produces a response which is approximately 1,7 times greater than the response of Synalar as measured by AUC values. It therefore appears that Synalar Forte produces maximum blanching, but that the concentration of fluocinolone acetonide in Synalar Forte is much greater than that required to produce this maximum blanching. Only by a direct comparison of various amounts of Synalar Forte applied to the test site could this suggestion be substantiated.

Comparing the results of the freshly prepared Synalar dilution with those obtained one month after manufacture there appears to be very little change in the blanching activity of the preparations. No physical tests or assays were performed on the preparations but visually the preparations appeared stable.

The results of the in vivo assessment of the 3 month old dilutions are depicted in figs. 20 and 21. χ^2 analyses of the results depicted in fig. 20 show no statistically significant differences between Synalar and Synalar/Aqueous Cream, but a few statistically significant differences between Synalar and Synalar/Buffered Cream in favour of the latter and statistically significant differences between Synalar and Synalar/ Emulsifying Ointment at most reading times in favour of Synalar. There

FIG. 20

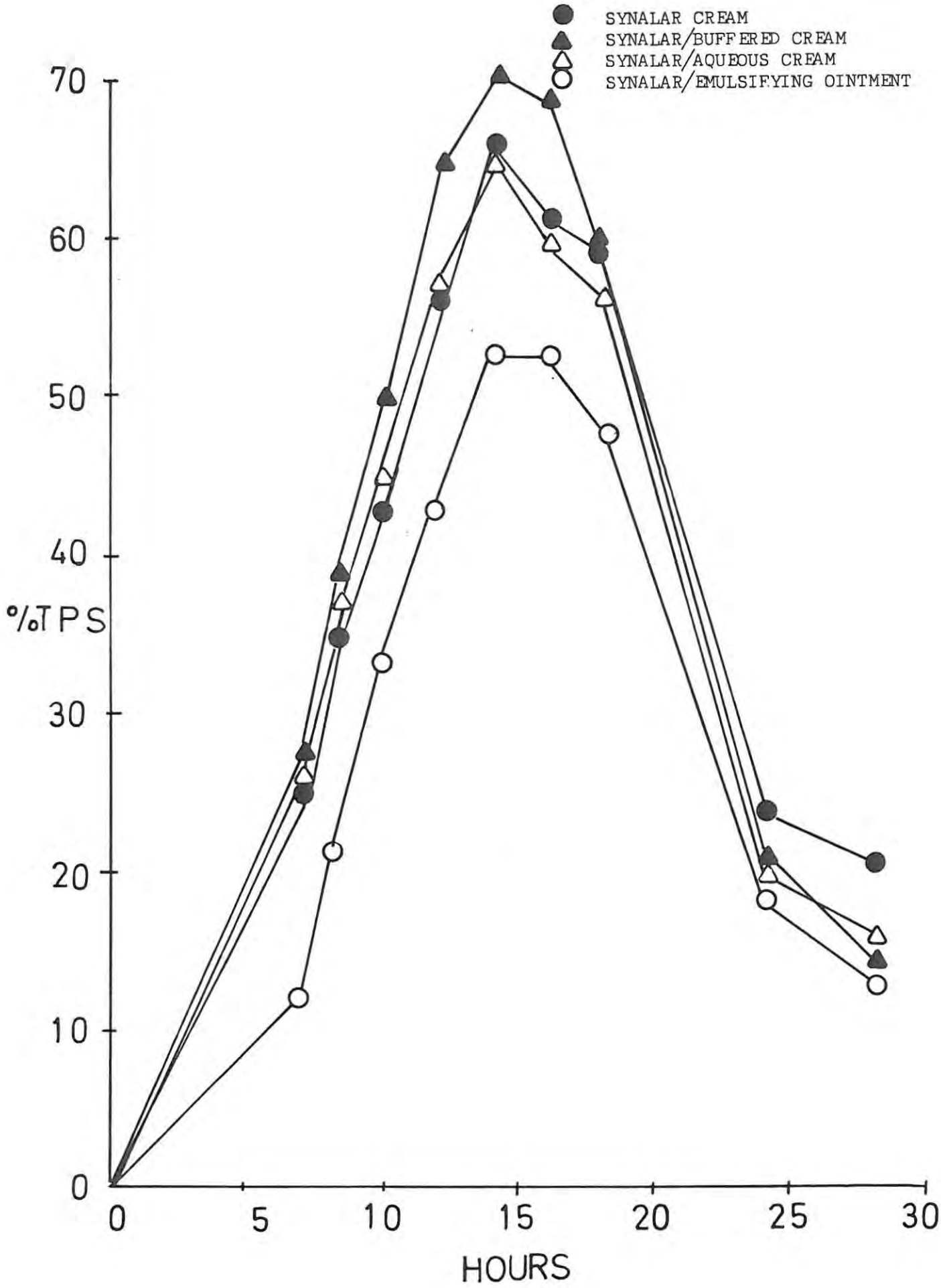
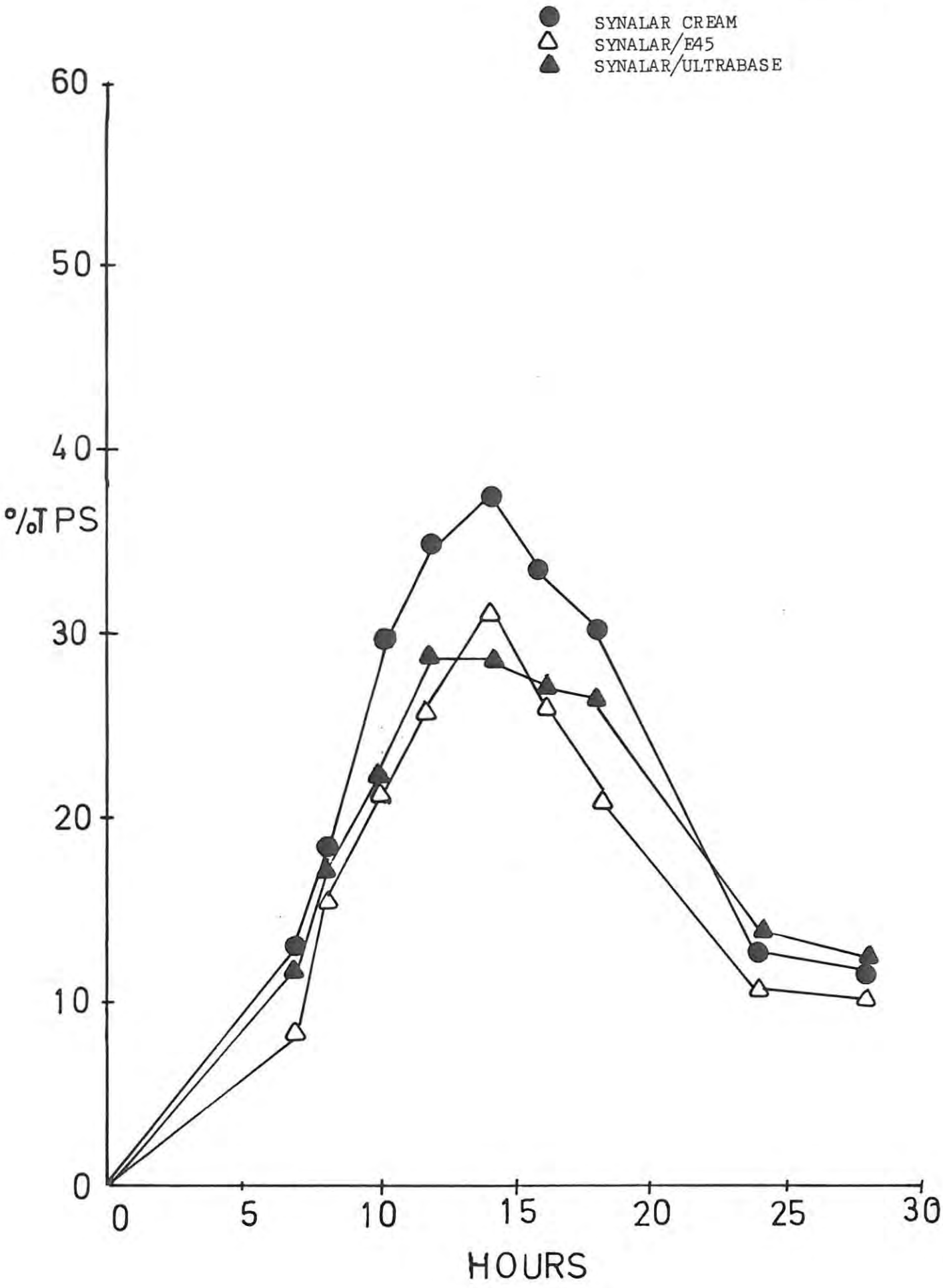

SYNALAR CREAM
SYNALAR/BUFFERED CREAM
SYNALAR/AQUEOUS CREAM
SYNALAR/EMULSIFYING OINTMENT
70
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

FIG.21


SYNALAR CREAM
SYNALAR/E45
SYNALAR/ULTRABASE
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

is a striking similarity between these results and the results depicted in fig. 18. In both trials Synalar/Buffered Cream produced the largest AUC value, Synalar and Synalar/Aqueous cream very similar values and Synalar/Emulsifying Ointment the smallest value.

