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Studies on Intravenously Infused Dexamethasone in Sheep

Z. Leish and B. A. Panaretto

Division of Animal Production, CSIRO,
P.O. Box 239, Blacktown, N.S.W. 2148.

Abstract

The metabolism of [1,2(n)-^{3}H]dexamethasone intravenously infused for periods of 4 and 8 days was examined in four Merino wethers.

On average 85·6±2·8 (s.e.m.) % of the total dose was recovered, 56·2±5·1 and 29·4±3·3% being excreted in urine and faeces respectively. In total 12·4% of the dose was associated with the unconjugated steroid fraction which represented 22% of the total urinary radioactivity.

Thin layer chromatography of the urinary unconjugated fraction revealed, apart from dexamethasone, five major and three minor radioactive components designated as I, III, IV, V, VIII and II, VII, IX respectively. Approximately 83% of the radioactivity associated with the unconjugated fractions was found in constituents more polar than dexamethasone with the radioactivity mainly confined to the principal metabolite IV, $R_f = 0{\cdot}24$ (dexamethasone, $R_f = 0{\cdot}45$; chloroform: formamide 50:1). The urinary constituent VI, $R_f = 0{\cdot}37$, was detected only during the second half of the 8-day infusion with the peak of excretion on day 6.

During the first 30 h of [^{3}H]dexamethasone administration the plasma radioactivity reached the level which remained relatively constant throughout the infusions. The initial high level of radioactivity detected in the plasma unconjugated fraction as well as dexamethasone during the first 24 h infusion declined over the next 2 days; this was followed by a small increase during the final period of infusions.

The radioactivity of the plasma unconjugated fraction was distributed, apart from dexamethasone, into six constituents (I, III, IV, V, VIII and IX) which when analysed by thin layer chromatography appeared to be qualitatively similar to those found in urine.

These results led to the conclusion that one or more products of dexamethasone metabolism might be biologically active and thus potentially important in inhibiting wool fibre growth in sheep.

Introduction

Dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione) is one of several potent synthetic glucocorticoids widely used clinically for their anti-inflammatory, anti-allergic and antirheumatic properties. Although dexamethasone is also extensively used to induce parturition in cattle and sheep (Fylling *et al.* 1973; Allen and Herring 1976) little attention has been given to its metabolism.

In early experiments dexamethasone had been observed to depress wool growth in sheep (Ferguson *et al.* 1965) but only recently was its efficacy as a defleecing agent evaluated and its plasma concentrations measured during its administration (Panaretto *et al.* 1975; Panaretto and Wallace 1978*a*).

In animals infused intravenously with dexamethasone over a period of 8 days, Panaretto and Wallace (1978*a*) reported reproducible plasma concentration profiles with a peak during the first 48 h of infusion followed by falling levels with a small increase during the final days of infusion. Subsequently it has been found that the

slow intravenous infusion of glucocorticoid analogues over an 8-day period caused wool shedding in a greater proportion of the total fibres than when the same dose was infused over a shorter period (Panaretto and Wallace 1978*b*).

In this paper we have compared some aspects of the metabolism of dexamethasone during two different infusion periods. Particular emphasis was given to the relation between plasma concentration profiles of the parent molecule and its metabolites during the 4- and 8-day infusion periods. Furthermore the qualitative, as well as quantitative, evaluation of metabolites and their subsequent isolation and identification would enable us to assess their inhibitory effects on wool fibre growth.

Materials and Methods

The Sheep, their Housing and Nutrition

Merino wethers, about 1½ years old, weighing 24–30 kg and having a fleece approximately 3 cm in depth, were housed individually in metabolism cages in air-conditioned quarters (22°C). The daily ration was 600 g of a mixture of chopped lucerne hay and ground oats (1:1) given as one meal. Drinking water was provided *ad libitum*.

Infusates, Infusion Technique and Collection of Blood, Urine and Faeces

[1,2(n)-^{3}H]Dexamethasone (specific activity 28 Ci mmol^{-1}, 71 mCi mg^{-1}; Radiochemical Centre, Amersham, England; reported purity 97–98%) and non-radioactive dexamethasone (Roussel UCLAF, France) were checked for radioactive and chemical purity by chromatography in several solvent systems before use (see p. 375).

Infusions were made using Harvard infusion pumps (series 932, Harvard Apparatus Inc., U.S.A.) through polyethylene tubing i.d. 0·86 mm, o.d. 1·52 mm (Dural Plastics, Australia) at a rate of 1·9 ml h^{-1}. The dose rates and duration of continuous intravenous infusions are given in Table 1.

