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USE OF CORTICOSTEROID ANTIBODIES FOR THE STUDY OF CORTICOSTEROID BIOSYNTHESIS *IN VITRO*

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SUMMARY

1. Radioimmunochromatographic analysis of adrenal incubates is a useful method for the characterization of uncommon and unidentified steroid fractions. In incubates of normal adrenals at least seven uncommon fractions were separated.
2. Uncommon and unidentified steroids may react also with antibodies of apparently high specificity. This observation may have practical consequences for *in vivo* studies.
3. Under pathophysiological conditions such as adrenal regeneration hypertension steroids may be produced by adrenocortical tissue which are not detectable in incubates of normal adrenals.

INTRODUCTION

The aim of the studies we are going to refer to was to make use of corticosteroid-antibodies for demonstrating the presence of steroids which are produced by adrenal tissue *in vitro*. By applying various types of antibodies of different specifications a radioimmunological analysis is possible which facilitates the identification of known steroids as well as the characterization of unknown substances on the basis of their similarities with known steroids.

In addition, by this procedure the characterization of steroid antibodies may be improved, since their specificity is not always satisfactorily established by their cross-reaction with steroids which are commercially available. By incubating adrenal slices it would be expected that uncommon steroids are produced which might interfere in various types of radioimmunoassays.

MATERIALS AND METHODS

Antibodies raised in our laboratory were used in this study. Rabbits and sheep were immunized with 21-hemisuccinate bovine serum-albumin complexes of different corticosteroids. As expected, antibodies of different specificities were obtained, and we used specific as well as non-specific antibodies.

The cross-reactions of various antibodies with commercially available steroids are summarized in Table 1.

The experimental procedure used for the characterization of steroids produced by adrenal tissue is given in Table 2.

ıarted rat adrenals were incubated for 4 h in
Ringer-bicarbonate-glucose-solution in the
e of tritiated deoxycorticosterone (DOC) as
ɔr.
me experiments other radioactive precursors
ritiated 18-OH-DOC were used.
bation medium was extracted with dichloro-
d, in addition, with ethyl acetate. At first,
was chromatographed in a propyleneglycol–toluene–system. The paper-strips were cut into pieces of 0·5–3 cm. and eluted with ethanol. Aliquots of the eluates were taken for measurement of radioactivity, other aliquots used for the radioimmunoassays. For the isolation of steroids parts of the eluates were further chromatographed in various paper- and thin-layer-systems.

RESULTS AND DISCUSSION

The following experiments were performed:

1. In 16 experiments, pooled adrenals of 5–25 untreated rats were incubated. In a separate experiment we incubated more than 200 rat-adrenals in order to obtain a large amount of the steroids.

2. In four experiments the zona glomerulosa tissue and the inner zones of the adrenal cortex were separately incubated.

3. In seven experiments adrenals which had regenerated after adrenal-demedullation according to Skelton[1] were incubated. The adrenals of these rats had been removed 6–8 weeks after demedullation. All animals used in that study were hypertensive (> 140 Hg mm systolic).

Twenty-hour chromatograms in a propyleneglycol-toluene-system—from the dichloromethane extract—resulted in radioactivity curves which are characteristic for the *in vitro* incubation of rat adrenals: the radioactivity peaks of 18-OH-corticosterone, aldosterone, 18-OH-DOC, and corticosterone could be identified (Fig. 1). In the figure curves are presented which were obtained by means of radioimmunoassays.

A non-specific antibody raised in a rabbit immunized simultaneously with cortisol, corticosterone, aldosterone, and deoxycorticosterone, reacted as expected with all four fractions i.e. 18-OH-corticosterone, aldosterone, 18-OH-DOC and corticosterone. Other peaks were discernible and one of them may be due to 19-OH-DOC.

Curves obtained with corticosterone and deoxycorticosterone antibodies are also included. The 18-OH-corticosterone fraction reacted with corticosterone antibodies.