χ^2 analyses of the blanching profiles depicted in fig. 21 shows no statistically significant differences between Synalar/E45 and Synalar but a few differences between Synalar and Synalar/Ultrabase in favour of the former. It is interesting to note that the AUC value of Synalar/E45 is slightly less than the AUC value of Synalar/Ultrabase, for in the two previous trials the AUC value of Synalar/E45 was greater than that of Synalar/Ultrabase. This could suggest a decrease in the blanching activity of Synalar/E45.

The Synalar dilutions were assessed again 9 months after manufacture. The results are depicted graphically in figs. 22 and 23. χ^2 analyses of the blanching profiles depicted in fig. 22 demonstrated equivalence between the blanching profiles of Synalar/Buffered Cream and Synalar/Aqueous Cream although the AUC value of Synalar/Buffered Cream was larger than that of Synalar Aqueous Cream. There were statistically significant differences between Synalar and Synalar/Buffered Cream at most reading times in favour of the former and Synalar/Emulsifying Ointment and Synalar/Aqueous Cream in favour of the latter at all reading times.

χ^2 analyses of the blanching profiles depicted in fig. 23 showed statistically significant differences between Synalar and Synalar/Ultrabase at all reading times and between Synalar/Ultrabase and Synalar/E45 at most reading times. Intercomparison of the results depicted in figs. 22 and 23 demonstrated that the smallest AUC value

FIG. 22

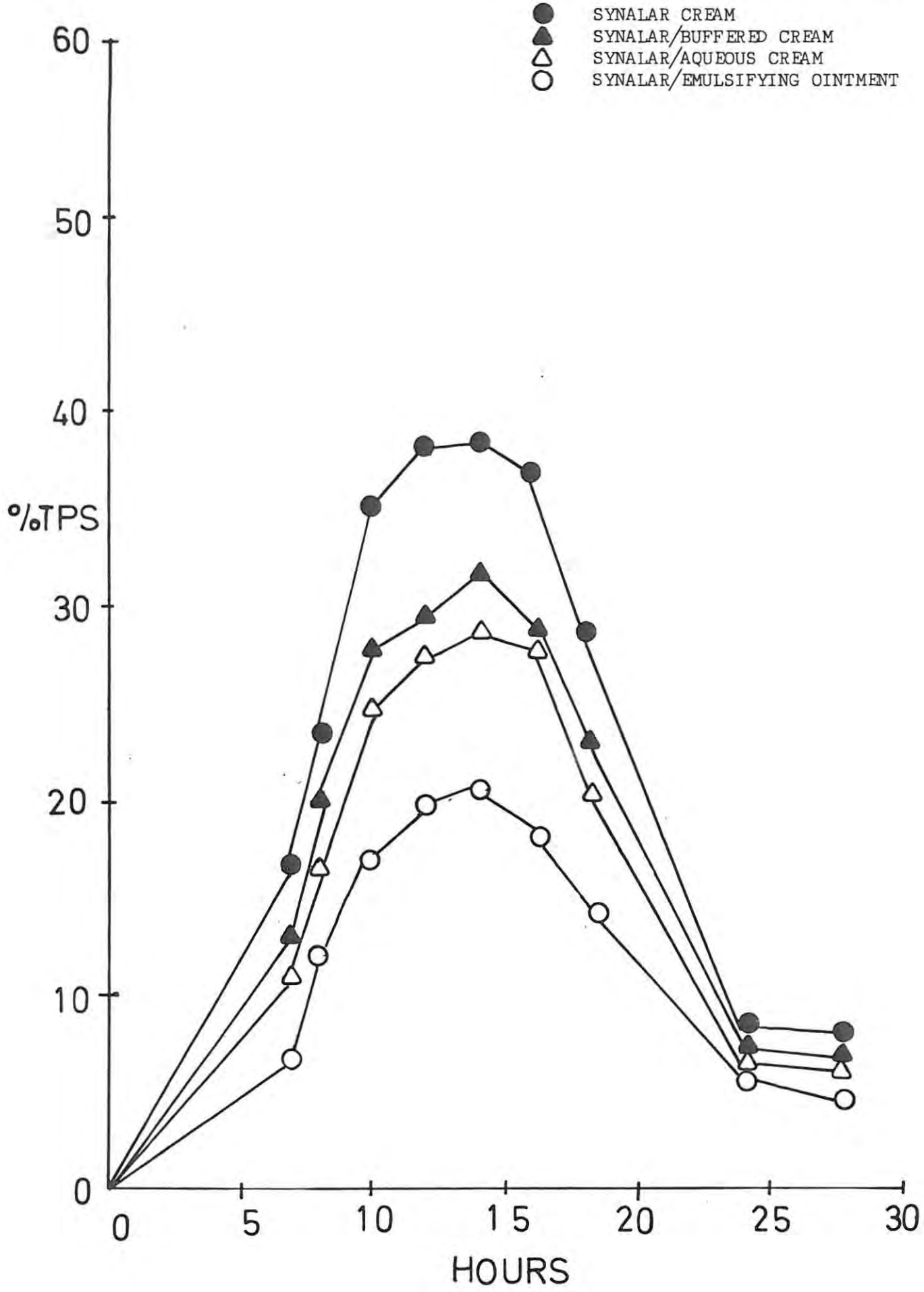
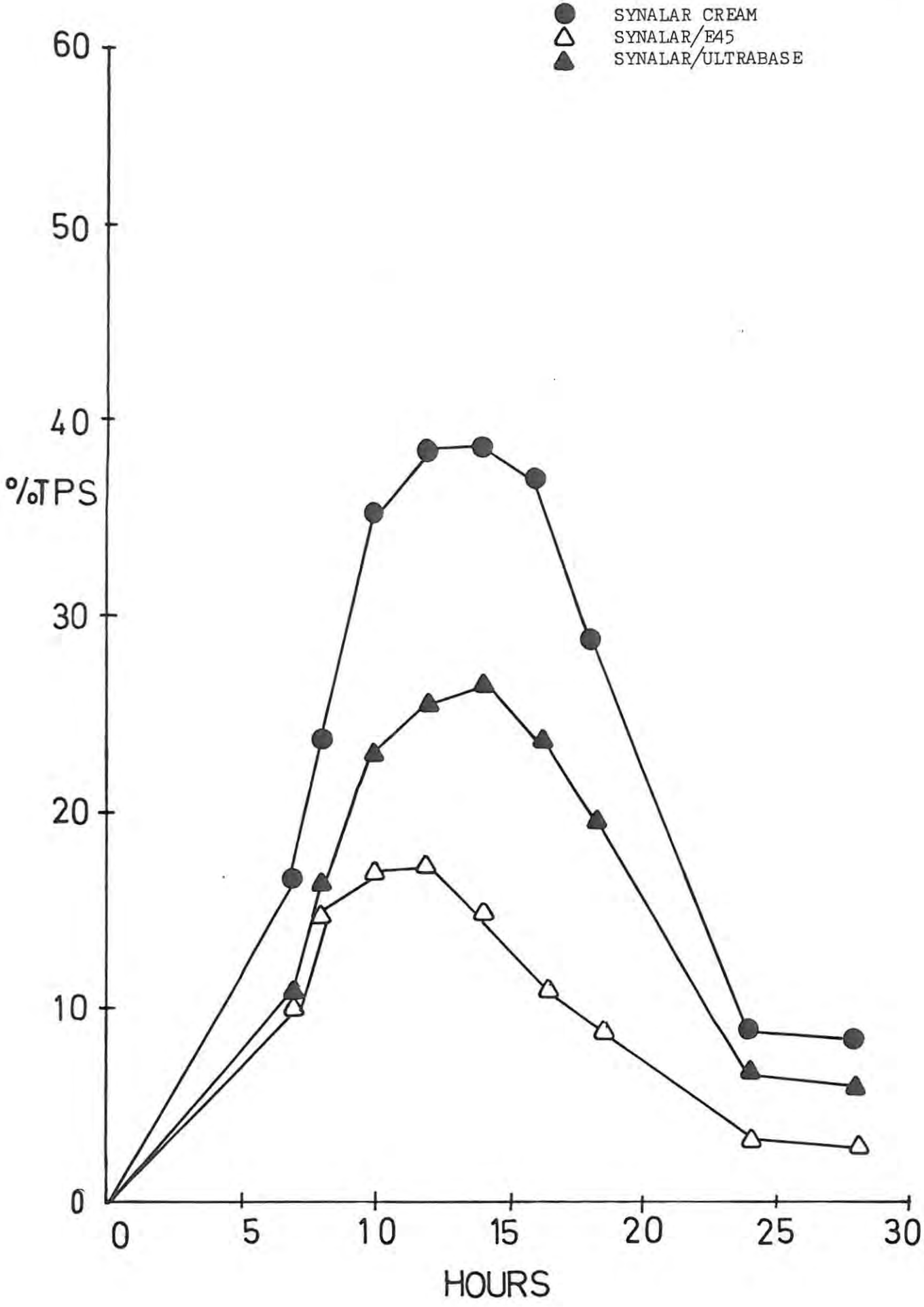