Table 1. Administration of [^{3}H]dexamethasone and unlabelled dexamethasone

Sheep No.	Body weight (kg)	Daily rate of infusion (μCi kg$^{-0.75}$)	(mg kg$^{-0.75}$)	Period of administration (days)	Total volume administered (ml)	Total dose (μCi)	(mg)
1564	25·0	6·48	1·90	4·25	195·3	307·9	90·3
1355	24·2	3·50	0·99	8·19	384·0	312·7	88·5
1572	29·7	23·55	1·83	4·00	178·5	1198·2	93·1
1916	28·8	12·47	0·99	7·75	359·0	1201·3	95·4

The mean quantity of dexamethasone given in these experiments was 7·8 mg kg$^{-0.75}$. This approximated the dose, 8·5 mg kg$^{-0.75}$, which was reported to cause the shedding of the whole fleece in the majority of sheep so treated (Panaretto and Wallace 1978*a*).

A mixture of radioactive and non-radioactive dexamethasone was dissolved daily in sterile pyrogen-free saline:ethanol solution (7:3, v/v) and administered to sheep by continuous intravenous infusion via a catheter inserted 10 cm into the jugular vein in the direction of the heart.

Heparinized blood samples (10–50 ml) were obtained twice daily at about 0900 and 1600 h either by venipuncture or from a cranially directed jugular catheter placed in the contralateral vessel.

Any haemoconcentration effect due to the withdrawal of large volumes of blood was monitored by the regular observation of haematocrit values using the method of Shutt and McDonald (1965). The blood was separated by centrifugation, 1-ml samples of plasma in duplicates were taken for the radioactivity measurements, and the remainder was stored at −20°C until further analysis.

Urine was collected into dry ice-chilled bottles which were changed twice daily. A screen prevented contamination of urine with faeces. The total radioactivity of urine samples was measured throughout experiments and remaining urine was stored at −20°C. Faeces were collected into plastic bags over periods of 24 h.

Radioactivity Measurements

A standard mixture containing p-terphenyl (0·3%, w/v) and 1,4-di-(2(4-methyl-5-phenyloxazolyl))-benzene (0·01%, w/v) in toluene was used for counting non-aqueous samples in a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3375).

Aqueous samples (1 ml) were diluted with 6·5 ml of distilled water and counted in a scintillation mixture consisting of six parts of Brydet X10 (Bryce and Co., Australia) and seven parts of the toluene-based scintillant described above. [^{3}H]n-Hexadecane was used as an internal standard to correct all samples for quenching.

Dry ground faeces were combusted by the oxygen flask technique (Downes and Till 1963). The combustion products were shaken with 20 ml of methyl cellosolve and 10 ml of the resulting solutions were mixed with 9 ml of the scintillant and counted for their radioactivities.

Extraction Procedure

Measured aliquots of plasma (15–25 ml) were washed two times with one volume of n-heptane. Sodium sulphate was added to the aqueous phase which was then extracted with chloroform (Haque *et al.* 1972) and subsequently with ethyl acetate. The chloroform and ethyl acetate extracts were concentrated under dry nitrogen at room temperature to a final volume of 0·3–1·0 ml. Using this extraction procedure the mean recovery of [^{3}H]dexamethasone added to plasma was 83%.

Urinary unconjugated steroids were extracted into a neutral fraction with ethyl acetate according to Franz *et al.* (1961) The ethyl acetate extracts were taken to dryness in a rotary evaporator and the residues were dissolved in an ethyl acetate:methanol mixture (1:1, v/v) followed by methanol to a final volume of 20 ml.

Chromatography

In our experiments a number of chromatographic systems for separation of dexamethasone metabolites was tested, e.g. silica gel-impregnated glass fibre sheets (ITLC-SG, supplied by Gelman Instrument Company, U.S.A.) using chloroform:formamide (50:1), chloroform:acetic acid (9:1) or chloroform:methanol (97:3) as solvent systems; t.l.c. Merck silica gel 60 (F_{254}) plates or plastic-backed silica gel sheets No. 13179 (Eastman Kodak Co., U.S.A.) developed in chloroform:dichloromethane:ethanol (8:1:1) or dichloromethane:acetone (8:2). Paper chromatography was performed on Whatman 3MM paper in ethyl acetate:chloroform:methanol:water (1:3:2:2) with the lower layer used as a mobile phase.

