STUDIES ON THE MECHANISM OF ACTION OF THE ADRENOCORTICOTROPIC HORMONE*

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It has been demonstrated that adrenocorticotropic hormone (ACTH) stimulates isolated, perfused adrenal glands (1) and adrenocortical tissue slices (2, 3) to release increased quantities of corticosteroid hormones. These findings are in accord with the well established fact that ACTH in vivo causes the adrenal cortex to secrete increased quantities of the adrenal steroid hormones. The present work was performed in an attempt to learn more about the mechanism by which ACTH effects this stimulation of the adrenal cortex. The first group of experiments indicated that ACTH acts primarily by increasing the rate of corticosteroid synthesis rather than by primarily causing release of the steroid hormones. Further investigation of the mechanism of action of ACTH revealed that the adrenal stimulation is accompanied by an increase of phosphorylase activity in the tissue. Data will be presented showing the relationship between phosphorylase activity and steroidogenesis.

Methods

Incubation of Slices—Adrenal cortices from steer glands were sliced free-hand and incubated at 37° in an isotonic salt medium in a manner similar to that previously described (3). A Ringer-bicarbonate medium was substituted for the saline-phosphate medium formerly used, and, instead of the whole glands being perfused with blood as earlier, the tissue slices were given a 45 minute preincubation, as described by Saffran and Bayliss (4). Commercial ACTH was added routinely to a final concentration of 0.1 unit per ml. of incubating medium; this was in the order of 1 unit per gm. of tissue slices.

Chemical Analysis—Corticosteroid hormones were extracted with ethyl acetate, and chromatography of these extracts was performed as described previously (3). For routine analyses, however, chromatography was not carried out, because it was found that ethyl acetate extracts, washed twice with water, could be assayed satisfactorily without further purification.

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In the experiments concerned with intracellular content and distribution of the corticosteroids, the hormones were determined with red tetrazolium dye by the technique of Mader and Buck (5). In all subsequent experiments the blue tetrazolium technique of Nowacrynski et al. (6) was used. For all analyses cortisone was used as a standard compound.\(^1\)

The chloroform-formamide solvent system of Zaffaroni and Burton (7) was used for filter paper chromatography of the steroids.

**Enzymatic Assays**—Glucose-6-phosphate dehydrogenase was assayed by the technique of Kelly et al. (8), and phosphoglucomutase by the method of Sutherland (9). Phosphorylase activity was measured by using the assay system of Sutherland and Wosilait (10), with the incubation time increased from 10 to 30 minutes.

For these assays, tissues were homogenized in a TenBroeck glass homogenizer or in a Potter-Elvehjem homogenizer; for some experiments the tissue was ground with sand in a mortar.

**EXPERIMENTAL**

**Intracellular Corticosteroid Content during ACTH Stimulation**—Experiments were performed to see whether ACTH caused a change in the intracellular corticosteroid content, because it was thought that, if ACTH acts primarily to increase corticosteroidogenesis, ACTH should increase the intracellular content of corticosteroids in the adrenal cortex. On the other hand, if ACTH acts primarily to stimulate the release of corticosteroids from the adrenal cell, then one would expect to find some decrease in the intracellular corticosteroid level. The intracellular content and distribution of corticosteroids in adrenal slices were determined under the following conditions. After 20 minutes incubation with and without ACTH added to the medium, adrenal slices were washed with Ringer's solution and homogenized in 5 ml. of solution containing 0.06 M sodium acetate buffer (pH 3.5) and 0.176 M sucrose. The resulting homogenate was centrifuged 45 minutes at 2-5\(^\circ\) at a force of approximately 6000 \(\times\) g or 30 minutes at 100,000 \(\times\) g, and the supernatant and sedimented portions were analyzed separately. Table I shows the results of a typical experiment; ACTH significantly increased the intracellular corticosteroid content and this increase occurred largely in the soluble fraction of the cells. Other experiments performed under the same conditions gave similar results. It was concluded that ACTH acted primarily on corticosteroid synthesis rather than on corticosteroid release.

