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CORTICOSTEROID AND CORTICOSTEROID METABOLITE LEVELS IN ANIMALS IMMUNIZED AGAINST CORTICOSTEROIDS

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SUMMARY

(1) Although the spontaneous mortality of rabbits immunized with corticosteroid and corticosteroid metabolite antigens increased, no certain biological effect of active or passive immunizations against corticosteroids could be established.

(2) The total plasma concentrations of antigen steroids always increased while the concentrations of antigen independent corticosteroids were frequently elevated.

(3) Elevations of free corticosteroid levels were estimated by equilibrium dialysis. These results were confirmed qualitatively, but not quantitatively, by other techniques, *e.g.* by estimating urinary-free steroids.

(4) One of the reasons for the discrepancy between the absence of biological effects and increased free steroid levels may be a disturbance of the dynamic equilibrium of plasma-bound and free steroids caused by an interaction between plasma proteins, antibody and antigen steroid.

INTRODUCTION

The essential role of corticosteroids in the maintenance of carbohydrate and mineral metabolism indicates that serious biological alterations could be expected as a consequence of active or passive immunizations against these steroids. However, the biological effects observed were different from those expected. The alterations which one might expect as biological consequences of immunization appeared irregularly, and the failure of reproducibility could only partly be explained by conditional factors. On the other hand, changes in corticosteroid levels, kinetics and excretion were observed, and this paper deals primarily with such effects with an emphasis on biological effectiveness of the immunization.

MATERIALS AND METHODS

Antigens

The antigens of corticosteroids, corticosteroid metabolites, sexual steroids and their metabolites were synthesized in our laboratory according to the method of Erlanger[1]. For corticosteroids (natural and synthetic) 3-oxime, 3.20-dioxime, 21-hemisuccinate for corticosteroid metabolites 20-oxime-bovine serum albumin (BSA) complexes were prepared. More details on the corticosteroid antigens used in our laboratory have been published elsewhere [2–5].

The following sexual steroid antigens were prepared and used in this study: for progesterone, 21-hemisuccinate-BSA; for testosterone, 3-oxime-BSA; for pregnandiol, 20-hemisuccinate-BSA; for 17-OH-pregnenolone, 20-oxime-BSA; for dehydroepiandrosterone, 17-oxime-BSA, for pregnantriol, 21-hemisuccinate-BSA.

A short description of the immunization. 0.5–1.0 mg, for sheep up to 3.0 mg antigen was dissolved in 0.5 ml of 0.9% saline solution, emulsified with 0.5 ml Freund's adjuvant (complete "Difco") and administered i.m. This procedure was repeated at intervals of 2–6 weeks [5].

Corticosteroid estimations

The radioimmunoassay techniques of plasma aldosterone, corticosterone, cortisol, compound S and urinary free aldosterone, tetrahydrocortisol- and tetrahydrocortisone-glucuronid estimations have been published elsewhere [2, 3, 5–10].

For estimation of urinary free corticosterone a chromatographic separation in propylene–glycol–toluene system was used prior to the radioimmunoassay step.

Equilibrium dialysis

Equilibrium dialysis microcell equipment purchased from the firm of Bachofer, Reutlingen, West Germany, was used. Plasma samples (0.1 ml) were equilibrated with 125,000 d.p.m. [H^3]-labelled radioactive ligand for 24 h at 4°C, the dialysis was performed for 2 h at 37°C.

RESULTS AND DISCUSSION

Spontaneous mortality

Two hundred and ninety-one New Zealand white rabbits, 21 sheep and 40 rats have been immunized against steroids, mainly corticosteroids and their metabolites, in our laboratory since 1970. This extensive experience has allowed us to make observations on the spontanous mortality following immunization.

The spontanous mortality was of two different types. The first type occurred in an early series of rabbits. Immunizations against aldosterone, DOC, cortisol and corticosterone together produced muscle weakness and occasional paralysis with a consequence of inability to eat and drink which frequently led to death of the affected animals. Abscesses were observed in this same series and the serum K was somewhat decreased. This syndrome resembled hypercorticism. Haning *et al.*[11] described very similar symptoms with 50% mortality of sheep immunized with an aldosterone antigen. Abscesses were also observed by Chapman[12] in sheep immunized against dexamethasone. This type of mortality was not repeated in later series, and we believe that this mortality was related to infections, climatic and other conditions of animal keeping.

Since 1975 the conditions under which the rabbits are kept have been very good. However, in the years 1975–1978, a second type of mortality appeared, which was characterized by a relatively sudden death. Due to the quick development, no biochemical characterization of this state was possible.