The best resolution was obtained on ITLC-SG sheets using chloroform:formamide (50:1) and this system was used in our investigations.

Chromatography of the urine and plasma extracts was carried out by spotting or streaking small aliquots of 10–20 μl and 100 μl respectively on ITLC-SG sheets with [^{3}H]dexamethasone and non-radioactive dexamethasone used as reference standards. The reference standards of dexamethasone were detected by short wave ultraviolet light (Δ^4-3 ketones) or spraying with alkaline blue tetrazolium (dehydroxyacetone side chain) (Recknagel and Litteria 1956).

The proportion of radioactivity retained in individual radioactive peaks was measured by cutting each developed sheet into 0·5 by 2·8 cm strips and counting each strip for radioactivity in the toluene-based scintillant. In some cases each strip was eluted with ethyl acetate:methanol mixture (1:1) or ethanol before counting. The radioactivity data thus obtained showed that no significant error was made by using the former procedure in calculations. The presented data represent the mean values for two to four separations.

Effects of Dexamethasone on Fibre Shedding

The effects of dexamethasone on wool fibre shedding were classified in a similar way to that used by Panaretto and Wallace (1978*b*). Briefly a shed wool fibre was defined as one that lay outside the fibre canal. The proportions of shed to continuous fibres which were observed, and included in our results, were present on all body regions. These estimates were purely subjective.

Results

The pattern of urinary and faecal excretion of ^{3}H label in 4- and 8-day infusions is shown in Figs 1*a* and 1*b* respectively. In total 85·6$\pm$2·8% of infused radioactivity was recovered in the urine and faeces within 2 days after the end of infusions. The

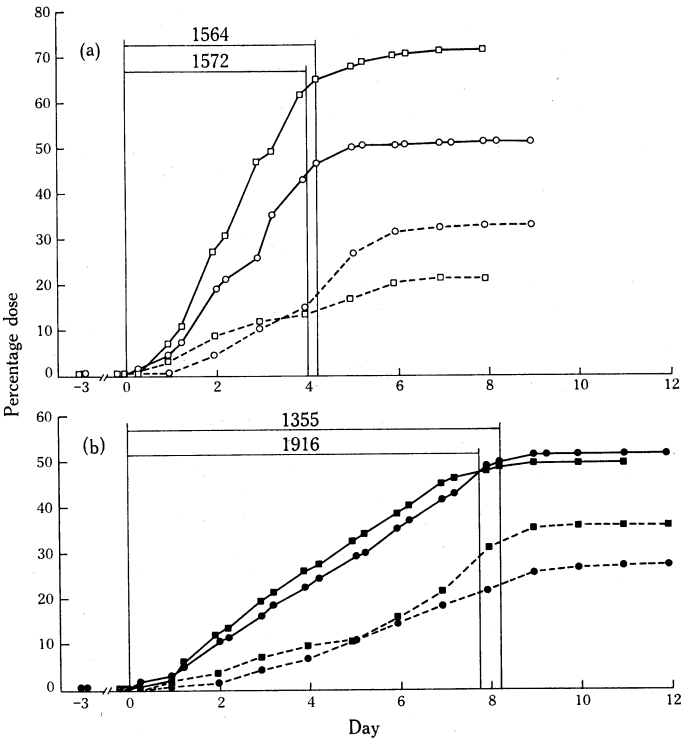

Fig. 1. Cumulative urinary and faecal excretion of radioactivity during (*a*) the 4-day infusion of [^{3}H]dexamethasone in sheep 1564 (○) and 1572 (□), and (*b*) the 8-day infusion of [^{3}H]dexamethasone in sheep 1355 (●) and 1916 (■). — Urinary excretion. - - - Faecal excretion.

