CONTRIBUTION TO THE EXISTENCE OF REGULATORY MECHANISMS AT THE ADRENAL LEVEL

By
Otto Linét

ABSTRACT

Rat adrenal glands atrophied by the administration of cortisol acetate in vivo were used as a model for the study of early metabolic processes occurring in vitro. Atrophied adrenals incubated in the presence of 14C-leucine incorporated subnormal quantities of this amino acid per mg of protein for the first 120 min. When the incubation lasted for a total period of 180 or 240 min a supranormal rise in the 14C-leucine incorporation was observed. Similar changes occurred with some delay with regard to corticosterone production as expressed per 100 mg of tissue. No differences in 14C-leucine incorporation were observed between the control and atrophied adrenals in vivo. Homogenates from atrophied glands incorporated 14C-leucine to a greater extent than the control homogenates. The in vitro incorporation of 14C-orotic acid into the RNA was also higher in atrophied adrenals. The in vitro use of actinomycin D, cycloheximide and amphenone indicated that corticosterone production depended on the incorporation of 14C-leucine. The addition of cortisol to the incubation media markedly decreased the enhancement of 14C-lysine incorporation into the protein of atrophied adrenals. These, as well as additional results suggest rebound phenomena: once atrophic adrenals are transferred to cortisol-free media, reparative processes begin after a delay period. Such phenomena seem to be mediated by regulatory mechanisms at the adrenal level.
Several reports of experimental findings may indicate the existence of a direct control of corticoid production at the adrenal level. Langecker & Laurie (1957) found that the adrenals of hypophysectomized rats pretreated with cortisone have a much lower response to exogenous ACTH than those from similar rats receiving no cortisone. It has been suggested that blood corticosterone levels may control its production in the rat by acting directly on the adrenal gland i.e., by inhibiting the action of ACTH at the adrenal level (Péron et al. 1960). The formation of corticoids by rat adrenals in vitro is reported to be inhibited by the addition of media in which other adrenals have been incubated, or by corticoid concentrations as low as 10−5M (Birmingham & Kurlents 1958). In keeping with these results, the in vitro synthesis of corticosterone is inhibited by the addition of cortisol, prednisolone or dexamethasone despite the addition of ACTH (Fekete & Görög 1963). Furthermore, corticosterone, cortisol and other steroids are reported to inhibit the incorporation of labelled precursors into the adrenal protein and RNA in vitro (Burrow et al. 1966; Ferguson et al. 1967; Morrow et al. 1967; Burrow & Morrow 1968).

Androgens and anabolic steroids administered in vivo to hypophysectomized rats partially decreased the morphological atrophy of the adrenal glands (e.g. Leonard 1944; Linèt & Bartová 1968). Nussdorfer & Mazzocchi (1970a,b) recently observed in autoradiographical studies, that after in vivo administration of corticosterone the incorporation of labelled orotate and leucine is inhibited in the adrenocortical cells of hypophysectomized, ACTH treated rats, along with alteration of several morphological features.

Our previous, in vitro experiments indicate (Linèt & Wadland 1970) that rat adrenals atrophied in vivo by cortisol administration incorporate a higher quantity of 14C-leucine than the control adrenals. Corticosterone production (µg/100 mg of tissue) was the same in both normal and atrophied adrenals. If the in vitro incubation lasted less than 180 min, the atrophied glands had neither an enhanced incorporation of 14C-leucine nor a normal production of corticosterone. Additional results suggested that atrophied adrenals may be used for metabolic studies occurring at the adrenal level. The present paper reports relevant findings.

MATERIALS AND METHODS

Adult, male rats of the Sprague-Dawley strain (Holtzman Company) were fed a Purina Lab Chow diet and tap water ad libitum. In experiments designated as D,G or J (see below), adult male rats of the Wistar-Lysolaje strain were used. Adrenal atrophy was induced by the subcutaneous administration of cortisol acetate in a daily dose of 1 mg/100 g body weight suspended in olive oil (0.1 ml/100 g) for 7 consecutive days. The control groups were treated with olive oil only. The animals were weighed regularly during each experiment.

24 hours after the last injection and between 9 and 11 a.m., the rats were sacrificed
by rapid decapitation (one animal from each group simultaneously). In the cold (4°C) pairs of adrenals were cleaned of extraneous fat, weighed and quartered. Quarters of each pair of adrenals from the same animal were placed in a flask containing 5 ml of Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, with 10 mg of glucose and then preincubated under 95% O₂/5% CO₂ in a Dubnoff metabolic shaker at 37°C for 60 min. The quarters were then transferred to another beaker with 5 ml of fresh KRB containing 1.0 μCi of L-[14C]-leucine with a specific activity of 30.5 mCi/μm (New England Nuclear Corp) or 1.0 μCi of L-[14C]-lysine with a specific activity of 120 mCi/μm (Institute for Radioisotope Research, Production and Utilization, Prague) and incubated in a similar manner. The times of incubation as well as any departures from this design, are indicated below.