Figure 1 shows the mortality during the period 1975–1978. A comparison made between control rabbits and animals immunized with antigens formed from sexual steroids and their metabolites showed the mortality of the corticosteroid-immunized rabbits to be relatively high. An especially high mortality was observed in those rabbits immunized against cortisol, THF, THB and allo-THB.

No spontanous mortality was observed in either sheep or rats. However, larger groups of animals would be necessary to elucidate the particular role of different corticosteroids and antigens.

Elevations in the total plasma corticosteroid concentrations

Longcope[13] was the first to describe elevated plasma oestrogen concentrations in sheep immunized with oestrogen antigen. Since then numerous reports have been published showing similar results in animals immunized against different sexual hormones. The earliest published reports of our group, from 1972–1974, described findings of elevated cortisol, corticosterone and aldosterone plasma concentrations in rabbits immunized with corticosteroid antigens. The great number of immunizations carried out during the last years has enabled us to obtain more definitive information on this topic. The following figures (Figs 2, 3 and 4) give data on plasma total cortisol, corticosterone, Cpd S and aldosterone concentrations in rabbits immunized with antigens to corticosteroids and their metabolites. It was often observed that not only did the concentrations of the hormones immunized against increase, but that increases in other corticosteroid levels were also seen. The aldosterone values in rabbits and sheep immunized with aldosterone antigens were especially high (Fig. 4). In rabbits the aldosterone values were up to 15 μg(!)/100 ml and in sheep up to 150 μg/100 ml. The cause of the enormously elevated aldosterone levels is not clear. One can speculate about the short half-life time of aldosterone. It is possible that the pooling effect of the antibodies (see later) is more effective in evoking high plasma concentrations of steroids which have short half-lives than of steroids which have long half-lives. The figures show that elevated cortisol, corticosterone and Cpd S levels were often found in rabbits immunized with antigens of different natural and synthetic corticosteroids.

Aldosterone levels changed only relatively slightly in animals immunized against other steroids when compared to those animals immunized against aldosterone itself. Elevated cortisol, corticosterone and Cpd S values were observed with a rather low frequency in rabbits immunized against corticosteroid metabolites. The rabbits immunized with antigens derived from sexual hormones and their metabolites had normal corticosteroid plasma levels. Since 1975, with optimal care being taken of the immunized rabbits, the plasma cortisol concentrations have become lower, indicating that environmental "stress" factors may additionally affect the hormone levels. Nishina *et al.*[14] immunized rabbits with cortisol antigens derivatized at the 6-C position, and found plasma cortisol levels which, while still in an elevated range, were lower than ours. These differences may be explained

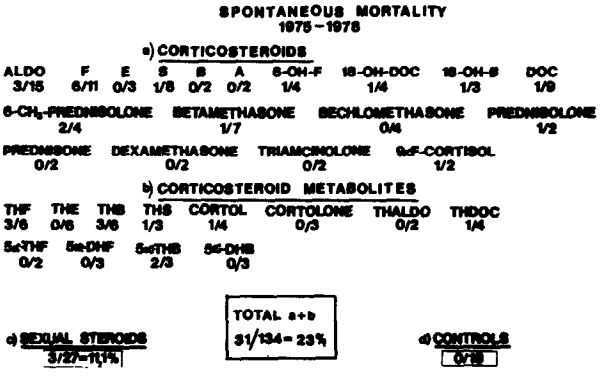

Fig. 1. Spontaneous mortality of rabbits between 1975 and 1978. The first figures of the fractional numbers indicate the mortality, the second one the total number of the immunized rabbits.

