STUDIES ON THE PERCUTANEOUS ABSORPTION OF BETAMETHASONE-17-VALERATE IN THE DOMESTIC PIG

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Thesis presented to the
Department of Pharmacology
Faculty of Medicine
University of Ottawa
in partial fulfillment
of the requirements
for the degree of
Master of Science.

Ottawa, Canada, 1969.

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ACKNOWLEDGEMENTS

The writer wishes to express his deep appreciation and gratitude to Dr. George M. Ling for his encouragement, direction and stimulation during this study. He also is grateful to Dr. MacConaill, and to other members of the Department of Pharmacology for their advice and helpful criticism during the course of this investigation; to Dr. R.K. Smiley, Head of the Department of Medicine, who offered valuable suggestions concerning the problems treated herein.

The writer also wishes to express his appreciation to Mrs. Anne Nunn for the expert typing of the thesis, and to Mrs. Janet Sreter, for her reliable assistance during the course of this study.

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ABSTRACT

The percutaneous absorption of ^{3}H betamethasone-17-valerate was studied in young domestic pigs. Our findings suggest that this animal may serve as a suitable model for studying the absorption of steroids from the skin. A single application of radioactive cream was applied to the skin and kept there for three days under occlusion. Urine proved to be the main excretory route of this topical steroid. Excretion in feces was ten times less. After three days occlusion, an appreciable amount of radioactivity was recovered from all tissues; highest concentrations were present in the liver. Plasma samples revealed significant levels of radioactivity as early as two hours after application and reached their highest concentrations at the end of 72 hours. After a single application, maximal levels of radioactivity were also observed to persist in plasma for a period of nine days in the presence of constant occlusion. In addition, pituitary adrenal axis suppression has been studied after non-tritiated betamethasone-17-valerate was applied with occlusion to the skin twice daily, daily and on alternate days. In all cases, adrenal suppression was evident after topical steroid therapy, as manifested by a distinct decrease in the plasma levels of endogenous cortisol. However, the application of 40 gm of steroid under occlusion every 48 hours caused less pituitary-adrenal axis suppression than that observed when 20 gm of the same steroid was applied daily. The above observations are discussed with reference to the clinical use in dermatology of topically applied steroids.

A. GENERAL INTRODUCTION

GENERAL INTRODUCTION

The addition of new and potent drugs have enabled the dermatologist to achieve marked progress in the treatment of skin diseases. In the early 1950's, topical corticosteroids were considered wonder drugs. Today, the beneficial therapeutic effects which are derived from the use of topical steroids in dermatology are unquestionable. However, within a short time after their introduction as therapeutic aids, these drugs began to lose some of the optimism which accompanied their early application. In the mid-1950's, disturbing reports of systemic and clinical side effects following topical use of corticosteroids started to appear in the medical literature.

Malkinson and Ferguson (1) were among the first to demonstrate the percutaneous absorption of a topical steroid. Using hydrocortisone labeled with C^{14} (hydrocortisone-C^{14}), they showed that radioactivity could be recovered in the urine of human subjects following the application of this steroid to normal skin. Scott and Kalz (2) used autoradiography to study the absorption of hydrocortisone-C^{14} ointment when this steroid was applied to the skin of patients. They demonstrated clearly the epidermal penetration of the hydrocortisone-C^{14} and its eventual access to the systemic circulation. These two groups of workers were the first to establish the percutaneous absorption of topical steroids into the systemic circulation. In 1955 Fitzpatrick et al. (3) and Livingood et al. (4) independently

reported sodium retention and edema after the topical administration only (and presumably due to percutaneous absorption) of fludrocortisone acetate. These were the first reports of clinical side effects from the use of a topical corticosteroid preparation.

In 1962 the use of occlusive dressings with Saran Wrap at the site of application had become an important ancillary measure in the management of skin diseases with topical corticosteroids. It was shown by McKenzie and Stoughton (5) (using vasoconstriction as an index of absorption) that Saran Wrap occlusive dressings could increase absorption of topical steroids one-hundredfold. These observations, together with the introduction of newer and more powerful topical steroids, prompted further study into the percutaneous absorption of topical steroids.

The use of topical steroids with the Saran Wrap occlusion technique has given rise to the frequent occurrence of occult systemic reactions. Several investigators have demonstrated pituitary-adrenal axis suppression as a direct result of the percutaneous absorption of topical steroids (6,7,8,9). Scoggins and Kliman (10) have stated that in some concentrations the suppressive percutaneous dose of a topical steroid (5 to 7.5 mg of triamcinolone acetonide/24 hours) is quite close to the suppressive oral dose. In all of the above reports, a definite lowering of plasma cortisol levels was observed. These levels would return to normal values within one or two days after

removal of the steroid ointment from the skin. Admittedly, these observations were made following the use of occlusive dressings and hence under conditions of facilitated percutaneous absorption. Even ophthalmic corticosteroid preparations have been associated with disturbing systemic effects. Nursall (11) recorded a significant eosinopenia following the use of Neodeltacortef[1] (0.25%) eye drops. Burch et al. (12) observed a decrease in the urinary 17-hydroxycorticosteroid excretion during treatment with dexamethasone eye drops (0.75 mg/day). These observations and others of the systemic effects of topical steroid therapy have been reviewed by Scholtz and Nelson (13) in 1965.

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[1] Upjohn Company trade name for neomycin sulfate 5 mg and prednisolone 2 mg.

B. OBJECTIVES OF THE PRESENT STUDY

The present study was designed with two objectives in mind. One of the objectives (Section I) was to study the distribution of labeled betamethasone-17-valerate following the topical application of this steroid.

The second objective (Section II) was to study and compare the effects of betamethasone-17-valerate on adrenal function (pituitary-adrenal axis suppression) when this steroid was applied to the skin of an experimental animal twice a day, daily and every second day over a four day treatment period.

To achieve these objectives, an animal of moderate size with a skin similar to that of the human being was required. The domestic pig (Sus Scrofa Domestica) was chosen as the experimental animal, since some similarities exist between human and porcine skins. They were similar in that both had a sparse concentration of hair, an epidermis with a well differentiated understructure, and a dermis with a rich population of elastic fibres. Dissimilarities also exist; the stratum corneum of the pig consists of more layers than that of the human, and the glandular distribution and structure were also found to be different from that of the human (Montagna et al. (14)).

SECTION I:

DISTRIBUTION OF TRITIATED BETAMETHASONE-17-VALERATE AFTER TOPICAL APPLICATION

INTRODUCTION

Labeled compounds are a very useful tool in studies of the distribution of topical steroids among various body compartments. Using labeled steroids, Malkinson (15) in 1957 demonstrated that although topically applied hydrocortisone and cortisone were absorbed in equal amounts, hydrocortisone had a greater clinical action. Feldman and Maibach (16) confirmed earlier work (1) showing that hydrocortisone-C^{14} appeared in the urine after application to normal human skin. Malkinson and Kirschenbaum (17) then studied triamcinolone-C^{14} acetonide in humans. They assessed the amount of radioactivity in urine, in blood and in feces and at the site of application. They also compared the degree of percutaneous absorption through normal skin and through stripped skin[1]; it was much greater through the stripped skin. The percutaneous absorption of tritiated betamethasone (as the 17-valerate) in man was studied by Butler (18). This interest in percutaneous absorption of drugs was extended by Malkinson (19) in his study of toxic substances (mercury, lead etc.) used in industry. By 1966 work in this field had achieved such importance that a critical and historical review of percutaneous absorption was made.

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[1] Stripping of the skin was done by repeated removals of stratum corneum layers with Scotch Tape(R).

Reiss (20) reviewed the different methods used in studying percutaneous absorption. His review was not limited to corticosteroids but extended to other drugs such as testosterone, estrogens and vitamins.

However, data on the plasma, fecal and tissue distribution of topically administered steroids were lacking, although Florini et al. (21) studied the plasma half-life, and the excretion of tritiated triamcinolone in dogs and rats, and the tissue distribution in the rat after intravenous administration.

In Section I of this investigation, the concentration and distribution of labeled betamethasone-17-valerate in plasma, urine, feces and tissues are examined after application to the skin of the domestic pig.

MATERIALS AND METHODS

1. Animals

Domestic piglets of either sex, 1½ to 2 months of age, and varying in weight from 20 to 40 pounds, were used in this study. The Animal House of the University of Ottawa Faculty of Medicine obtained them from a local farmer. All pigs were kept in a large double metabolic cage which permitted separate collection of urine and feces.

2. General Methodology

The pigs were anesthetized with Halothane[1]. A 15 x 15 cm area of skin on the back was prepared for application of the cream by shaving and rubbing with sandpaper. The radioactive betamethasone cream was then applied to the prepared area and immediately covered with Saran Wrap[2] and a protective dressing. The pigs were kept in metabolic cages for the predetermined observation period; this was 72 hours for the first seven pigs and 9 days for an eighth.

At preselected times, 1 ml blood samples from an ear vein were collected in heparinized tubes. The sample was

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[1] Halothane: trade name for 2-bromo-2-chloro-1,1,1-trifluorethane with 0.01% thymol as the stabilizer. Hoechst Pharmaceuticals, Montreal, Quebec.

[2] Saran Wrap: Dow Chemical Corporation.

centrifuged and the plasma was removed and stored at -10°C until analyzed. The total 24-hour urinary excretions and extractions of fecal material were measured and 0.2 ml samples were taken for further analyses.

At the end of the observation period, the pigs were sacrificed by overdose of the anesthetic. They were then exsanguinated to minimize contamination of the tissues and organs removed during autopsy. Samples of skin, of fat and of muscle, (from anterior and posterior sites) and of bone marrow were taken and weighed. Thyroid, thymus, heart, lungs, liver, gall bladder, spleen, kidneys, adrenals, pancreas, brain, testes or ovaries and uterus were extirpated and weighed. A sample of about 50 mg was taken from each organ and tissue for evaluation of radioactive content. Small pieces of the Saran Wrap and the skin at the site of application were also removed for analysis.

Urine and plasma samples were also investigated. NCS solubilizer[1] was added to the samples of Saran Wrap and skin (0.4 ml) and to all organ and tissue samples (0.2 ml) to digest them. The radioactive steroid was extracted from feces with methanol.

Each processed sample was dissolved in 10 ml of a

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[1] NCS solubilizer: a quartenary ammonium base, 0.6 N in toluene; obtained from Nuclear Chicago Corporation, Des Plaines, Illinois.

modified Bray's solution[1]. Radioactive determinations were made with a Nuclear Chicago Mark-I liquid scintillation counter.

3. Preparation of the Tritiated Betamethasone-17-Valerate Cream

a) Labeling the Steroid

Pure crystals of betamethasone-17-valerate[2] were supplied by Schering Corporation. 49.5 mg were sent to New England Nuclear Corporation[3] to be tritium labeled by catalytic exchange. A methanolic solution was returned with a total activity of 165 mCi corresponding to a specific activity of 3.3 mCi/mg.

b) Purification

The tritiated material returned by New England Nuclear Corporation required further purification. A separate sample was taken for each experiment. On the day preceeding application to the skin, the following procedure was carried out:

i) Distribute 0.5 cc of the methanolic solution evenly across a silicagel thin layer chromatography plate[4].

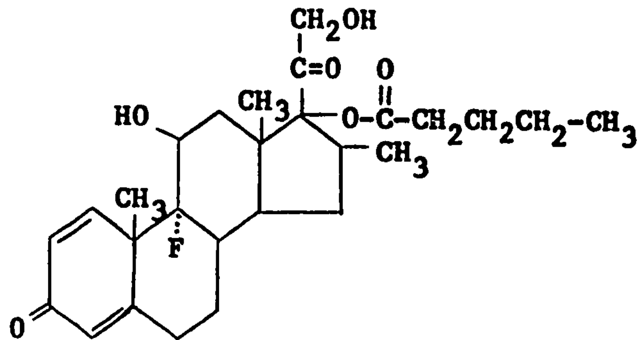
---

[1] Modified Bray's solution: 1 gallon dioxane
400 gm naphthalene
28 gm PPO
1.2 gm POPOP

[2] See chemical structure page 18a.