Following the incubation, the media were decanted and the adrenal quarters were washed with 2 ml of KRB. These washes were pooled with the incubation media and the concentrations of corticosterone were determined fluorometrically (van der Vies et al. 1960; Bartová & Hocman 1965) by means of an Amino-Bowman spectrophotofluorometer. The incorporation of [14C]-leucine or [14C]-lysine into the adrenal protein was estimated according to Burrow et al. (1966), by means of a Tri-Carb liquid scintillation counter and internal standards in order to correct for quenching. Counting was performed in Bray's scintillation mixture (Bray 1960). The amount of adrenal protein was determined by the method of Lowry et al. (1951).

The following experiments were designed for the studies indicated:

A. – The time-dependency of [14C]-leucine incorporation and corticosterone production in vitro (Figs. 1 and 2). Pairs of adrenals from both control and cortisol-treated rats were incubated in the presence of [14C]-leucine for 30, 60, 120, 180 and 240 min after 60 min of pre-incubation.

B. – In vivo experiment (Table 1). Groups of control and cortisol-treated rats were anaesthetized with 5 mg/100 g body weight of pentobarbital given intraperitoneally 24 hours after the last dose of cortisol. [14C]-leucine in a dose of 2 μCi/100 g and dissolved in 0.2 ml/100 g of saline solution was then rapidly injected to the tail vein. The incorporation of [14C]-leucine into the adrenal protein was measured 10 min after the injection.

C. – Hence the increase of in vitro [14C]-leucine incorporation into the atrophied adrenals might have been due to a larger ratio of surface area to tissue, the adrenals from the control rats were cut into 8 pieces whereas the atrophied adrenals were quartered. Incubation with labelled leucine lasted for 180 min.

D. – [14C]-leucine incorporation into adrenal homogenates involving no transport of amino acid (Table 2). 6 pairs of control and 6 pairs of atrophied adrenals were homogenized in 2 ml of ice cold KRB. A 0.3 ml aliquot of each homogenate was added to 0.7 ml of KRB containing 1.0 μCi of [14C]-leucine (Burrow & Morrow 1968) and each mixture was incubated for 4 hours.

E. – The effect of the preincubation time on the in vitro [14C]-lysine incorporation. Pre-incubation of both the control atrophic adrenals was carried out for 120 min. Incubation with 1 μCi of [14C]-lysine lasted for 120 min.

F. – The in vitro effects of actinomycin D and cycloheximide, as inhibitors of protein synthesis, and amphenone (3,3-bis/paraaminophenyl/-2-butaneone), as an inhibitor of steroidogenesis (Kibeltis & Ferguson 1964) (Table 3). Four groups of adrenal pairs were always used: (1) control, (2) control with addition of an inhibitor, (3) cortisol-atrophied, (4) cortisol-atrophied with the addition of an inhibitor. Cycloheximide or amphenone was present only during the 180 min incubation period, while actinomycin D was present during both the pre-incubation (60 min) and the incubation
periods. The concentration of the inhibitors (expressed per ml of KRB) were as follows: actinomycin D - 20 μg (Burrow et al. 1966); cycloheximide - 15 μg; amphenone - 1 mM (Farese 1966). We have confirmed the finding of the latter author, that actinomycin D does not significantly affect the fluorescent method for the determination of corticosterone.

G. - The effect of cortisol added to the incubation media in vitro on the 14C-lysine incorporation into the cortisol-atrophied adrenal glands. Three groups of adrenal pairs were used: (1) control, (2) cortisol-atrophied, (3) atrophied, with the addition of cortisol at a concentration of 11.2 × 10⁻⁴ M. This concentration is twice as high as the concentration used by Morrow et al. (1967) and which inhibited 14C-leucine incorporation into the normal adrenal by 44% in vitro. Cortisol was dissolved in 0.1 ml of propylene glycol and added to both the pre-incubation and incubation beakers. Propylene glycol only (0.1 ml) was added to the beakers of groups 1 and 2. After pre-incubation for 60 min, each adrenal was incubated with 1 μCi of 14C-lysine for 180 min (Table 4).

H. - The effect of corticosterone accumulation in the media during in vitro incubations (Table 5). Quartered adrenals from untreated animals were incubated for 180 min without any change of media or with a change of each 60 min. In both groups, the pre-incubation periods lasted for 60 min. Corticosterone concentrations were determined in all the fractions of the second group and the sum of these determinations was compared with the corticosterone production of the first group.

