Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 197–202. Pergamon Press. Printed in Great Britain

HIGH TITRE ANTISERUM SPECIFIC TO ALDOSTERONE*

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(*Received* 14 *May* 1973)

SUMMARY

A highly sensitive and specific antiserum was obtained by immunizing a rabbit with an aldosterone-carboxymethoxime bovine gamma globulin conjugate. Immunological analysis of the antiserum was performed 6, 9, 13 and 23 weeks after first immunization.

Over the observed period the antibody titre ranged from 4000 to 40,000 and antibody concentration was between 410 and 3540 μg/ml antiserum. Association constants were in the order of 10^9 l./mole.

When antiserum from week 23 was used, 50% displacement of [^{3}H]-aldosterone bound to the antibody was achieved by the addition of 15 pg unlabeled aldosterone, indicating very high sensitivity. The same effect was seen with sera from week 6, 9 and 13 when 32, 23 and 21 pg of unlabeled aldosterone were added.

The minimal amount of hormone which produced a statistically significant decrease in [^{3}H]-aldosterone bound to the antibody was 3 pg with antiserum from week 6, whereas sera from week 9, 13 and 23 allowed the detection of 2 pg alsosterone.

Over the experimental period antisera showed cross reaction with cortisol ranging from 0·0007 to 0·0010% and with corticosterone from 0·12 to 0·06%.

The high sensitivity and high specificity of the antiserum allowed the determination of plasma aldosterone in the presence of other naturally occurring steroid hormones.

INTRODUCTION

Sensitive and specific antisera are required for the measurement of aldosterone in plasma since plasma concentrations of aldosterone are extremely low and plasma concentrations of the other steroid hormones such as cortisol and corticosterone are higher by one or two orders of magnitude. The use of an antiserum of high sensitivity and high specificity may allow the determination of plasma aldosterone in the presence of other steroid hormones.

We have previously succeeded in measuring aldosterone concentration in plasma and in urine in the presence of other naturally occurring steroid hormones by using highly sensitive and specific antisera [1, 2]. This report describes the production and characterization of the most sensitive of these antisera obtained by immunizing a rabbit with an aldosterone protein conjugate.

EXPERIMENTAL

Materials

D-Aldosterone (crystalline) was obtained from Ciba-Geigy AG, Basel, Switzerland. [1, 2-^{3}H]-Aldosterone (54 mCi/mmol) was purchased from New England Nuclear, Dreieichenhain, West Germany. Cortisol, corticosterone, deoxycorticosterone (DOC), progesterone and testosterone were from Sigma Chemical Company, St. Louis, U.S.A. 18-OH-corticosterone and 18-OH-deoxycorticosterone were kindly supplied by Ciba-Geigy AG. 1-Ethyl-3(3-dimethyl-aminopropyl)-carbodiimide HCl was purchased from Sigma Chemical Company. Lysozyme was obtained from Sigma Chemical Company. Carboxymethoxylamine hemichloride was from Aldrich-Europe, Beerse, Belgium. Freund's complete adjuvant and killed mycobacterium tuberculosis were obtained from Difco Laboratories, Detroit, U.S.A. Dextran-coated charcoal was prepared by adding 2500 mg charcoal (Norit A from Sigma Chemical Company) and 250 mg dextran (dextran 70 from Pharmacia Fine Chemicals, Uppsala, Sweden) to 100 ml phosphate buffer (0·15 M NaCl, 0·01 M K_2HPO_4; pH 7·4). Instagel from Packard Instrument GmbH, Frankfurt, West Germany was used as a counting solution. Statistical analysis were performed by Student's-*t*-test. Elemental analysis was done by the Schwarzkopf Microanalytical Laboratory, Woodside, New York.

Preparation of the steroid protein conjugate

Aldosterone-carboxymethoxime was prepared according to Erlanger *et al.* [3]. However, to prevent

* Supported by Deutsche Forschungsgemeinschaft.

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alkaline denaturation of the aldosterone molecule, the reaction of carboxymethoxylamine with the steroid was allowed to proceed at pH 9–10. 100 mg aldosterone and 200 mg carboxy-methylamine were dissolved in 20 ml of absolute ethanol. The solution was adjusted to pH 9–10 by dropwise addition of 5% NaOH and heated over a reflux in a rotatory evaporator flask for 60 min. The solution was evaporated to a volume of 2–3 ml under air stream and diluted with 5 ml distilled water. After extraction with ether (3·10 ml) concentrated hydrochloric acid was added dropwise to the aqueous phase under vigorous stirring. The resulting precipitate was immediately redissolved in 2 ml methanol and the methanol solution was added to 20 ml phosphate buffer containing 200 mg bovine gamma globulin (BGG). This was followed by the addition of 500 mg carbodiimide. After 12 h at room temperature, the solution was dialysed for 48 h against 6 changes of 2 l phosphate buffer. The lyophilized conjugate was stored at 4°C.