[3] New England Nuclear Corporation, Boston, Massachusetts

[4] Prepared plates were provided by Dr. D.S. Layne, Department of Biochemistry, University of Ottawa.



9α-fluoro-11β,21-dihydroxy-16β-methyl-
17α-valeryloxypregna-1,4,diene-3,20-dione

(BETAMETHASONE-17-VALERATE)

(Celestoderm by Schering Corporation)

ii) On either side of the plate, deposit a drop of unlabeled ("cold") betamethasone-17-valerate dissolved in ethanol to act as a tracer.

iii) Insert the plate into a jar containing a mixture of 100 ml of ethyl acetate and 50 ml of cyclohexane. Leave until the solvent front rises to about 5 cm from the top.

iv) Remove the plate and allow to air dry. Stain the sides of the plate with phosphomolybdic acid. Heat the stained sides only. The level to which the cold betamethasone-17-valerate tracer has migrated shows up as a pair of dark spots on each margin of the plate.

v) Take samples of silicagel 1 cm long from a 5 mm wide strip along the centre of the plate. Add each sample to a vial containing 10 ml toluene and measure the radioactivity in the liquid scintillation counter.

vi) The highest activity should be in those samples from the same level as the two black spots indicating the presence of "cold" betamethasone-17-valerate. This confirms that the radioactive material is betamethasone-17-valerate.

vii) Remove the silicagel from the full width of this area of highest activity and dissolve the purified tritiated beta-methasone-17-valerate in ethyl acetate. Filter through fritted glass to remove the silicagel, and evaporate filtrate to dryness. The final product consists of fine crystals of pure tritiated betamethasone-17-valerate. The average yield was approximately 2.7%.

c) Incorporation into Cream Base

i) Dissolve the purified betamethasone-17-valerate in 50 ml of ethyl acetate.

ii) Dilute a 50 µl aliquot to 10 ml with modified Bray's solution and measure its radioactivity. From this, the total activity of the remaining material can be determined by multiplying by 1000[1].

iii) Evaporate the remainder of the original solution to dryness.

iv) Add 10 gm of the cream base[2] to the residue. Heat the mixture to 55°C and stir thoroughly to incorporate the steroid into the cream.

Approximately 9 gm of the steroid cream prepared as above was applied to the back of each pig. This contained about 60 µg of purified tritiated betamethasone-17-valerate. The total activity applied was calculated by subtracting the activity in the residue from the activity originally present in the purified material.

4. Calculations

The Channels Ratio Method was used to correct all

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[1] Strictly 999 but error of counting is greater than error of approximation.

[2] Cream base used in Celestoderm Cream, courtesy of Schering Corporation, Montreal, Quebec.

samples for variations in counting efficiency. A standard tritium quench correction curve was established using a series of tritium samples with known activity and increasing amounts of methanol added; thus successive samples in the series had increasing quenching. Channel A was adjusted to record the lower 30% of the tritium energy spectrum and channel B recorded the upper 70% of the spectrum. Channel C registered all counts. The sample with lowest quenching had a B:A ratio of 2.3 and as the quenching increased, the B:A ratio decreased. The curve obtained when the B:A ratio was plotted against the efficiency of counting[1] (Eff) is the Tritium Correction Curve.

Once this curve had been obtained, the counting efficiency in any sample containing tritium could be derived from the B:A ratio and the standard curve. The counts per minute (CPM) and the efficiency (Eff) being known, the total disintegrations per minute (DPM) could be calculated as:

$$DPM = \frac{CPM}{Eff}$$

All results were expressed as DPM.

5. Statistics

Since in a number of cases, the percentage of the absorbed dose showed wide variations and yet only positive results were meaningful, these measurements were considered as

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[1] $\frac{\text{CPM measured}}{\text{DPM of sample}}$

being lognormally distributed. The arithmetic means and standard errors were calculated for the logarithms of these measurements, and these statistics reconverted to the original percentage scale giving geometric means with the equivalent standard errors.

RESULTS

Table I shows the distribution of that part of the applied dose which was recovered. 12.2% remained at the site of application. Of the other 87.8%, 20% could be accounted for in the plasma, the liver and other organs, and the total urinary and fecal excretions over the observation period. This left a deficit of 68% of the original dose unaccounted for. As no attempts had been made in this study to determine the amount of radioactivity in the total muscle mass, skeleton or in the other areas of the skin, it is therefore probable that the remaining 68% of the total activity applied was localized in such structures, although some may have been excreted by sweat glands or lost during expiration.

TABLE I

Recovery of ^{3}H Betamethasone-17-Valerate after topical application with 3 days of occlusion.

	% of Total DPM Applied
Unabsorbed portion	12.2 ± 3.5 †
Sampled compartments	20.0 ± 3.5
Total recovery	32.0 ± 5.0

† Geometric Mean ± Equivalent Standard Error

Table II presents in more detail the distribution among the compartments sampled of the total activity recovered at the end of the 72 hour period. The plasma contained 0.57%. The urine voided during the 3 day period contained 8.5% while the feces accounted for only 0.72%. The liver retained 2.6% of the total dose leaving 3% to be distributed among the other organs sampled.

TABLE II

Distribution of absorbed ^{3}H Betamethasone-17-Valerate after topical application with 3 days of occlusion.

	% of Total DPM Applied
Plasma	0.57 ± 0.12 †
Urine	8.5 ± 2.2
Feces	0.72 ± 0.56
Liver	2.6 ± 1.2
Organs	3.0 ± 0.9

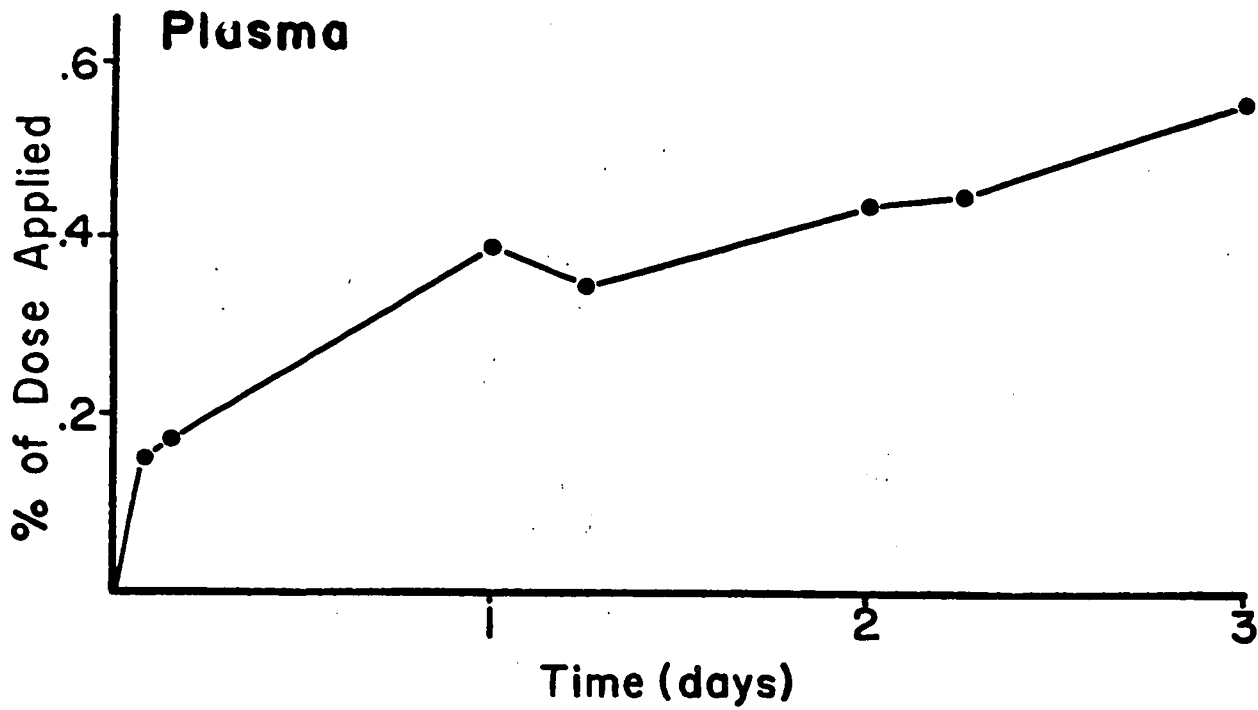
† Geometric Mean ± Equivalent Standard Error

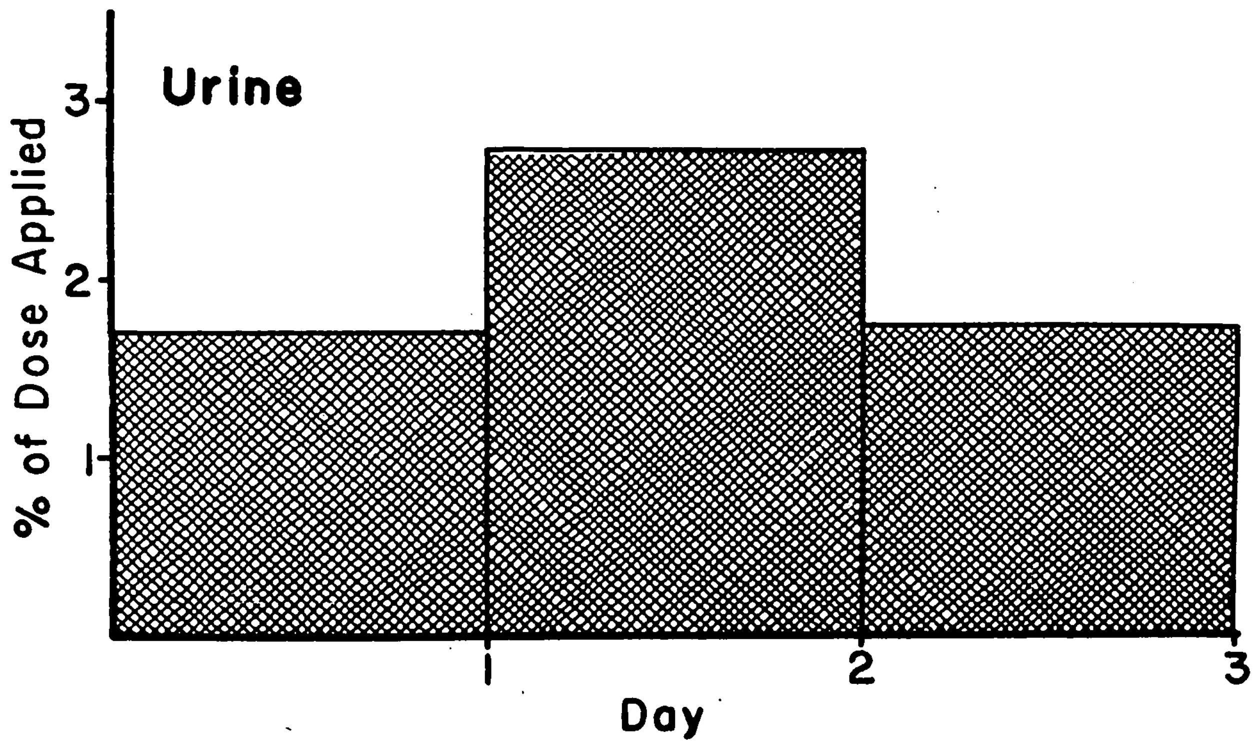
The evaluation of the tissue distribution of the labeled betamethasone-17-valerate presented certain difficulties. Severe and irregular quenching occurred presumably from the use of a potent quartenary ammonium base tissue dissolving reagent and from the yellow colour of the chemically treated tissues.