SYNALAR CREAM
SYNALAR/BUFFERED CREAM
SYNALAR/AQUEOUS CREAM
SYNALAR/EMULSIFYING OINTMENT
60
50
40
%TPS
30
20
10
0
0
5
10
15
20
25
30
HOURS

FIG.23


SYNALAR CREAM
SYNALAR/E45
SYNALAR/ULTRABASE
60
50
40
30
20
10
0
%TPS
0
5
10
15
20
25
30
HOURS

was that produced by the Synalar/E45 dilution and that this AUC value was very much less than the AUC value produced by the Synalar/Ultrabase dilution. It can be noted that in the assessment of the freshly prepared Synalar dilutions (fig. 17), Synalar/E45 produced a much larger AUC value than Synalar/Ultrabase and that on closer examination of all 4 in vivo assessments of Synalar/E45 its AUC decreases successively over the 9 month testing period indicating a decrease in blanching activity.

Because of the decrease in response of the Synalar/E45 dilution over the 9 month testing period it was decided to assess the blanching ability of this dilution again, 14 months after manufacture.

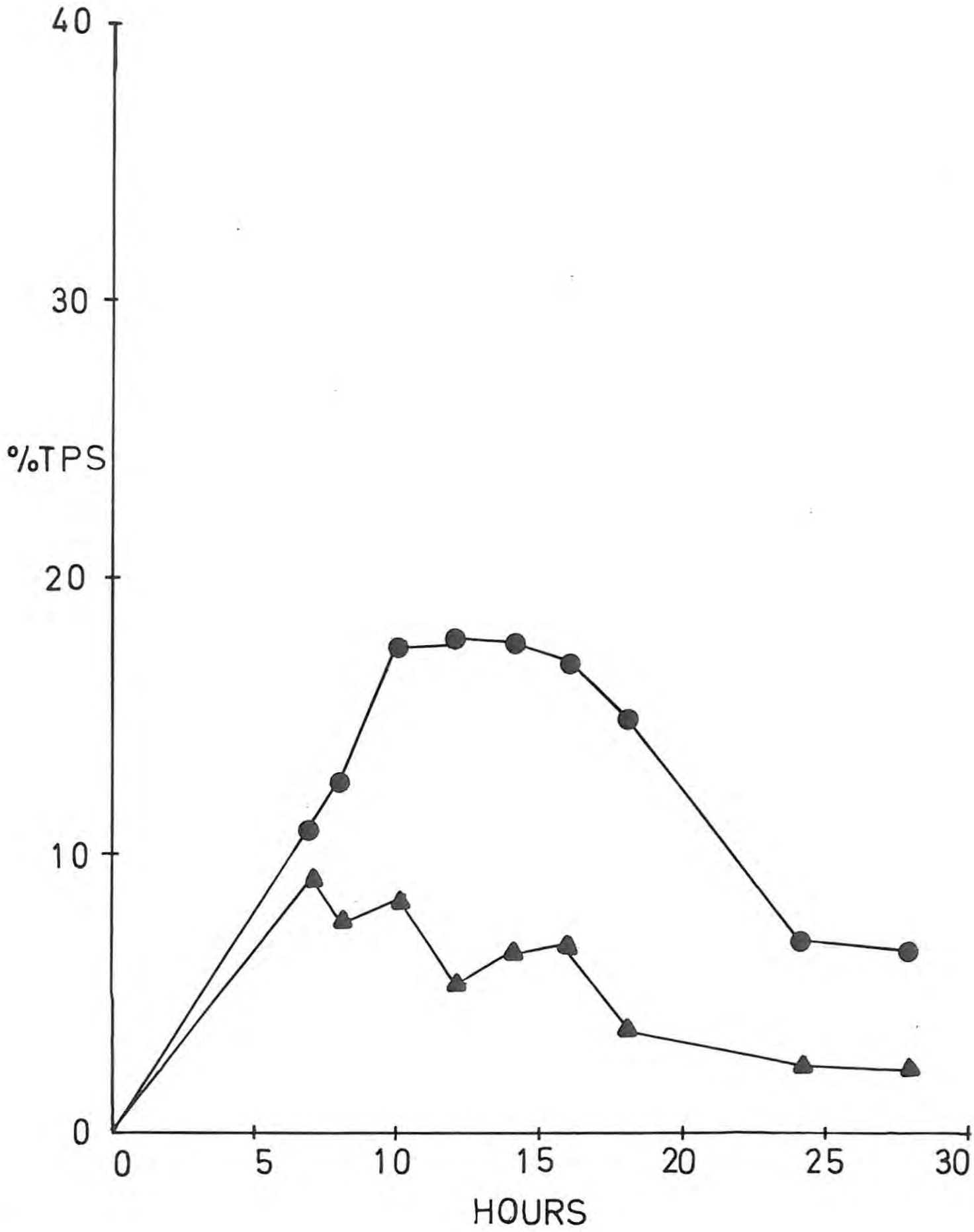
The results of this trial are depicted graphically in fig. 24. χ^2 analyses of the blanching profiles depicted in fig. 24 show statistically significant differences between Synalar and Synalar/E45 in favour of the former at all reading times. The blanching profile obtained for this dilution was irregular with no general trend, similar to that normally obtained for placebo bases.

It is apparent from the blanching profile and AUC value of the 14 month old Synalar/E45 dilution that this preparation has virtually no blanching activity. This correlates with the analytical results in which no fluocinolone acetonide was found to be present in the 14 month old Synalar/E45 dilution. (See section 4.4).

3.2.7 Conclusion

From the blanching profiles of all the in vivo assessments of the Betnovate dilutions it is apparent that 50% extemporaneous dilution

FIG. 24


SYNALAR CREAM
SYNALAR/E45
40
30
%TPS
20
10
0
0
5
10
15
20
25
30
HOURS

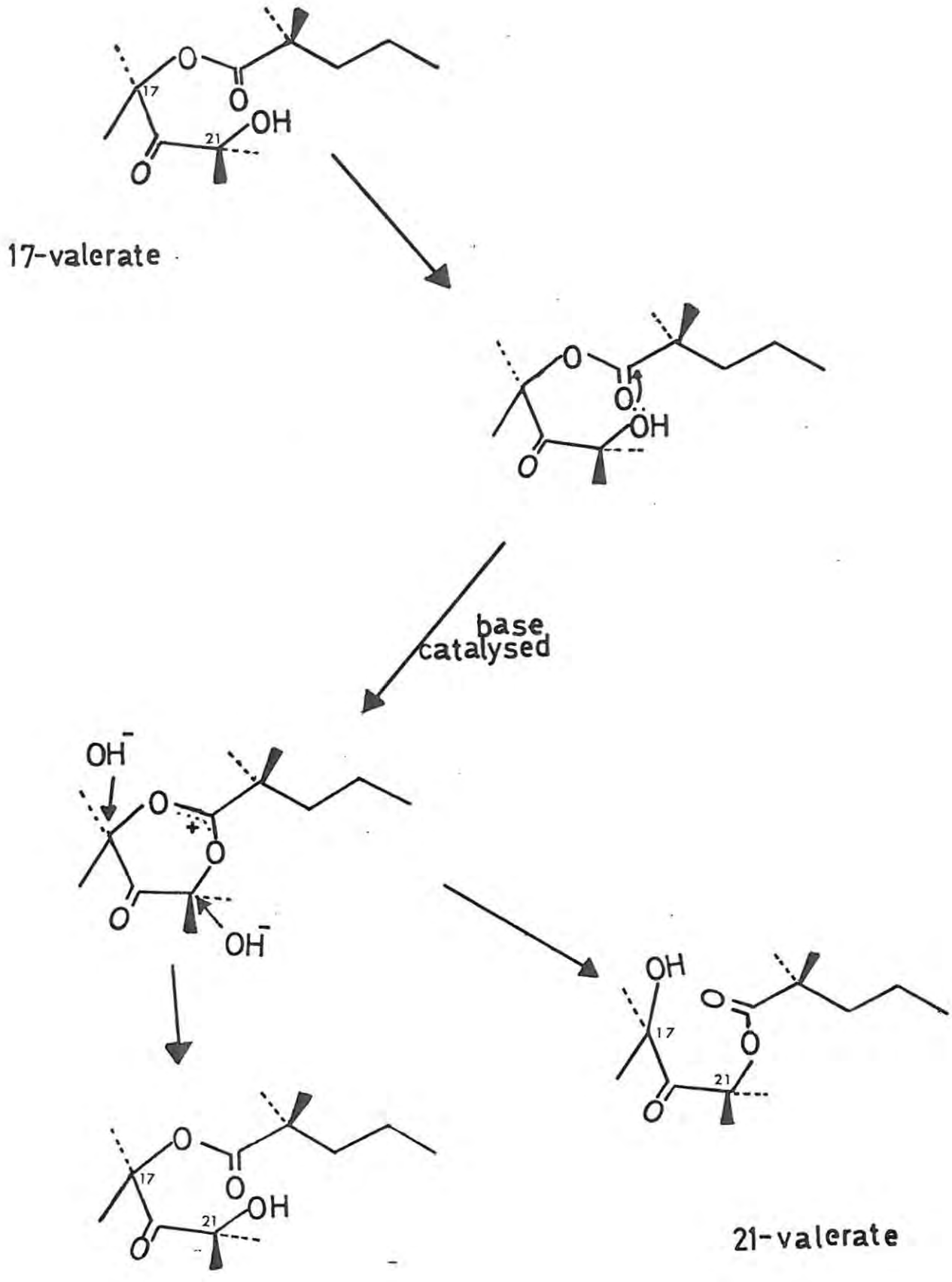
of Betnovate cream produced a preparation of similar release characteristics, blanching ability and potency to that of Betnovate cream. Barry noted similar results and suggested that there is a discrepancy between mathematical and physicochemical theory and dermatological practice.[131]

