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# PRODUCTION AND CHARACTERISTICS OF HIGHLY SPECIFIC ANTIBODIES TO ALDOSTERONE

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SUMMARY

The 3-carboxymethoxime of aldosterone 18,21-diacetate has been prepared and after enzymic deacetylation the product has been covalently linked to bovine serum albumin. This immunogenic complex when injected into rabbits (6), produced antibodies of high specificity and avidity in all animals within 16 weeks, as assessed by radioimmunoassay. The importance of assessing specificity at several different levels of response is stressed. All antibodies showed a reduction of cross-reactivity as the response (% free) increased but there was great variability in the rate of change of cross-reactivity with the magnitude of the response. Specificity appeared to remain unchanged, or to fall slightly, 21 weeks after primary immunization.

The ability of make steroids immunogenic by covalent linkage to a protein carrier has been established[1]. With reference to aldosterone, many groups[2–5] including our own[6] have succeeded in raising antibodies to the 3-carboxymethoxime–BSA complex and/or the 18,21-disuccinyl–BSA complex. Antibodies induced by either hapten have shown similar specificity with regard to corticosteroids of related structure. On theoretical grounds it might be expected that linkage to the protein carrier at C-3 which exposes characteristic structures on the C- and D-rings would produce more specific antibodies than linkage at C-18 and/or C-20, (A-ring exposed). This does not seem to be true and perhaps the lack of difference of specificity has been due to the failure to purify and crystallise the hapten before attachment to the carrier. In the present paper we have endeavoured to prepare a pure monocarboxymethoxime under acidic conditions. Antisera of high specificity and high avidity have been obtained. However, we also show how specificity is a relative phenomenon and how different antibodies with an adequate relative potency at one level of response may be quite inadequate at another.

## MATERIALS AND METHODS

### *Materials*

Carboxymethoxylamine hemihydrochloride was obtained from R. N. Emanuel, Wembley, Middx., U.K. and pseudocholinesterase (horse serum, Type IV) from Sigma (London) Chemical Co., Kingston, Surrey, U.K. D-aldosterone, 18-hydroxycorticosterone (18-OH Cpd B) and 18-hydroxy-11-deoxycorticosterone (18-OH DOC) were generous gifts from Ciba, Horsham, Sussex, U.K. All other unlabelled steroids were purchased from Sigma, BDH Ltd. (Poole, Dorset, U.K.) or Koch–Light (Colnbrook, Bucks, U.K.). Organic solvents (Analar grade) were from B.D.H. Ltd. Reagents for the radioimmunoassay procedure were as previously described[6]. NCS solubiliser (hyamine hydroxide) was supplied by Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, U.K.

Thin-layer chromatography (t.l.c.) plates were prepared with Kieselgel $GF_{254}$ at 0·3 mm thickness (Merck, Darmstadt, Germany). t.l.c. Systems were; system M, ethyl acetate–n-hexane–acetic acid (87:20:5 by vol.) and system E, ethyl acetate–n-hexane–acetic acid (72:13·5:4·5:10 by vol.)[7].

### *Preparation of aldosterone-3-carboxymethoxime*

To minimise dioxime formation, the 18,21-diacetate of aldosterone was prepared by reacting aldosterone (100 mg; plus 0·01 μCi[$^3$H]-aldosterone) in pyridine (9·9 ml) with acetic anhydride (5 ml) at room temperature for 16 h. The resulting solution was evaporated to dryness *in vacuo*. Carboxymethoxylamine hemihydrochloride (51 mg) in the minimum volume of M-NaOH was adjusted to pH 5 with acetic acid and added to the 18,21-diacetate residue dissolved in ethanol (9 ml). After 18 h in the dark at room temperature the solution was evaporated *in vacuo* and the residue was dissolved in water (20 ml). The pH of the solution was adjusted to 8 and was extracted with ethyl acetate (2 × 100 ml) to remove unreacted diacetate. The aqueous phase was acidified with M-acetic acid (6 ml) and re-extracted with ethyl acetate (3 × 100 ml). The organic extract was washed, dried and evaporated *in vacuo* to yield a gummy residue (138 mg) which in solution absorbed at $\lambda_{max}$ 249 nm, $E^{1\%}_{1cm.}$ 355. Two spots were found on t.l.c. (system M) each of which showed U.V. absorption and stained blue with alkaline blue tetrazolium.

