IN VITRO MODIFICATION OF RAT ADRENAL ZONA FASCICULATA/RETICULARIS FUNCTION BY THE ZONA GLOMERULOSA

By

G. P. Vinson and Barbara J. Whitehouse

ABSTRACT

Following earlier findings, the possibility that the zona glomerulosa of the rat adrenal cortex may modify inner zone function was investigated. In a series of in vitro experiments designed to test this hypothesis it was found that:

1. Inner zone preparations gave significantly more steroid than either glomerulosa tissue incubated alone, or (for the first 40 min of a two hour incubation), an equivalent amount of whole adrenal tissue. Whole tissue yields were greater than glomerulosa alone only at the 120 min point. At no time did the whole tissue output approach the total anticipated by addition of separate inner zone and glomerulosa yields.

2. In 20 min incubations, inner zone steroid output generally could be depressed by the addition of a glomerulosa preincubation medium (PIM), or a steroid extract of a glomerulosa PIM, or by the addition of 100 ng aldosterone. Inner zone PIM had less effect.

3. Higher concentrations of aldosterone gave increased inhibition of inner zone corticosteroid production. The results indicated that only a part of the total corticosterone output was aldosterone sensitive in this way.

4. More specific analysis of the steroids showed that yields of corticosterone from endogenous precursors were depressed by aldosterone, but deoxycorticosterone and 18-hydroxydeoxycorticosterone were not. In the same incubations the presence of aldosterone had no effect on the formation of these three products from [3H]pregnenolone.

In total the results suggest that the presence of the glomerulosa may affect inner zone function by inhibiting the secretion of corticosterone formed from endogenous precursors. It is possible that aldosterone is the agent of this effect.
In a series of recent studies on the functions in vitro of the rat adrenal cortex, differences have been described between the mechanisms of steroid hormone biosynthesis and response to ACTH found in the zona glomerulosa and in the inner zones (Whitehouse & Vinson 1971, 1972; Vinson & Whitehouse 1973a,b).

In particular it has been found that the zona glomerulosa contains a mechanism for the control of secretion of corticosterone independent of other compounds. In contrast, in the inner zones the rates of formation of all products vary together.

It might be expected that in incubations of whole tissue the steroid output would mirror the inner zone results, since the inner zones are the major sources of steroid (cf. Baniukiewicz et al. 1968). However, there is a curious anomaly in the findings, since incubation of minced whole tissue shows a glomerulosa pattern of steroid output, in particular the formation of corticosterone may be suppressed when other compounds are unaffected (Vinson & Whitehouse 1969a,b, 1973a,b; Whitehouse & Vinson 1971, 1972). One possibility is that in some way the presence of the zona glomerulosa in such incubations modifies inner zone functions. This paper presents results of some preliminary experiments which test this hypothesis.

MATERIALS AND METHODS

Animals
Rats were obtained when required directly from commercial suppliers and maintained only for periods of up to a few days in the Department of Physiology, Queen Elizabeth College. When required for collection of adrenocortical tissue they were killed by stunning and rapidly decapitated. All animals were female Wistars of approximately 200 g body weight and provided 55 ± 7 mg adrenal tissue.

Tissue
Adrenals were collected and stored for brief periods on ice. They were trimmed clean of fat, and where necessary, separated into capsules (zona glomerulosa) and inner zones by squashing between glass plates. Whole adrenal tissue was prepared for incubation by mincing with a pair of fine scissors.

Incubations
Tissue from individual rats was incubated in 1 ml Krebs bicarbonate Ringer (pH 7.4) solution at 37°C under an atmosphere of 95% O₂, 5% CO₂. Under these conditions no significant change in pH occurs during the period of incubation.

Expt. 1. – Whole tissue from 6 rats was incubated in separate vessels and compared with 6 incubations each of glomerulosa and inner zone tissue alone. Samples (1%v) of each incubation medium were withdrawn at 20 min intervals over a two hour period. Corticosteroid concentration was estimated by competitive protein binding (CPB, see below) and plotted against time.
Expt. 2. – Inner zone tissue from 6 rats was incubated in 1 ml Ringer in which glomerulosa tissue had previously been incubated for 20 min (glomerulosa pre-incubation medium, PIM), or in Ringer with the addition of the residue from an ethyl acetate extract of PIM and compared with controls. This experiment was performed three times and on two occasions a further set of flasks received the addition of 100 ng aldosterone (expts. 2 a, b, c).

Expt. 3. – For comparison with the effects of glomerulosa PIM, inner zone tissue was incubated in an inner zone preincubation medium, prepared similarly, and compared with controls.