The diluting medium that had the greatest effect on Betnovate cream is E45 cream. The alkalinity of this base[116] induces the conversion of betamethasone 17-valerate to the isomer betamethasone 21-valerate. A possible mechanism for this conversion is shown in fig. 25.

It is not known how much of the 17-valerate will be converted to the 21-valerate as this will depend on the relative stabilities of the 2 isomers. If the stabilities of the 2 isomers are similar, then, because of the 2 possible modes of opening of the cyclic intermediate by base attack, both isomers will be expected to be present in similar amounts.

From the *in vivo* assessment of the Betnovate/E45 dilution it appears that the conversion takes place between 9 and 14 months after manufacture, as 9 months after manufacture the area under the blanching profile of Betnovate/E45 was larger than the corresponding value for Betnovate cream. However 5 months later this value was found to be less than that of Betnovate, indicating a decrease in activity. It is known that betamethasone 21-valerate has a lower blanching activity than betamethasone 17-valerate.[53] It is certain that this conversion does not take place within 6 months as no betamethasone 21-valerate was found when the Betnovate dilutions were assayed by HPLC 6 months after manufacture. (See section 4.5).

FIG. 25



Because of the similarity between the responses of extemporaneously prepared 50% dilutions of Betnovate and commercially available Betnovate cream it is economically advantageous to dilute Betnovate cream to this strength. No one base appears to be superior to the others.

Unlike Betnovate, where the nature of the diluting base has very little effect on the original preparation, the nature of the base used to dilute Synalar cream to 50% of its original strength does affect the release and potency of the original preparation.

It appears that dilution of Synalar cream with Buffered Cream B.P.C. results in a base of excellent release characteristics, as this preparation twice produced an AUC value greater than the corresponding value for Synalar (figs. 18 and 20). Dilution with Aqueous Cream B.P. also results in a base of good release characteristics as can be seen from the assessments in which the AUC values of Synalar/Aqueous Cream dilution were found to be similar to that of Synalar full strength (figs. 18 and 20).

Dilution of Synalar cream with Ultrabase results in a base of release characteristics similar to that of the commercial Synadone (0,01% fluocinolone acetonide). There appears to be no difference in the release of fluocinolone acetonide from either Synadone or a 50% dilution of Synalar with Ultrabase. It is suspected that Ultrabase may contain propylene glycol and therefore, it may be possible that when Synalar cream is diluted with this base that the steroid is over-solubilised resulting in a decrease in response.

The use of E45 cream as the diluting medium initially produces a base of relatively good release characteristics, but with time the alkalinity[116] of E45 cream appears to alter the fluocinolone acetonide molecule resulting in a progressive decrease in blanching activity.

The effect of alkali on the fluocinolone acetonide molecule is not known. It is however, highly unlikely that the steroid nucleus collapses under basic conditions. It is more probable that basic conditions may alter one or more of the side chains attached to this nucleus. Any modification of these side chains may alter the properties of fluocinolone acetonide accounting for a decrease in blanching activity.

Modification of the side chain may result in a molecule of different polarity to that of fluocinolone acetonide. Thus when a Synalar/E45 dilution containing a converted molecule is assayed by HPLC (see section 4.4) the modified molecule may have a different retention time to that of fluocinolone acetonide, as was previously seen in the analysis of the aged Betnovate/E45 dilution (see section 4.5). It would therefore be possible for this compound to either remain on the column or to be eluted at the same rate as one of the suspected preservatives. This may result in the inability to assay or identify this compound.

The progressive decrease in blanching activity of the Synalar/E45 dilution suggests that there may be a side chain modification which begins soon after preparation.

4. ANALYTICAL

4.1 INTRODUCTION

Steroids have been assayed by a number of different analytical methods. These methods include spectrophotometry,[132] colorimetry,[133,134] gas chromatography[135,136] and other types of separations[137] including high performance liquid chromatography (HPLC).

Spectrophotometry and colorimetry both suffer from the same drawbacks in that the assay is non-specific for individual compounds and that there are many substances which interfere with the assay, especially in the case of colorimetry.[138,139]

Paper chromatography, thin layer chromatography and column chromatography are generally applicable for steroidal analysis as they are simple, inexpensive and do not require derivitization of the steroid.[140] However, all these methods have the same inherent limitations with regard to speed of separation, ease of quantitation and separation efficiency.[141]

Gas-liquid chromatography has become a popular method for steroidal analysis because of its speed, relative ease of quantitation and high resolution capabilities. However, the method suffers from one major limitation in that many steroids have either a low volatility or are too thermally labile for direct chromatographic analysis. For these steroids, derivitization techniques are required, but, because of the complexity of the steroid molecule, it is often difficult to obtain complete derivitization which is necessary for quantitation.

The analysis, separation and quantitation of pharmaceutical products by HPLC has been an extremely active area.

The analysis of steroids by HPLC was first reported by Siggia and Dishman[140] in 1970. They reported the separation of a number of structurally similar androgens, estrogens, progestins and adrenal corticosteroids employing a liquid ion exchange resin used as a reverse-phase packing. Henry et al.[142] reported on the separation of steroids using both partition and ion exchange packings.

Since these two early reports, there have been numerous publications[143-148] using liquid chromatography as a method of steroidal analysis and it has been recently recognised by the U.S.P.[132] and National Formulary[149] as the method of choice. Liquid chromatography is particularly well suited for analysis of extracts of steroids from tablets, ointments and creams, because of the very high degree of selectivity which enables separation of the steroid from the excipients.

Bailey and Brittain[150] used an extremely simple method of extracting the steroid from the formulation. The formulation was partitioned between iso-octane and methanol. The steroid partitioned in the methanol layer which was suitable for injection. A recent publication makes use[148] of an even simpler extraction by dispersing the formulation in hot water/ methanol or acetonitrile. These crude extracts significantly reduce the length of time required to assay a formulation, but tend to reduce the column life.

Because of the speed, selectivity and simplicity of the procedure, HPLC was chosen as the method of analysis for this study.

4.2 THE ANALYTICAL SYSTEM

A reverse phase system was chosen as it has wide application in the field of pharmaceutical analysis.

The reverse phase system employs a non-polar stationary phase, usually composed of silica coated with high molecular weight hydrocarbons or substituted hydrocarbons. The mobile phase is generally a mixture of distilled water and miscible organic solvents e.g. methanol, acetonitrile, etc.

The column employed in this study was the Varian Aerograph MicroPak CH-10 column which was packed with porous silica (Li Chrosorb Si-60, a product of E. MERCK, Darmstadt.) of 10 micron average diameter, with a thin uniform layer of octadecylsilyl-groups chemically bonded to the silica through a stable siloxane bond. The column length was 25 cm with an inner diameter of 0,21 cm.

The column efficiency is approximately inversely proportional to the square of the flow velocity but by increasing the column temperature from 25°C to 50°C the efficiency of the column can be approximately doubled.