I. - The interactions of anabolic steroids with cortisol acetate in vivo (Table 6). Groups of animals received daily doses of (A) 0.2 ml olive oil, (B) 2 mg of 19-nortestosterone 17β-phenylpropionate (NTPP), (C) 1 mg of cortisol acetate, (D) 2 mg of NTPP and 1 mg of cortisol acetate; all doses are expressed per 100 g body weight and were given sc in 0.1 ml of olive oil. In another experiments, methandienolone (17α-methyl-17β-hydroxyandrossta-1,4-dien-3-one) or stanozolol (17α-methyl-17β-hydroxy-5α-androstan-/3,2-c/-pyrazole), both in a dose of 2 mg/100 g/day, were ad-

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**Table 1.**

Effect of in vivo cortisol acetate administration (1 mg/100 g/day × 7) on in vivo 14C-leucine incorporation into the rat adrenal protein (mean ± se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Final body weight (g)</th>
<th>Adrenal weight (mg/pair)</th>
<th>Adrenal protein (mg/pair)</th>
<th>Leucine incorporation (cPM/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>289 ± 4</td>
<td>43.0 ± 2.44</td>
<td>3.11 ± 0.18</td>
<td>420 ± 30</td>
</tr>
<tr>
<td>Cortisol</td>
<td>236 ± 61</td>
<td>26.8 ± 1.381</td>
<td>1.96 ± 0.091</td>
<td>375 ± 25</td>
</tr>
</tbody>
</table>

Twenty four hours after last dose of cortisol, animals were anaesthetized with Nembutal and injected intravenously with 2 μCi 14C-leucine per 100 g body wt. 10 min later the radioactivity of the adrenal protein was estimated. 6 animals were used in each group.

1) Significant at P < 0.01 against control.
ministered orally instead of NTPP. All the in vivo treatments lasted for 7 consecutive days. Animals were sacrificed 24 hours after the last in vivo dose and the adrenals were incubated in vitro.

J. – Incorporation of 14C-orotic acid into the RNA and DNA in vitro by cortisol-atrophied adrenals (Table 7). Pairs of both control and atrophied adrenals were pre-incubated for 60 min and then incubated for 180 min in the presence of 0.5 µCi of 2–14C-orotic acid with a specific activity of 47.0 mCi/mM (Institute for Radioisotope Research, Production and Utilisation, Prague). After incubation, the adrenal pairs were placed in an ice-cold buffered sucrose solution (0.25 M sucrose, 20 mM Tris-HCl, pH 7.6, 10 mM Mg\((\text{CH}_3\text{COO})_2\), 40 mM NaCl, 100 mM KCl) and homogenized with a motor-driven teflon pestle. Nucleic acids were extracted with a modified procedure (Ogur & Rosen 1950). This technique was shown to give the same results as that of Schmidt-Thanhäuser (Riman et al. 1958). Aliquots (2 ml) of the final homogenate extract were used to determine 14C-orotic acid incorporation by scintillation counting. Amounts of RNA and DNA in the adrenal homogenates were estimated spectrophotometrically by absorption at 260 nm and expressed as DNA-P and RNA-P (Riman et al. 1958).

**Fig. 1.**

Effect of incubation time on the in vitro 14C-leucine incorporation into protein of control and cortisol-atrophied adrenals (mean ± se). Pairs of adrenals from both control and cortisol treated animals were incubated in the presence of 1µCi of 14C-leucine after 60 min of pre-incubation. The periods of incubation are given on the abscissa. Results are expressed as cpm per mg of adrenal protein. The cortisol induced adrenal atrophy was similar in magnitude to those introduced in the Results. Brackets indicate number of adrenal pairs used for the determination of each mean.
Effect of incubation time on the in vitro corticosterone production by control and cortisol-atrophied adrenals. Results are expressed as μg of corticosterone produced per 100 mg of adrenal tissue. See legend of Fig. 1 for further details.

Each experiment was repeated at least twice. In experiments where more than 2 groups of animals were used simultaneously, statistical evaluation was performed by analysis of variance and by mean values concordance (Roth et al. 1962).

RESULTS

In vivo administration of cortisol acetate markedly decreased the final body and adrenal weight as well as the adrenal protein content (Table 1). The average decreases (in %) to the corresponding controls from all experiments were $81.3 \pm 1.58$, $60.3 \pm 1.29$ and $56.8 \pm 1.49$ for body weight, adrenal weight and adrenal protein content respectively (mean $\pm$ se). In all instances these differences between the control and cortisol treated groups were significant
Table 2.
Effects of in vivo cortisol acetate administration (1 mg/100 g/day X 7) on adrenal weight, adrenal protein, and in vitro 14C-leucine incorporation into protein of rat adrenal homogenates (mean±se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total adrenal weight (mg)</th>
<th>Adrenal protein (mg/flask)</th>
<th>Leucine incorporation (cpm/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>220.7</td>
<td>2.85±0.11</td>
<td>225±10</td>
</tr>
<tr>
<td>Cortisol</td>
<td>134.8</td>
<td>1.28±0.152</td>
<td>295±251</td>
</tr>
</tbody>
</table>

Adrenal homogenates were prepared by homogenising all the control and all the cortisol-atrophied adrenals in 2ml of ice-cold KRB each. A 0.3 ml aliquot of each homogenate and 0.7 ml KRB were incubated in the presence of 1.0 μCi of 14C-leucine for 240 min. 6 animals were used in each group.