Expt. 4. – To assess the duration of any aldosterone effect, inner zone tissue was incubated in the presence and absence of 100 ng aldosterone and samples of the incubation medium were withdrawn at 20 min intervals as for expt. 1.

Expt. 5. – Inner zone tissue was preincubated for 20 min and then incubated in Ringer containing aldosterone in concentrations from 100 ng to 1 μg/ml to assess the dose-response relationship.

Expt. 6. – Inner zone tissue was incubated with \([7\alpha-\text{H}]\)pregnenolone (1 μCi, 69 Ci/mMol) in the presence of 150 ng aldosterone per ml, and mass and isotope content of corticosterone, 18-hydroxydeoxycorticosterone and deoxycorticosterone were estimated (see below).

Extraction and quantitation of steroids

In expts. 1–5, 1% aliquots of the incubation media were mixed with 1 ml methanol and centrifuged. An aliquot of the supernatant (0.1 ml) was reduced to dryness for assay.

Total (transcortin-binding) corticosteroid was estimated using the competitive protein binding method (CPB) previously described (Vinson 1974). This method is based on conventional techniques using 1% dog plasma as a source of transcortin.

In expt. 6, steroids were extracted from the incubation medium with ethyl acetate and subjected to analysis using the paper and gas chromatographic methods previously described (Vinson & Whitehouse 1969a).

Quantitative results for production of steroid from endogenous precursors were all expressed as ng of steroid produced by tissue from individual animals over the period of incubation. Yields of steroid from the radioactive precursors were expressed as percentages of the added substrate.

RESULTS

In these experiments individual batches of rats were purchased as required for each experiment. Within each batch corticosteroid output was reasonably even, and for each experiment comparison of cortisol and experimental groups is valid within the statistical conventions. Considerable variation occurred between different batches of animals, which may possibly be due to differences in origin, genotype, seasonal variation in diet, reproductive status etc. Comparison of results from control or experimental groups in different experiments is therefore invalid and possibly misleading.
Yields of corticosteroid (ng/adrenal pair) measured by competitive protein binding produced by separated fasciculata/reticularis (Fascic/retic) and glomerulosa (glom.) preparations from pairs of adrenals and by whole adrenal pairs over a period of two hours incubation. For comparison of whole tissue with fasciculata/reticularis (Fascic/retic), $P < 0.05$ at 20 and 40 min, comparison of glomerulosa with whole tissue $P < 0.01$ at 120 min. Values are means of 6 experiments ± se.

Expt. 1. – Changes with steroid output with time are shown in Fig. 1. In general following the initial rapid secretion of steroid there is little evidence of continued output of steroid in either the inner zone or glomerulosa incubations. In contrast, with whole tissue there is evidence of continued output of steroid throughout the two hour period. It is notable, however, that for at least the first 40 min, inner zone was greater than whole tissue output, itself not significantly different from the glomerulosa incubations. Only in the final sample at 120 min was the whole tissue greater than the glomerulosa yield.

Expt. 2. – The results of three trials with the effects of glomerulosa PIM and its steroid extract on inner zone steroid output are shown in Table 1. In the first trial, expt. 2 a the glomerulosa preincubation medium depressed inner zone output. The steroid extract of the PIM gave no significant change. On the second trial (expt. 2 b) the glomerulosa PIM incubations gave yields of steroids similar to the control, while the steroid extract of the PIM significantly depressed steroid output. In this case too the presence of 100 ng aldosterone had a similar effect. On the third trial (expt. 2 c) all three experimental groups showed a significant depression of inner zone steroid output. It should be noted that even in the first two trials where significant depression of steroid output was not always achieved, the total amount of

![Graph showing corticosteroid yield over time](image-url)
Corticosteroid production by inner zone adrenal tissue incubated in Krebs-Ringer bicarbonate for two hours under control conditions, or in the presence of glomerulosa preincubation medium, or its lipid extract, or with the addition of 100 ng aldosterone. Values are ng produced per adrenal pair (means ± se, n = 6 in each case).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+glomerulosa pre-incubation medium (GPIM)</th>
<th>+lipid extract of GPIM</th>
<th>+100 ng aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 2a</td>
<td>627 ± 106</td>
<td>276 ± 74*&lt;sup&gt;a&lt;/sup&gt;</td>
<td>447 ± 84</td>
<td>1183 ± 183*</td>
</tr>
<tr>
<td>2 b</td>
<td>2197 ± 408</td>
<td>2591 ± 309</td>
<td>901 ± 197*&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1183 ± 183*</td>
</tr>
<tr>
<td>2 c</td>
<td>1276 ± 83</td>
<td>861 ± 157*</td>
<td>489 ± 191†</td>
<td>723 ± 170**</td>
</tr>
</tbody>
</table>

* P < 0.05  ** P < 0.02  † P < 0.01.