4.3 THE METHOD OF QUANTITATION

HPLC with concomitant automation used with the measurement and calculation procedure developed for gas chromatography is a highly accurate and precise analytical technique.

All preparations were assayed by this method. Use was made of the internal standard (I.S.) method of quantitation in which the sample peak of interest is quantitated relative to the internal standard peak. This method compensates for varying injections volumes and day to day instrumental changes.

There are two basic ways of relating the analogue signal to concentration, namely peak height and peak area.

The peak height ratios were compared to the peak area ratios for the same injection. The date obtained from this comparison are listed below:

Injection Number	$\frac{\text{Peak Height Sample}}{\text{Peak Height I.S.}}$	$\frac{\text{Peak Area Sample}}{\text{Peak Area I.S.}}$
1	1,93	0,99
2	2,00	1,11
3	1,94	1,07
4	2,00	0,83
5	1,91	0,95
Average value	1.96	0,99
Standard Deviation	0,0018	0,012

The greater standard deviation obtained from the peak area ratios is due to the difficulty encountered by the electronic integrator in determining when the slope changes sufficiently for it to start and stop calculating area under the peak. This difficulty is increased when broad peaks are encountered by the integrator.

It was therefore decided that in all subsequent quantitative assessments, peak height measurements would be used as the method of quantitation.

4.4 THE HPLC ASSESSMENT OF FLUOCINOLONE ACETONIDE IN SYNALAR FORMULATIONS

Bailey and Brittain[150] and Coleman[151] used a reverse phase C18 column as the stationary phase and methanol/water as the mobile phase in the separation of fluocinolone acetonide from other extracted ingredients of Synalar cream. However, it was found that neither of these two sets of conditions were suitable for the analysis of the Synalar cream used in this study. The chromatogram of the Synalar cream used in this study showed an additional peak compared to the chromatogram of the Synalar cream illustrated by Bailey and Brittain[150] and this peak overlapped with the fluocinolone acetonide peak. Changes in solvent concentration, flow rate, and pH were used in attempts to achieve separation, but without success.

The use of the less polar solvent, ethanol effected separation of the fluocinolone acetonide but the resultant peak was broad and did not lend itself to quantitation. Changes of flow, pH and solvent as well as the addition of 1% tetrahydrofuran to the mobile phase did not improve the shape of the fluocinolone acetonide peak. However, by increasing the column temperature good separation was obtained with relatively narrow peaks. The increase in temperature, in addition, had the effect of reducing the assay time. It was found that the optimum column temperature was 44^{o}C. A temperature above this caused overlapping of the peaks and below this the peaks tended to broaden.

Assay

Reagents:

(1) Water ; Distilled

(2) Methanol ; Reagent Grade (Merck)

(3) Iso-Octane ; Reagent Grade (Merck)

(4) Ethanol ; Reagent Grade (Merck)

(5) Norethisterone ; Internal Standard. Accurately weigh out approximately 0,12 g of norethisterone authentic specimen and transfer to a 50 ml volumetric flask. Make up to volume with methanol.

(6) Standard Preparation. Accurately weigh out approximately 2,5 mg of fluocinolone acetonide authentic specimen and transfer to a 50 ml volumetric flask. Add 1 ml internal standard solution and make up to volume with methanol.

Procedure:

(1) Accurately weigh out approximately 10 g of formulation into a 50 ml separating funnel. The formulation is placed into a 10 ml plastic disposable syringe immediately prior to the assay being performed. The syringe is weighed before and after extruding the formulation.

(2) Add 15 ml of methanol.

(3) With the aid of gentle heat and agitation dissolve the formulation in the methanol.

(4) Add 50 ml of iso-octane.

(5) Shake, allow to separate and run off the lower methanol layer into a second separating funnel.

(6) To the iso-octane mixture in the first separating funnel, add 15 ml methanol. Shake, allow to separate and add the methanol layer to the second separating funnel. Gentle heat may be necessary to cause the layers to separate.

(7) Allow the combined methanol layer to stand for 15 minutes. This achieves further separation of the iso-octane layer.

(8) Run the lower methanol layer into a 50 ml volumetric flask.

(9) Add 1 ml of the internal standard solution.

(10) Make up to 50 ml with methanol.

(11) Inject 2 μl into the HPLC.

Conditions for Use of HPLC

Solvent: 33% Ethanol/67% Water.

Column: CH-10 Micro Pax 25 cm x 2 mm (Varian).

Column Temperature: 44^{o}C.

Flow Rate: 50 ml/hr.

Detector: 238 nm.

SENSITIVITY. Detector 0,2

C.D.S. 4

Recorder 5 mv.

Calculations

Linear regression was performed on the calibration plot of peak height ratio vs concentration of fluocinolone acetonide.

The equation of this curve was found to be

$$y = 0,0285\ x$$

with a correlation coefficient of r = 1,01.

As an example, the data obtained from the analysis of Synalar/Aqueous Cream is presented below.

Weight of Preparation Taken	Peak Height Fluocinolone Acetonide mm	Peak Height Internal Standard mm	Ratio
ASSAY 1 10,81653 g	18 24 25 24	26 35 36 36	0,692 0,685 0,694 0,666
ASSAY 2 11,28620 g	32 29 30 27	43 40 40 36	0,744 0,725 0,750 0,750
ASSAY 3 11,31682 g	12 19 22 23	16 25 30 31	0,750 0,760 0,733 0,742

ASSAY	AVERAGE RATIO	STANDARD DEVIATION	COEFFICIENT OF VARIATION	% PURITY
1	0,684	0,014	2,046	88,76
2	0,742	0,012	1,617	92,28
3	0,746	0,012	1,609	92,54
			AVERAGE % PURITY	= 91,19

It had been intended to assay the Synalar dilutions immediately after manufacture, 6 months later and again after one year. However, difficulty was experienced in the development of this assay due to the inability to isolate the fluocinolone acetonide peak. As a result, the assay was finally accomplished 14 months after the preparations had been manufactured. All the preparations were found to be within limits apart from the Synalar/ E45 dilution (see later). The average percentage purity from three individual assays of each preparation are listed below.

PREPARATION	AVERAGE PERCENT PURITY
Synalar/Ultrabase	92,30
Synalar/Buffered Cream	91,42
Synalar/Emulsifying Ointment	91,11
Synalar/E45	0,00

In the Synalar/E45 dilution no fluocinolone acetonide was found to be present. It is suggested that the fluocinolone acetonide, due to the alkalinity of E45 cream has been converted to some other compound. The conditions listed above will however, be suitable for the assessment of the amount of fluocinolone acetonide present in a freshly prepared dilution of Synalar/E45.

The chromatograms for the above assessments are shown on pages 99 and 100.

4.5 THE HPLC ASSESSMENT OF BETAMETHASONE 17-VALERATE AND BETAMETHASONE 21-VALERATE IN BETNOVATE FORMULATIONS

Assay

Reagents:

(1) Water: Distilled.

(2) Methanol: Reagent Grade (Merck).

(3) Iso-Octane: Reagent Grade (Merck).

(4) Toluene: Analytical Grade. Internal Standard.

(5) Standard Preparation. Accurately weigh out approximately 10 mg betamethasone 17-valerate authentic specimen and transfer to a 50 ml volumetric flask. Add 100 µl internal standard (toluene) and make up to volume with methanol.

Procedure

The procedure is identical to that previously described for the Synalar dilutions but in this case 100 μl of toluene is used as the Internal Standard. (Page 91)

Conditions for Use of HPLC

Solvent: 63% Methanol/37% Water.

Column: CH-10 Micro Pax 25 cm x 2 mm (Varian).

Detector: 254 nm.

SENSITIVITY. Detector 0,01

C.D.S. 4,00

Recorder 5 mv.

The Betnovate dilutions were assayed both 6 and 14 months after manufacture. Three separate assays were performed on each formulation with a minimum of 5 injections per formulation.

The average % purity of each dilution determined 6 and 14 months after manufacture are listed below and the respective chromatograms are shown on pages 101 and 102.

PREPARATION	AVERAGE % PURITY AFTER 6 MONTHS	AVERAGE % PURITY AFTER 14 MONTHS
Betnovate/Ultrabase	95,17	90,22
Betnovate/Emulsifying Ointment	90,11	91,33
Betnovate/Aqueous Cream	97,14	104,16
Betnovate/Buffered Cream	92,31	91,60
Betnovate Half Strength	92,55	96,49
Betnovate/Betnovate base	98,46	103,22
Betnovate/E45	95,02	62,94

As can be seen from the above Table, 14 months after manufacture it was found that the amount of betamethasone 17-valerate in the Betnovate/E45 dilution was well below the accepted limits. In addition the chromatogram of this dilution showed a shoulder on the betamethasone 17-valerate peak. It was suspected that this shoulder was betamethasone 21-valerate, and therefore, the retention time of this shoulder was compared to the retention time of an injection of betamethasone 21-valerate. The two retention times were shown to be the same. As the conditions listed above did not effect a good separation of the 17- and 21-valerates and as it was desired to quantitate the amount of each isomer present a new assay was developed.

