Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 673–676. Pergamon Press. Printed in Great Britain

TRITIATED 18-HYDROXYDEOXYCORTICOSTERONE: BINDING IN RENAL, CARDIAC AND HEPATIC CYTOPLASM, AND IN PLASMA FROM ADRENALECTOMIZED RATS

P. J. FULLER and J. W. FUNDER
Medical Research Centre, Prince Henry's Hospital, Melbourne 3004, Australia

(*Received* 6 *February* 1976)

SUMMARY

Hypersecretion of 18-hydroxydeoxycorticosterone (18-OHDOC) may be implicated in some cases of low renin hypertension and in salt-sensitive hypertension in Dahl rats. To investigate the mechanism of action of the steroid, the binding of tritiated 18-OHDOC was studied in cytoplasmic fractions of kidney, liver and heart. Binding was displaceable by excess unlabelled 18-OHDOC, but to sites with affinity too low for a physiological role as 18-OHDOC receptors. Similarly, no specific high affinity plasma binding protein could be demonstrated; by equilibrium dialysis, high capacity albumin binding was shown to be ~65%. The weak mineralocorticoid effects of 18-OHDOC would therefore appear to be mediated by occupation of physiological aldosterone receptors, and not by occupation of physiological receptors specific for 18-OHDOC itself.

INTRODUCTION

18-Hydroxydeoxycorticosterone (18-OHDOC) is a normal secretion product of the adrenal cortex, and has been shown capable of eliciting Na^+ transport in both the toad bladder [1] and the rat kidney [2]. The adrenal secretion rate and circulating plasma levels of 18-OHDOC have been shown to be elevated in some patients with hypertension [3, 4], in rats with adrenal regeneration hypertension [5], and in rats of the salt dependent hypertensive strain developed by Dahl [6].

The possibility of a role for 18-OHDOC in the pathophysiology of blood pressure elevation has led to an examination of its affinity for renal adrenocorticoid receptors [7], and more latterly to the development of radioimmunoassay methods for measuring its plasma levels [8]. The previous study of the affinity of 18-OHDOC for renal adrenocorticoid receptors was by indirect means, by examining the ability of the unlabelled steroid to compete with tritiated aldosterone, tritiated dexamethasone and tritiated corticosterone for their renal binding sites. With the advent of tritiated 18-OHDOC of high specific activity two further studies on its properties became feasible. First, the possibility of specific receptor sites for 18-OHDOC, distinct from mineralocorticoid and glucocorticoid receptors, becomes a testable proposition. Secondly, the binding of 18-OHDOC to plasma proteins, including low affinity binding to albumin, can be studied, and the role of such binding in the electrolyte activity of the steroid determined.

MATERIALS AND METHODS

Tritiated 18-OHDOC [^{3}H]18-OHDOC, 51 Ci/mmol) was obtained from Amersham–Searle (U.K.). Unlabelled 18-OHDOC was the kind gift of Dr. J. C. Melby, Boston, U.S.A. and unlabelled dexamethasone (DM) the generous gift of Merck, Sharp & Dohme (Australia). Unlabelled aldosterone (A), corticosterone (B), oestradiol (E_2), progesterone (P) and dihydrotestosterone (DHT) were all Calbiochem, chromatography grade. Other reagents used were analytical grade.

Mature male Sprague–Dawley rats (body weight 120–160 g) were used in all experiments. Rats were adrenalectomized 1–5 days before use; after operation they were maintained on pellets and 0.9% NaCl *ad libitum*. Rats were anaesthetized with Nembutal®, and killed by exsanguination into syringes wet with Na^+ heparin, 5000 U/ml. After exsanguination rats were perfused via the abdominal aorta with 20 mls of ice-cold incubating solution (Na^+ 133 K^+6 Cl^-134 $H_2PO_4^-$6 Ca^{2+} 1 Mg^{2+} 0.5 Tris–HCl 5 glucose 5—all in mM; pH 7.4). Kidneys and hearts were removed and trimmed and placed in ice-cold incubating solution to await slicing or homogenization.