Figure 1 shows the time course of the mean plasma and

FIGURE 1. Time course of the mean levels of plasma and urinary ^3H Betamethasone-17-Valerate for the first five pigs.

FIGURE 1





urine activity for the first five pigs. The curve for plasma demonstrates that significant activity was present as early as 2 hours after application with a steady increase thereafter, attaining the highest value at 72 hours. The peak of radioactivity excreted in the urine was during the second day.

Figures 2 to 6 inclusive give a graphic illustration of the correlation between the urinary excretion of activity and the urine volume for each individual pig. The maximum daily excretion of radioactivity varied from pig to pig. It is of particular interest that in pig #1 (Fig. 2) the very low amount of DPM excreted during the first day was associated with a low volume of urine voided during that period. There was some tendency for the total activity excreted to be higher with the higher urine volumes, but this correlation was not clear-cut. The highest daily excretion of radioactivity was never seen as late as the third day of collection, whereas the plasma values were always at the maximum at 72 hours.

In order to evaluate the reproducibility of our experimental results, two pigs (numbers 6 and 7) of approximately the same size and weight (14.3 and 12.2 kgm) were topically treated at the same time with radioactive cream containing 380 million and 360 million DPM respectively. Figure 7 summarizes these findings. The excretions of radioactivity were very similar in both pigs with a maximum daily urinary excretion in the first

FIGURES 2 TO 6. **Correlation between the urinary excretion of activity and the urine volume for each pig.**