1) Significant at P < 0.05 against control.
2) Significant at P < 0.01 against control.

at P < 0.01. For this reason these estimations will not be described in detail.

Changes in 14C-leucine incorporation with time during the in vitro incubations are shown in Fig. 1. The incorporation by the atrophied adrenals was less than that by the controls during the initial 120 min of incubation. When the in vitro experiments lasted for 180 min or more, a sharp rise in the incorporation by atrophic tissue was noted. In contrast, the incorporation into the protein of the control glands reached a plateau after incubation for 120 min. Corticosterone production by atrophied adrenals was markedly less than that of the control adrenals during the first 120 min of incubation, but no such difference was detected after 120 min (Fig. 2). No changes could be detected between the control and atrophied glands with regard to the in vivo incorporation of 14C-leucine (Table 1).

When the control adrenals were cut into 8 fragments instead of 4, 14C-leucine incorporations into the protein (cpm/mg) were 6130±510 and 11 275±600 for the control and atrophied adrenals respectively. Adrenal homogenates from atrophied adrenal glands incorporated more 14C-leucine than homogenates from the control glands (Table 2). If the pre-incubation period was increased from 60 to 120 min, subsequent (120 min) incubation significantly enhanced the 14C-lysine incorporation into the protein of the atrophied adrenals (controls: 1048±65 cpm/mg; atrophied: 1832±214 cpm/mg).

Results obtained in vitro after the additions of the inhibitors to the incubation media are shown in Table 3. Actinomycin D decreased the 14C-leucine
Table 3.
Effect of actinomycin D, cycloheximide and amphenone on in vitro $^{14}$C-leucine incorporation and corticosterone production in adrenals from rats treated in vivo with cortisol acetate (mean±SE).

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal protein (mg/pair)</th>
<th>Leucine incorporation (cpM/mg of protein)</th>
<th>Corticosterone production (µg/pair)</th>
<th>Corticosterone production (µg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>2.53±0.08</td>
<td>6820±785</td>
<td>3.13±0.45</td>
<td>8.96±1.45</td>
</tr>
<tr>
<td>B Control + actinomycin D</td>
<td>2.68±0.14</td>
<td>3835±450</td>
<td>1.49±0.12</td>
<td>4.14±0.16</td>
</tr>
<tr>
<td>C Cort.</td>
<td>1.81±0.09</td>
<td>10 440±315</td>
<td>1.72±0.32</td>
<td>8.68±1.42</td>
</tr>
<tr>
<td>D Cort. + actinomycin D</td>
<td>1.65±0.14</td>
<td>4895±410</td>
<td>1.06±0.04</td>
<td>4.55±0.08</td>
</tr>
<tr>
<td>Significance at $P &lt; 0.05$</td>
<td>A &gt; C and D</td>
<td>A &gt; B</td>
<td>A &gt; B,C and D</td>
<td>A &gt; B and D</td>
</tr>
<tr>
<td></td>
<td>B &gt; C and D</td>
<td>C &gt; A,B and D</td>
<td>D &lt; B and C</td>
<td>C &gt; B and D</td>
</tr>
</tbody>
</table>