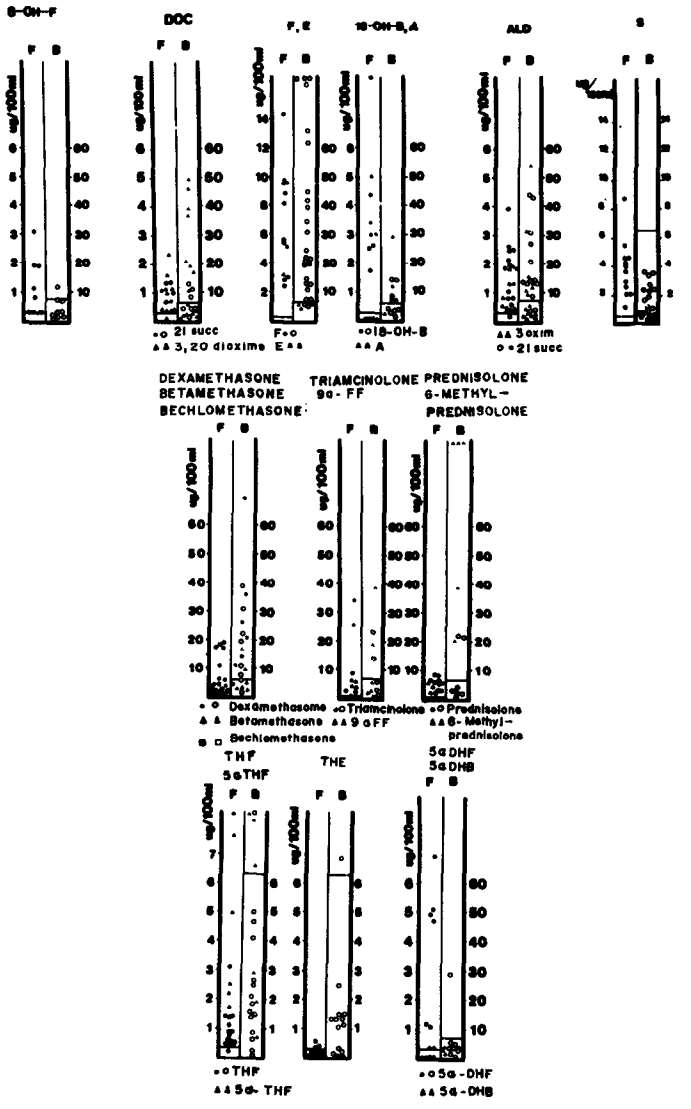

Fig. 2. Total plasma cortisol and corticosterone concentrations of rabbits immunized against corticosteroids and corticosteroid metabolites. Top left to right—rabbits immunized against 6-OH-cortisol (6-OH-F); DOC; cortisol and cortisone (F, E); 18-OH-corticosterone and 11-dehydrocorticosterone (18-OH-B, A); aldosterone (Ald) and compound S (S). For DOC and Ald the types of antigens are especially indicated. Centre—rabbits immunized against 9α-fluorocortisol (9α-FF) and other synthetic corticosteroids. Bottom—rabbits immunized against tetrahydrocortisol (THF); allo-tetrahydrocortisol (5α-THF); tetrahydrocortisone (THE); allo-dihydrocortisol (5α-DHF); and allo-dihydrocortcosterone (5α-DHB). The horizontal lines in the columns indicate the upper limit of the normal range.

by differences of the antigen structures. However, in our experiments no indication of the role of different antigen types, such as 3-oxime, 3,20-dioxime, 20-oxime and 21-hemisuccinate, could be obtained. The following factors can be considered as possible causes of the increase in plasma corticosteroid concentrations:

(a) Pooling of endogenous steroids by the antibodies in the vascular bed. (b) Increased production of steroids by the adrenals. (c) Alterations in corticosteroid metabolism and elimination.

The following data support the role of "pooling" mechanism:

(1) The disappearance of radioactive corticosteroids is delayed in animals immunized actively and passively againse the same steroid [5, 15, 16].

(2) The high degree of correlation between titers and plasma antigen corticosteroid concentrations.

On the other hand, there are data arguing against the exclusive role of "pooling" mechanism:

(1) Elevated corticosteroid concentrations in rabbits immunized with corticosteroid metabolite antigens (the corticosteroid metabolite antibodies do not bind with unmetabolized corticoids).

(2) Elevations, with individual differences, in the antigen independent corticoid levels.

Figure 5 shows that correlation of a high degree could be calculated when plasma concentrations of

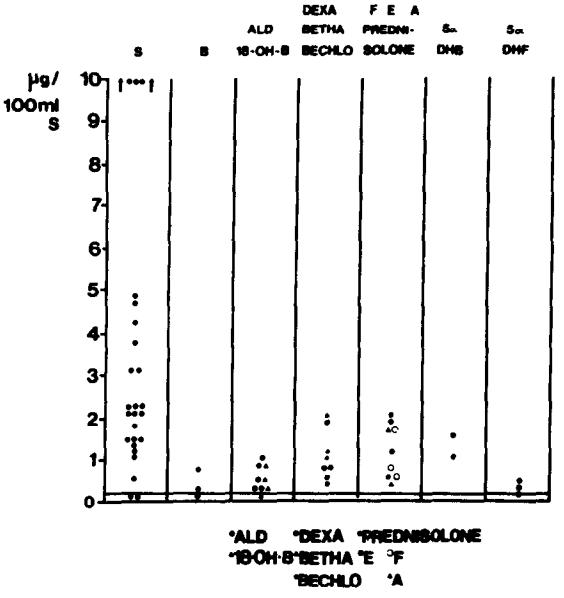

Fig. 3. Total plasma compound S concentrations in rabbits immunized against compound S and other corticosteroids and corticosteroid metabolites.