Deacetylation of the carboxymethoxime was carried out using pseudocholinesterase[8, 9]. The crude carboxymethoxime was dissolved in 0·1 M-

NaOH (3 ml) and pseudocholinesterase (16 mg) in phosphate buffer (5 ml) at pH 7·4 added. The reaction volume was made up to 50 ml with buffer and the solution was incubated at 37°C for 16 h. Unchanged di-acetate was removed by acidifying with M-HCl to pH 3·4 and extracting with benzene (2 × 50 ml). Subsequent extraction with ethyl acetate (3 × 60 ml) yielded a gummy residue (113 mg; $\lambda_{max}$ 248 nm, $E^{1\%}_{1cm.}$ 374) which on t.l.c. (system E) gave two spots ($R_F$ 0·31; 0·37) both absorbing in the U.V. region and staining blue with alkaline blue tetrazolium. Attempts to resolve the two components and crystallise the products were unsuccessful.

*Preparation of the immunogen aldosterone-3-carboxymethoxime–BSA*

The deacetylated product, regarded mainly as forms of aldosterone-3-carboxymethoxime, was conjugated to BSA by the mixed acid anhydride method of Erlanger *et al.*[10]. This product in dimethylformamide (2·5 ml), tributylamine (83 μl) and iso-butyl chloroformate (23 μl) at −10°C was allowed to stand for 30 min and was then added to a stirred solution of BSA (204 mg) in water (5 ml), M-NaOH (0·2 ml) and dimethylformamide (3·6 ml). The mixture became clear at 7 min and was allowed to stand at 4°C for 16 h. The product was transferred to a gel-filtration column (Sephadex G25; 50 cm. × 2 cm. i.d.) and the column was eluted with water. Fractions 9–14 (5 ml fractions) contained protein and also absorbed U.V. with $\lambda_{max}$ 250 nm; these fractions were combined and lyophilised to give 210 mg of protein–steroid conjugate. Radioassay of the lyophilised solid, using NCS solubiliser with suitable internal standards indicated a molar ratio steroid:protein of 30:1. As indicated by t.l.c., fractions 21–37 contained unchanged carboxymethoxime.

*Production and testing of antibodies*

The immunogenic complex was homogenised in water with an equal volume of Freund's adjuvant and was administered intradermally into the backs of 6 hybrid rabbits (females of 3–3·5 kg) (Norfolk Rabbits Ltd.). The injection was made at 20–30 sites so that each animal received 0·5 ml of the emulsion. Initially each rabbit received 500 μg of the complex; this was followed by secondary injections (500 μg) at 4 weeks, and 100 μg at 11 weeks, 18 weeks and subsequently every three months. Sample bleeds were made from the ear vein periodically, starting 6 weeks after the initial injection; the plasma was separated and stored at −20°. Antibody dilution curves were prepared by diluting the anti-plasma in phosphate buffer containing 0·15% gelatin; samples of diluted anti-plasma (100 μl) were incubated with [$^3$H]-aldosterone (100 μl; 11 fmol) in the absence (100 μl buffer) or in the presence (100 μl; 111 fmol) of non-radioactive aldosterone. The incubation was at 4°C overnight and free and bound radioligands were separated by direct extraction of the free component into toluene scintillator; this technique has been fully validated and described in detail by Jowett *et al.*[6].