FIGURE 2

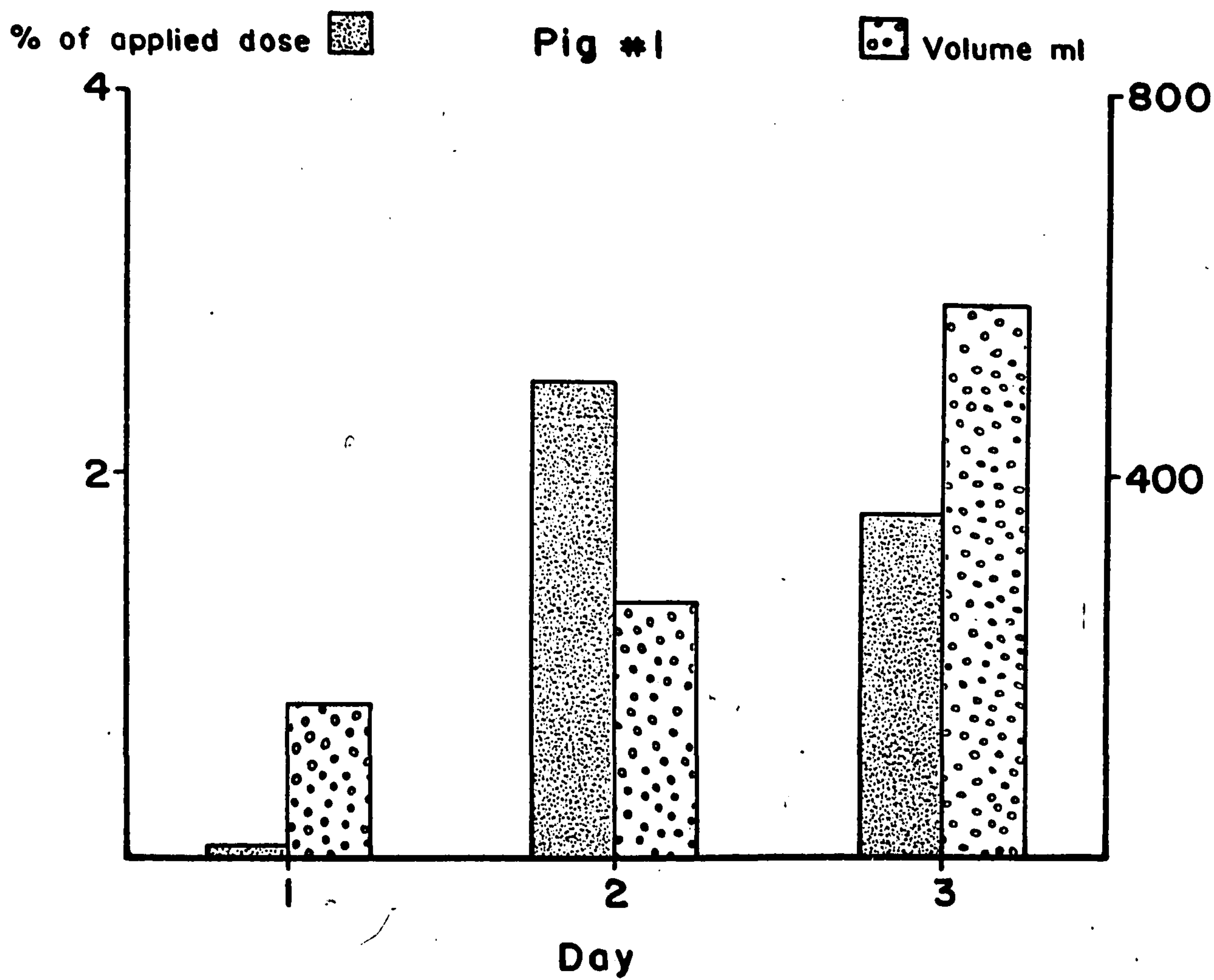


FIGURE 3

URINE

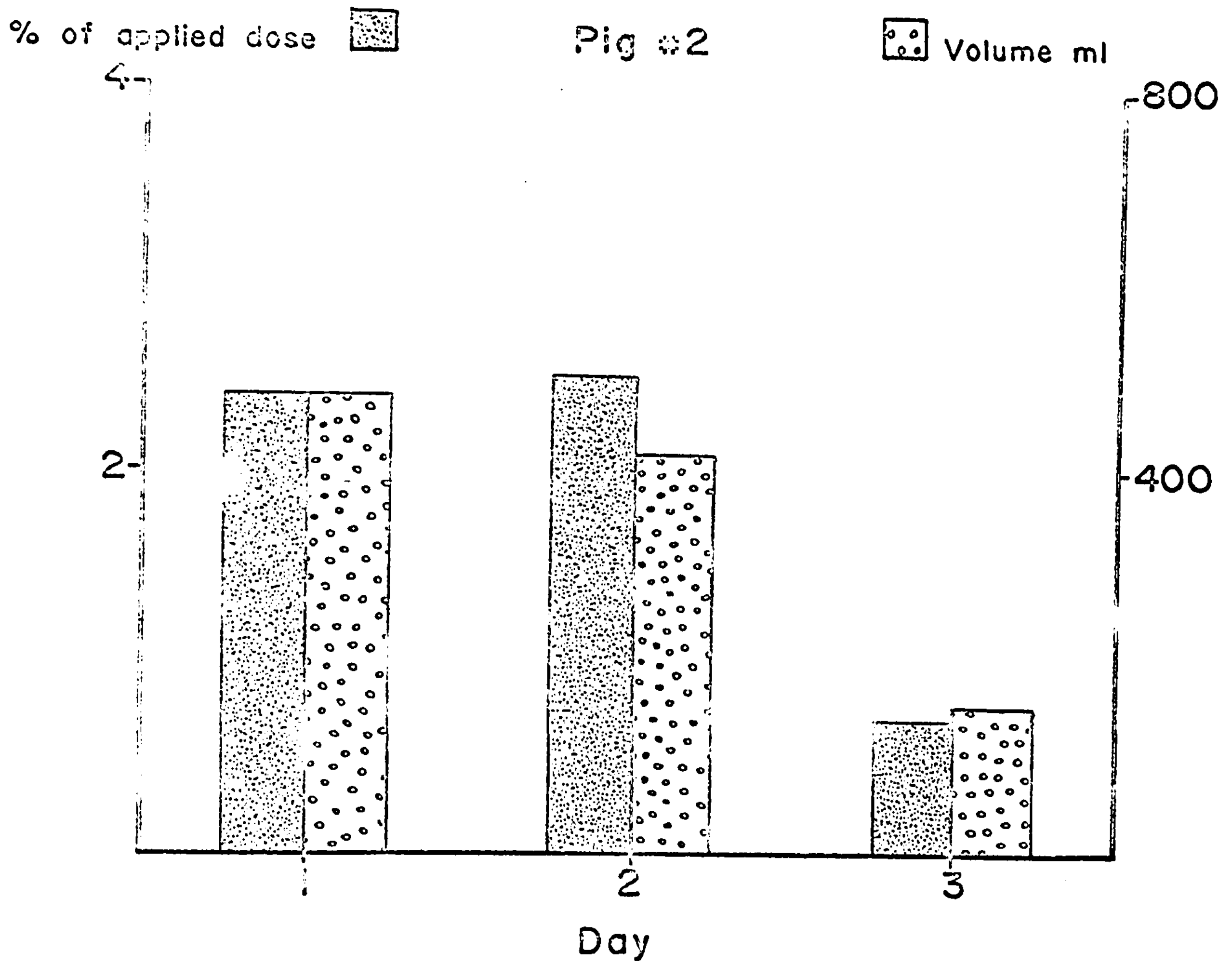


FIGURE 4

URINE

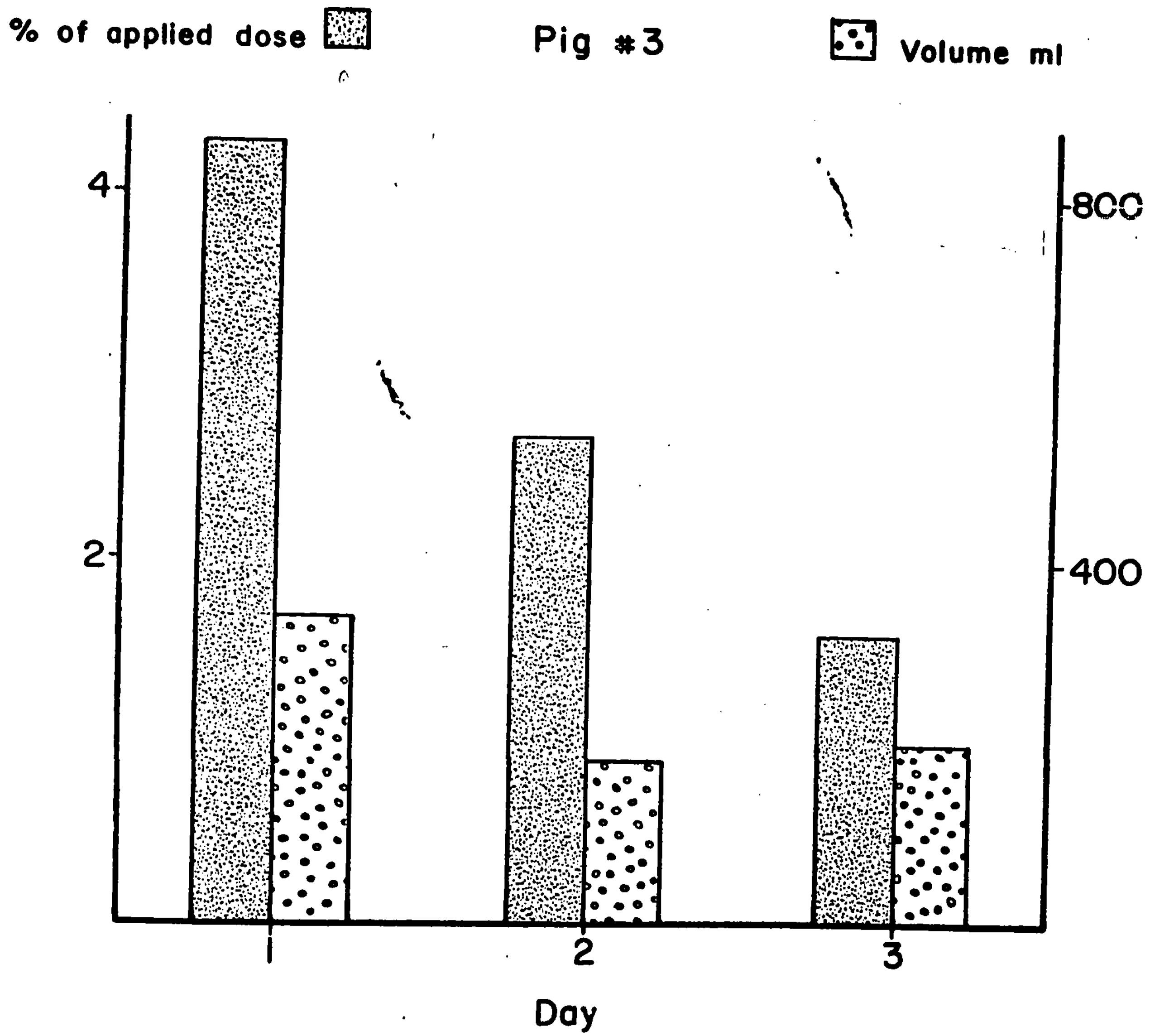


FIGURE 5

URINE

Pig # 4

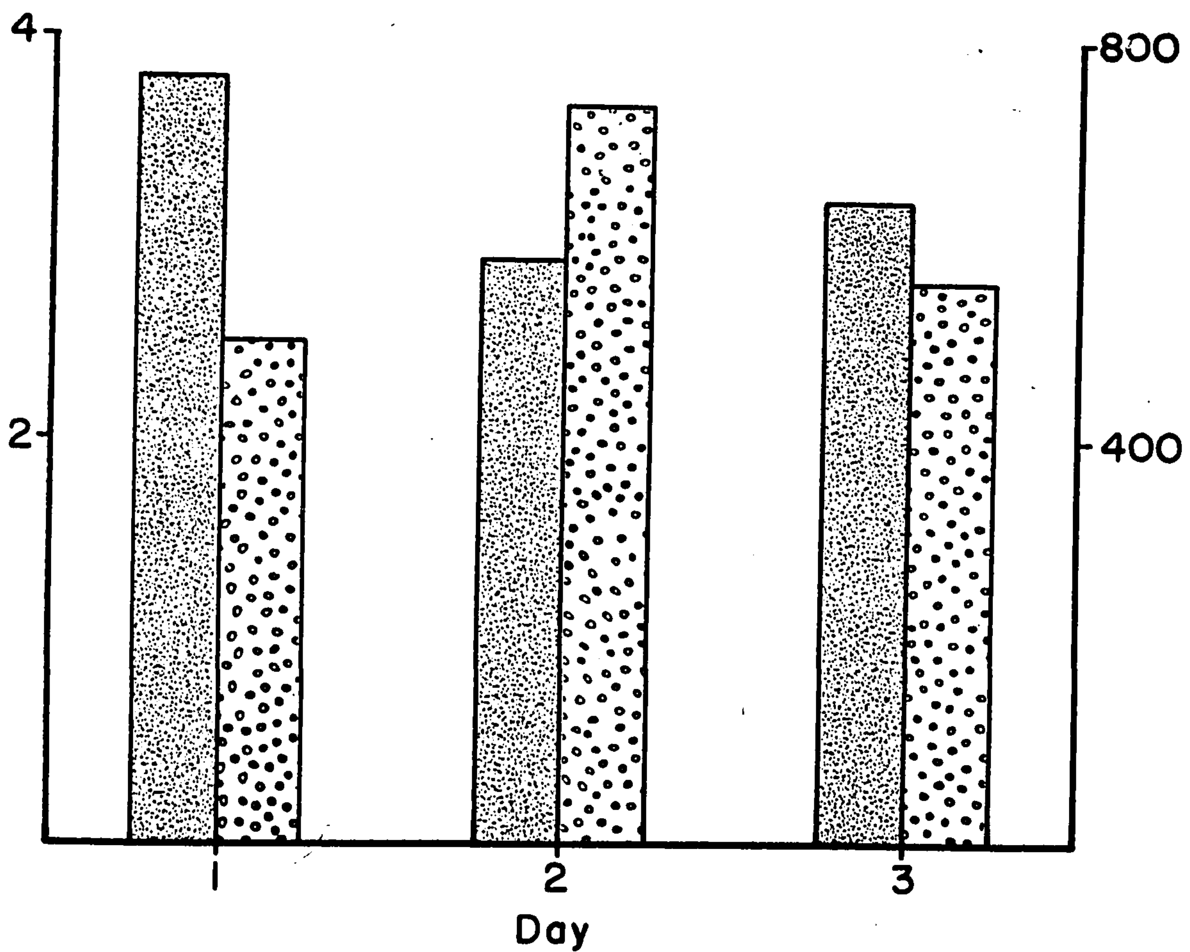


FIGURE 6

URINE

Pig # 5

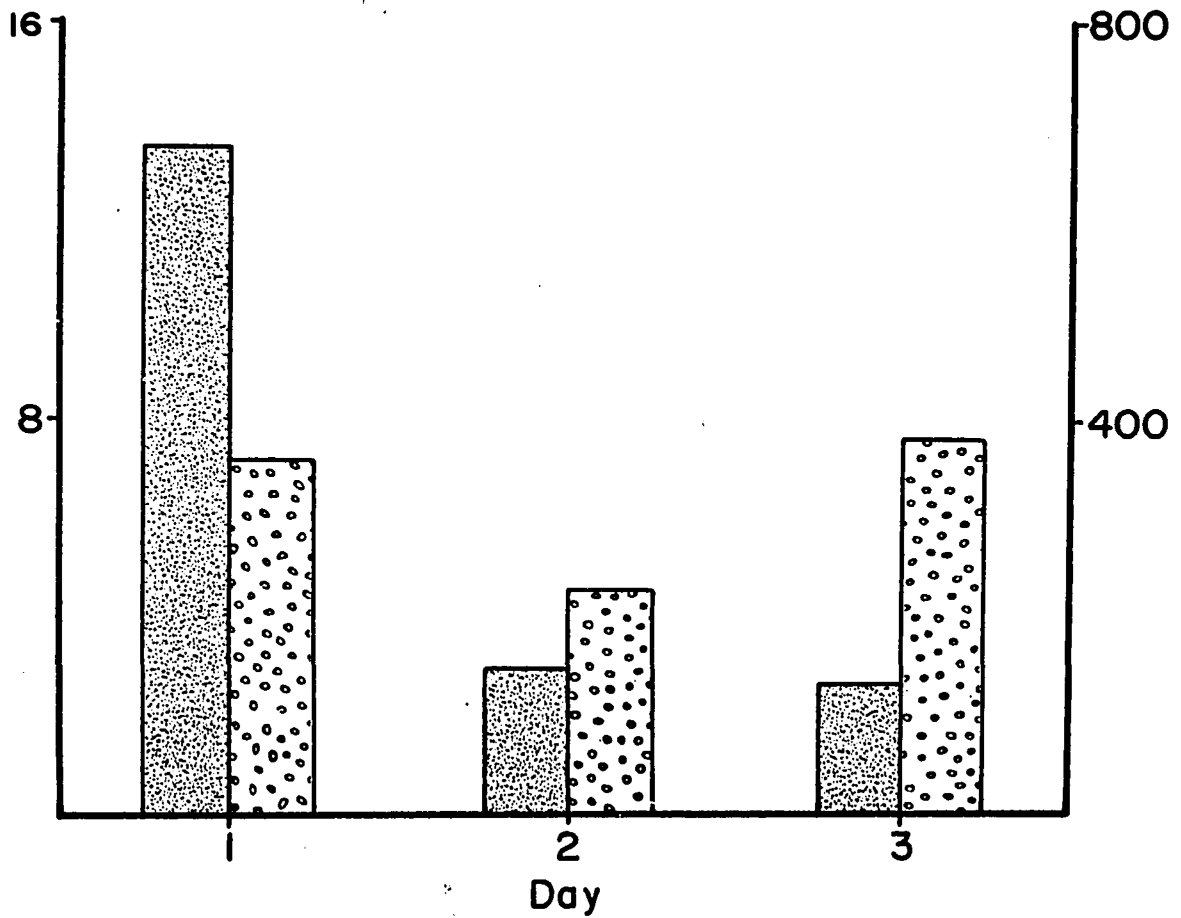
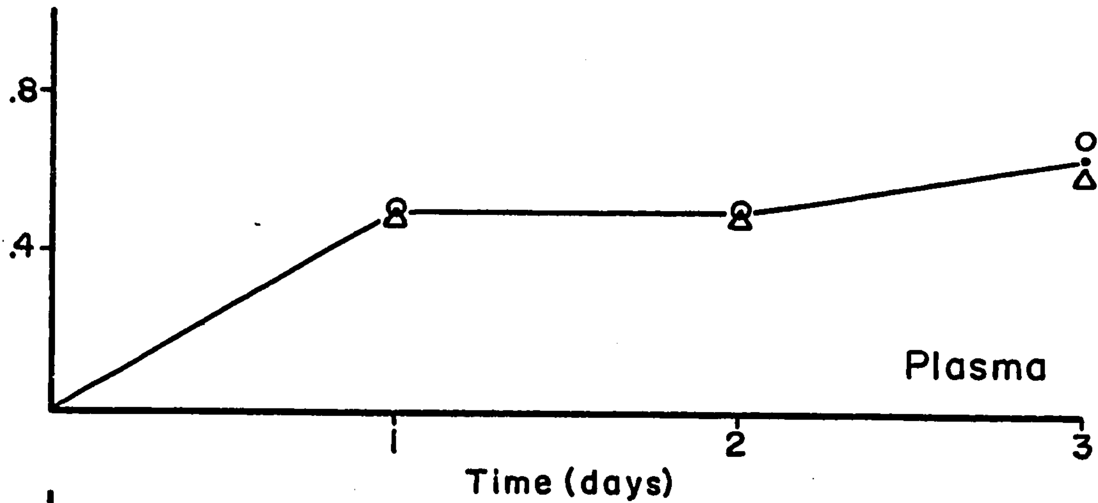
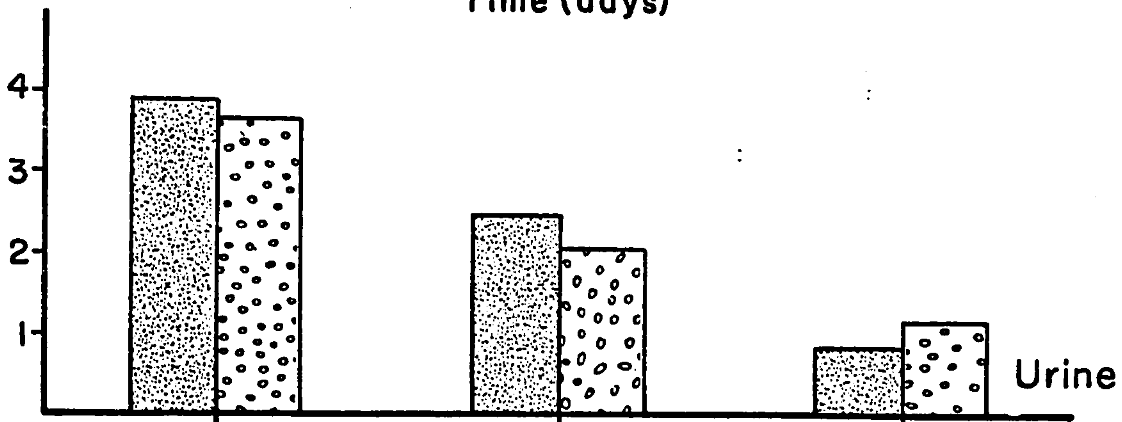