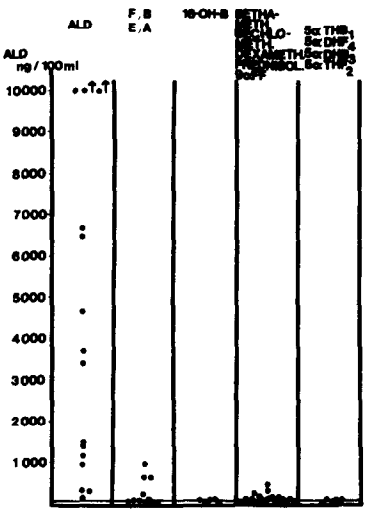

Fig. 4. Total plasma aldosterone concentrations in rabbits immunized against aldosterone and other corticosteroids and corticosteroid metabolites.

the antigen-corticosteroids were plotted against titers established with [H^3]-labelled variants of the same steroid. However (at the bottom of the figure) when concentrations of the antigen-independent steroids were plotted against apparent titers obtained with the radioactive variant of the same antigen independent steroids the correlations are rather moderate.

The following techniques are available for investigation of the production of adrenal steroids:

(1) Histological studies. They indicate a hyperfunction [5, 17].

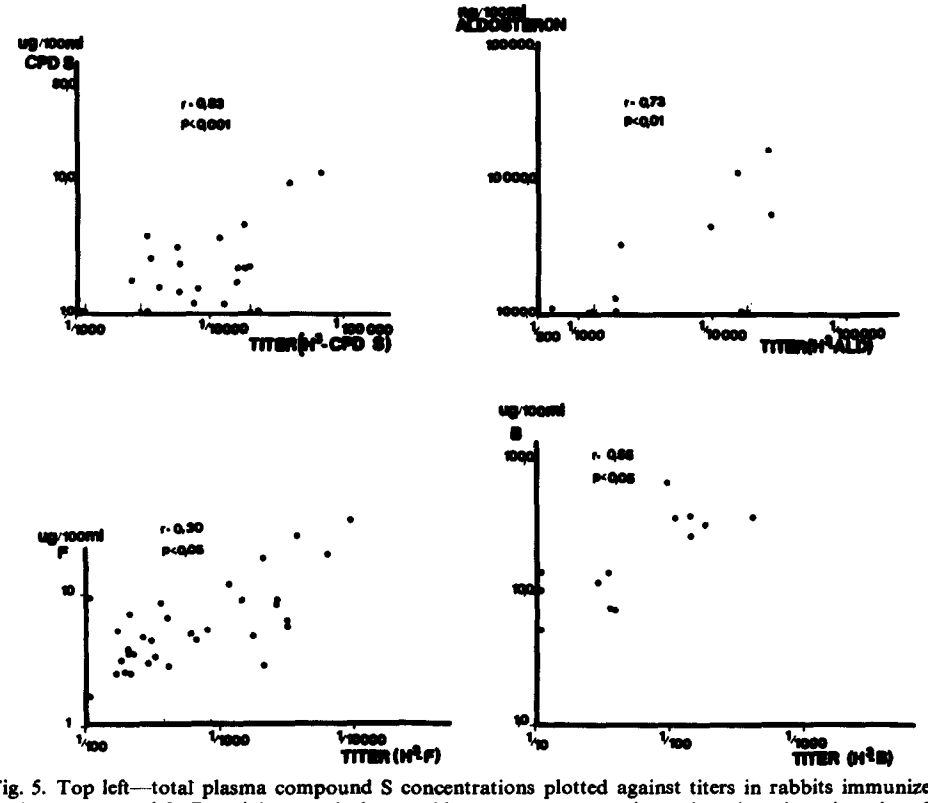

Fig. 5. Top left—total plasma compound S concentrations plotted against titers in rabbits immunized against compound S. Top right—total plasma aldosterone concentrations plotted against titers in rabbits immunized against aldosterone. Bottom left—total plasma cortisol concentrations plotted against titers (obtained with [H^3-F) in rabbits immunized against other steroids than F. Bottom-right—total plasma corticosterone concentrations plotted against titers (obtained with [H^3]-B) in rabbits immunized against other steroids than B.