Table 1. Cross-reactions of steroid-antibodies used in this study (values in %)

CROSS REACTIONS	S 2 ALDO	S3 ALDO	R 26 ALDO	R 7 ALDO	R 19 B	R 24 DOC	ANTIGEN
»SPECIFIC« ANTIBODIES							
F	0.04	0.08	-	0.37	0.46	-	
B	2.4	0.28	0.66	3.4	100	6.07	
DOC	4.2	-	-	0.12	16.8	100	
ALDO	100	100	100	100	0.36	0.0	
SUBST. S	-	-	-	-	5.6	21.2	
18-OH-DOC	6.04	0.12	0.03	-	10	1.64	

	R 119 F	R A THS x	R 28 xx F-B-DOC-ALDO	ANTIGEN
»UNSPECIFIC« ANTIBODIES				
F	100	0.2	67.8	
B	135	-	19	
DOC	140	-	100	
ALDO	2	-	5.9	
SUBST. S	128	0.57	-	
18-OH-DOC	140	-	63.3	
THF	-	25	-	INCUBATION WITH
THE	-	100	-	^{x} H^3-THE
THS	-	33	-	^{xx} H^3-DOC

A strong reaction was seen between the 18-OH-DOC fraction and the corticosterone antibody, and that fraction reacted also with the deoxycorticosterone antibody. Studies are now in progress to make use of corticosterone antibodies for determining 18-OH-DOC when no specific 18-OH-DOC antibodies are available.

The less specific aldosterone antibody of sheep No. 2, reacted not only with aldosterone but also with other fractions, at least to a certain degree. Immediately before the 18-OH-DOC fraction a new fraction was detected. In other incubation experiments the separation of this fraction from the 18-OH-DOC was less distinct, and the material ran in the initial part of the 18-OH-DOC zone. The material bound also with the aldosterone antibody of high specificity raised in sheep No. 3, but hardly, if at all, with the somewhat less specific aldosterone antibodies raised in rabbits Nos. 7 and 26. This fraction is interesting for two reasons:

1. Since the most specific aldosterone antibody available to us reacted strongly with that fraction, the unidentified fraction may interfere with the aldosterone-radioimmunoassay if the isolation procedures are not complete.

2. Aldosterone and the unidentified material may have similar chemical structures.

For the isolation of the steroid, additional chromatographies were performed (Fig. 2).

In a Bush B5 system the unidentified material could be further separated from other steroids. In the dichloromethane–ethanol thin-layer system the curves of the aldosterone-like immunoactivity corresponded exactly to the radioactivity.

The radioimmunochromatographic analysis of the incubation medium from zona glomerulosa tissue