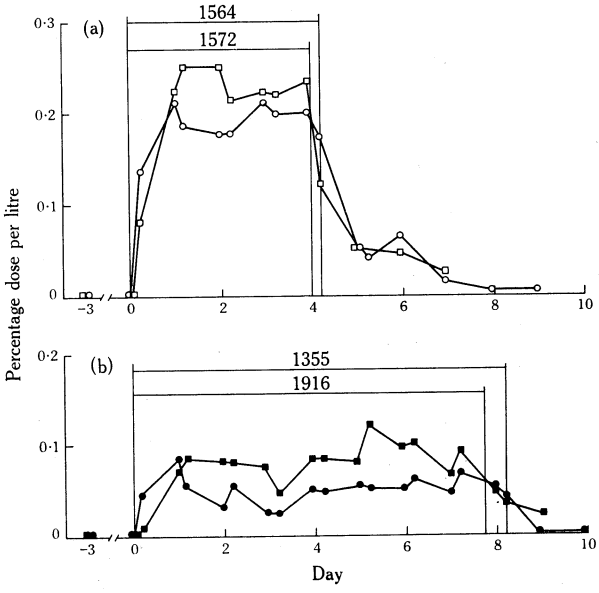

Fig. 2. Percentage of the total dose in 1 litre of plasma during (*a*) the 4-day infusion of [^{3}H]dexamethasone in sheep 1564 (○) and 1572 (□), and (*b*) the 8-day infusion of [^{3}H]dexamethasone in sheep 1355 (●) and 1916 (■).

urinary and the faecal excretion averaged 56·2±5·1 and 29·4±3·3% of the total dose respectively. In sheep No. 1572 a higher proportion of the dose was excreted in the urine (71·6%) and less in the faeces (21·2%) compared with that in the other sheep.

The level of radioactivity in plasma was measured throughout the infusions (Figs 2*a* and 2*b*). In all sheep the maximal level of radioactivity was reached during the first 30 h of infusion. In three out of four sheep plasma radioactivity then remained relatively constant throughout the infusions. In the fourth animal, sheep 1355, radioactivity declined during the 2nd and 3rd days of infusion before reaching relatively stable levels.

All urine samples collected were analysed for the presence of unconjugated steroids. The radioactivity found in the unconjugated fraction steadily increased over the period of infusions. 12·5 and 12·3% of the total dose was accounted for in the unconjugated fraction extracted from urine of animals infused for 4 and 8 days respectively. These values represented on average 22% of the total urinary radioactivity.

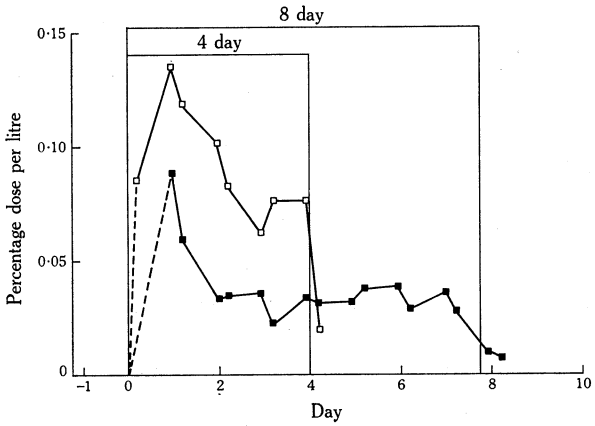

Fig. 3. Percentage of the total dose in 1 litre of plasma associated with the unconjugated fraction during the 4- and 8-day infusions of [^{3}H]dexamethasone in sheep 1572 (□) and 1916 (■) respectively.

The curves representing radioactivity in the plasma unconjugated fraction over 4- and 8-day infusion periods are presented in Fig. 3. Peak radioactivity concentrations characterized the first 24 h of the 4-day infusion and then they steadily decreased over the next 2-day period. Slightly higher values were again recorded on the last day of infusion. A similar pattern was observed during the first half of the 8-day infusion, and this was followed by relatively steady levels of radioactivity throughout the second half of the infusion.

Further examination of all unconjugated urinary fractions by thin layer chromatography revealed, apart from dexamethasone ($R_f = 0{\cdot}45$; chloroform: formamide 50:1), five major (I, III, IV, V and VIII) and three minor components (II, VII and IX). The constituent designated as VI ($R_f = 0{\cdot}37$; chloroform:formamide 50:1) was detected only in the second half of the 8-day infusion. The typical separation of the radioactivity into individual peaks is illustrated in Fig. 4.

In total 83% of the unconjugated fraction was accounted for in the radioactive components with a polarity greater than that of dexamethasone. Throughout the infusions the principal radioactive peak IV ($R_f = 0{\cdot}24$; chloroform:formamide 50:1) was associated with more than 50% of the radioactivity found in the unconjugated fraction.

The relative distribution of ^3H label in the major radioactive constituents of the urinary unconjugated fraction is presented in Figs 5*a* and 5*b*. The cumulative percentages of the radioactivity excreted as metabolites IV and V are expressed as one value due to an imperfect separation. It was estimated that approximately 10% of the radioactivity of the unconjugated fraction was confined to peak V.