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<tr>
<th>Group</th>
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<th>Corticosterone production (µg/pair)</th>
<th>Corticosterone production (µg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>2.73±0.18</td>
<td>5540±700</td>
<td>3.82±0.27</td>
<td>9.51±1.11</td>
</tr>
<tr>
<td>B Control + cycloheximide</td>
<td>3.08±0.11</td>
<td>325±20</td>
<td>1.37±0.11</td>
<td>3.88±0.09</td>
</tr>
<tr>
<td>C Cort.</td>
<td>1.64±0.03</td>
<td>11 005±1310</td>
<td>2.53±0.16</td>
<td>10.95±0.22</td>
</tr>
<tr>
<td>D Cort. + cycloheximide</td>
<td>1.42±0.11</td>
<td>695±95</td>
<td>0.77±0.10</td>
<td>3.49±0.44</td>
</tr>
<tr>
<td>Significance at $P &lt; 0.05$</td>
<td>A &gt; C and D</td>
<td>A &gt; B and D</td>
<td>A &gt; B,C and D</td>
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<th>Leucine incorporation (cpM/mg of protein)</th>
<th>Corticosterone production (µg/pair)</th>
<th>Corticosterone production (µg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>2.76±0.22</td>
<td>4110±218</td>
<td>3.02±0.43</td>
<td>7.97±1.05</td>
</tr>
<tr>
<td>B Control + amphenone</td>
<td>2.62±0.20</td>
<td>4519±639</td>
<td>0.70±0.08</td>
<td>2.03±0.33</td>
</tr>
<tr>
<td>C Cort.</td>
<td>1.35±0.11</td>
<td>7287±253</td>
<td>1.80±0.29</td>
<td>8.32±0.85</td>
</tr>
<tr>
<td>D Cort. + amphenone</td>
<td>1.39±0.04</td>
<td>8491±881</td>
<td>0.29±0.04</td>
<td>1.51±0.27</td>
</tr>
<tr>
<td>Significance at $P &lt; 0.05$</td>
<td>A &gt; C and D</td>
<td>A &lt; C and D</td>
<td>A &gt; B,C and D</td>
<td>A &gt; B and D</td>
</tr>
<tr>
<td></td>
<td>B &gt; C and D</td>
<td>B &lt; C and D</td>
<td>C &gt; B and D</td>
<td>C &gt; B and D</td>
</tr>
<tr>
<td></td>
<td>D &lt; B</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pairs of quartered adrenals from each experimental group were pre-incubated in KRB for 60 min. Incubation was then carried out for 180 min in the presence of 1 µCi of $^{14}$C-leucine. Actinomycin D was added to obtain a final concentration of 20 µg/ml of KRB during pre-incubation and incubation periods. Cycloheximide and amphenone were added to obtain a final concentration of 15 µg/ml and 1 mM/ml of KRB resp., during the incubation period only. 4 animals were used in each group. Contr. = control, cort. = cortisol acetate.
Table 4.
Effect of cortisol in vitro on 14C-lysine incorporation into adrenals from rats treated in vitro with cortisol acetate (1 mg/100 g/day × 7) (mean ± se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal protein (mg/pair)</th>
<th>Lysine incorporation (cpm/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  Control</td>
<td>3.15 ± 0.17</td>
<td>1494 ± 120</td>
</tr>
<tr>
<td>B  Cortisol acetate</td>
<td>1.90 ± 0.11</td>
<td>3330 ± 325</td>
</tr>
<tr>
<td>C  Cortisol acetate + addition of cortisol</td>
<td>1.88 ± 0.07</td>
<td>432 ± 58</td>
</tr>
<tr>
<td>Significance at P &lt; 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A &gt; B and C</td>
<td></td>
<td>A &gt; C</td>
</tr>
<tr>
<td>B &gt; A and C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After pre-incubation in KRB for 60 min, pairs of quartered adrenals from each experimental group were incubated for 180 min in the presence of 1 μCi of 14C-lysine; where indicated, cortisol was added at a concentration of 11.2 × 10⁻⁴ M during both the pre-incubation and incubation periods. 6 animals were used in each group.

incorporation into the protein of both the control and cortisol-atrophied adrenals to approximately the same extent; corticosterone production was decreased to a similar extent. Cycloheximide markedly decreased the incorporation by control and atrophied glands; nevertheless, the remaining activity was higher

Table 5.
Effect of corticosterone accumulation during in vitro incubations on corticosterone production (mean ± se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal weight (mg/pair)</th>
<th>Corticosterone production (μg/pair)</th>
<th>Corticosterone production (μg/100 mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>33.7 ± 1.19</td>
<td>3.60 ± 0.65</td>
<td>10.51 ± 1.68</td>
</tr>
<tr>
<td>B</td>
<td>35.1 ± 2.12</td>
<td>2.80 ± 0.35</td>
<td>8.09 ± 1.13</td>
</tr>
</tbody>
</table>

A Pairs of quartered adrenals were pre-incubated in KRB for 60 min; after incubation for 180 min, the corticosterone production was estimated.

B Pairs of quartered adrenals were pre-incubated in a similar manner. After each 60 min of incubation, the media were decanted and replaced with fresh KRB. Corticosterone was estimated in all the separate fractions; the results presented in Table 5 represent the total sum of these values. Six adrenal pairs were used in each group.
Table 6.
Effects of the in vivo administration of cortisol acetate (1 mg/100 g/day × 7) and NTPP (2 mg/100 g/day × 7) on in vitro 14C-leucine incorporation and corticosterone production by rat adrenals (mean±se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Final body weight (g)</th>
<th>Adrenal weight (mg/pair)</th>
<th>Adrenal protein (mg/pair)</th>
<th>Leucine incorporation (cpm/mg of protein)</th>
<th>Corticosterone production (μg/pair)</th>
<th>Corticosterone production (μg/100 mg of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>272 ± 10</td>
<td>38.6 ± 2.32</td>
<td>2.76 ± 0.25</td>
<td>4671 ± 285</td>
<td>3.01 ± 0.36</td>
<td>7.76 ± 0.61</td>
</tr>
<tr>
<td>B NTPP</td>
<td>263 ± 8</td>
<td>40.0 ± 4.60</td>
<td>3.06 ± 0.54</td>
<td>8793 ± 528</td>
<td>3.19 ± 0.76</td>
<td>7.93 ± 1.80</td>
</tr>
<tr>
<td>C Cortisol</td>
<td>198 ± 6</td>
<td>21.9 ± 0.30</td>
<td>1.54 ± 0.06</td>
<td>8595 ± 915</td>
<td>1.50 ± 0.40</td>
<td>6.83 ± 1.78</td>
</tr>
<tr>
<td>D Cortisol + NTPP</td>
<td>221 ± 4</td>
<td>26.8 ± 0.45</td>
<td>2.50 ± 0.30</td>
<td>6720 ± 108</td>
<td>2.35 ± 0.66</td>
<td>8.77 ± 2.49</td>
</tr>
<tr>
<td><strong>Significance at P &lt; 0.05</strong></td>
<td><strong>A &gt; B and C</strong></td>
<td><strong>A &gt; C and D</strong></td>
<td><strong>A &gt; C and D</strong></td>
<td><strong>A &lt; B,C and D</strong></td>
<td><strong>C &lt; A and D</strong></td>
<td><strong>C &lt; A and D</strong></td>
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<td>B &gt; C and D</td>
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<td>C &lt; D</td>
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After pre-incubation in KRB for 60 min pairs of quartered adrenals from each experimental group were incubated for 180 min in the presence of 1 μCi of 14C-leucine. Each group included four animals. NTPP = 19-nortestosterone 17β-phenylpropionate.
Table 7.