The number of steroid molecules which had been incorporated into one molecule of BGG was determined by using tracer amounts of [^{3}H]-aldosterone: 15 molecules aldosterone were coupled to one molecule BGG.

Cortisol-carboxymethoxime was prepared under the same conditions as described for aldosterone-carboxymethoxime. Elemental analysis of the cortisol-carboxymethoxime showed that quite definitely a monosubstituted steroid-carboxymethoxime was formed. A composition of 64·24% C, 8·20% H and 3·31% N was found. The theoretical composition of a disubstituted cortisol molecule would be 59% C, 7·1% H and 5·5% N, of a monosubstituted steroid 63·4% C, 7·6% H and 3·2% N.

Immunization

Lyophilized conjugate (1 mg) was emulsified in 1 ml complete Freund adjuvant and injected into the hind foot pads of a male New Zealand white rabbit. The animal was given a booster dose 3, 7, 11 and 17 weeks after first immunization with 0·5 mg antigen in 0·5 ml of the same adjuvant. The boost at week 3 was given in the hind foot pads, boosts at weeks 7, 11 and 17 were given subcutaneously. Blood was collected through the central ear artery every 14 days, beginning 4 weeks after first immunization.

Immunological analysis

The antiserum was diluted with phosphate buffer containing 1 mg/ml lysozyme. The diluted antiserum 0·5 ml was incubated with 4400 d.p.m. [^{3}H]-aldosterone overnight at 4°C. Bound hormone was separated from free by adding 0·25 ml dextran-coated charcoal. The samples were centrifuged for 30 min at 2500 ***g*** and the supernatant was decanted into 10 ml of a counting solution. The samples were counted in a liquid scintillation counter with a counting error of less than 1 per cent. An external standard was employed for quenching correction.

Standard curves were performed by adding increasing amounts of unlabeled aldosterone (0, 5, 10, 20, 50, 100, 200, 400 and 1000 pg in 0·02 ml phosphate buffer) to 0·5 ml antiserum and 4400 d.p.m. [^{3}H]-aldosterone.

The specificity of the antiserum was determined by testing the ability of steroids other than aldosterone to displace [^{3}H]-aldosterone bound to the antibody. Sensitivity and specificity of the antiserum were examined 6, 9, 13 and 23 weeks after first immunization.

Displacement experiments were performed at antiserum dilutions at which 40–50% of 4400 d.p.m. [^{3}H]-aldosterone were bound to the antibody.

RESULTS

Sensitivity

Sensitivity of an antiserum may be expressed by the slope of the semilog standard curve. The steeper the standard curve the greater the sensitivity. Figure 1 shows standard curves of sera collected 6, 9, 13 and 23 weeks after the first injection with antigen. Almost parallel curves were obtained with antisera taken after week 9, 13 and 23, whereas antiserum from week 6 gave a standard curve which was less steep than those obtained with the other sera. From these standard curves the amount of unlabeled aldosterone necessary to displace 50% of [^{3}H]-aldosterone bound to the antibody was calculated. A displacement of 50% was achieved with serum from week 6 on the addition of 32 pg unlabeled aldosterone. 23 pg unlabeled hormone had to be added when serum from week 9 was used. The same effect was seen with serum obtained at week 13 on the addition of 21 pg aldosterone. 15 pg of unlabeled aldosterone produced a 50% decrease of [^{3}H]-aldosterone bound to the antibody when serum collected 23 weeks after first immunization was examined.

Sensitivity of an antiserum may also be expressed as the least detectable amount of unlabeled aldosterone producing a statistically significant decrease in [^{3}H]-aldosterone bound to the antibody. A statistically significant decrease ($P < 0{\cdot}01$) in % [^{3}H]-aldosterone bound to the antibody was observed with sera from week 9, 13 and 23 on the addition of 2 pg unlabeled aldosterone, whereas with serum from week 6 a statistically significant decrease ($P < 0{\cdot}005$) was seen first on the addition of 3 pg unlabeled aldosterone.