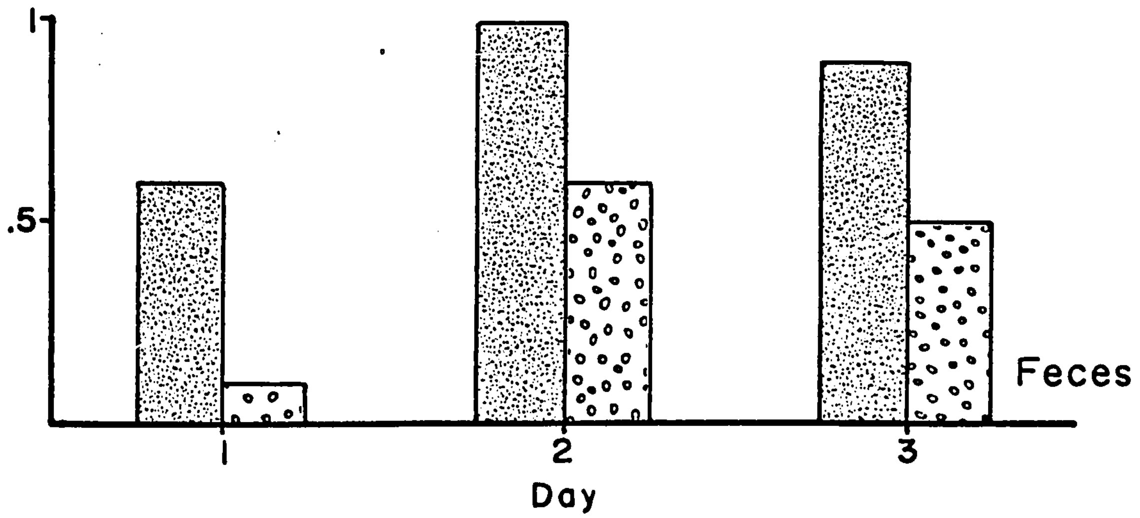
FIGURE 7. Result of treating two pigs with equivalent doses of ^{3}H Betamethasone-17-Valerate in order to evaluate the experimental reproducibility of results.

% of Applied Dose ○ Pig #6 Δ Pig #7

FIGURE 7







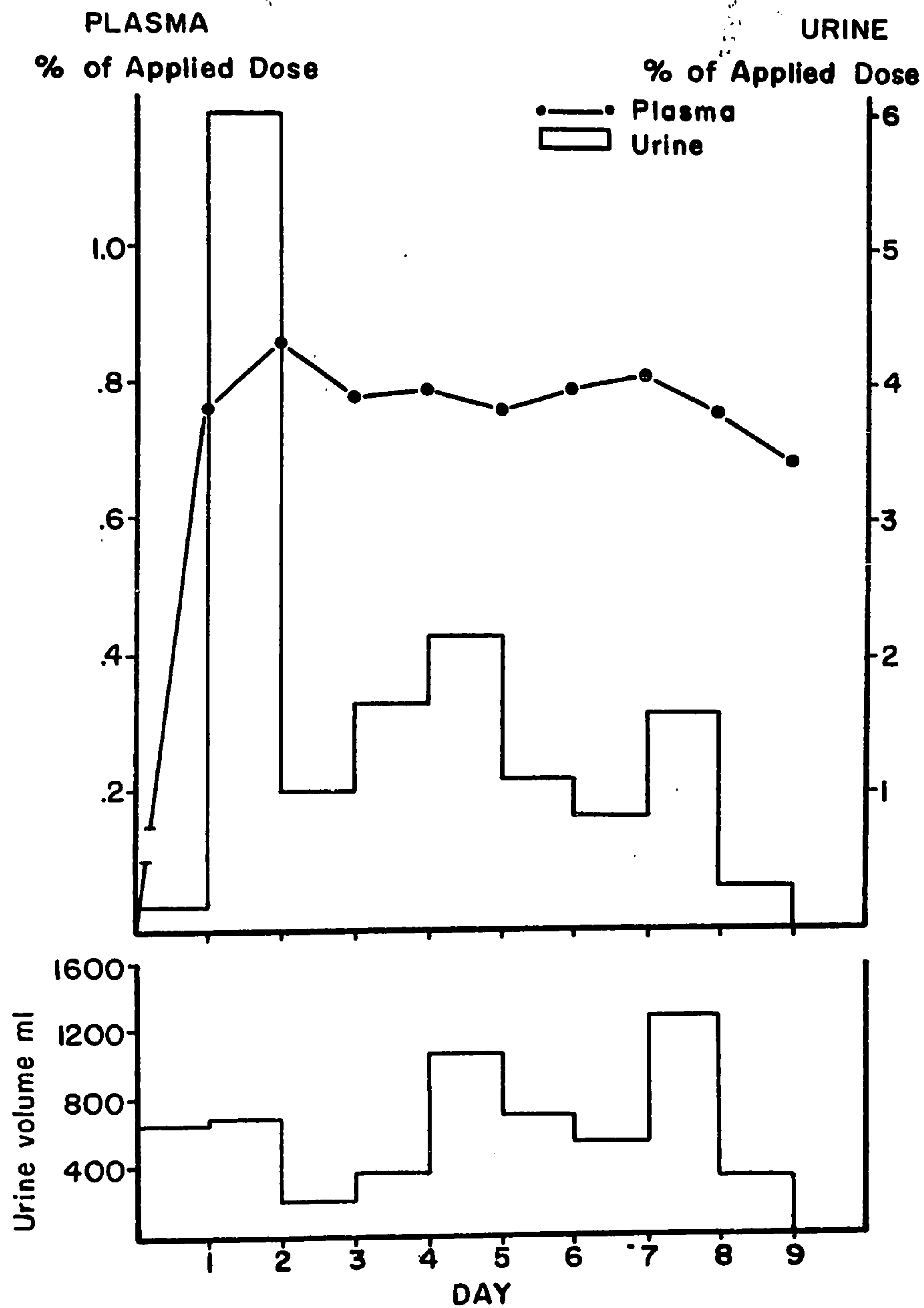
day and maximum fecal activity on the second day. In the urine, pig 6 excreted 3.9% during the first 24 hours while pig 7 had a value of 3.7% during the same period. Plasma levels of radioactivity were also similar, again reaching their highest values at 72 hours; at this time, pigs 6 and 7 had recorded values of 0.7 and 0.6% respectively. It is of interest that in both these pigs the urine output of activity again passed its peak level before the plasma had attained its highest value.

Since the level of radioactivity in the plasma seemed to be still rising during the third day, it was necessary to determine whether further increases in plasma radioactivity would appear over a more prolonged period. To test this, the occlusion and observation periods were prolonged in pig #8 for a total of 9 days. Plasma samples and total urine excretion were collected each day; their activity is shown in Figure 8. The highest radioactivity in plasma (0.87%) occurred on the second day. This level remained steady, with minor fluctuations, until the 9th day. Urinary activity, however, showed marked variations. The peak daily excretion (6% of the total DPM applied) occurred on the 2nd day; excretion then fell off markedly decreasing to a final value of 0.3% on the 9th day.

In the lower graph of Figure 8, we see that there is a definite parallelism between urinary radioactivity excreted and daily volumes voided over the last 7 days.

FIGURE 8. Levels of radioactivity in plasma and urine during nine days of occlusion, and correlation between urinary radioactivity and daily volumes voided.

FIGURE 8



DISCUSSION

The results obtained in this section of our study clearly indicate a) that betamethasone-17-valerate is absorbed from the skin of the domestic pig, and b) that marked levels can be found in plasma within two hours of application. Since similarities exist between porcine and human skin, it is probable that a similar rate of percutaneous absorption can also occur in man; however, histological dissimilarities have also been pointed out by Montagna (14) and further comparative studies of the absorptive characteristics of the skin of man and the pig are needed.

Plasma levels of radioactivity showed an initial rapid increase in the first two hours. This initial entry of the labeled steroid into circulation may be due, in part, to rapid absorption via the follicular pathway as described by Feldman and Maibach (16), and, in part, to the experimental condition of the animal's skin which was slightly irritated to produce hyperemia, often associated with inflammatory reactions of the skin. Comparison of the histological analysis of normal skin to that of a skin treated slightly with sandpaper (five strokes in either direction) did not reveal any appreciable differences. Macroscopically, mild erythema was observed when the skin was irritated. No appreciable damage to the stratum corneum was visible under microscopic examination. The thickness of the stratum corneum was similar

in both pigs, but the sandpaper irritated skin seemed to have lost some of the very small loosely arranged tags projecting from the outermost layer of the stratum corneum. This area was also treated with Saran Wrap and clear evidence has been presented by McKenzie and Stoughton (5) demonstrating that such a procedure can enhance the absorption of topical steroids one-hundredfold.

Over the next 2-3 days, plasma levels continued to rise, reaching a maximum on the third day and persisted until the 9th day. Vickers (23) has described a stratum corneum reservoir for topically applied steroids which is capable of releasing the steroid when permissive conditions such as occlusion are in constant operation. Carr and Wieland (24) confirmed the presence of this corticosteroid reservoir may be acting as a regulator allowing only small quantities of steroid to be released over a period of time into the systemic circulation. This can be contrasted to the initial rapid entry of steroid into the circulation through the follicular pathway. It is also possible that the consistently high plasma levels of radioactivity may have been the result of release from tissue stores. Cope (25) demonstrated an exchange of steroid location in tissues, suggesting that they are held there for a short time and are then released, with or without chemical change. This view has also been discussed by Tait et al. (26). Such an exchange has been shown to exist between erythocytes and plasma (27,28). Florini et al. (21)

noted that after the intravenous administration of a labeled steroid, this steroid was rapidly taken up by muscle where the concentration soon equalled that in blood. After two hours, the amount of steroid in the blood was less than that found in muscle, supporting the possibility that the labeled steroid or its metabolic products may be more firmly bound to muscle than to blood proteins.

The percentage of betamethasone-17-valerate excreted in the urine of the pig was 8.5%. This value is similar to that observed in man. Studies by Butler (18) have shown that urine is the main excretory route of betamethasone-17-valerate. In her studies, a patient with pemphigus, who had 20% of the body area occluded for 3 days, excreted 18.5% of the total applied dose. In our experiments, 0.72% of the total applied dose was recovered in feces and this again is in agreement with reports in man (18) where similar low percentages (1.6%) were found in feces after intravenous or oral administration of tritiated betamethasone-17-valerate.

As was mentioned earlier, the evaluation of the tissue distribution of the labeled betamethasone-17-valerate presented certain difficulties in this investigation. Quenching problems were encountered, resulting mainly from the use of NCS solubilizer, which is a potent quartenary ammonium base, tissue dissolving reagent. The yellow colour produced by the dissolved tissues also contributed to the quenching. The problems of interpretating

tissue radioactivity have been studied by Braunsberg et al. (29). The current method used to determine radioactivity in tissues is very similar to that used by Watanabe et al. (30). The main difference was in our use of NCS solubilizer rather than of hyamine hydroxide. Another example of disturbing problems encountered was the variation in counting efficiencies from tissue to tissue and from animal to animal in similar organs. Thus no detailed conclusions could be drawn from our tissue studies. The greatest activity was definitely concentrated in the liver (2.6% of total dose applied) and the total mean recovery in the other organs studied was 3.0%.

During the course of these studies, an unexpected finding appeared. Why did the peak in urinary radioactivity occur before the plasma had reached its maximum values? At this time, a full explanation of this peculiar finding is not possible. One can only speculate as to the reasons explaining this interesting phenomenon. A careful analysis of the methodology involved reveals that no relation can possibly be made between plasma levels of radioactivity and urinary levels with reference to time sequence. The reason is that plasma samples consisted of the amount of urine voided over a 24-hour period. The possibility exists that more meaningful data could have emerged from a study of urine samples obtained from an indwelling urinary bladder catheter. This approach would permit a closed correlation between urinary radioactivity and plasma levels. This study is planned for the future.