Effects of *in vivo* administration of cortisol acetate (1 mg/100 g/day \(\times 7\)) on *in vitro* \(^{14}\)C-orotic acid incorporation into adrenal RNA and DNA (mean \(\pm\) se).

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal weight (mg/pair)</th>
<th>RNA-P ((\mu g/100) mg of tissue)</th>
<th>DNA-P ((\mu g/100) mg of tissue)</th>
<th>CPM/RNA-P/ adrenal pair</th>
<th>(^{14})C-orotic acid incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CPM/10 (\mu g) of RNA-P</td>
<td>CPM/100 mg of RNA-P</td>
</tr>
<tr>
<td>Control</td>
<td>35.8 (\pm) 1.90</td>
<td>28.9 (\pm) 1.68</td>
<td>47.9 (\pm) 3.0</td>
<td>466 (\pm) 26</td>
<td>425 (\pm) 36</td>
</tr>
<tr>
<td>Cortisol</td>
<td>23.7 (\pm) 1.41(^2)</td>
<td>28.5 (\pm) 1.56</td>
<td>53.8 (\pm) 3.9</td>
<td>349 (\pm) 29(^2)</td>
<td>522 (\pm) 30(^1)</td>
</tr>
</tbody>
</table>

In both groups, pairs of quartered adrenals were pre-incubated in KRB for 60 min, and then incubated for 180 min in the presence of 0.05 \(\mu\)Ci of \(^{14}\)C-orotic acid. 6 animals were used in each group.

\(^1\) = significant at \(P < 0.05\) against control.

\(^2\) = significant at \(P < 0.01\) against control.
in the atrophied tissue. Corticosterone production was also decreased in both the control and atrophied adrenals. Amphenone considerably decreased the corticosterone production by the control and atrophied glands; incorporation of $^{14}$C-leucine, however, remained unaffected.

In the latter experiments the atrophied adrenals produced less corticosterone per pair then did the control pairs. When the production was calculated per 100 mg of tissue, no differences could be detected (Table 3, see also Fig. 2). The presence of cortisol in the media during both pre-incubation and incubation periods markedly decreased the enhanced $^{14}$C-lysine incorporation in the atrophied adrenals (Table 4). Table 5 shows, however, that corticosterone accumulation in the medium which might cause an inhibiton of corticosterone production in normal adrenals had no such effect on the in vitro corticosterone production.

As indicated in Table 6, decreases in total body weight, adrenal weight and adrenal protein content, as well as increases in $^{14}$C-leucine incorporation found in the cortisol-treated animals, were counteracted by the coadministration of NTPP. The decrease in adrenal corticosterone production however, was not influenced. It should be noted that NTPP alone enhanced leucine incorporation, as did cortisol acetate. Less pronounced, but similar effects were obtained if instead of NTPP methandrostenolone or stanozolol was administered together with cortisol – with the single exception, that methandrostenolone plus cortisol decreased corticosterone production (in $\mu$g/100 mg tissue).

No difference in the nucleic acid concentration could be detected between control and cortisol-atrophied adrenals. The $^{14}$C-orotic acid incorporation into RNA-P expressed as cpm/pair of adrenals was lower in the atrophied glands (Table 7); when the incorporation was expressed as cpm/10 $\mu$g of RNA-P or as cpm/RNA-P/100 mg of tissue, higher values were obtained for the atrophied adrenals. No such differences could be detected in this respect for DNA-P.

DISCUSSION

The incorporation of $^{14}$C-leucine into the adrenal protein of control adrenals (Fig. 1) was found to be nearly linear with regard to time up to 120 min of the incubation in vitro; after 120 min, however, no significant increments were detected. Similar findings have been described for the in vitro incorporation of $^{14}$C-glycine into adrenal protein (Bransome & Reddy 1963). In comparison with the control adrenals, the atrophied gland initially incorporated low amounts of labelled leucine; unexpectedly, this incorporation was markedly enhanced after incubation for 180 min (Fig. 1). Furthermore, in vivo findings (Table 1) suggest that this dramatic, supranormal increase in $^{14}$C-leucine incorporation occurs only under in vitro conditions. It is of interest that the
increase in $^{14}$C-leucine incorporation was found to precede the \textit{in vitro} enhancement of corticosterone production by atrophied adrenals (Fig. 2).