Assay of Betnovate/E45 After 14 Months

It was found that an acetonitrile/water mixture proved to be effective in causing the separation of the betamethasone 17-valerate and the betamethasone 21-valerate. Initially, difficulties were encountered when using this mixture as the mobile phase as it was found that, when the steroid was dissolved in either methanol or acetonitrile and injected onto the column, there was a large baseline shift due to a refractive index change of the mobile phase. Changes in refractive index of the liquid in the cell changes the amount of light reflected at the quartz-liquid interfaces of the cell. This change in light reflected is indistinguishable from absorbed light and results in a shift of the dectector base line.

This could have been overcome by extracting the steroid with the mobile phase, but it was felt that the extraction would be more efficient using methanol. The detector wavelength was therefore altered from 254 nm to 234 nm at which the change in refractive index was not detected.

The chromatogram (page 100) was considerably shortened and improved by increasing the column temperature to 40°C.

Assay

Reagents:

(1) Water: Distilled.

(2) Methanol: Reagent Grade (Merck).

(3) Acetonitrile: Reagent Grade (Merck).

(4) Iso-Octane: Reagent Grade (Merck).

(5) Medroxyprogesterone Acetate: Internal Standard. Accurately weigh out approximately 0,45 g of medroxyprogesterone acetate authentic specimen and transfer to a 50 ml volumetric flask. Add 20 ml acetonitrile and dissolve medroxyprogesterone acetate with shaking. Make up to volume with methanol.

(6) Standard Preparation: Accurately weigh approximately 10 mg of both betamethasone 17-valerate and betamethasone 21-valerate Authentic Specimens and transfer to a 50 ml volumetric flask. Add 1 ml of internal standard and make up to volume with methanol.

Procedure

The procedure is identical to that previously described for Synalar (page 91) but in this case the internal standard is medroxyprogesterone acetate.

Conditions for Use of HPLC

Solvent: 45% Acetonitrile/55% Water.

Column: CH-10 Micro Pax 25 cm x 2 mm (Varian).

Column Temperature: 40°C.

Flow Rate: 70 ml/hr.

Detector: 234 nm.

SENSITIVITY. Detector 0,2

C.D.S. 4,0

Recorder 5 mv.

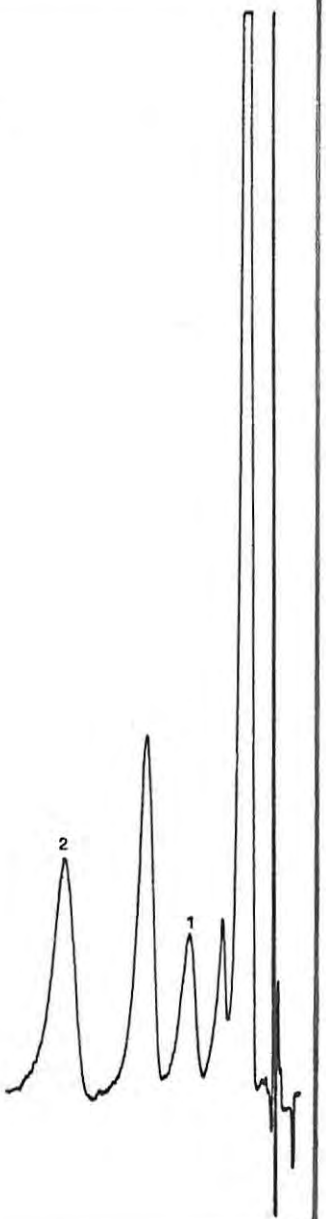
Two individual assays were performed with five injections per assay. The average results for these assays are listed below.

ASSAY	MASS OF PREPARATION	AMOUNT OF B 17-v	AMOUNT OF B 21-v
1	10,9310	3,50 mg	3,15 mg
2	10,5125	3,25 mg	2,95 mg

HPLC allows for a rapid, precise, reliable and reproducible method of steroid analysis with the additional advantage that it is specific and can therefore be used for stability indications. Unfortunately it is not without its drawbacks, for the cost of the instrument, columns and solvents are high.

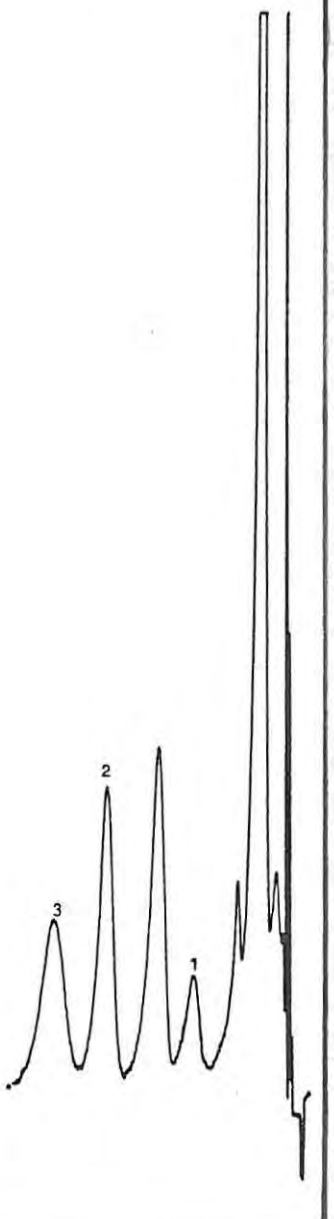
SYNALAR/EMULSIFYING OINTMENT

1 FLUOCINOLONE ACETONIDE
2 INTERNAL STANDARD



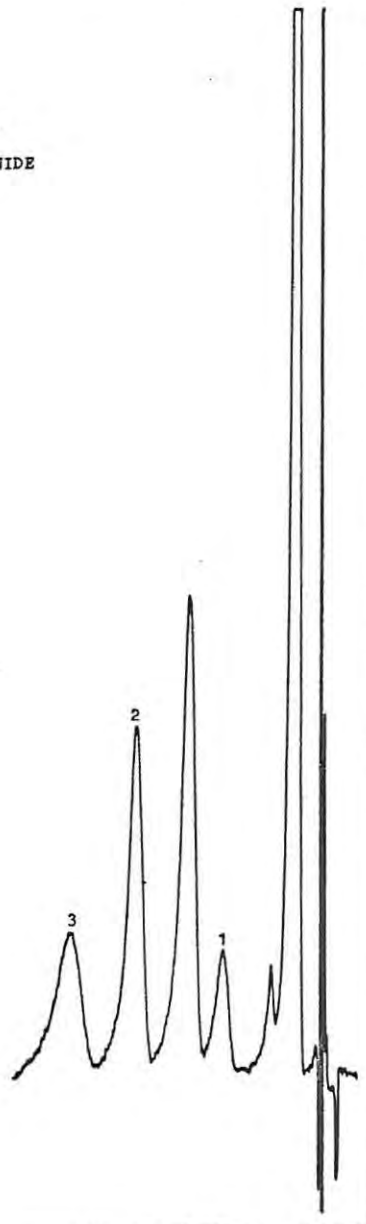
SYNALAR/BUFFERED CREAM

1 FLUOCINOLONE ACETONIDE
2 CHLOROCRESOL
3 INTERNAL STANDARD



SYNALAR/AQUEOUS CREAM

1 FLUOCINOLONE ACETONIDE
2 CHLOROCRESOL
3 INTERNAL STANDARD

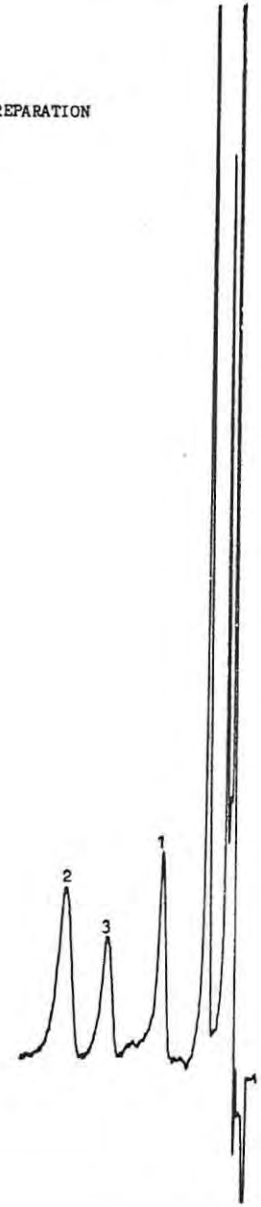


SYNALAR/ULTRABASE

1 FLUOCINOLONE ACETONIDE
2 INTERNAL STANDARD

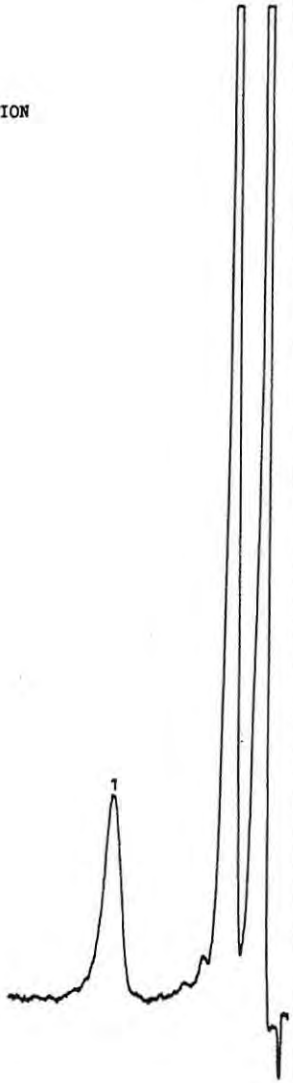
BETNOVATE/E45 14 MONTHS AFTER PREPARATION