Corticosteroid extracted was certainly less than the anticipated combined outputs of inner zones and glomerulosa (cf. Fig. 1).

Expt. 3. – In contrast to the results of expt. 2, when inner zone tissue was incubated in inner zone preincubation medium incomplete summation of the two outputs did occur (Fig. 2).

Yields of corticosteroids (ng/adrenal pair/2 h) from rat adrenal inner zones preparations under control conditions and in the presence of an inner zone (fasciculata/reticularis) pre-incubation medium (Fascic/retic P.I.M). Values are means of 6 experiments ± se.
Fig. 3.
Yields of corticosteroids (ng/adrenal pair) from rat adrenal inner zone preparations under control conditions (C) and in presence of 100 ng aldosterone (Ald) at different time intervals. Comparison of C vs aldosterone addition at 20 min, \( P < 0.01 \).
Values are means of 6 experiments ± se.

Fig. 4.
Yields of corticosterone (ng/adrenal pair) produced by rat adrenal inner zone preparations in presence of varying concentrations of aldosterone. Incubation time was 20 min. Values are means of 6 experiments ± se.
Yields of corticosterone (B), deoxycorticosterone (DOC) and 18-hydroxydeoxycorticosterone (18-OH-DOC) (ng/adrenal pair/2 h) formed from endogenous precursors by rat adrenal inner zone preparations (measured by GLC) under control (C) conditions and in presence of 150 ng/ml aldosterone (+Ald.). Comparison of corticosterone produced in control conditions and in presence of aldosterone, $P < 0.05$. Values are means of 6 experiments ± se.

**Expt. 4.** – The effects of a single addition of 100 ng/ml aldosterone to the incubation medium significantly depressed corticosterone output in the 20 min but not in the subsequent samples (Fig. 3).

**Expt. 5.** – The curve of steroid output against Log aldosterone concentration shows that there appears to be a relationship between the two parameters even though the curve is somewhat shallow (Fig. 4).

**Expt. 6.** – In the presence of 150 ng/ml aldosterone the suppression of steroid output was confined to corticosterone; 18 OH-DOC and DOC were unaffected (Fig. 5). In contrast, yields of all three compounds from $[^3]$Hpregnenolone were unaffected by the procedure (Fig. 6).
Yields of \(^{3}H\)corticosterone (B), \(^{3}H\)deoxycorticosterone (DOC) and \(^{3}H\)18-hydroxydeoxycorticosterone (18-OH-DOC) formed from \(^{3}H\)pregnenolone by rat adrenal inner zone preparations in the same incubations as for Fig. 5, expressed as percentages of the added precursor. Values are means of 6 experiments ± se.

**DISCUSSION**

In any experiment in which closed systems of incubation are used reservations may be expressed about the nature of 'steroid output', since an equilibration occurs between steroid in the medium and tissue which can affect further synthesis, thus making the interpretation of the physiological significance of *in vitro* findings difficult. However, in general it is found that the tissue incubated under these circumstances reflects *in vivo* function reasonably well in synthesising steroid both from endogenous and from added radioactive precursors, and in responding to stimuli such as ACTH (e.g. Saffran & Bayliss 1953; Saffran et al. 1967; Vinson & Whitehouse 1970).

With these reservations in mind the present experiments suggest that aldosterone can inhibit corticosterone production *in vitro*. From Fig. 1 it may be seen that the production of CPB corticosteroid (largely corticosterone) by the zona glomerulosa is approximately 50% of the inner zone output. This is in agreement with other observations from these laboratories, using GLC techniques (Whitehouse & Vinson 1971). The whole tissue output on the other hand is considerably less than the sum of the two components, and for at least the first 40 min it is also significantly less than the isolated inner zone output. Similar observations have been made by Baniukiewicz et al. (1968) and Tait et al. (1970). It is clear that throughout the incubation, the capacity of the
inner zone tissue to produce steroid is suppressed, presumably by a zona glomerulosa product. The suppression is most marked in the early stages. The results shown in Table 1 indicate that a lipid extract of a glomerulosa pre-incubation medium, or the addition of aldosterone (in a concentration which is within the capacity of the glands to secrete) are as efficient as the preincubation medium itself in inhibiting corticosteroid output. Even where suppression by the preincubation medium is not significant the total product is considerably less than the sum of the product of the two components incubated separately. Maximally the inhibition obtained is of the order of 50%o. On the other hand inner zone preincubation medium does not inhibit further steroid production by fresh tissue and the product of the two batches of tissue partially summate (Fig. 2). The degree of inhibition of steroidogenesis by 100 ng per ml of aldosterone seems to be somewhat variable and in Fig. 3 at 20 min it is only of the order of 20%o (still statistically different however). This may be partially explained by the wide variation in in vitro steroid secretion seen in these animals. Each batch purchased from the supplier and kept and treated together as a group gave uniform and reasonably consistent results. However, as will be seen from the figures there was a considerable between-group variation. At present we have no explanation for this since each batch had the same origin and while there is a possibility of differences in genotype, age, diet etc., these were not apparent. Another point may be relevant here: inspection of Fig. 4 shows that while the response to aldosterone appears to be dose-related, the curve is shallow. It is clear that it tends to flatten, and will not reach zero output. This means that only a fraction of the corticosterone produced is sensitive to aldosterone: in the experiments illustrated in Fig. 4 this would seem to be about 30%o. Obviously the size of this fraction may vary and contribute to the inter-experiment variability. It would be interesting to know what factors determine sensitivity to aldosterone.