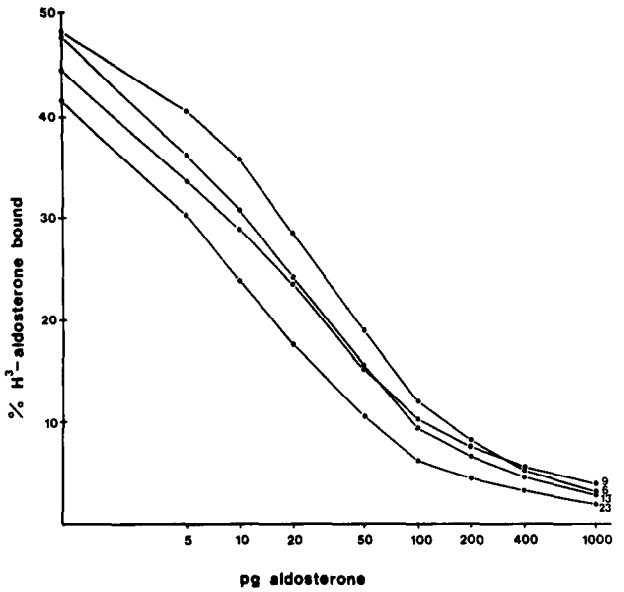

Fig. 1. Standard calibration curves of antisera obtained 6, 9, 13 and 23 weeks after first immunization.

The results of displacement experiments adding 2, 3 and 5 pg unlabeled aldosterone are summarized in Table 1.

Specificity

Specificity of the sera was calculated from displacement experiments according to Abraham [4]. If x pg of aldosterone are required to displace 50% of the [^{3}H]-aldosterone bound to the antibody and y equals the pg of steroid S, required to displace 50% of the [^{3}H]-aldosterone bound to the antibody, then the % cross reaction of the steroid S is $(x/y) \times 100$. The results are summarized in Table 2.

The antiserum showed very low cross reaction with cortisol, testosterone and progesterone. Higher cross reaction was observed with deoxycorticosterone. Except for serum collected at week 6 the other sera

Table 1. Sensitivity of the antiserum obtained 6, 9, 13 and 23 weeks after first immunization. The effect of 2, 3 and 5 pg unlabeled aldosterone on the % [^{3}H]-aldosterone bound to the antibody. Each value represents the mean of 6 individual measurements ($\bar{X} \pm$ S.D.)

pg aldosterone added / Weeks after first immunization	0	2	3	5
6	43·6% ± 1·1%	42·7% ± 0·7%	40·5% ± 0·8%	37·8% ± 1·0%
9	43·1% ± 0·7%	40·1% ± 0·7%	38·3% ± 1·2%	34·5% ± 0·6%
13	46·0% ± 1·2%	42·2% ± 1·3%	38·9% ± 0·5%	36·0% ± 1·2%
23	42·1% ± 1·1%	37·7% ± 0·8%	34·8% ± 0·8%	30·5% ± 0·9%

Table 2. Cross reaction (%) of the antiserum with various steroid hormones 6, 9, 13 and 23 weeks after first immunization. Cross reaction (%) was calculated from displacement experiments according to Abraham [4]

Steroids	Weeks after first immunization 6	9	13	23
Cortisol	0·0010	0·0007	0·0010	0·0008
Corticosterone (B)	0·12	0·09	0·09	0·06
Deoxycorticosterone (DOC)	0·18	0·10	0·20	0·10
18-OH-B	0·030	0·025	0·035	0·020
18-OH-DOC	0·065	0·055	0·075	0·045
Progesterone	0·0040	0·0025	0·0030	0·0015
Testosterone	0·0030	0·0009	0·0035	0·0015
Cortisol-carboxy-methoxime	0·0040	0·0030	0·0045	0·0025

Table 3. Association constants (K_0), antibody concentrations and antibody titre of the antiserum at different time after first immunization. The values were calculated from the aldosterone displacement experiments as described by Nisonoff and Pressman [5]

Weeks after first immunization	Titre*	K_0 (l./mole)	Antibody concentrations (μg/ml serum)†
6	4000	$4{\cdot}6 \times 10^9$	410
9	8000	$2{\cdot}1 \times 10^9$	960
13	40,000	$4{\cdot}1 \times 10^9$	3540
23	20,000	$4{\cdot}1 \times 10^9$	1140

* Antibody titres are expressed as reciprocal antiserum dilutions at which 0·5 ml of diluted antiserum binds 40–50% of 4400 d.p.m. [^{3}H]-aldosterone.

† These values are based on an assumed antibody molecular weight of 150,000.

showed lower cross reaction (< 0·1%) with corticosterone. Both 18-OH-DOC and 18-OH-corticosterone consistently cross reacted to a lesser extent (< 0·08%).