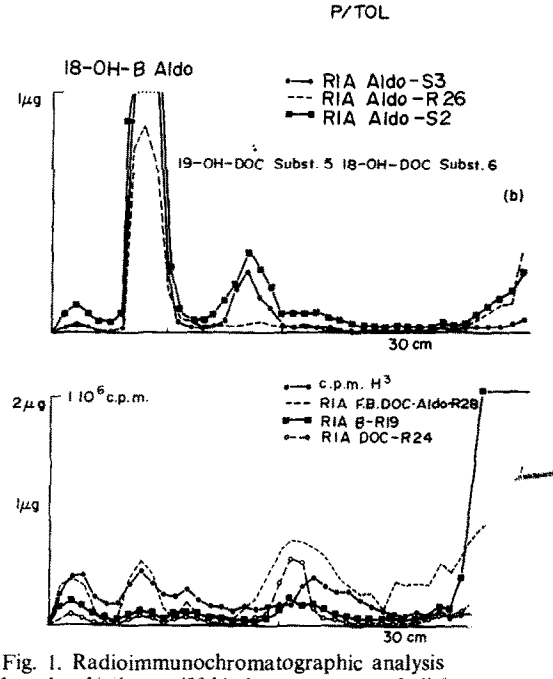
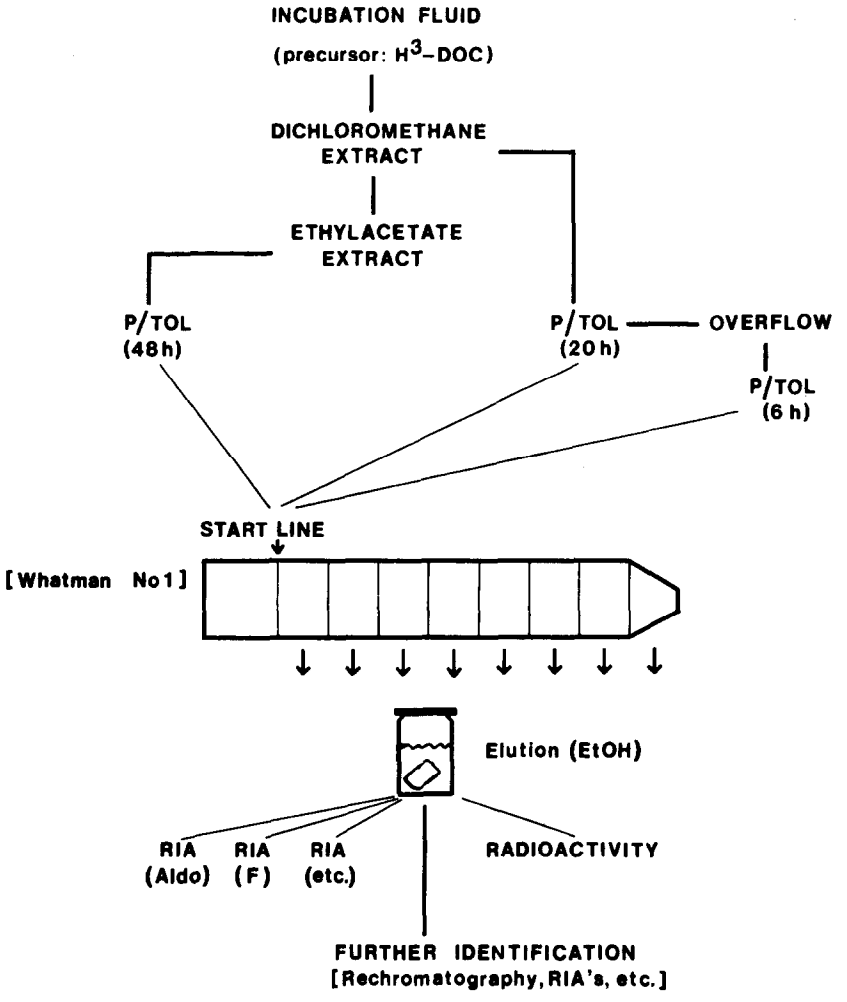


Fig. 1. Radioimmunochromatographic analysis
leneglycol/toluene (20 h)-chromatogram of dic
ane extract of adrenal incubation fluid from up
(20 Mio c.p.m. 1,2-H^3-DOC was added as pre

propy-
rometh-
ated rats

Table 2. Flow-sheet of the experimental procedure



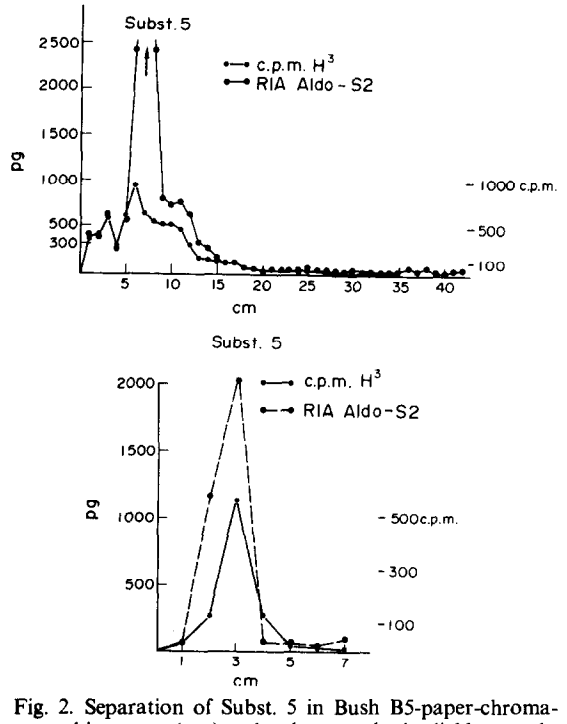


Fig. 2. Separation of Subst. 5 in Bush B5-paper-chromatographic system (top) and, subsequently, in dichloromethane–ethanol (100:5) thin-layer-system (bottom).

revealed that the unidentified material was produced by these cells. No corresponding fraction could be demonstrated in the incubates from inner zones of the adrenal cortex (Fig. 3).