The results illustrated in Figs. 5 and 6 indicate that the inhibitory effect is exerted only on corticosterone from endogenous sources and not on conversion of radioactive pregnenolonone: furthermore 18-hydroxydeoxycorticosterone and deoxycorticosterone are unaffected.

In any incubation system without a continuously flowing medium, the possibility arises that the continued production of hormones may be modified by the accumulation of end products. From time to time this possibility has been raised in adrenocortical literature, but as a whole the data seems somewhat contradictory and fragmentary. Perhaps the best documented aspect is the interaction of glucocorticoids with the adrenal response to ACTH. In vivo experiments in rats and dogs suggest that a high (0.4–1 µg/ml) circulating concentration of glucocorticoids may partially inhibit the response to ACTH (Langdecker & Lurie 1957; Péron et al. 1960; Black et al. 1961; Hill & Singer 1968). On the other hand Yates et al. (1961) and Barthe & Desaulles (1971)
could find no effect of corticosterone or dexamethasone respectively in similar studies in rats. In the absence of ACTH, experiments in vitro have also given conflicting evidence on the effect of glucocorticoids on continued glucocorticoid production, some investigators have found inhibition (e. g. Birmingham & Kurlents 1958) while others did not (Vinson 1966; Kahnt & Neher 1966). On the other hand the in vitro evidence does suggest that rat adrenal products inhibit the response to ACTH. For example it has long been recognised that a pre-incubation period is essential to observe the effects of ACTH in vitro (e. g. Saffran & Bayliss 1953; Bakker & de Wied 1961). It is possible that the elimination of preformed products may also contribute to the special advantages offered by superfusion or isolated cell suspension techniques in the study of ACTH effects in vitro (e. g. Tait et al. 1967; Saffran et al. 1967; Sayers et al. 1971). In the absence of ACTH too, changing the incubation medium periodically or continuously may well enhance the yield of corticosteroids (Birmingham & Kurlents 1958). There is also a striking contrast between the rates of corticosterone production using whole tissue preparations recorded in the superfusion experiments of Tait et al. (1970) and by Vinson & Whitehouse (1969b) using a conventional incubation procedure. In the former experiments, corticosterone output was maximal during the first 30 min of incubation and thereafter declined. In the latter experiment corticosterone output was near zero in the first 30 min and only reached a maximum after 1 h. This only applied to unlabelled corticosterone; that formed from [14C]progesterone was produced maximally from the start. Similar delays before maximum corticosterone production rate is reached have also been shown using adrenal tissue from the duck (Vinson & Whitehouse 1969b), rabbit (Vinson et al. 1971) and in a lizard, Tiliqua rugosa (Vinson et al., in press). The best explanation for the contrast between superfusion and conventional incubation results and between labelled and unlabelled corticosterone production rates in the latter is that in a conventional incubation flask an adrenal product inhibits the early release of corticosterone from endogenous sources. This applies not merely to corticosterone produced in response to an acute ACTH stimulus, but also to 'basal' output, i. e. output already established by ACTH stimulation in vivo.

The effects of aldosterone reported here occur at a concentration which is within the capacity of the gland to produce. It is possible that the presence of aldosterone may account for differences between superfused and conventionally incubated adrenals and also for the difference in steroidogenic capacity between whole tissue and separated zones.

ACKNOWLEDGMENT

We are most grateful to the Wellcome Trust for a research grant (to GPV).
REFERENCES

Baniukiewicz S., Brodie A., Flood C., Motta M., Okamoto M., Tait J. F., Tait S. A. S.,
Black W. C., Crampton R. S., Verdesca A. S., Nedeljkovic R. I., & Hilton J. G.:

Received on January 30th, 1975.