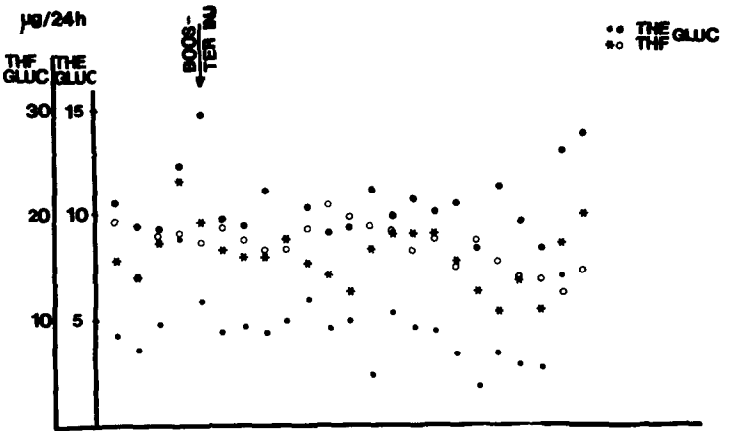

Fig. 6. THF- and THE-glucuronide (Gluc) excretion [7] in 2 rabbits immunized with cortisol antigen prior and after a booster injection.

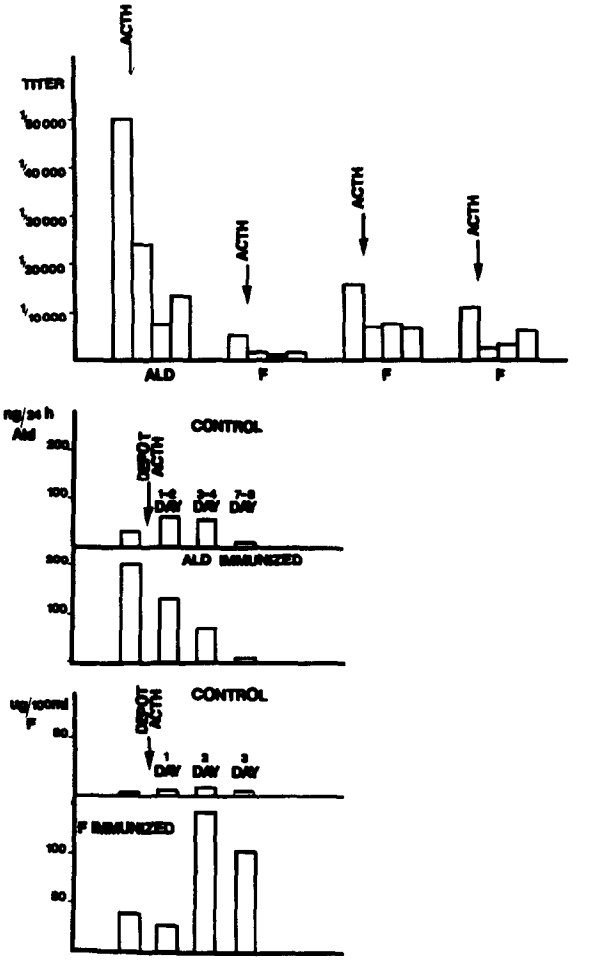

Fig. 7. Top—titers measured in a 24-h interval prior and after administration of "depot" ACTH (50 I.U. Synacthen-depot). In rabbits immunized against cortisol (F) and aldosterone (Ald). Centre—urinary free aldosterone excretion in controls and aldosterone (Ald) immunized rabbits prior and after administration of "depot" ACTH (typical experiment). Bottom—alterations of plasma cortisol in control and cortisol (F) immunized rabbits prior and after administration of "depot" ACTH (typical experiment).