On the basis of R_F-values as well as reactions with antibodies it was highly probable that the material did not correspond to 17-iso-aldosterone, 11-dehydro-aldosterone, tetrahydro-aldosterone, tetrahydro-corticosterone, and to the tautomers of aldosterone. In various experiments no parallelism between the amounts of the unidentified substance and of aldosterone could be found.

Several attempts were made at the identification of the material, which is a steroid since it is formed from radioactive DOC. Incubation of a pool of 213 adrenals yielded a large amount of material which after chromatographic isolation and further purification was submitted to mass-spectrometry. However, no satisfactory result could be achieved. In the first experiments we found a molecular line at 368, but later experiments revealed that this was caused by an impurity.

After dichloromethane-extraction the incubation fluid was reextracted with ethylacetate (Fig. 4). At

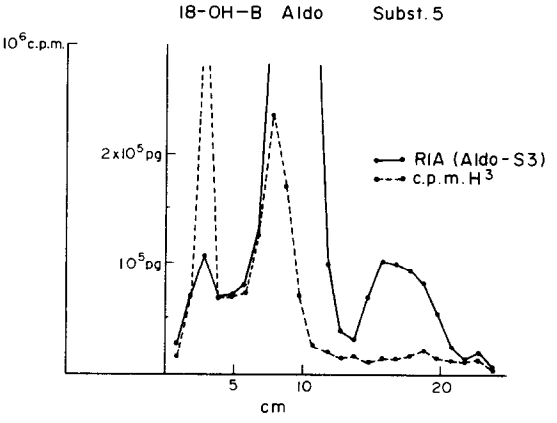


Fig. 3. Radioimmunochromatographic analysis of the incubation fluid of the zona glomerulosa cells. Propyleneglycol–toluene system, 20 h.

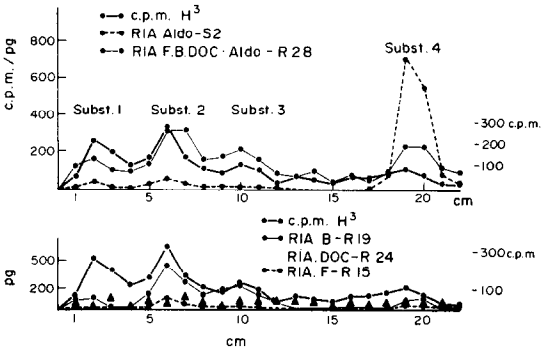


Fig. 4. Radioimmuno-chromatographic analysis of an ethyl acetate extract of incubation fluid which was made in addition to the dichloromethane extraction. Propyleneglycol–toluene system, 48 h.

least four different fractions were demonstrable in the propyleneglycol-toluene chromatogram. The fractions were radioactive-formed from tritiated DOC and gave immunoreactions with non-specific and specific antibodies.

By repeated chromatography it was shown that the bulk of those fractions was homogeneous. In the last chromatographic steps the radioactivity curves corresponded to the radioimmunological reactions. Considering the chromatographic behaviour and the similarities in the radioimmunological reactions the second fraction seems to be identical with 18-OH-corticosterone and the fourth one with aldosterone. These two corticosteroids remained in the aqueous phase after the first dichloromethane extraction. The first fraction is perhaps a tetrahydro-derivative which reacts with the non-specific tetrahydro-deoxycortisol-antibody. However, we have no idea of the identity of the third fraction.

The analysis of a 6-h propyleneglycol–toluene chromatogram performed with the run-off of the 20-h chromatogram of dichloromethane extracts resulted in corticosterone, 11-dehydro-corticosterone and in a large less polar fraction at the front giving intense reaction with the non-specific antibodies. This may be caused by the DOC and progesterone present in that fraction. Surprisingly, three not very distinct subfractions could be separated by means of reactions with aldosterone and corticosterone antibodies. A further separation of these fractions was not carried out.