*Determination of cross reactivity*

Steroids of structure similar to that of aldosterone were dissolved in ethanol at a concentration of 1 mg ml$^{-1}$ and subsequently diluted with assay buffer to give a series of concentrations which would span the response range of the radioimmunoassay. We were particularly concerned to determine relative potency at the lower end of the dose-response curve, since, as described previously[6], relative potency varies with the magnitude of the response and is maximal at the lower end of the response curve. The relative potency[13, 14] of a cross-reactant at any response level (in this case, % free) is given by the equation:

$$\text{relative potency (\%) at } y\%\text{ free} = \frac{\text{concentration of aldosterone that produces } y\%\text{ free}}{\text{concentration of cross-reactant that produces } y\%\text{ free}} \times 100.$$

## RESULTS

All six rabbits produced antibodies within six weeks. By 16 weeks titres ranging from 1/24,000 to 1/180,000 (final dilution) were obtained, which, in all cases, gave a useful response between 0 and 111 fmol (0–40 pg) of aldosterone per tube. A typical response curve is shown in Fig. 2.

*Relative potency at 16 weeks*

At the 50% response level each antibody showed very similar relative potencies compared with other physiologically relevant corticosteroids, as shown in Table 1. However, this value does not provide a true index of cross reactivity since the slope of the response obtained with a cross reactant differs widely, both with different compounds in any one antiplasma and with the same compound in different antiplasmas. This is illustrated in Table 2 and Fig. 1. Relative potency for corticosterone changes by a factor of 22 (rabbit 4) to 64 (rabbit 6) whereas the relative potency for cortisol changes by a factor of 4 (rabbit 4) to 93 (rabbit 6) from the 50% to 25% response level. Between a 30% and 25% response the relative potency for cortisol changes by only a factor of 1·6 in rabbit 4, whereas it changes by a factor of 9·3 in rabbit 6.

*Antibody characteristics as a function of time*

We have studied rabbits 4, 5 and 6 in greater detail to obtain information on relative potency, titre and affinity constant as a function of time. In rabbit 6 (Table 3) the relative potency is minimal at 16 weeks at any given response level, both for

Table 1. Relative potency (%) of common corticosteroids at the 50% free level (Aldosterone = 100). Note similarity in all 6 rabbits

| Steroid | Rabbit 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Cortisol | <0·0007 | <0·0008 | <0·0003 | 0·0002 | <0·0009 | <0·0003 |
| Corticosterone (B) | 0·0070 | 0·0026 | 0·0080 | 0·0057 | 0·0059 | 0·0034 |
| 18-OH B | | | 0·0093 | | | 0·0364 |
| Deoxycorticosterone (DOC) | 0·0070 | 0·0081 | 0·1500 | 0·0270 | 0·0120 | 0·0280 |
| 18-OH DOC | | | 0·0467 | | | 0·1500 |
| Cortisone | 0·0012 | <0·0010 | <0·0006 | <0·0030 | <0·0020 | <0·0006 |
| Testosterone | 0·0005 | <0·0010 | 0·0050 | <0·0030 | <0·0020 | 0·0028 |
| Progesterone | 0·0036 | 0·0043 | 0·0187 | <0·0030 | 0·0020 | 0·0050 |

Table 2. Relative potency (%) of cortisol and corticosterone at specified points at low levels of response (% free aldosterone) in rabbits 4 and 6

| Response | Rabbit 4 Cortisol | Rabbit 4 Corticosterone | Rabbit 6 Cortisol | Rabbit 6 Corticosterone |
|---|---|---|---|---|
| 50% | 0·0002 | 0·0057 | <0·0003 | 0·0034 |
| 35% | 0·0004 | 0·0185 | 0·0009 | 0·0196 |
| 30% | 0·0005 | 0·0413 | 0·0030 | 0·0740 |
| 25% | 0·0008 | 0·1259 | 0·0278 | 0·2174 |