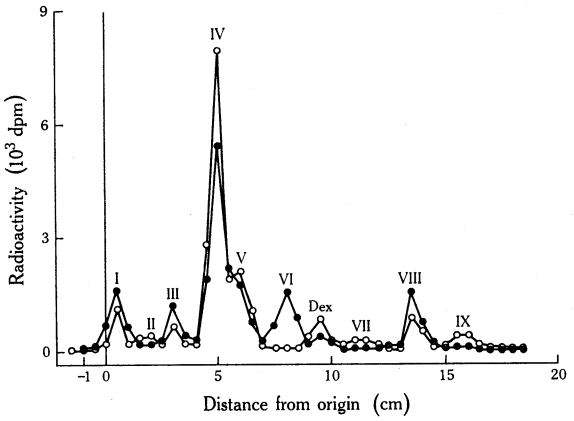

Fig. 4. The chromatographic pattern of radioactivity in the ethyl acetate extract of urine from sheep 1564 (○) and 1355 (●) infused with [^{3}H]dexamethasone (Dex) for 4 and 8 days respectively. Urine samples were collected on day 1 (sheep 1564) and on day 5 (sheep 1355). (ITLC-SG sheets were developed in chloroform:formamide 50:1, v/v.)

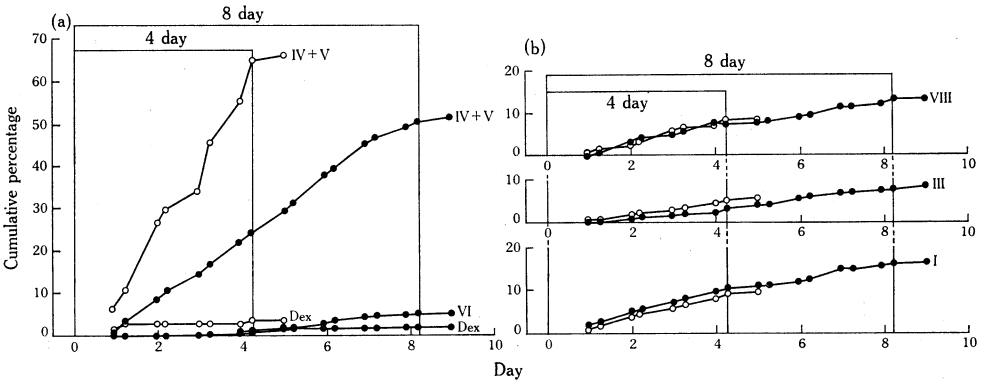

Fig. 5. Radioactivity found in the individual components expressed as the cumulative percentages of the total urinary unconjugated fraction during the 4- and 8-day infusions of [^{3}H]dexamethasone in sheep 1564 (○) and 1355 (●) respectively. The urinary unconjugated fraction was separated on ITLC-SG sheets developed in chloroform:formamide (50:1, v/v) solvent system into (*a*) dexamethasone (Dex), components IV + V and VI, and (*b*) components I, III and VIII.

In both 4- and 8-day infusions a small proportion of radioactivity of the unconjugated fraction (<3%) was excreted as the component with R_f corresponding to the reference standard of dexamethasone. The only qualitative difference between the 4- and 8-day infusions with respect to urinary output of the radioactive components was that constituent VI excreted in the second half of the 8-day infusion (Fig. 5*a*) was not detected during the 4-day infusion.

It is interesting to note the differences in the distribution of radioactivity in individual peaks, mainly peaks IV + V (Fig. 5*a*) and peak I (Fig. 5*b*), during the 4-day infusion compared with those during the 8-day infusion.

The free steroid fractions extracted from plasma were analysed for the presence of components already detected in urine. The results are presented in Figs 6 and 7. The original peak of radioactivity associated with dexamethasone was followed by a

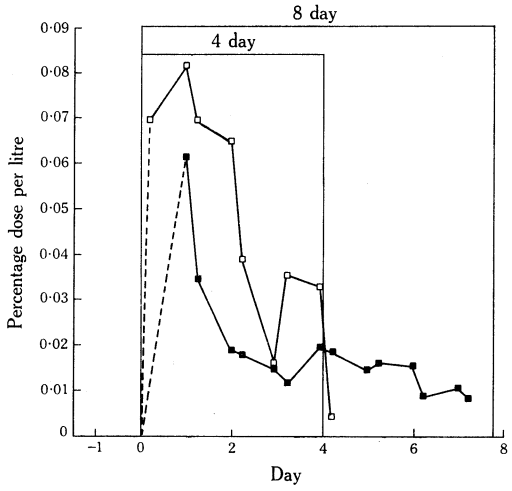

Fig. 6. Percentage of the total dose in 1 litre of plasma associated with dexamethasone during the 4- and 8-day infusions of [^{3}H]dexamethasone in sheep 1572 (□) and 1916 (■) respectively.