Table 3 summarizes the fractions found in normal

Table 3. Fractions formed in normal adrenal incubates

		R_{Aldo}	max. RIA	Identity
Ethylacetate Extract P/Tol 48 h "A"	Subst. 1	0.1	THS	TH-Derivative
	Subst. 2	0.3	B	18 OH B ?
	Subst. 3	0.55	F-B-DOC-Aldo	?
	Subst. 4	1.0	Aldo	Aldo ?
Dichlormethane Extract P/Tol 20 h "B"	18-OH-B	0.25	B	
	Aldo	1.0	Aldo	
	19-OH-DOC	1.43	F-B-DOC-Aldo	
	Subst. 5	2.0	Aldo	?
	18-OH-DOC	2.59	B + DOC	
	Subst. 6	3.76	F-B-DOC-Aldo	?
	B	4.59	B	

		R_f	max. RIA	Identity
Runoff of "B" P/Tol 6 h	B	0.24	B	
	A	0.47	F-B-DOC-Aldo	
	Subst. 7	0.67	B	?
	Subst. 8	0.8	B	?
	Subst. 9	0.87	B	?
	DOC / Prog.	0.63–0.93	DOC	

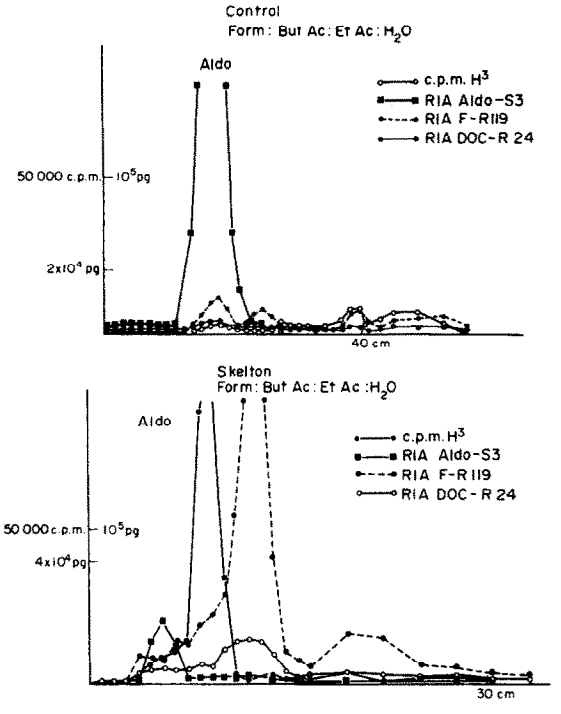


Fig. 5. Radiochromatographic analysis from experiments of control (top) and Skelton-hypertensive rats (bottom). Chromatography: formamide – butylacetate – ethylacetate – water system (rechromatography of the aldosterone fraction of propyleneglycol–toluene system).

adrenal incubates. The table contains data on the chromatographic behaviour of the substances and suggestions for their identity. By means of autoradiography, Sheppard *et al.*[2] found unidentified steroids formed from [^{14}C]-progesterone. Two substances found by these authors ran similarly to our substances 3 and 6 in the propyleneglycol–toluene system. Repeated efforts to identify substance 5 was unsuccessful. Not enough material was available for further studies of the other unidentified substances.

In the incubation media of regenerated adrenals after enucleation further unidentified steroid fractions might be found, since it is doubtful whether the observed increase of DOC and 18-OH-DOC is responsible for the hypertension which develops after adrenal enucleation.

In those animals plasma-renin activity was decreased, indicating an increase in total mineralocorticoid activity. Chromatographic analysis of the incubation fluid of adrenals removed from rats with adrenal regeneration hypertension revealed differences from that of control incubates.

However, using either [^{3}H]-DOC or [^{3}H]-18-OH-DOC as precursors, a radioactive material was found with a chromatographic behaviour in propyleneglycol-toluene similar to aldosterone. In the aldosterone fraction De Nicola *et al.*[3] have also described an unidentified material formed from [^{3}H]-DOC in adrenals regenerated after enucleation. The same fraction has reacted with a non-specific cortisol antibody but hardly with a specific aldosterone antibody. Both radioactivity and peak reacting with an unspecific cortisol antibody could be separated from aldosterone in a formamide–butyl acetate–ethyl acetate–water system (Fig. 5). Further efforts to identify these fractions will be made.

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3. De Nicola A. F., Oliver J. T. and Birmingham M. K.: *Endocrinology* **83** (1968) 141–148.