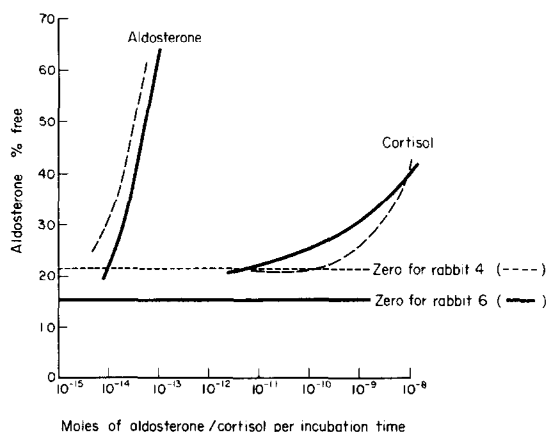


Fig. 1. Response curves for aldosterone and cortisol in antiplasma from rabbit 4 and rabbit 6. Zero = response when no inert steroid present. Note the difference in the curvature of the cortisol response for the two antiplasmas.

cortisol and corticosterone, and it shows a considerable and progressive deterioration with time, especially at the lower response levels. In contrast, the titre is highest at 21 weeks and the equilibrium constant as judged by the initial slope of a Scatchard plot slightly increases with time. Plasma was obtained at 16 and 21 weeks after primary immunization from rabbits 4 and 5. Relative potency at any response level and the equilibrium constant remained unchanged although the titre had increased slightly as with rabbit 6.

*The effect of cross-reacting steroids in the presence of aldosterone*

All the data presented so far have involved cross-reacting steroids in the presence of only tracer quantities of aldosterone, as is the usual procedure. However, Goebel and Kuss [16] claim to have shown with one particular antibody raised against oestriol, that the cross-reactivity of oestrone and oestradiol increases with the addition of increasing amounts of oestriol. This contradicts predictions from theory [13, 14]. Therefore we have repeated our cross-reactivity experiments in the presence of inert aldosterone, both with 50 ng cortisol per tube and also with the three most important cross-reactants (cortisol, corticosterone and deoxycorticosterone) added simultaneously in physiologically relevant amounts. Figure 2 confirms that, for our most useful antibody (rabbit 4) the presence of a relatively large amount of cortisol does not perturb the response curve to aldosterone.

Table 3. Relative potency (%), titre (final dilution) and equilibrium constant for rabbit 6 as a function of the time since the primary immunization

| Response | 16-week bleed Cortisol | 16-week bleed Corticosterone | 21-week bleed Cortisol | 21-week bleed Corticosterone | 31-week bleed Cortisol | 31-week bleed Corticosterone |
|---|---|---|---|---|---|---|
| 50% | <0·0003 | 0·0034 | 0·0006 | 0·0121 | 0·0006 | 0·0710 |
| 35% | 0·0009 | 0·0196 | 0·0086 | 0·2250 | 0·2670 | 0·5450 |
| 30% | 0·0030 | 0·0740 | 0·0606 | 0·6400 | 0·9000 | 1·0590 |
| 25% | 0·0278 | 0·2174 | | 2·2727 | | 1·7060 |
| Titre | $\frac{1}{60,000}$ | | $\frac{1}{90,000}$ | | $\frac{1}{30,000}$ | |
| Equilibrium Constant ($LM^{-1}$) (4°) | $7{\cdot}7 \times 10^{10}$ | | $9{\cdot}36 \times 10^{10}$ | | $1{\cdot}58 \times 10^{11}$ | |