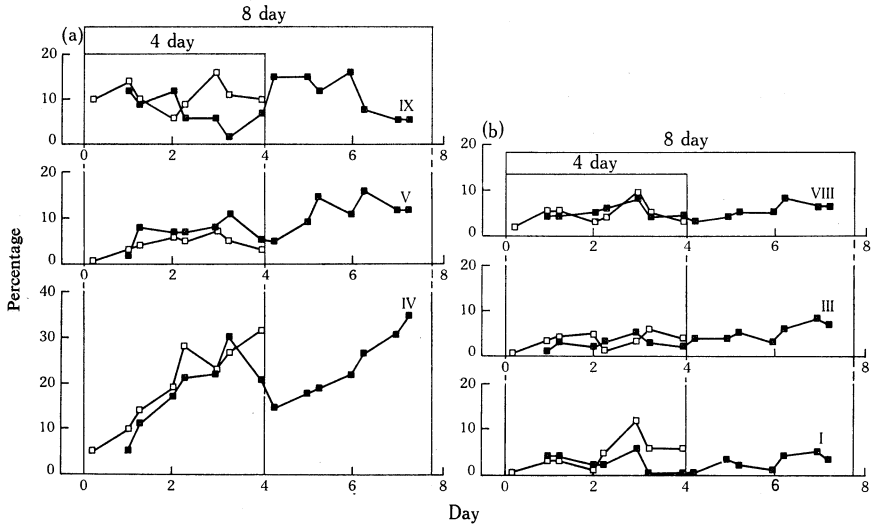

Fig. 7. Radioactivity found in the individual components expressed as the percentage of the plasma unconjugated fraction during the 4- and 8-day infusions of [^{3}H]dexamethasone in sheep 1572 (□) and 1916 (■) respectively. The plasma unconjugated fraction was separated on ITLC-SG sheets developed in chloroform:formamide (50:1, v/v) solvent system into (*a*) components IV, V and IX, and (*b*) components I, III and VIII.

steep decline over a period of 2 days (Fig. 6). Slightly higher values were observed on the last day of the 4-day infusion and in the second half of the 8-day infusion. At the end of both infusions 33% of the ^{3}H label accounted for in the plasma unconjugated fraction was found in the principal polar constituent IV (Fig. 7*a*). It is

significant that one of the major plasma components, IX (16% of the radioactivity of the free fraction), was barely detectable in the urine fraction (<3%).

During the 4-day infusion the level of component V reached about 7% of the radioactivity associated with the free fraction compared with 16% detected during the last 3 days of the 8-day infusion.

In these experiments none of the sheep treated with dexamethasone shed *all* their wool fibres, so casting their fleeces. However, in all cases inspection of the wool approximately 3–4 weeks after infusion showed that animals infused for 8 days had a much greater proportion of shed fibres relative to the continuous ones than those animals treated for 4 days.

Discussion

The results of this investigation indicate that intravenously infused dexamethasone undergoes several metabolic transformations which lead mainly to metabolic products more polar than the parent drug. In this work only free steroids were studied and it was found that in addition to dexamethasone the six radioactive constituents detected by thin layer chromatography in plasma appear qualitatively similar to those found in urine.

The further metabolism of dexamethasone probably entails typical conjugation reactions yielding glucuronides and sulphate esters.

The high level of radioactivity found in both plasma and urinary conjugated fractions points to extensive elimination processes through biosynthetic conjugation reactions (Starnes 1966).

It has been concluded from studies *in vitro* that the presence of the 1,4-diene-3-one and fluorine atom in the 9α position inhibited enzymic reduction of the A ring of the molecule (Glenn *et al.* 1957; Florini *et al.* 1961). In the absence of A ring reduction, the major metabolites of those synthetic corticosteroids that have been studied (e.g. betamethasone and triamcinolone) were formed by the relatively slow reaction of 6β-hydroxylation and reduction of the ketone group at C-20 (Florini *et al.* 1961; Butler and Gray 1970). Oxidation of the 11β-hydroxyl group and removal of the side chain resulting in 17-oxosteroids have also been reported (Butler and Gray 1970).