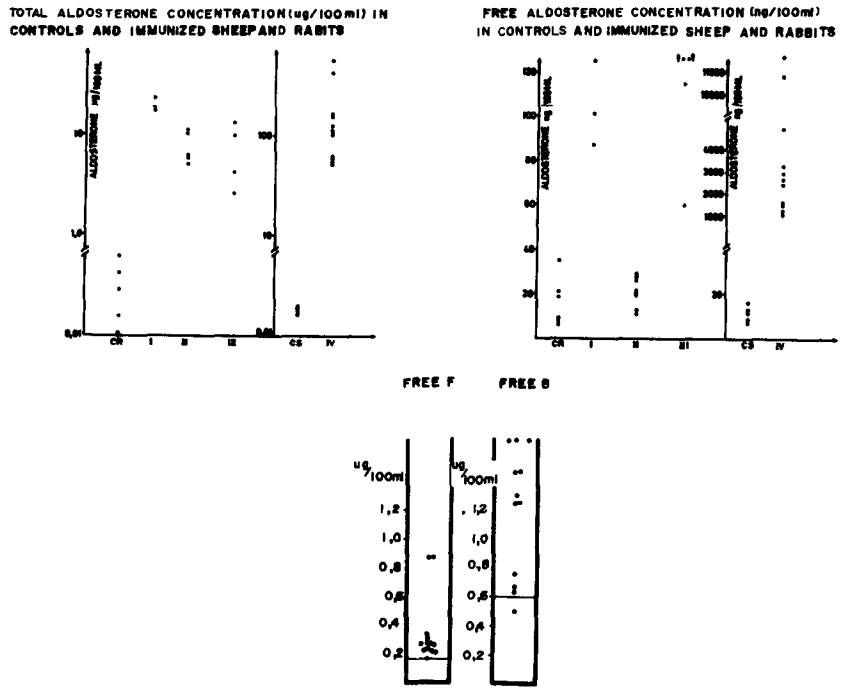

Fig. 8. Top—total and free plasma aldosterone concentrations in controls and aldosterone (Ald) immunized sheep and rabbits. Control rabbits (CR): I—aldosterone 21-hemisuccinate antigen. II—aldosterone 3,20-dioxime antigen. III—aldosterone 3-oxime antigen (rabbit experiments). Control sheep (CS): IV—aldosterone 21-hemisuccinate antigen (sheep). Below—plasma free cortisol (F) and corticosterone (B) concentrations in rabbits immunized against other steroids than F, B. The total plasma F- and B-concentrations of the animals have been increased (see Fig. 2). Horizontal lines: upper limits of normal ranges.

(2) Adrenal venous blood and peripheral blood corticosteroid estimations: evaluation uncertain.

(3) "Secretion rate"—has not been used.

(4) Study on the *in vitro* production of surviving adrenal tissue: no alteration in rats immunized passively with aldosterone antiserum [18]. No data on active immunization.

(5) Estimation of tropic hormones. Renin occasionally decreased [19], mostly unchanged (unpublished data of our laboratory). No data on ACTH.

One has to be cautious, however, concerning the application of morphological findings to the judgement of the adrenal function. In the early sixties, we have noted that rats showing apparent histological equivalents of hyperglucocorticoidism did not have elevated corticosterone levels [20]. It was also shown that 2–3 weeks after adrenal demedullation, or after chronic ACTH treatment, the *z. glomerulosa* of adrenal cortex was either normal or hypertrophied while aldosterone production was decreased [21]. Therefore, functional studies are also necessary. Unfortunately, the presence of antibodies hinders the application of techniques using peripheral or adrenal venous blood sampling. The "secretion rate" technique would probably be better. However, the large fraction of corticosteroids eliminated extra-renal in rats and rabbits may cause problems in evaluation of the results.

Alterations in the *corticosteroid metabolism* could also cause changes in the plasma corticosteroid concentrations. To study this problem THF, THB, aldosterone-18-glucuronide and TH-aldosterone estimations were made in urine samples of rabbits immunized against the corresponding unmetabolized steroids. No significant alterations were found. Figure 6 shows typical data.

Effect of ACTH in immunized rabbits

It had been shown that water-soluble ACTH caused higher cortisol and corticosterone plasma values in rabbits immunized against cortisol and corticosterone than in the controls [15, 16]. Figure 7 shows results of experiments obtained after administration of a "depot" ACTH preparation. The urinary free aldosterone values of control rabbits showed a transient increase followed by the well-known decline of aldosterone excretion. The pre-ACTH aldosterone excretion was increased in rabbits immunized with aldosterone antigen. No further increase was obtained after administration of "depot" ACTH. This was probably due to the fact that aldosterone production was already maximally stimulated. The bottom field of the figure shows plasma total cortisol values. The cortisol values estimated after ACTH were significantly higher in the cortisol immunized than in the control rabbits.