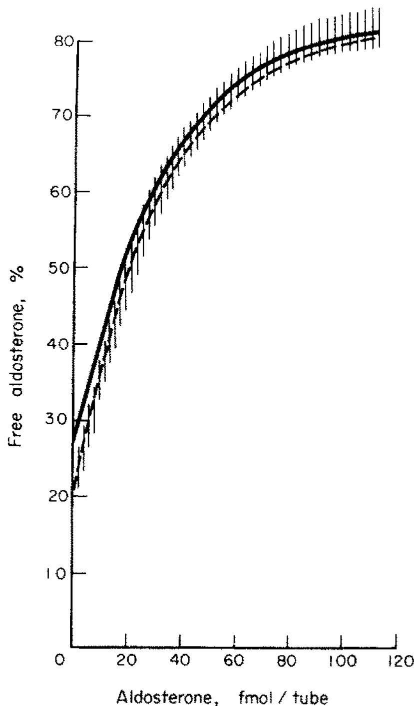


Fig. 2. 95% confidence limits for 4 consecutive response curves using antiplasma from rabbit 4 are shown by the vertical hatching. The interrupted line (------) represents the response obtained in the presence of 50 ng (138 pmol) cortisol; the solid line represents the response obtained in the presence of 25 ng (69 pmol) cortisol, 2·5 ng (7·2 pmol) corticosterone and 50 pg (151 fmol) deoxycorticosterone.

It also shows that the mixture of cross-reactants described above produces a slight increase of response at the lower end of the response curve, as predicted from the cross-reactivity for corticosterone shown in Table 2, but as the amount of aldosterone is increased the curves converge, as expected from theoretical analysis.

### DISCUSSION

There seems little doubt that careful purification of the 3-carboxymethoxime prior to coupling to carrier protein is capable of yielding antibodies of much greater specificity than those prepared without a specific purification step[2–6]. For example, the relative potency for cortisol of our previous best antibody varied from 0·03 to 0·01% over the response range of 30–70% free [$^3$H]-aldosterone. In contrast, using a very similar immunisation schedule, our best antibody produced with the purified hapten described above shows a relative potency which varies from 0·0005 to less than 0·0002% over the same response range. At the 50% response level, improvement in cross-reactivity with other corticosteroids was also seen, ranging from about 25-fold for cortisone to 200-fold for testosterone and progesterone. The particular precautions taken during the purification which appear relevant are the initial 18,21-diacetylation to prevent C-20 oxime formation and prevention of alkaline degradation by maintaining acid conditions throughout the preparative procedure. Similar improvement of relative potency appears to have been achieved by Vetter *et al.*[11] who used controlled, mildly alkaline conditions for 3-carboxymethoxime preparation and by Mackenzie and Clements[12] who used a mildly acid reaction mixture and chromatographic purification of the product, which behaved as a single spot on their chromatography system. On the other hand, we obtained a double product which may represent *cis* and *trans* isomeric forms.

Detailed comparison of relative potency with that described by the above workers is difficult because relative potency varies as a function of the response metameter, as predicted by a theoretical analysis, assuming a single species of binding site[13, 14]. Therefore, unless relative potency is estimated at all levels of response it is impossible to fully characterise antibody specificity. In fact some of our antibodies show a much greater curvature at the lower end of the response curve than others. Thus, if specificity is considered at the 50% response level all of our antibodies appear similar but at the 25% response level gross dissimilarities are revealed (see Table 2). Presumably variations in the heterogeneity of binding sites in the antiplasma are responsible for these differences, despite similarity in the equilibrium constants (see Table 3). Full characterisation of relative potency is important because, for the successful radioimmunossay of aldosterone in biological fluids without chromatography, an antibody with a maximal cross reactivity for cortisol of less than 0·001% at any response level is required (all other corticosteroids are either present in relatively low concentration or can be removed by a simple chemical partition). Furthermore, in order to measure aldosterone concentration in small volumes of plasma, maximum sensitivity is required and this entails full use of the lower part of the response curve.

In view of the data reported by Abraham[15] for antisteroid antisera prepared in adult ewes, we had expected specificity to increase up to 12 months after the primary immunisation. However, in the three antiplasmas we tested there was either a peak at 16 weeks or a plateau between 16 and 21 weeks, despite an increase of titre and an unchanged or slight increase of equilibrium constant. This may represent a difference between the immunological behaviour of rabbits as compared to sheep.

There would appear to be little reason why our findings are not, in principle, applicable to the development and testing of highly specific antibodies to other haptens.

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