Kidney slices of 230 μm thickness were made with a Sorvall® tissue chopper; slices from 2 animals were pooled for each experiment. The pool was then divided into the appropriate number of aliquots, and each aliquot of slices was incubated with [^{3}H]18-OHDOC in the presence or absence of competing unlabelled steroids for 30 min at 25°C. At the end of the period of incubation the slices were drained, and then homogenized in 1.5 ml of 0.25 M sucrose–3 mM $CaCl_2$ for 2 s with Polytron P-10 (speed setting 2). The homogenates were centrifuged at 30,000 *g* for 30 min to yield a high speed supernatant (HSS) containing soluble cytoplasmic proteins. Homogenization and all steps subsequent were carried out at 0–4°C.

Aliquots of HSS (vol. 1 ml) were passed through G-50 (fine) Sephadex minicolumns of bed vol. 3.6 ml to separate protein bound from residual free steroid. This procedure has been extensively employed previously in similar studies [7, 9, 10]. Aliquots of the excluded vol. of the Sephadex minicolumns were counted in a Packard 3375 liquid scintillation spectrometer, efficiency 45%, using 1 ml of aqueous sample with 10 ml of counting solution (1 l toluene, 500 ml Teric X10, 0.15 g POPOP, 8.26 g PPO). Protein concentrations were determined by the method of Warburg and Christian [11].

In the studies on the cardiac and hepatic binding of [^{3}H]18-OHDOC, tissues were homogenized in incubating solution, and the homogenates centrifuged for 30 min at 30,000 *g*. Aliquots of the HSS were incubated with [^{3}H]18-OHDOC, with and without competing unlabelled steroids, for 90 min in ice. At the end of this period of incubation separation of bound and free steroid, and subsequent steps, were as described above.

Two series of studies on the plasma binding of 18-OHDOC were made. In the first, [^{3}H]18-OHDOC was incubated for 30 minutes at 37°C with plasma diluted 1:60 with 0.9% NaCl solution, in the presence and absence of competing unlabelled steroids. At the end of this period the incubates were passed through G-50 (fine) Sephadex, under which conditions only relatively high affinity ($K_{diss} < 10^{-6}$ M) binding of ligand is detected. In the second series of experiments upon the plasma binding of 18-OHDOC, plasma was diluted 1:5 in 0.9% NaCl and 500 μl aliquots pipetted into dialysis sacs of 8/32 Visking cellulose tubing. Sacs were suspended in 10 mls of 0.9% NaCl containing [^{3}H]18-OHDOC alone, or together with competing unlabelled steroids. Dialysis was allowed proceed for 18 h at 37°C with constant agitation: at the end of this period aliquots of the solutions inside and outside were taken for counting, and the extent of plasma binding determined.

RESULTS

Figure 1 shows the binding of [^{3}H]18-OHDOC in kidney slices over a range of concentrations from 2.5×10^{-8} M to 1.6×10^{-9} M. Open circles represent the binding in the absence of competing unlabelled 18-OHDOC; solid circles represent the binding in the presence of $\geq$ 100-fold unlabelled 18-OHDOC. 'Specific', in the sense of displaceable, binding of [^{3}H]18-OHDOC can be seen; that this 'specific' binding is to sites with low affinity is shown by the Scatchard [12] plot of displaceable binding (insert, triangles). One component of this low affinity binding system for 18-OHDOC may well be mineralocorticoid receptors, which in two laboratories [7, 13] have been reported to show a K_{diss} (25°C) $\approx 1.4 \times 10^{-7}$ M for 18-OHDOC. No additional high affinity, limited capacity binding sites can be demonstrated in cytoplasmic fractions from rat kidney slices.

Similarly, neither in cardiac nor hepatic cytoplasmic extracts can a high affinity, limited capacity binding system be demonstrated (Figs. 2 and 3). As in the kidney, 'specific' in the sense of displaceable binding of [^{3}H]18-OHDOC can be seen; as in the kidney, Scatchard plots of the 'specific' binding show that it is to sites with a low affinity for 18-OHDOC. In all three tissues, no accurate description of the low affinity binding of 18-OHDOC can be attempted given the range of concentrations of [^{3}H]18-OHDOC chosen. By the same token, because the range chosen covers physiological levels, the absence of high affinity binding strongly suggests that no physiological receptors for 18-OHDOC exist in kidney, liver or heart.