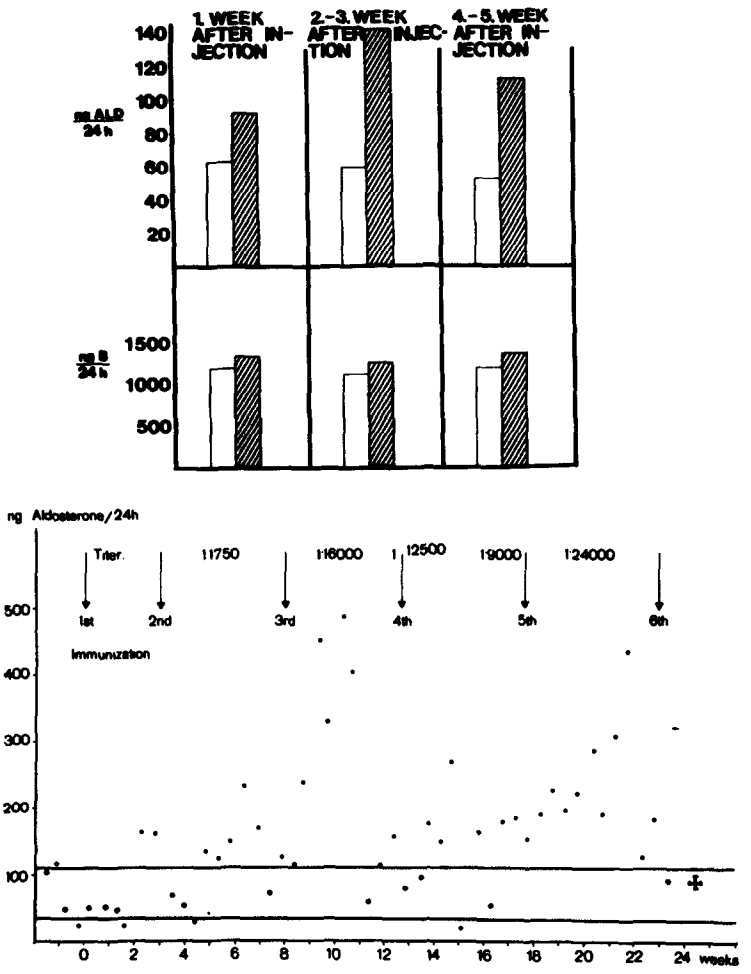

Fig. 9. Top—free urinary aldosterone (Ald) and free corticosterone (B) excretion in rabbits immunized against aldosterone (dashed column) and in control rabbits injected with Freund's adjuvant saline mixture (open column). The columns represent average values of 4 immunization periods (3 rabbits). Bottom—free urinary aldosterone excretion during 24 weeks of immunization of a rabbit immunized against aldosterone.

At the top of the figure an interesting observation, *i.e.* the decrease of titers after injecting ACTH, can be seen. It is possible that the binding sites of antibodies are partly saturated by the elevated corticosteroid concentrations evoked by ACTH.

Free corticosteroids in plasma and urine

To answer the question on the extent of plasma corticosteroids bound with proteins *equilibrium dialysis studies* in the plasma and estimations of urinary free steroids were performed. Figure 8 indicates that increased total corticosteroid concentrations are accompanied by apparently increased plasma free steroid levels. Indices of elevated free corticosteroid levels have also been published by Nieschlag *et al.*[22]. At the bottom, antigen-independent apparent free steroid concentrations are given. They are elevated, too. As a consequence of the contradictions between the apparent free steroid concentrations and other findings obtained by Hilier *et al.*[23] in animals immunized against sexual steroids, criticism of the applicability of the equilibrium dialysis method has arisen. One of these criticisms concerned the uncertainty of measurements of minimum amounts of radioactivity. For aldosterone, however, the bound fraction is relatively small and the previously mentioned methodological failure can hardly account for such erratic results.

Figure 9 shows that the rabbits immunized with an aldosterone antigen excrete significantly higher amounts of free aldosterone than the controls, especially in periods of the highest expected titers, 7–21 days after the last antigen administration. Similar patterns are seen at the bottom of the figure which shows the alterations of the free urinary aldosterone excretion in one rabbit during the 7 month duration of immunization.

Therefore, the measurement of urinary free aldosterone confirmed qualitatively, but not quantitatively, the results obtained by equilibrium dialysis, both indi-

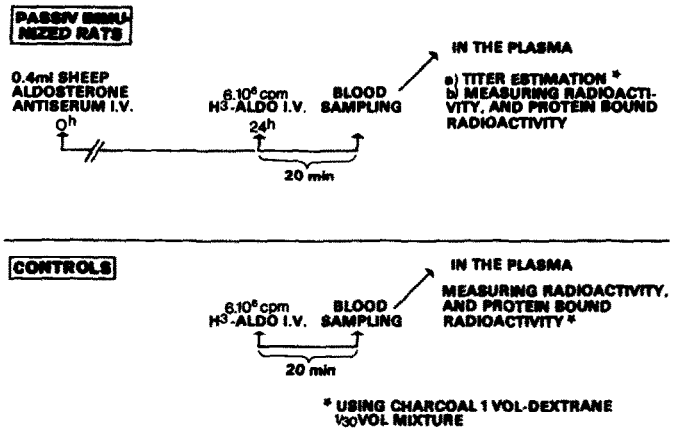

Fig. 10(a). Charcoal solution = 5 gm Norit A, in 80 ml, pH 8.0, 0.05 M borate buffer containing 0.6% human γ-globulin) plus 1/30 vol. dextran solution (100 mg dextran T70) dissolved in 80 ml γ-globulin buffer solution.