The result of the experiment in which control adrenals were cut into 8 instead of 4 fragments indicated that the access of $^{14}$C-leucine into tissue apparently did not limit the rate of incorporation. It appears unlikely therefore, that the smaller size of the quarters from cortisol-atrophied adrenals was responsible for the enhanced incorporation of labelled leucine. Furthermore, the increased incorporation of radioactive aminoacid was also found in homogenates from atrophied glands (Table 2). In this case, amino acid transport should not be involved (Burrow & Morrow 1968). When the pre-incubation period lasted for 120 min instead of 60 min, an enhancement of $^{14}$C-lysine incorporation into the adrenal protein during the incubation was detected as early as after 120 min. On the other hand, when a 60 min pre-incubation period was used, the enhancement was detected only after 180 min of incubation (Fig. 1). It is possible that the sum of the times of the pre-incubation and incubation periods is related to the enhanced incorporation of the labelled precursors into atrophied adrenals.

Actinomycin D is a potent inhibitor of DNA-dependent RNA synthesis and secondarily inhibits protein synthesis. The recent report of Bransome (1969) however, limits the value of this antibiotic as an experimental tool, because its actions frequently appear to be non-specific manifestations of toxicity. Several reports have indicated that actinomycin D decreases the base line production of corticoids by adrenal fragments \textit{in vitro} (for references, see Bransome 1969) as well as the incorporation of labelled amino acids (Burrow et al. 1966; Ferguson et al. 1967). In our experiment, actinomycin D decreased corticosterone production and $^{14}$C-leucine incorporation not only in the control, but also in the atrophied adrenals (Table 3). Similar results (Table 3) were obtained with cycloheximide, which interferes more directly with protein synthesis: this antibiotic markedly decreases both amino acid incorporation and steroid production \textit{in vitro} by normal adrenals (Burrow et al. 1966; Davis & Garren 1968). It should be emphasized that the enhancement of $^{14}$C-leucine incorporation into the cortisol-atrophied adrenals is sensitive to both actinomycin D and cycloheximide, although the mechanisms of action of both inhibitors are different. Furthermore, despite morphological and metabolic differences, the control and atrophied adrenals reacted to both inhibitors in a similar manner.

\textit{In vitro} results with amphenone (Table 3) clearly demonstrate that inhibition of steroidogenesis does not influence $^{14}$C-leucine incorporation by normal or atrophied adrenal tissue. These and other findings (Figs. 1 and 2; Table 3) suggest that $^{14}$C-leucine incorporation may influence corticosterone production in both control and atrophied adrenals. On the other hand, a decrease in steroidogenesis does not appear to inhibit amino acid incorporation. The

\begin{table}
\begin{tabular}{|c|c|c|}
\hline
\textbf{Experiment} & \textbf{Control} & \textbf{Atrophied} \\
\hline
\textbf{Amino acid incorporation} & 50 & 75 \\
\hline
\textbf{Steroid production} & 25 & 50 \\
\hline
\end{tabular}
\end{table}
steroidogenic effect of ACTH appears to be related to adrenal protein syn-
thesis (Ferguson 1963; Farese 1964).

The addition of cortisol during both the pre-incubation and the incubation
periods inhibited the in vitro enhancement of 14C-lysine incorporation into the
protein of atrophied adrenals (Table 4). This result may be related to the
reported inhibitions by some steroids, including cortisol, of in vitro incor-
porations of amino acids into the normal rat adrenals (Burrow et al. 1966;
Ferguson et al. 1967; Morrow et al. 1967; Burrow & Morrow 1968). On the
other hand, the results shown in Table 5 suggest that the in vitro enhancement
of corticosterone production by atrophied adrenals cannot be explained on the
basis of a feedback inhibition in the control adrenal glands. If such an inhibi-
tion is to be detected, more adrenal tissue and a smaller volume of incubation
media must be used (Birmingham & Kurlents 1958).

Of the 3 anabolic steroids studied in vivo, only depot injectable NTPP
significantly counteracted the cortisol-induced decreases in total body weight,
in adrenal weight, and in adrenal protein content. Unexpectedly, the enhance-
ment of 14C-leucine incorporation in vitro by atrophied adrenals was diminished
by NTPP, given simultaneously, although this steroid alone increased the
in vitro incorporation (Table 6). It is of interest that testosterone added in vitro
to the incubation media affects radioactive amino acid incorporation by adrenal
tissue (Burrow & Morrow 1968). Both methandrostenolone and stanozolol when
combined with cortisol in vivo behave in a similar manner but are less active
than NTPP. In an earlier paper, we postulated (Linét & Bartová 1966) that
the adrenal atrophy induced by glucocorticoids is mediated not only via ACTH
but also by a direct catabolic effect on adrenal tissue and that only the latter
effect is affected by anabolic agents.