In a previous study [7] it was shown that 18-OHDOC had an affinity for transcortin ~1% of that of corticosterone. In the absence of radiolabelled 18-OHDOC, two questions remained unanswered; (i) the possible existence of specific high affinity plasma binders for 18-OHDOC, having a low affinity for corticosterone, (ii) the extent of low affinity, high capacity albumin binding. Diluted plasma from adrenalectomized rats was incubated with [^{3}H]18-OHDOC, and the high affinity ($K_{diss} < 10^{-6}$ M) binding estimated by Sephadex chromatography (Fig. 4). Binding of [^{3}H]18-OHDOC was minimally displaced by dexamethasone, oestradiol, aldosterone or dihydrotestosterone. Both corticosterone and progesterone, however, are better competitors for high affinity

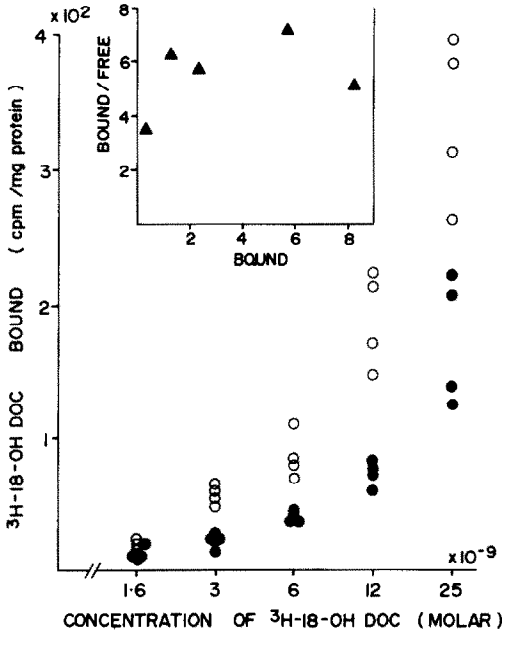

Fig. 1. Binding of [^{3}H]18-OHDOC in renal cytoplasm. Kidney slices from adrenalectomized rats were incubated over a range of concentrations for 30 min at 25 C with [^{3}H]18-OHDOC either alone (open circles) or in the presence of $\geq$100-fold unlabelled 18-OHDOC (closed circles). Each circle represents an individual determination. Mean values for specific binding at each concentration of [^{3}H]18-OHDOC are plotted on linear coordinates by the method of Scatchard (Insert, triangles).

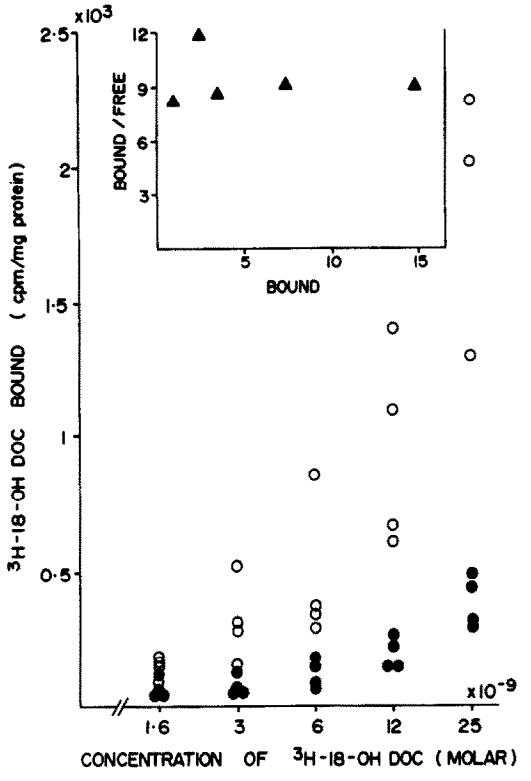

Fig. 2. Binding of [^{3}H]18-OHDOC in cardiac cytoplasm. High speed supernatant of cardiac homogenates from adrenalectomized rats was incubated over a range of concentrations for 90 min at 4 C with [^{3}H]18-OHDOC either alone (open circles) or in the presence of $\geqslant$100-fold unlabelled 18-OHDOC (closed circles). Each circle represents an individual determination. Mean values for specific binding at each concentration of [^{3}H]18-OHDOC are plotted on linear coordinates by the method of Scatchard (Insert, triangles).