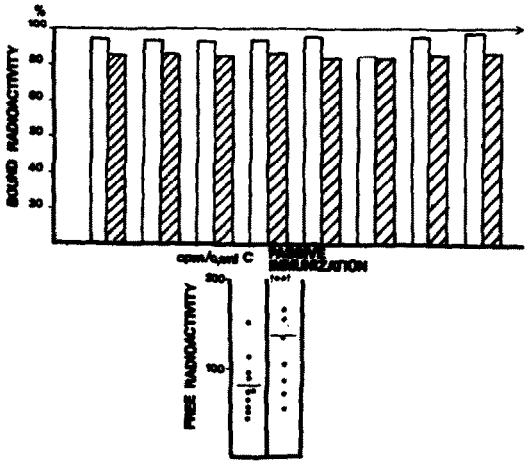

Fig. 10(b) Top—see text. Bottom—Dextran charcoal adsorbed radioactivity (c.p.m./0.1 ml) in control (C) and passive immunized rats according to the protocol of Fig. 10(a). Note that the total radioactivity measured in control animals was 121 c.p.m./0.1 ml on average in the immunized rats 2049.44 c.p.m./0.1 ml. The difference is significant: $P < 0.01$. Similar experiments: the following aldosterone plasma concentrations were estimated 24 h after passive immunization with aldosterone antibody. Total = 176–1027 ng/100 ml (controls up to 40), free aldosterone = 17–68 ng/100 ml (in controls up to 20).

cating an increase in the free corticosteroid concentrations. However, the study on the biological effects of immunization resulted in negative, or uncertain findings: (a) Body weight curve, urine volume: unchanged (studies in active and repeatedly passive immunized animals). (b) Blood and urine electrolytes: in general no changes. (c) Surviving time of adrenalectomized rats: when rats were passively immunized against aldosterone 24 h prior to surgery, no alteration was observed. (d) Blood pressure: mild elevation in rats actively immunized against aldosterone, no effect after single or repeated passive immunization of rats with aldosterone antiserum, no influence of Goldblatt-type rat hypertension of passive immunization against aldosterone (unpublished observations in our laboratory).

Nothing is known about the reasons for the absence of biological effects. One of the possibilities is the inhibition of steroid molecules in attending receptors by the presence of antibodies. This possibility is not supported by any experimental data yet. Another possibility is that the dynamic equilibrium between the protein bound and unbound protein fractions in the plasma is disturbed by the antibodies. In the last part of this paper we should like to describe an experiment supporting this theory.

An attempt was made in our laboratory to estimate directly aldosterone in unprocessed native human plasma samples with radioimmunoassay. This was done according to the description of Al-Dujaili and Edwards[24] but using [H^3]- instead of I^{125}-labelled aldosterone. To our surprise, in one part of the

samples, negative values were estimated: the bound radioactivities were found to be significantly higher than the 0 intercept of the calibration curve.

This surplus of [H^3]-aldosterone binding was found only in the presence of antibody and disappeared when the plasma samples were preheated at 60°C for 35 min, indicating the role of plasma protein fractions, *e.g.* aldosterone binding globulin. Note that in these radioimmunoassay experiments a powerful adsorbent mixture of charcoal and dextran was used. It seems that an interaction between aldosterone, certain plasma proteins and antibody occur, and the binding of aldosterone to plasma proteins became especially pronounced by this interaction.

We suppose that similar interactions can also happen in animals immunized against aldosterone, disturbing the dynamic equilibrium of protein bound and unbound steroid fractions. This problem was investigated in rats passively immunized with sheep aldosterone antiserum. This was done because previous experiments had shown that passive immunization was also able to evoke some basic effects of the immunizations: both the total plasma aldosterone and the apparent free aldosterone concentrations were found to be increased [5 and Böttger, Vecsei unpublished].

Figure 10 (a and b) shows the experimental protocol and the results. The open columns in the top section of the figure indicate the observed bound radioactivities. The dashed columns indicate the expected bound radioactivity of the same plasma samples. The expected bound radioactivity was calculated by means of the quotient of titers of injected antiserum and the plasma samples taken from the rat. The last titer was significantly less, due to *in vivo* dilution and decomposition. The results confirmed our hypothesis that the plasma protein(s), aldosterone and antibody interaction occurs also under circumstances of a passive immunization *in vivo*. As a side-product of the experiment the bottom section of Fig. 10b shows the apparent free radioactivity adsorbed by charcoal–dextran mixture in passive immunized and control rats. The values of passively immunized rats are significantly higher.

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