SECTION II:

ADRENAL FUNCTION STUDIES AFTER TOPICAL STEROID APPLICATION

INTRODUCTION

When first introduced into clinical practice, topical steroids were applied several times a day. This form of therapy though beneficial, gave rise to some undesirable and disturbing side effects (3,4,6,7,8,9). Similar unwanted reactions also resulted from the oral use of corticosteroids when these were administered several times a day. To minimize these side effects, Reichling and Kligman in 1961 (31) proposed an 'alternate-day' systemic corticosteroid therapy. Harter et al. (32) dramatically illustrated the decrease in overt side effects using an 'intermittent' oral corticosteroid dosage regimen; this was not associated with any loss of therapeutic efficacy. These authors gave 80 mg of prednisone orally the morning of every second day instead of 10 mg of prednisone orally four times a day. With this treatment schedule, they demonstrated that the normal diurnal variation in the urinary 17-hydroxycorticosteroid excretion was maintained. A regression of Cushingoid features was also observed in these patients along with other beneficial changes. Recently, Ackerman and Nolan (33) have found that the adrenal cortex responsiveness after alternate-day corticosteroid therapy was greater than that observed in patients who received daily steroid therapy and was equal to that of the controls. Saxena and Crawford (34) applied the Harter intermittent steroid therapy regime in cases of nephrosis and observed pronounced results in reduction of side effects.

In view of several reports of pituitary-adrenal axis suppression due to the percutaneous absorption of topical steroids, it was of interest to find out whether a similar intermittent regime of topically applied steroid therapy would also reduce systemic effects. To this end, the following working hypothesis was adopted: in the domestic pig, steroids applied to the skin on alternate-days under occlusive dressings will be associated with fewer systemic effects (as reflected in pituitary-adrenal axis suppression) than the daily topical application of steroids.

The most accurate index of adrenal suppression is considered to be the level of plasma cortisol. Thus, a sensitive method of corticoid analysis in pig plasma was needed. Man and the pig are described as having similar circulating corticosteroids, i.e., both cortisol and corticosterone (36); however, there is no data as to which preponderates in the pig. In humans, the competitive protein binding (CPB) radio-immuno-assay originally proposed by Murphy (37) has proved to be very sensitive, particularly with low levels of plasma corticoids and this method was adapted to the domestic pig.

This method utilizes the steroid binding properties of corticosteroid-binding-globulin (CBG, transcortin). Effective modifications have increased the original sensitivity one-hundredfold, making it possible to measure quantities as low as 200 pg (38). The principle behind the CPB analysis is described fully by Murphy (37). When conditions are chosen so that the CBG present

in the tracer solution has reached saturation with the tracer steroid, a dynamic equilibrium exists between radioactive steroid bound to CBG and the free steroid in the deproteinized plasma sample. The radioactive steroid may be either cortisol or corticosterone, since they are in a state of dynamic equilibrium. As the sample steroid content increases, the amount of tracer bound CBG decreases proportionately. After separation of the protein bound and unbound fractions, the bound tracer is measured. Quantitation is accomplished by measuring the displacement of the tracer caused by known concentrations of a standard steroid. The standard curve can be obtained from a plot of percentage of tracer bound against amount of steroid added; however, a more useful linear relationship is obtained if the reciprocal of the percent tracer bound is used. This is most easily achieved by plotting the time required to reach a pre-set count of radioactivity versus the amount of steroid added. This is possible since the time required to count the tritiated protein bound fraction to a pre-set number of counts increases as the bound fraction labeled with tritium decreases. Murphy (39) as well as Metivier et al. (40) and Brisson et al (41) have applied the CPB analysis method to human plasma samples.

MATERIALS AND METHODS

1. General Methodology

All pigs used in this study were first secured in restraint jackets and placed in metabolic cages for a day. The next morning, a 15 cm square area on the back was shaved and excess hair was removed by gentle brushing with sandpaper of medium grade. A control blood sample for determination of plasma cortisol, white blood cell and eosinophil counts were taken. The steroid cream[1] was then applied and kept under Saran Wrap occlusion covered with a protective dressing.

Three regimes of therapy with a treatment period of four days were compared. In the first group of animals, 40 g of Celestoderm Cream[1] was applied at 9:30 a.m. every second day during a four day treatment period. The second group received 20 g daily and the third regime of therapy consisted of twice-daily applications of 10 g of Celestoderm Cream. Any cream remaining from the previous applications was gently wiped off before a new application was made. At the end of the four day treatment period, the study was extended for another two days of observation.

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[1] Celestoderm Cream 0.1% supplied by Schering Corporation of Canada. The active steroid component of this cream is Betamethasone-17-Valerate.

Daily blood samples were taken from the tail vein. Eosinophil and white blood cell counts were done immediately, while 0.5 ml of plasma was stored at $-10^{o}C$ for later determination of the cortisol levels by the Murphy Method.

2. Animals

Domestic piglets of either sex, weighing from 25-36 pounds, were used in this study. Throughout the duration of the experiment, the pig was kept in a jacket specifically designed to facilitate the collection of blood samples and the handling during the applications of steroid cream. The pig remained relatively still when suspended in the jacket, thus eliminating the need for an anesthetic. The jacket is illustrated in Figure 9.

3. Murphy Semi-Micro Method for Cortisol Determination (42)

a) Reagents

i) Ethanol, absolute (Fisher Scientific).

ii) Florisil 60-100 mesh[1] (Fisher Scientific).

The Florisil was first washed with distilled water until the supernatant was clear and free from small particles. Then it was spread on a tray and dried slowly in a warm oven.

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[1] A silicate used for adsorption.

FIGURE 9. Restraining jacket used to facilitate handling of pigs during the application of the steroid cream and the collection of blood samples.

iii) Modified Bray's Solution[1].

b) Standards and Stock Solutions

i) Cortisol[2]. A 1 mg/ml stock solution in ethanol. An aliquot from this stock solution was diluted to 100 ng/ml with pure ethanol for use in each assay.

ii) Corticosterone-1,2-^{3}H. Nuclear Chicago Corporation provided this labeled steroid with a specific activity of 39.1 Ci/mMole or 113 μCi/μg. The solvent (methanol) was evaporated from the original bottle containing the tritium labeled steroid. Sufficient ethanol is then added to give a final activity of 10 μCi/ml.

iii) Corticosteroid Binding Globulin (CBG) Stock. Add 5 ml of fresh human plasma to 50 ml of distilled water in a volumetric flask. Then add 0.6 ml of the 10 μCi/ml solution of corticosterone-1,2-^{3}H and make to 100 ml with distilled water.

c) General Procedure

i) Collection of samples.

- Collect 1.5 ml of blood at 9:30 a.m. from the tail vein of the pig, allowing the drops of blood to drip into a heparinized tube.
- Centrifuge the blood at 3000 rpm in a clinical centrifuge.

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[1] Modified Bray's solution: 1 gallon dioxane
400 gm naphthalene
28 gm PPO
1.2 gm POPOP.

[2] Cortisol (Hydrocortisone) obtained from Sigma Chemicals.

- Pipette 0.5 ml of plasma into a test tube and store at -10°C. It keeps for several weeks at this temperature.

ii) Deproteinization.

- Pipette 1.00 ml of ethanol to the sample tubes containing 0.5 ml plasma samples.
- Mix and centrifuge for 4 minutes at 3000 rpm.
- With a 1 ml measuring pipette, remove duplicate 0.3 ml aliquots of supernatant. At this time, prepare standard cortisol samples by adding to 4 tubes 0.1, 0.2, 0.3 and 0.4 ml of the 100 ng/ml solution, thus giving 10, 20, 30 and 40 ng of cortisol respectively. A fifth tube is taken for the blank.
- Evaporate to dryness the contents of all tubes (standards and samples) at 45°C in a stream of air.

iii) Quantitation.

- Equilibration
  - Once dry, add 1 ml of CBG stock solution to the residue in each of the test tubes.
  - Shake the test tubes by hand for 30 seconds.
  - Add 0.5 ml of CBG stock solution to 10 ml of Bray's solution in a scintillation bottle. This bottle will serve as a control at a later stage.
  - Incubate all tubes at 45°C for 10 minutes.
  - Shake again.
  - Cool tubes in a cold bath (10°C) for at least 10 minutes.

The assay may be interrupted for up to one hour at this stage.

- Separation
  - Add a special "Murphy spoonful" (38) of Florisil (∿160 mg) to each test tube.
  - Shake mechanically for 2 minutes.
  - Return to the bath for 10 minutes to allow the Florisil to settle.
  - Pipette 0.50 ml of the supernatant to 10 ml of Bray's solution in a scintillation bottle.

iv) Calculation

- For each bottle, measure the time required for 10,000 counts in the scintillation counter.
- Plot the time required to count the standards against the standard weights of cortisol. This is the "standard corticoid curve".
- The amount of corticoid in the plasma samples can then be read from this standard curve. The number of nanograms in the sample is numerically equal to the number of micrograms of corticoid per 100 ml of plasma (Fig. 10).

4. Hematology

Both white blood cell counts and absolute eosinophil counts were carried out by the standard methodology described by Wintrobe (43).

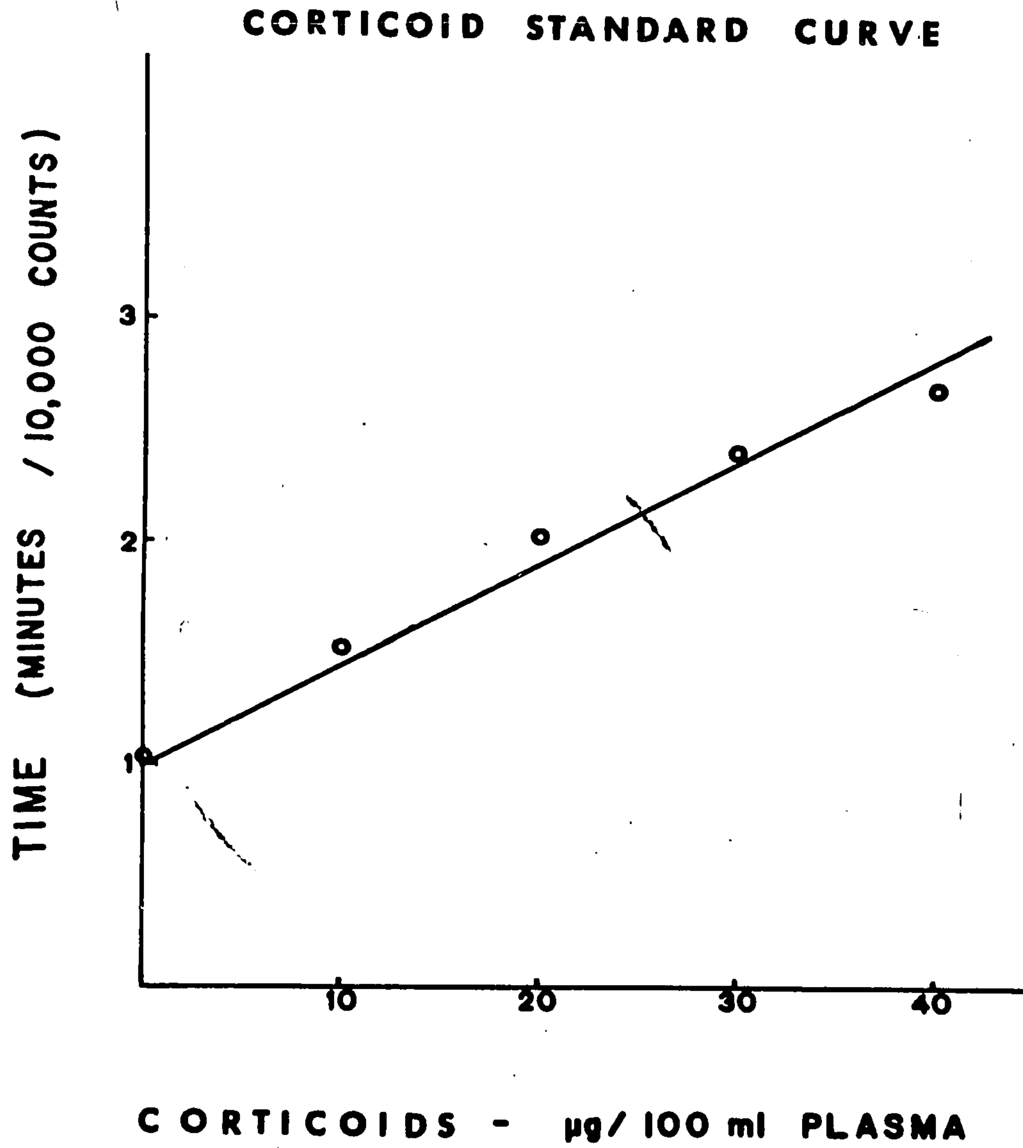

FIGURE 10. Corticoid standard curve.