In previous studies three unconjugated polar metabolites have been reported in human urine after a single dose of labelled dexamethasone (Haque *et al.* 1972). Chromatographic studies of a polar metabolite detected in rat urine indicated that this metabolite is likely to be 6-hydroxy-dexamethasone (Rice *et al.* 1974).

These findings allow speculation about the identity of the principal plasma as well as urinary component IV reported here; it will most likely prove to be 6β-hydroxy-dexamethasone.

A comparison of our results regarding polarity of component VIII with those of Dumasia *et al.* (1976) who studied the metabolism of dexamethasone in the horse and provisionally identified one metabolite, indicates that the component VIII might be 11-dehydrodexamethasone. However, the identities of all detected radioactive components, especially IV, V and VI which are being currently investigated, remain to be established.

Previous studies have shown similarities in the main routes of excretion of natural and synthetic glucocorticosteroids (Tredger *et al.* 1973; Rice *et al.* 1974) but the partition of glucocorticosteroids and their metabolites between urine and faeces varied in different species.

In our experiments we could recover about 86% of the total dose of dexamethasone. Our results indicated that 66% of this was present in urine and the rest in faeces. These values were similar to those of Lindner (1972) for the excretion of cortisol and its metabolites in sheep. Thus for both the natural hormone cortisol and its synthetic analogue the sheep can be placed between the rat on the one hand which shows a predominant faecal route of excretion (Rice *et al.* 1974) and man on the other hand with mainly urinary excretion (Peterson and Pierce 1960).

The plasma profiles of radioactivity associated with dexamethasone (Fig. 6) are compatible with plasma dexamethasone profiles in sheep measured by radioimmunological assay (Panaretto and Wallace 1978*a*) where results showed peak concentrations during the first 48 h infusion which were succeeded by continuously declining levels until the final days of infusion when a terminal increase in dexamethasone concentration was frequently seen.

The present study clearly established that a steep decline of dexamethasone concentration in plasma is caused by extensive metabolism of the parent compound.

Parke (1976) has suggested that many drugs are able to enhance their own metabolism either by stimulating enzymes concerned in their metabolism or by increasing the rate of synthesis of enzyme proteins. He also suggested that induction of drug metabolizing enzymes was first preceded by a period of enzyme inhibition. Since steroids of all kinds, including corticosteroids, have been shown to increase the activity of microsomal drug-metabolizing enzymes (Conney 1967; Parke 1971) which also mediate the oxidative metabolism of steroids, it is possible that dexamethasone is able to enhance its own metabolism. The degree of enhancement is likely to be reflected in the increased urinary excretion of component IV and in the excretion of unchanged dexamethasone during the first days of a 4-day infusion.

Kinetics of particular metabolizing enzymes [low Michaelis constants for the hydroxylation of steroids (Parke 1971); different substrate concentrations] probably determined the quantitative differences in urinary excretion between the 4- and 8-day infusions.

It is interesting to note the relationships of the levels of dexamethasone and its metabolites in plasma and wool fibre shedding. Wool fibre shedding was always greater after the 8-day infusion of glucocorticoid than in animals infused for only 4 days (this paper; Panaretto and Wallace 1978*a*, 1978*b*). During the second half of the 8-day infusion the falling dexamethasone level in plasma (Fig. 6) was steadily replaced by increasing levels of metabolites, especially constituents IV and V (Fig. 7*a*). On the last 2 days of the infusion only 30% of the radioactivity of the unconjugated fraction was accounted for in dexamethasone. Of the remainder, components IV and V comprised on average 30 and 15% respectively.

One of the explanations for the greater wool fibre shedding observed after the longer infusions might be that a prolonged exposure of the follicles to even the low and progressively decreasing levels of dexamethasone caused the observed effect on wool fibre growth. Other reasons could be that one or more dexamethasone metabolites and not the parent drug, or both dexamethasone and its metabolites, are biologically active.

Further studies including isolation, identification and testing of dexamethasone metabolites will establish to what degree these compounds inhibit wool fibre growth. Already the biological activities of the principal metabolite IV are currently being investigated in this laboratory.

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