The ability of a steroid-oxime derivate to displace [^{3}H]-aldosterone bound to the antibody was determined by cross reaction studies with cortisol-carboxymethoxime which had been prepared under the same conditions as described for the reaction of carboxymethoxylamine with aldosterone (pH 9–10). Cortisol-carboxymethoxime was always 3–4 times more potent in displacing [^{3}H]-aldosterone bound to the antibody than cortisol (Table 2).

Displacement studies with aldosterone incubated at different pH values

To determine whether the described changes during steroid-carboxymethoxime preparation had also changed the immunological characteristics of the antiserum, displacement experiments were performed using both aldosterone which had been incubated under strong alkaline conditions [3] and steroid which had been exposed for 60 min at pH 9–10. When antiserum from week 23 was used 18 pg aldosterone incubated at pH 10 had to be added to displace 50% of the [^{3}H]-aldosterone bound to the antibody. With aldosterone incubated under strong alkaline conditions [3] the same effect was seen on the addition of 45 pg.

Association constants, antibody concentration and antibody titre

The association constants (K_0) and antibody concentrations were calculated from the aldosterone displacement data according to Nisonoff and Pressman [5].

Association constants, antibody concentrations and antibody titres are summarized in Table 3.

Over the observed period antibody titre ranged from 4000 to 40,000 and antibody concentrations were between 410 and 3,540 μg/ml antiserum. Association constants showed minimal changes and were always in the order of 10^9 l./mole.

Equilibrium studies

The ability of the antibody aldosterone complex to reach equilibrium was studied by incubating 4400 d.p.m. [^{3}H]-aldosterone and 0·5 ml diluted antiserum for 1, 3, 6, 12, 24 and 48 h at 4°C.

Figure 2 shows, that after 12 h at 4°C almost maximal binding of [^{3}H]-aldosterone to the antibody was achieved. No marked differences in the conditions necessary to reach equilibrium were observed between sera collected 6, 9, 13 and 23 weeks after first immunization.

DISCUSSION

Aldosterone carries two oxo-groups at C_3 and C_{20}. Thus, the reaction of aldosterone with carboxymethoxylamine could lead to the formation of either an aldosterone-3-monocarboxymethoxime or to a steroid-3,20-dicarboxymethoxime or even to an aldosterone-20-monocarboxymethoxime. Elemental analysis of a cortisol-carboxymethoxime which had been prepared under the same conditions as described for aldosterone (pH 9–10) showed that quite definitely a monosubstituted steroid-oxime was formed. This and the observation that pregnenolone carrying only a keto-group at C_{20} could not be converted to a steroid-carboxymethoxime [6] supports the belief that under the conditions described in this study most probably an aldosterone-3-carboxymethoxime was formed. Thus, conjugation of the steroid-oxime to the carrier protein through C_3 was achieved.

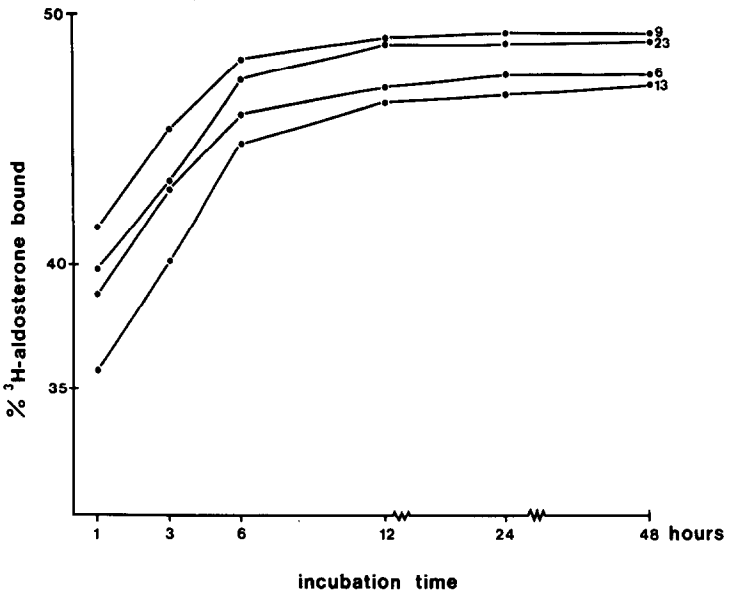

Fig. 2. The ability of antisera obtained 6, 9, 13 and 23 weeks after first immunization to bind [^{3}H]-aldosterone after 1, 3, 6, 12, 24 and 48 h at 4°C.

It is known since Beiser *et al.* [7] that the specificity of antisera increases when the position of conjugation between steroid and carrier protein is remote from the distinguishing functional groups of the steroid molecule. Since configurational changes among individual cortico steroids occur at ring C and D, position C_3 seems to be the ideal point of conjugation. Similar results were reported by Midgley and Niswender [8].