RESULTS

1. Adrenal Suppression by Dexamethasone

Before investigating the effects of intermittent topical steroid therapy on adrenal function, a study was made of the Murphy "semimicro" technique in the evaluation of dexamethasone-induced adrenal suppression in the domestic pig. A pig was injected intramuscularly with four mgs of dexamethasone* (in the form of Decadron, 4 mg/cc, from Merck, Sharp and Dohme) at 11:20 a.m. A control sample of plasma (4 mcgs of corticoids/100 ml of plasma) was taken before the injection of Decadron. Subsequent samples for corticoid determinations were taken at 2:00 p.m. and 8:00 p.m. on that same day, and then 2:00 p.m. on the subsequent two days. The results are shown in Fig. 11. It is evident that the adrenal suppression which produced plasma corticoid levels as low as 0.2 and 0.8 ug/100 ml of plasma can be measured by this method. A return to normal values was seen within 24 hours, and an apparent rebound phenomenon was recorded at 48 hours.

2. Comparison of Different Regimes of Therapy

Having established the usefulness and sensitivity of this method for plasma corticoid determination in the domestic pig, we then undertook the second phase of our study, namely a comparison of different regimes of therapy using topical steroids with reference to various parameters of adrenal function.

Figure 12 illustrates the fluctuations in the levels of plasma corticoid for each individual pig throughout the duration

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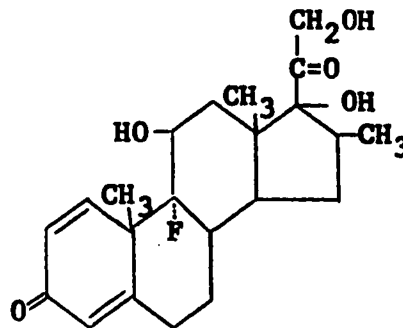
* See chemical structure page 54a.



16α-methyl-9α-fluoroprednisolone

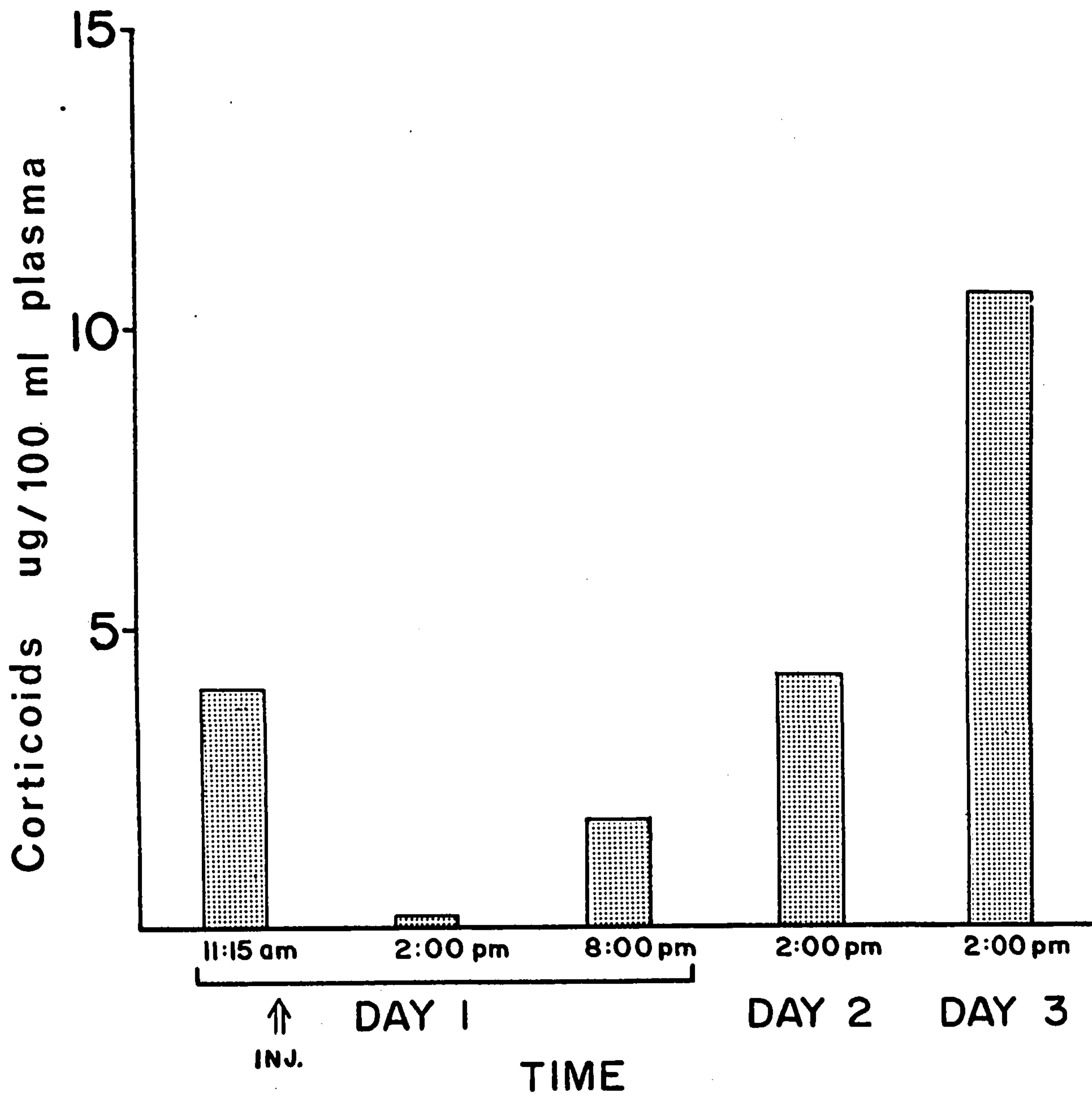
(DEXAMETHASONE)

(Decadron by Merck, Sharp and Dohme)

FIGURE 11. Dexamethasone suppression test in the pig.

FIGURE 11

DEXAMETHASONE SUPPRESSION TEST IN THE PIG



of the experiment. The plasma corticoid value obtained at 9:30 a.m. after a 24-hour period of adaptation was considered as the control value and is expressed as 0 in Figure 12. All other plasma corticoid values are expressed as a percentage deviation from control. This figure contrasts the results obtained in two groups of pigs subjected to different regimes of therapy. The upper three graphs refer to the first group in which 40 g of Celestoderm cream (0.1%) was applied on the morning of day 0 and day 2 and removed entirely on the morning of day 4. This regime of topical steroid therapy on alternate days produced a decrease in endogenous plasma corticoids levels; but this lowering was not constant. In each of the three pigs studied, at varying times during the treatment period, levels higher than the control value were recorded. In contrast, the lower three graphs represent the second group in which 20 g of Celestoderm cream was applied daily from day 0 - day 3 inclusively and the steroid cream was removed entirely on the morning of day 4. The results obtained in this group indicate a definite lowering of plasma corticoid values below the control value; however, in this group the decrease is constant throughout the treatment period and it is only on day 5 that levels begin to increase once again. The maximum negative deviation from control values occurred in the first group in pig number 785 on the morning of day 3 and was -56.6%. In a second group, pig number 795 showed the greatest negative deviation from the control value of plasma corticoids on the morning of the fourth day and this was recorded as -91%.