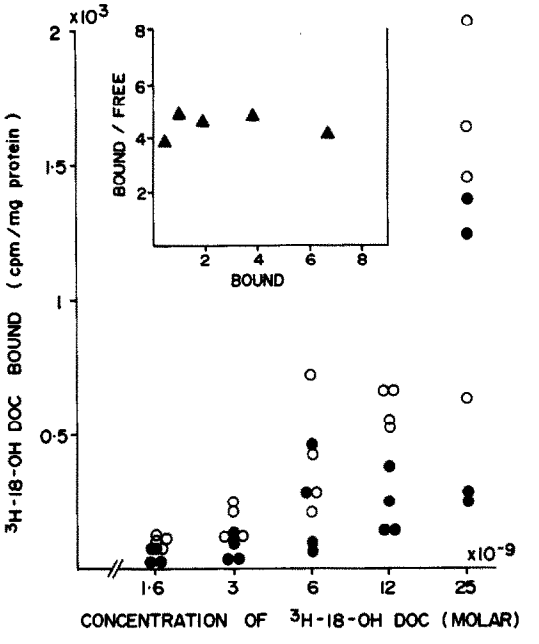

Fig. 3. Binding of [^{3}H]18-OHDOC in liver cytoplasm. High speed supernatant of liver homogenates from adrenalectomized rats was incubated over a range of concentrations for 90 min at 4 C with [^{3}H]18-OHDOC either alone (open circles) or in the presence of $\geqslant$100-fold unlabelled 18-OHDOC (closed circles). Each circle represents an individual determination. Mean values for specific binding at each concentration of [^{3}H]18-OHDOC are plotted on linear coordinates by the method of Scatchard (Insert, triangles).

[^{3}H]18-OHDOC plasma binding sites than unlabelled 18-OHDOC itself. The existence, therefore, of a physiologically significant set of plasma binding sites, with a high affinity for 18-OHDOC and a low affinity for corticosterone, appears unlikely.

The extent of low affinity binding of [^{3}H]18-OHDOC was measured by the technique of equilibrium dialysis. Under these conditions ~65% of the steroid was bound (Fig. 5); that this binding was truly to high capacity, low affinity sites is shown by the inability of a large excess of corticosterone, 18-OHDOC or dihydrotestosterone to compete for tracer binding.

DISCUSSION

The results of the series of experiments detailed above can be summarized as follows. First, [^{3}H]18-OHDOC is bound in cytoplasmic fractions from rat, kidney, liver and heart. Secondly, this binding, although displaceable by excess unlabelled 18-OHDOC, is of low affinity ($K_{diss} \geqslant 2.5 \times 10^{-8}$ M) and therefore cannot be to physiological 18-OHDOC receptors. Thirdly, there appears to be no high affinity plasma binding protein specific for 18-OHDOC in adrenalectomized rat plasma. Finally, in rat plasma at 37°C, between 60 and 70% of 18–OHDOC is bound to high capacity binding sites, presumably on SA.

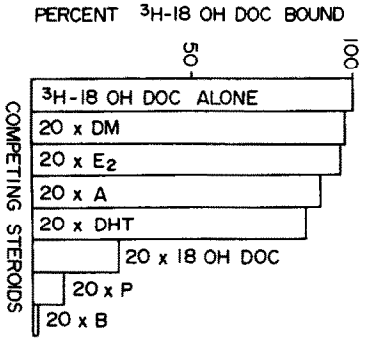

Fig. 4. High affinity plasma binding of [^{3}H]18-OHDOC. Plasma from adrenalectomized rats was diluted 1:60 with 0.9% NaCl and incubated for 30 min at 37 C with [^{3}H]18-OHDOC 2.5×10^{-9} M either alone, or in the presence of 20-fold (5×10^{-8} M) unlabelled competitor. DM = dexamethasone, E_2 = oestradiol, A = aldosterone, DHT = dihydrotestosterone, P = progesterone, B = corticosterone. At the end of the period of incubation bound [^{3}H]18-OHDOC was separated from residual free steroid by Sephadex chromatography. Each bar represents the mean of at least two determinations.