That the antiserum described in this study was obtained with an antigen linked through position C_3 may be one reason for the higher specificity of our antiserum as compared with antisera obtained using aldosterone-21-hemisuccinate protein conjugates [9, 10]. The original method of steroid-oxime preparation takes place under strong alkaline conditions [3]. Since aldosterone may undergo configurational changes under these conditions [11] we allowed the reaction of carboxymethoxylamine with the steroid to proceed at pH 9–10.

Displacement experiments showed that the ability of the antiserum to bind aldosterone was markedly reduced when steroid was used incubated under strong alkaline conditions, whereas steroid incubated at pH 9–10 was nearly as potent in displacing [^{3}H]-aldosterone bound to the antibody as standard aldosterone preparations. This is evidence that under strong alkaline conditions configurational changes of the steroid molecule may occur. This also indicates that the antiserum described in this study most probably was induced with a chemically unaltered antigen. As reported briefly [12], this might explain why antisera obtained with aldosterone-carboxymethoxime protein conjugate prepared according to Erlanger *et al.* [3] were both less sensitive and less specific than the antiserum described in this study [13–18].

It is of particular interest that the 'oxime-interface' between steroid and carrier protein seems to be part of the antigenic determinant since cortisol-oxime was more potent in displacing [^{3}H]-aldosterone bound to the antibody than cortisol.

Our results indicate that sera from a single rabbit collected at different time intervals after first immunization differ both in sensitivity and specificity. This is in contrast to the results of Africa and Haber [19] who found no changes in antibody specificity in an antiserum from a single rabbit immunized with an aldosterone-hydrazone protein conjugate.

The high specificity of the antiserum described allowed the determination of aldosterone concentration in plasma and in urine in the presence of other naturally occurring steroid hormones [1, 2]. The high sensitivity of the antiserum offers an opportunity to measure aldosterone concentrations in extracts from 0·25 and 0·10 ml plasma. In addition to the high antibody specificity, the use of such little plasma volumes may be of additional advantage in avoiding cross reaction in the assay system.

REFERENCES

1. Vetter W., Vetter H. and Siegenthaler W.: *Acta endocr., Copenh.* **74** (1973) 548–557.
2. Vetter W., Vetter H. and Siegenthaler W.: *Acta endocr., Copenh.* **74** (1973) 558–567.
3. Erlanger B. F., Borek F., Beiser F. M. and Lieberman S.: *J. biol. Chem.* **228** (1957) 713–727.
4. Abraham G. E.: *J. clin. Endocr. Metab.* **29** (1969) 866–870.
5. Nisonoff A. and Pressman D. J.: *J. Immun.* **80** (1958) 417–428.
6. Vetter W.: unpublished observation.
7. Beiser S. M., Erlanger B. F., Agate F. J. and Lieberman S.: *Science* **129** (1959) 564–565.
8. Midgley A. R. and Niswender G. D.: *Acta Endocr., Copenh.* **147** (1970) 320–331.
9. Haning R., McCracken J., St. Cyr M., Williams G. and Abraham G.: *Steroids* **20** (1972) 73–88.
10. Ito T., Woo J., Haning R. and Horton R.: *J. clin. Endocr. Metab.* **34** (1972) 106–112.
11. Schmidlin J., Anner G., Billeter J. R., Hensler K., Ueberwasser H. and Wieland P. and Wettstein A.: *Helv. chim. Acta* **40** (1957) 2291–2321.
12. Vetter W., Haber E., Freedlender E., Beckerhoff R. and Siegenthaler W.: *Verh. Dtsch. Ges. Inn. Med.* **78** (1972) 1607–1610.
13. Vetter W., Freedlender E. and Haber E.: *Acta Endocr., Copenh. Suppl.* **159** (1972) 32.
14. Mayes D., Furuyama S., Kem D. C. and Nugent C. A.: *J. clin. Endocr., Metab.* **30** (1970) 682–685.
15. Bayard F., Beitins I. A., Kowarski A. and Migeon C. J.: *J. clin. Endocr., Metab.* **31** (1970) 1–6.
16. Farmer R. W., Roup W. G., Pellizzari E. D. and Fabre L. F.: *J. clin. Endocr., Metab.* **34** (1972) 18–22.
17. Bands P., Ekins R. P. and Slater J. D. H.: *Acta endocr., Copenh. Supp.* **155** (1971) 94.
18. McCaa R. E., McCaa C. S., Read D. G., Bower J. D. and Guyton A. C.: *Circulat. Res.* **31** (1972) 473–480.
19. Africa B. and Haber E.: *Immunochemistry* **8** (1971) 479–486.