1 BETAMETHASONE 17-VALERATE
2 BETAMETHASONE 21-VALERATE
3 INTERNAL STANDARD



SYNALAR/E45 14 MONTHS AFTER PREPARATION

1 INTERNAL STANDARD



BETNOVATE/EMULSIFYING OINTMENT

FLOW 90 ml/hr

1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE

BETNOVATE HALF STRENGTH

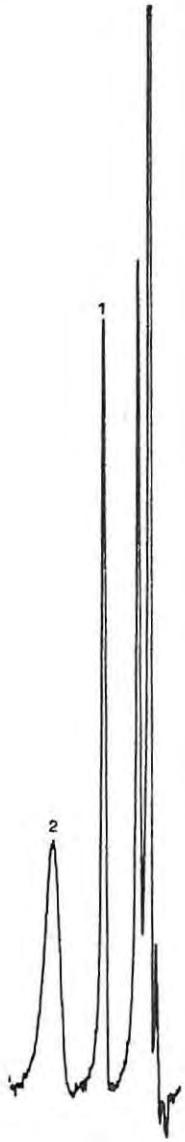
FLOW 90 ml/hr

1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE

BETNOVATE/ULTRABASE

FLOW 80 ml/hr

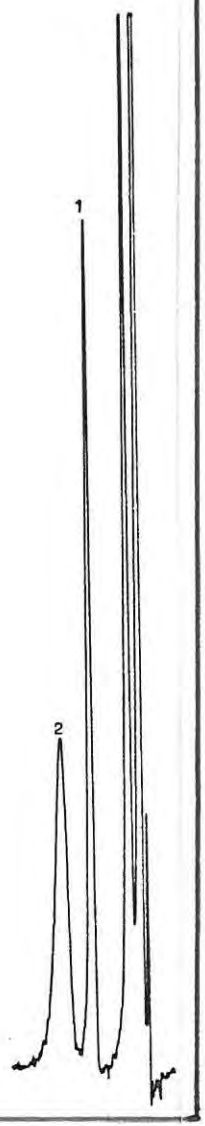
1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE



BETNOVATE/AQUEOUS CREAM .

FLOW 80 ml/hr

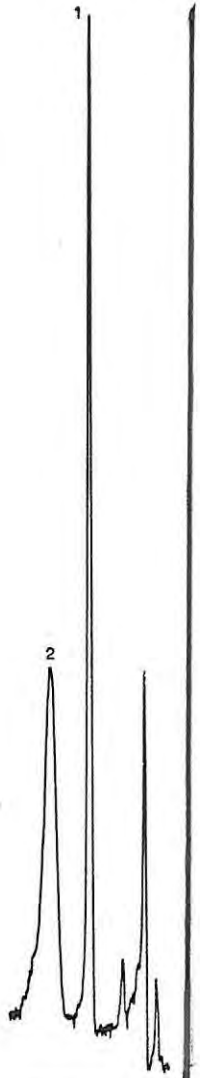
1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE



BETNOVATE/BUFFERED CREAM

FLOW 80 ml/hr

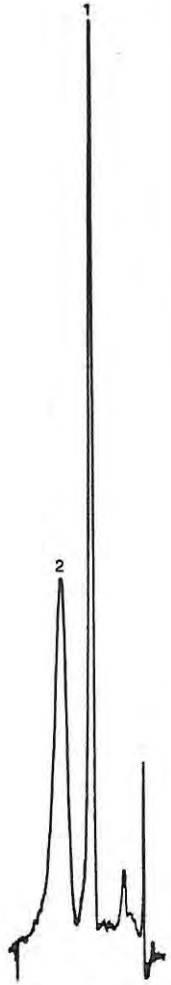
1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE



BETNOVATE/BETNOVATE BASE

FLOW 90 ml/hr

1 INTERNAL STANDARD
2 BETAMETHASONE 17-VALERATE



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APPENDIX A

% TPS VERSUS TIME FOR ALL THE IN VIVO ASSESSMENTS.

ABBREVIATION	PREPARATION
B	BETNOVATE CREAM
B/A	BETNOVATE/AQUEOUS CREAM
B/B	BETNOVATE/BUFFERED CREAM
B/Bb	BETNOVATE/BETNOVATE base
B/H	BETNOVATE HALF STRENGTH
B/U	BETNOVATE/ULTRABASE
B/E	BETNOVATE/EMULSIFYING OINTMENT
B/45	BETNOVATE/E45
S	SYNALAR
S/A	SYNALAR/AQUEOUS CREAM
S/B	SYNALAR/BUFFERED CREAM
S/E	SYNALAR/EMULSIFYING OINTMENT
S/U	SYNALAR/ULTRABASE
S/45	SYNALAR/E45
S/D	SYNADONE CREAM
S/F	SYNALAR FORTE
A	AQUEOUS CREAM
B/C	BUFFERED CREAM
B/b	BETNOVATE BASE
E	EMULSIFYING OINTMENT
U	ULTRABASE
45	E45
S/b	SYNALAR BASE

TIME	7	8	10	12	14	16	18	24	28	
FIG. 1										
2 STRIPES	17,4	25,0	35,9	40,0	37,6	28,3	19,2	3,7	2,2	
3 STRIPES	21,9	29,4	46,1	49,4	42,6	32,4	23,2	8,0	5,2	
4 STRIPES	25,9	33,0	49,3	55,0	49,1	37,4	28,2	6,7	4,3	
5 STRIPES	30,0	35,7	55,1	58,0	49,3	38,2	28,5	6,3	4,1	
FIG. 2										
5 STRIPES	34,8	40,2	51,7	55,9	54,0	52,0	42,8	8,1	7,6	
6 STRIPES	39,2	45,3	55,9	60,6	56,6	55,6	46,5	9,1	7,9	
7 STRIPES	38,7	44,9	56,6	62,1	58,9	55,7	47,3	8,8	8,4	
8 STRIPES	41,6	44,4	56,2	61,3	57,9	56,9	48,3	10,8	9,6	

TIME	4,5	6,5	9	10	12	14	16	18	24	28
FIG. 3										
2 HOURS	2,5	7,3	11,2	11,4	14,2	15,1	12,0	10,2	1,9	0,01
4 HOURS	7,6	9,3	18,1	25,5	30,3	32,2	27,6	24,8	7,3	3,7
6 HOURS		16,2	22,1	29,6	38,6	40,9	37,2	31,7	6,8	4,6
8 HOURS			25,9	32,7	43,7	50,7	46,2	40,1	12,1	7,2

TIME	9	11	12,5	14	16	18	20	22	24	36
FIG. 4										
8 HOURS	31,7	35,2	53,2	58,2	61,5	51,2	46,2	40,8	32,7	5,8
10 HOURS		38,9	52,8	62,3	69,5	55,9	51,1	45,6	36,3	5,5
12 HOURS			56,9	65,0	68,5	61,2	54,1	46,2	36,4	6,5