Anabolic steroids were not found to enhance the low corticosterone content
of cortisol-atrophied adrenals in vivo (Linét & Bartová 1966). The present
results show that none of the steroids studied is able to enhance the low
in vitro corticosterone production by atrophied adrenals. These results support
the view that anabolic steroids do not improve the deterioration of adrenal
function despite definite inhibition of adrenal atrophy.

In summary, data of the present study support the view (Linét & Wadland
1970) that increases in amino acid incorporation and corticosterone production
which occur in cortisol-atrophied adrenals during in vitro incubation should
be considered as a rebound phenomenon. After the atrophied adrenals are
transferred to cortisol-free media, reparative processes occur or, in other
words, some kind of de-repression occurs. Since the enhancement of protein
synthesis is of the utmost importance for damaged cell metabolism, it is
logical that an enhanced amino acid incorporation into adrenal protein may
be a primary step in the de-repression. Moreover, this enhancement is followed
by an increase in corticosterone synthesis, from low levels.
On the other hand, such a view must take into consideration some alternative suggestions. The enhancement, in vitro, of labelled leucine and lysine incorporation by atrophied adrenals could possibly be due only to a passive process, i.e. damage to the adrenal structure caused by cortisol administration. Several observations do not support this possibility: 1) the incorporation is not enhanced a) during the initial period of the in vitro incubation, b) in vivo, c) when cortisol is present in pre-incubation and incubation media, and d) if the in vivo cortisol administration lasts for 4 days only despite the production of marked adrenal atrophy (Linét & Wadland 1970); 2) $^{14}$C-leucine incorporation is enhanced in adrenal homogenates; 3) the changes in $^{14}$C-leucine incorporation are followed by similar changes in corticosterone production; 4) $^{14}$C-orotic acid incorporation into RNA is also increased (Table 7), suggesting an enhancement of RNA synthesis. Although steroidogenesis is maintained in vitro by rat adrenals for 20 hours (Laplante & Stachenko 1966) and although in vitro preparations of rat adrenals respond to ACTH after incubation for 180 min (Morrow et al. 1967), we cannot completely discount arteficial conditions of the in vitro incubations.

The expressions used for the incorporation of radioactive amino acids deserve consideration. The expression cpm/mg of adrenal protein, has been commonly used in the relevant literature (i.e. by Burrow et al. (1966), Ferguson et al. (1967), Morrow et al. (1967), Burrow & Morrow (1968)). In critical experiments of this report only this expression was used for several reasons. In each experiment a) the degree of adrenal atrophy is of the same magnitude and the adrenal protein content correlates very closely with the adrenal weight and b) control adrenals are always included. If amino acid incorporation were expressed as cpm per pair of adrenal glands the incorporation after 180 min of incubation would be of the same magnitude for both the control and atrophied glands. This, however, was not so for a period of 240 min of incubation (Fig. 1) where the incorporation, if expressed in absolute terms is higher in the atrophied tissue. Furthermore, if these absolute values (cpm/adrenals pairs) were to be used under the conditions shown in Fig. 1, the per cent enhancement of incorporation after 120 min of incubation would be negligible in the control group, but high in the cortisol-atrophied group. Similar considerations are also valid for corticosterone production expressed as $\mu$g/adrenal pair (Fig. 2). Therefore such expressions in respect to time-course changes should be given due consideration, especially as both estimates are very low in the atrophied glands during the first 120 min of incubation.

The close relation between endogenous corticoid production in the adrenal cortex and adrenaline biosynthesis in the adrenal medulla (Wurtman 1966) suggests that the enhanced incorporation of $^{14}$C-leucine by cortisol-atrophied adrenals might be explained by metabolic processes occurring solely in the medulla. Our previously reported findings, however, do not support this sug-
gestions. The administration of cortisol acetate (2 mg/100 g body weight × 8) produces marked adrenal atrophy in rats, but does not influence adrenal concentrations of either adrenaline or noradrenaline. Furthermore, in acute experiments, the intravenous administration of prednisone does not interfere with the uptake of tritiated noradrenaline by rat adrenals (Linët & Hertting 1966).

Bransome (1968) has reported that the sensitivity of the adrenal to ACTH with regard to in vivo growth as followed by adrenal weight gain and net synthesis of DNA, RNA and protein, is proportionately greater in dexamethasone-suppressed guinea pils that in normal animals. This finding is somewhat similar to our present observations.

Further experimental work is necessary for a more complete elucidation of the regulatory mechanisms at the level of the adrenal cortex. The use of cortisol-atrophied adrenals seems to be a suitable model for the study of early recovery processes occurring in vitro in the absence of cortisol in the media. Similar processes which occur after the withdrawal of exogenous glucocorticoids in vivo are of long duration, apparently due to the persistence of the administered corticoids, and for other reasons, which are more complex.

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