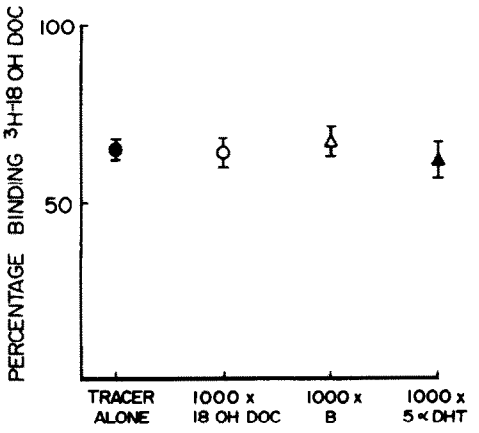

Fig. 5. High capacity plasma binding of [^{3}H]18-OHDOC. Plasma from adrenalectomized rats was diluted 1:5 with 0.9% NaCl; vol. of 0.5 ml were incubated for 18 h at 37 C in dialysis sacs suspended in 10 mls of 0.9% NaCl containing [^{3}H]18-OHDOC 2.5×10^{-10} M alone, or with 1000-fold corticosterone (B), 5α-dihydrotestosterone (5α-DHT), or unlabelled 18-OHDOC. The means and standard deviations of separate determinations using plasma from six different rats are plotted.

The conclusions reached by a previous study [7] using unlabelled 18-OHDOC were that (i) markedly elevated plasma levels of 18-OHDOC could occupy a proportion of the mineralocorticoid receptor population, and (ii) this receptor occupation and Na^+ retention would not turn off the production of 18-OHDOC as it is not subject to the normal feedback mechanisms operating to regulate mineralocorticoid secretion.

The findings in the present study, taken together, serve to delineate more precisely the possible pathophysiological role of 18-OHDOC in low renin hypertension, or in the hypertension of salt sensitive rats of the Dahl strain. The level of plasma binding (~65%) is only marginally greater than for aldosterone, and much less than for corticosterone or DOC. This indicates that, as for aldosterone, a relatively large fraction of circulating 18-OHDOC is available for target tissue binding. In the absence of specific 18-OHDOC receptors in kidney, heart or liver, target tissue binding is presumably to receptors for other steroids. The weak mineralocorticoid action of 18-OHDOC, therefore, would appear to be mediated by occupancy of mineralocorticoid receptors under conditions where its secretion rate is elevated.

Acknowledgements—This work was supported by Grants from the National Health and Medical Research Council of Australia, and the National Heart Foundation of Australia. The technical assistance of Ms. Jenny Hood and Ms. Julie Mercer, the figures drawn by Ms. Anne Hayres, and the typing of Ms. Leonie Tippett and Ms. Elaine Griffiths are gratefully acknowledged.

REFERENCES

1. Porter G. A. and Kimsey J.: *Endocrinology* **89** (1971) 353–357.
2. Birmingham M. K., McDonald M. L. and Rochefort J. G.: In *Functions of the Adrenal Cortex* (Edited by K. W. McKerns) Vol. 11. Appleton-Century Crofts, New York (1968) pp. 647–689.
3. Melby J. C., Dale S. L. and Wilson T. E.: *Circ. Res.* **28/29** (1971) suppl. 2. 143–152.
4. Melby J. C., Dale S. L., Grekin R. J., Gaunt R. and Wilson T. E.: *Recent Prog. Horm. Res.* **28** (1972) 287–351.
5. Spark R. F.: *New engl. J. Med.* **287** (1972) 343–349.
6. Rapp J. P. and Dahl L. K.: *Circ. Res.* **28** suppl. 2 (1971) 153–159.
7. Feldman D. and Funder J. W.: *Endocrinology* **92** (1973) 1389–1396.
8. Edwards C. R. W., Biglieri E. G., Martin V. I., Taylor A. A. and Batte F. C.: *J. Endocr.* **63** (1974) 29.
9. Funder J. W., Feldman D. and Edelman I. S.: *Endocrinology* **92** (1973) 944–1004.
10. Marver D., Goodman D. and Edelman I. S.: *Kidney Int.* **1** (1972) 210–223.
11. Warburg O. and Christian W.: *Biochem. Z.* **310** (1942) 384–421.
12. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
13. Palem-Vliers M., Genard P. and Van Cauwenberge H.: *Res. Steroids* **7** (1976) (in press).