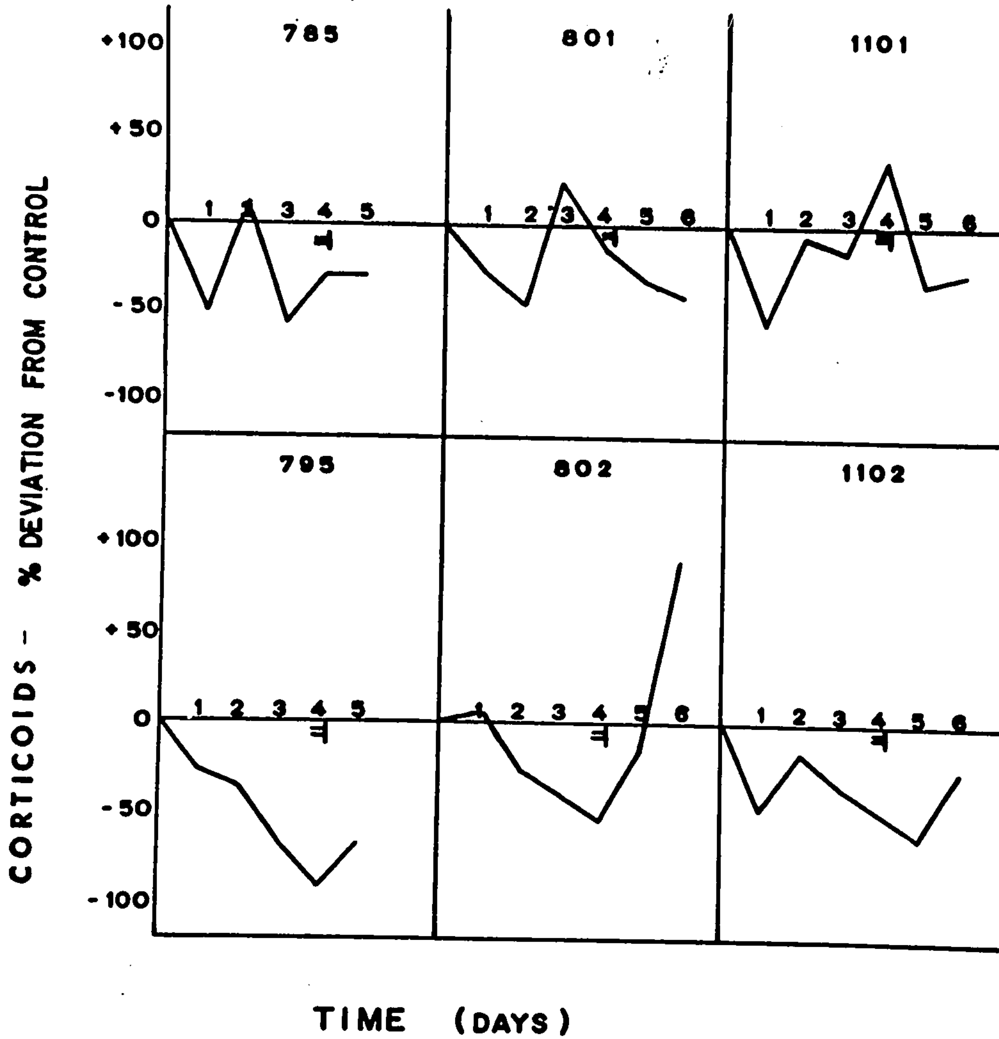


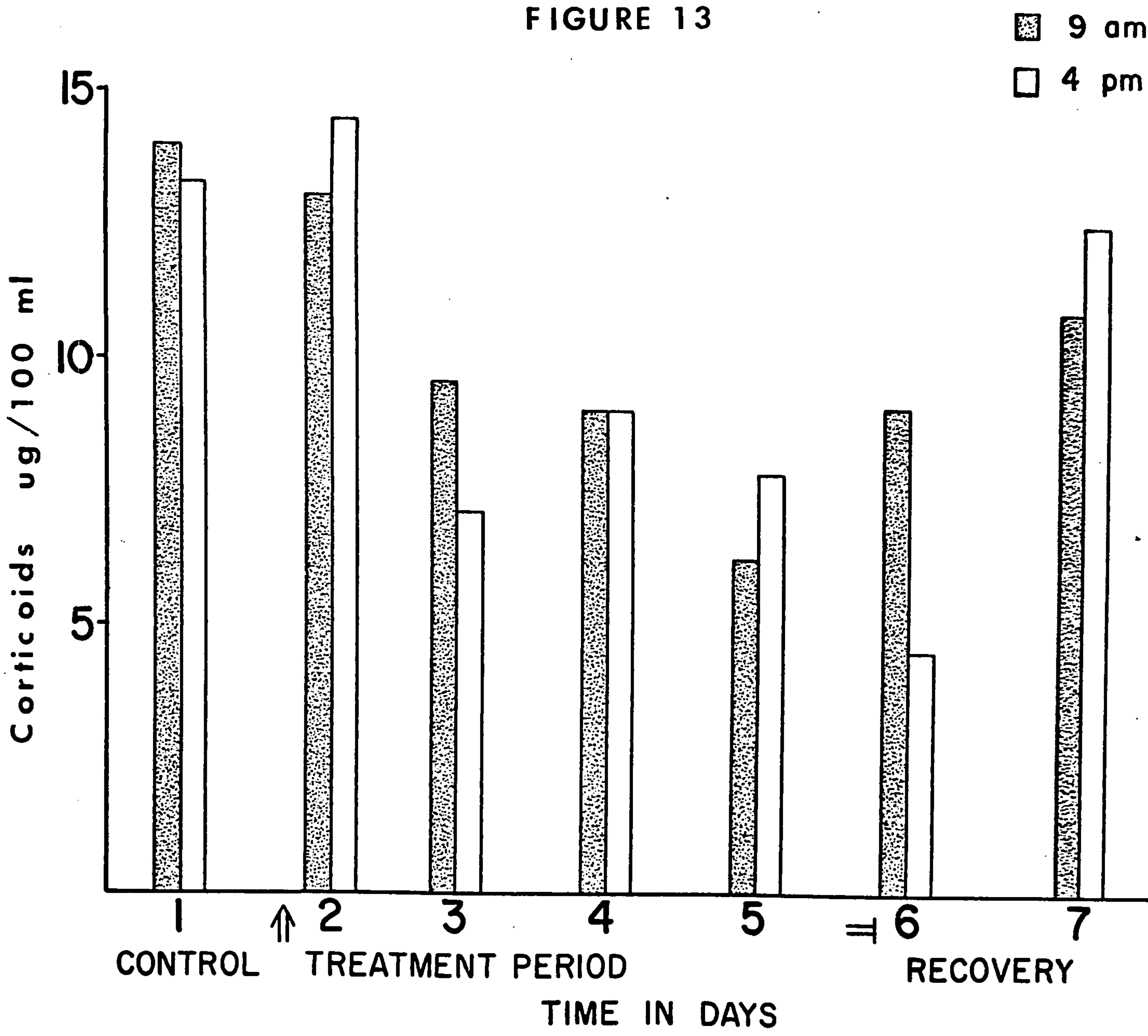
FIGURE 12. Fluctuations in levels of plasma corticoids, contrasting the results in two groups of pigs subjected to different regimes of therapy (see page 54 in text).

Using the same methodology, a third regime of therapy was studied. Because of illness in this group of animals, only one case is reported. In this pig, 10 g of Celestoderm cream 0.1% was applied to the back at 9:00 a.m. and 4:00 p.m. on four successive days. Plasma corticoid values were also determined at these times. After removal of the steroid cream, the observation period was continued for 2 days. Figure 13 illustrates the results obtained in this experiment. A definite adrenal suppression is observed with a decrease in endogenous corticoid values within 48 hours after removal of the steroid cream. This observation is similar to that obtained in the group where the Celestoderm cream was applied daily. In both instances the adrenal suppression obtained is constant and at no time during the treatment period do the corticoid levels go above control values.

Figure 14 illustrates the changes that occur in the hematological picture of individual pigs. The eosinophils and white blood cells are expressed as absolute counts per cu mm. Forty grams of Celestoderm cream 0.1% were applied to pigs 801 and 1101 on day 0 and on day 2 and completely removed on the morning of day 4. Similarly, 20 g of Celestoderm cream 0.1% were applied to pigs 802 and 1102 daily from day 0 to day 3 and removed on the morning of day 4. As expected, in all pigs a general neutrophilia was observed. In 3 out of 4 cases, the white blood cell count/cu mm had more than doubled by day 4. However, the variations in eosinophil counts were not quite as clear. Pigs numbers 801 and 802 showed minor changes during the treatment period. The

FIGURE 13. Plasma cortisol levels obtained in one pig using a third regime of therapy, i.e. celestoderm cream applied twice a day.

FIGURE 13



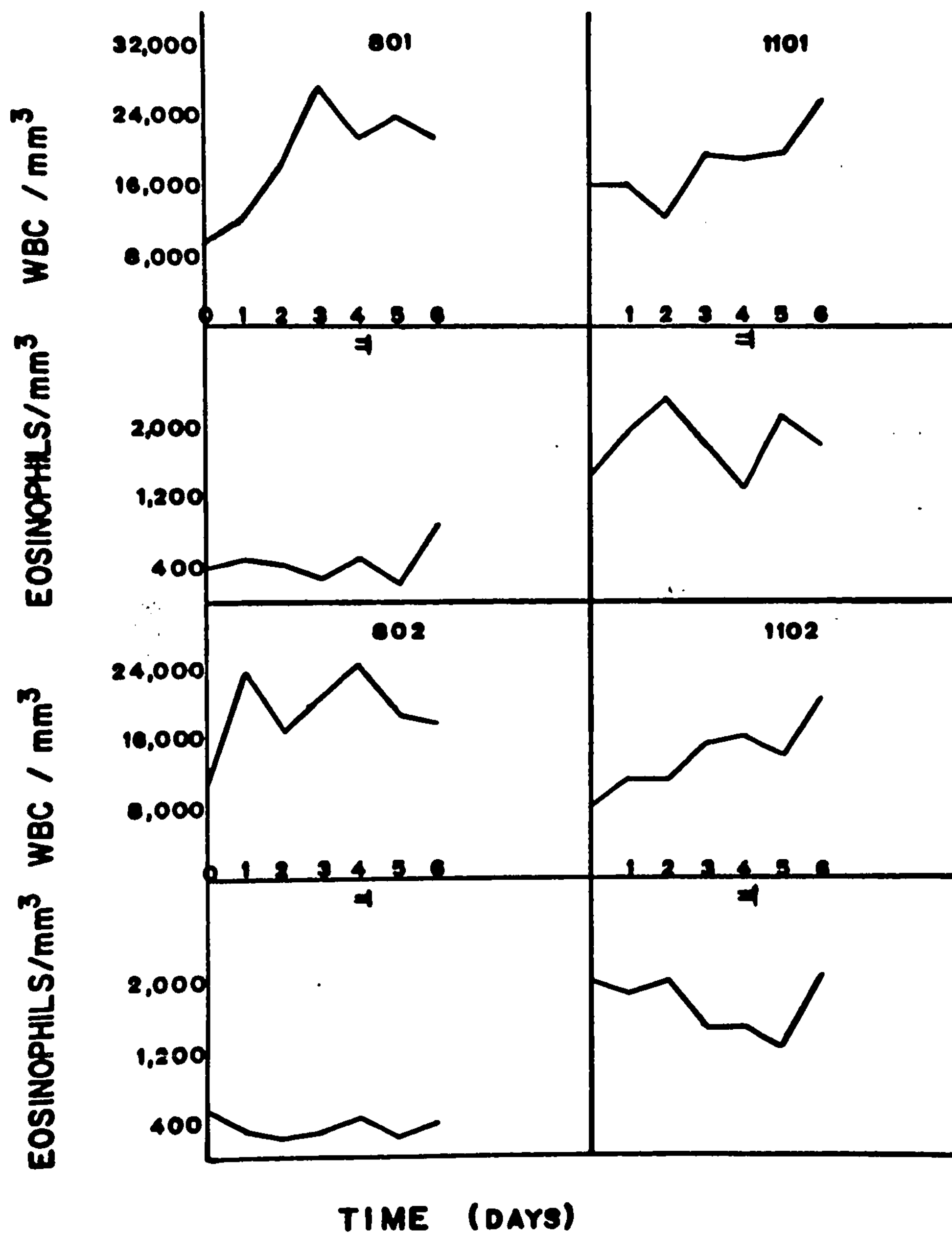


FIGURE 14. Hematological changes (eosinophil and white blood cell counts) for each pig after different regimes of application of Celestoderm cream 0.1%.

fluctuations in the eosinophil count of pig number 1101 was erratic. Only one pig (number 1102) out of the four showed a definite decrease in the amount of eosinophils per cu mm from the original value of 2,000 down to a low of 1,311 on the morning of day 5. This decrease of eosinophils was fairly constant with a final increase beyond control values on the morning of the 6th day.

DISCUSSION

The results of the second part of our studies indicate that some degree of pituitary-adrenal axis suppression occurs when a total daily dose of 20 g of Celestoderm cream (0.1%) is applied under occlusion on the back (15 x 15 cm area) of the domestic pig. Analysis of these results is also of interest. In the group of pigs under-going alternate day topical steroid cream therapy, lowering of endogenous corticoid levels in plasma did occur but this was not constant. In each pig of this group, there was a point during the treatment period at which the plasma corticoid levels were higher than the control values. Plasma corticoid values obtained on the morning of day 1 and 3 of the pigs number 785 and 1101 indicate adrenal suppression since the bi-phasic curves in these animals show low levels at the end of 24 hours after application of the topical steroid. Conversely, at day 2 and 4, the plasma corticoid levels are increased approximately 48 hrs after the application of the steroid cream suggesting a rebound in adrenal function. A comparison of the results obtained in pigs given applications on alternate days with those of three other pigs submitted to applications of 20 g of Celestoderm cream 0.1% daily, reveals some interesting findings.

In the latter experiments, the lowering in endogenous plasma corticoid levels (reflecting pituitary-adrenal suppression) is more constant, and the decrease persists throughout the treatment period. It is only on the first and on the second day of recovery (day 5 and day 6), that plasma corticoid levels are increased. An exception to this general finding is pig number 1102

which did show a mild increase in plasma corticoid levels on the morning of day 2; however this increase failed to reach control values. These findings indicate that the daily application of topical steroids evokes a more persistent pituitary-adrenal axis suppression as manifested by decreasing levels of endogenous plasma corticoid. Similar changes in plasma corticoid levels were also observed in one additional pig which was treated for 4 days with 10 g of Celestoderm cream 0.1% applied twice a day on every other day.

A finding of technical importance also emerged from these studies. The Murphy "semimicro" competitive protein binding radio-assay had proved to be a rapid and sensitive method for determining plasma corticoid levels in the domestic pig. The usefulness of this method for the determination of plasma corticoid values in other experimental animals and in man has been well demonstrated (44).

The hematological parameters in this study did not appear to be as sensitive an index of adrenal function as the plasma cortisol determinations.

As expected, high dosages of exogenous steroid produced a neutrophilic response in all four pigs. No marked difference in this neutrophilic response was observed between the two groups with different regimes of therapy. In two out of the four pigs studied, the eosinophils showed very minimal changes, the third animal showed a definite decrease in the amount of eosinophils per cu mm throughout the treatment, with a return back to normal levels during the recovery period. The fourth pig showed an

initial increase followed by a marked decrease. Here again, no consistent significant differences between the two groups under study could be seen.

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