TIME	7	8	10	12	14	16	18	24	28
FIG. 5									
B	5,1	6,9	7,9	9,7	10,2	12,5	10,2	3,7	1,9
B/A	9,9	6,6	8,6	11,1	11,9	15,2	9,9	0,8	0,8
B/Bb	6,2	6,2	6,2	8,3	8,0	8,6	8,3	1,2	0,9
B/H	5,1	6,9	7,9	9,7	10,2	12,5	10,2	3,7	1,9
B/U	4,7	6,2	7,4	8,2	9,3	7,0	4,7	1,6	1,2
FIG. 6									
B	17,3	26,7	40,7	49,8	50,6	43,2	35,0	9,1	6,2
B/A	9,9	12,3	34,2	37,9	40,0	33,7	30,0	9,9	7,0
B/Bb	11,1	15,4	32,1	38,0	40,7	36,1	25,9	5,5	4,9
B/H	10,7	19,8	35,8	39,1	43,2	37,4	30,9	6,6	5,3
B/U	7,3	12,8	31,6	38,0	37,6	35,0	25,2	6,8	5,5
FIG. 7									
B	22,2	31,2	41,2	50,5	54,5	43,9	35,7	16,4	11,6
B/A	18,1	23,0	35,7	44,2	44,4	35,1	30,0	12,4	10,6
B/Bb	16,9	24,8	36,9	44,7	44,9	35,7	29,3	11,6	8,7
B/H	16,4	24,1	33,3	41,4	43,5	31,9	25,7	10,4	8,3
B/U	15,8	21,8	32,3	40,5	41,8	33,9	28,3	12,3	8,8
FIG. 8									
B	25,9	32,9	42,9	49,2	47,2	37,4	24,3	5,9	3,2
B/45	25,0	28,1	36,5	45,0	42,8	33,7	23,9	6,5	3,9
B/B	24,0	29,0	40,3	46,4	46,2	35,5	34,6	8,5	4,0
B/E	22,4	26,7	36,8	45,3	42,8	35,6	28,0	9,9	5,6
Bb	7,3	6,8	2,0	4,3	1,5	2,3	1,8	1,0	0,5

TIME	7	8	10	12	14	16	18	24	28
FIG. 9									
B	22,2	34,6	43,4	44,0	43,6	39,5	31,9	12,1	8.4
B/A	29,2	35,8	43,4	47,5	47,2	37,9	30,5	10,3	6,3
B/Bb	24,4	32,7	40,7	41,9	42,1	33,2	25,8	8,4	5,7
B/H	23,2	31,5	41,9	41,9	41,1	31,6	23,9	8,7	4,3
B/U	27,2	35,8	43,4	45,3	44,2	36,0	28,9	9,5	4,5
FIG. 10									
B	25,6	31,6	42,5	48,8	47,5	35,1	28,6	11,7	8,0
B/45	30,0	35,3	43,0	45,2	43,6	29,3	19,7	9,8	3,6
B/B	23,7	29,5	37,6	39,7	39,4	30,3	24,2	8,8	4,5
B/E	22,5	29,2	38,1	44,2	38,3	30,0	24,4	9,4	4,2
U	4,2	5,6	5,3	4,7	5,3	4,7	3,6	2,5	2,2
FIG. 11									
B	29,0	37,7	51,5	63,9	64,5	54,0	50,2	17,3	15,4
B/A	29,9	36,8	53,5	65,6	61,8	52,8	50,0	19,4	13,5
B/Bb	22,6	33,7	44,1	59,7	58,3	49,7	45,5	13,2	12,2
B/H	27,4	37,2	50,3	60,8	60,4	50,7	48,0	12,8	10,4
B/U	31,6	39,6	55,9	64,9	63,2	53,5	51,4	14,9	14,2
FIG. 12									
B	28,3	31,3	39,4	50,7	47,4	43,5	29,6	8,8	4,8
B/45	22,6	25,0	30,2	43,9	43,2	44,6	33,3	11,3	8,0
B/B	24,3	29,6	37,5	53,5	49,4	45,9	35,9	10,6	6,3
B/E	16,7	20,4	25,0	39,8	38,7	42,4	29,3	9,6	8,0
E	8,0	7,6	7,1	5,0	2,1	1,5	1,0	1,1	0,7

TIME	7	8	10	12	14	16	18	24	28
FIG. 13									
B	19,3	25,8	42,4	52,2	49,8	44,9	29,1	5,1	4,0
B/A	29,5	30,4	51,0	54,6	53,1	46,9	32,0	6,1	3,4
B/Bb	27,6	34,1	52,4	59,9	57,5	53,2	34,1	6,7	3,6
B/H	26,3	30,6	48,5	55,8	55,3	48,5	33,8	6,1	3,2
B/U	25,7	29,7	48,3	53,7	55,6	46,7	31,7	5,3	3,4
FIG. 14									
B	19,3	31,3	45,8	54,2	50,3	34,4	27,3	5,0	1,8
B/45	22,6	34,6	47,7	55,6	52,6	36,7	27,2	4,1	3,1
B/B	19,0	30,2	44,5	49,7	49,5	37,0	26,0	4,4	2,9
B/E	16,7	25,5	41,2	47,9	46,6	33,9	25,8	2,1	2,0
FIG. 15									
B	24,3	31,5	44,4	49,6	51,4	42,6	30,7	8,2	6,0
B/45	24,5	26,7	28,5	40,9	44,0	47,3	21,8	5,6	4,7
FIG. 16									
S	12,2	16,9	28,9	35,0	37,7	33,3	30,0	12,7	11,6
S/A	12,8	13,3	21,1	28,9	32,2	27,8	25,6	11,9	8,9
S/B	10,6	14,2	18,6	26,1	32,5	28,6	29,4	12,5	12,0
S/E	10,8	11,9	20,0	25,8	31,4	26,1	22,8	7,7	6,4
B/C	3,5	3,0	2,8	2,5	2,3	2,5	2,5	0,25	0,25

TIME	7	8	10	12	14	16	18	24	28
FIG. 17									
S	20,2	24,2	39,3	50,0	53,6	52,8	49,4	20,0	16,7
S/45	12,3	17,3	25,0	39,8	41,0	42,7	39,4	15,2	13,0
S/D	8,9	13,5	20,5	29,0	28,3	27,9	25,6	10,7	8,7
S/U	9,5	12,9	20,0	27,3	28,9	28,9	26,5	12,7	9,5
A	11,5	6,2	4,6	4,6	0,8	0,8	0,8	2,3	2,3
FIG. 18									
S	26,4	32,5	47.4	58.9	55,6	50,6	49,4	19,7	17,9
S/A	29,0	33,0	52,5	57,1	61,1	50,3	48,2	22,5	18,5
S/B	33,3	42,2	57,1	66,2	62,6	53,5	50,3	21,5	18,9
S/E	20,3	22,2	36,9	49,7	48,1	43,3	41,7	23,1	18,3
45	2,7	4,2	6,9	3,4	2,1	2,1	1,7	0,4	2,1
FIG. 19									
S	11,6	17,6	29,2	40,6	39,4	37,9	35,5	15,5	13,3
S/F	24,6	32,5	48,2	64,9	65,8	60,5	56,4	30,7	28,9
S/45	13,7	18,3	26,9	37,7	39,8	35,1	30,7	13,7	12,9
S/U	9,8	13,4	23,5	33,3	34,9	31,0	27,1	10,6	9,6
S/b	2,9	3,9	2,3	4,2	1.3	1,0	0,0	0,3	0,3

FIG. 19 continued

TIME	30	33	48
S	12,9	11,6	5,3
S/F	27,1	25,1	12,9
S/45	11,8	8,2	3,1
S/U	8,6	7,8	2,8
S/b	0,7	0,3	0,7

TIME	7	8	10	12	14	16	18	24	28
FIG. 20									
S	20,8	28,1	36,6	47,2	56,1	52,5	50,6	20,3	17,2
S/A	25,8	35,6	45,0	56,4	65,3	59,7	56,1	19,4	15,3
S/B	23,1	31,3	42,5	54,7	60,3	58,6	50,8	17,5	11,9
S/E	11,7	19,4	33,1	43,1	52,2	52,2	47,8	18,1	12,2
FIG. 21									
S	12,2	16,9	28,9	35,0	37,8	33,3	30,0	12,8	11,7
S/45	8,3	15,3	21,7	26,4	31,7	26,4	21,4	10,8	10,0
S/U	11,4	16,7	21,4	28,6	28,6	26,4	26,7	13,3	11,7
FIG. 22									
S	16,7	22,8	35,1	38,5	38,6	36,7	28,4	8,4	8,0
S/A	10,8	16,2	24,5	27,5	28,4	27,8	20,4	8,3	7,5
S/B	10,4	18,5	27,4	28,9	32,0	30,5	24,5	8,0	7,0
S/E	6,3	11,5	16,7	19,3	20,8	18,9	15,2	6,0	4,5
FIG. 23									
S	16,7	22,8	35,1	38,5	38,6	36,7	28,4	8,4	8,0
S/U	9,3	16,1	22,6	25,4	26,4	23,7	19,7	6,3	5,6
S/45	9,3	14,4	16,9	17,4	14,9	11,1	9,5	3,0	2,6
FIG. 24									
S	11,1	12,8	17,5	17,7	17,7	16,9	14,8	6,4	6,8
S/45	9,1	7,4	8,4	5,1	6,4	6,8	3,7	2,1	2,1