**Factors Influencing Corticosteroid Synthesis by Homogenates**—The char-

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\(^1\) The tetrazolium dye techniques measure the reducing value of the corticosteroids, which is a function of the \(\alpha\)-ketol side chain; thus they do not necessarily indicate the quantity of biologically active steroids present.
acteristics of adrenal homogenates were investigated in order to delineate some of the conditions and factors influencing corticosteroid synthesis. The homogenates were prepared from adrenal slices which were incubated for 30 minutes in a Ringer's solution with or without ACTH added, washed with cold 10 per cent sucrose, blotted, and homogenized with enough 10 per cent sucrose to make a 50 per cent homogenate; this was centrifuged at 5° at 350 × g for 6 minutes. Aliquots of the supernatant fluid (0.4 ml.) were used for incubation. Potassium phosphate buffer (0.1 M, pH 7.4) was added to a final concentration of 2 × 10⁻² M, and nicotinamide to a final concentration of 2.8 × 10⁻² M; addition of other components was made at the same time. The tubes were shaken for 30 minutes at 37° with air as the gas phase and analyzed for their corticosteroid content. Table II shows the data of such an experiment. As reported by Reich and Lehninger (11), the addition of fumarate to the homogenates greatly increased the production of corticosteroids; other experiments confirmed this repeatedly and, moreover, showed that the additions of acetate, acetoacetate, or cholesterol to the homogenates did not influence the production of corticosteroids. It can be seen in Table II that the addition of TPN² together with fumarate enhanced corticosteroid production, whereas the addition of the same concentration of TPN alone had no effect. In relation

<table>
<thead>
<tr>
<th>Control slices</th>
<th>ACTH-treated slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimented fraction</td>
<td>Soluble fraction</td>
</tr>
<tr>
<td>4.5</td>
<td>1.9</td>
</tr>
<tr>
<td>3.5</td>
<td>4.6</td>
</tr>
<tr>
<td>4.0</td>
<td>4.3</td>
</tr>
<tr>
<td>4.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Mean values</td>
<td>4.0</td>
</tr>
<tr>
<td>Sums of means of soluble and sedimented fractions</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Table I

Effect of ACTH on Intracellular Corticosteroids

The content of corticosteroids was determined in the soluble and sedimented (45 minutes at 6000 × g) fractions of adrenal slices at the end of 20 minutes incubation with and without ACTH. The values are in micrograms of corticosteroids per gm. (wet weight) of adrenal tissue.

²TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.
to this finding, Sweat and Lipscomb (12) have reported that fumarate stimulates 11β-hydroxylase activity of enzyme preparations from the adrenal cortex by maintaining TPN in the reduced form, the reduction being mediated by a transhydrogenase. Inasmuch as TPN and fumarate af-

**TABLE II**

*Production of Corticosteroids by Adrenal Homogenates*

The values are expressed as micrograms of corticosteroids produced per gm. of adrenal tissue (wet weight) during a 30 minute incubation of the homogenates. All the values represent the mean of duplicate incubations. In homogenate experiments of this type, zero time values of corticosteroids were usually in the range of 15 to 40 γ per gm. of tissue. In this particular experiment the initial value was 33 γ of corticosteroids per gm. of adrenal tissue.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Corticosteroid formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Fumarate (4 × 10⁻² M)</td>
<td>99</td>
</tr>
<tr>
<td>&quot; (4 × 10⁻²) + TPN (4 × 10⁻⁴ M)</td>
<td>136</td>
</tr>
<tr>
<td>TPN (4 × 10⁻⁴ M)</td>
<td>2</td>
</tr>
<tr>
<td>TPNH (6 × 10⁻³ M)</td>
<td>22</td>
</tr>
</tbody>
</table>

**TABLE III**

*Influence of Various Additions on Corticosteroid Production of Adrenal Homogenates*

The values are expressed as micrograms of corticosteroids produced per gm. of adrenal tissue (wet weight) during a 30 minute incubation period. All the values represent the mean of duplicate incubations. Homogenates were fortified with TPN, 4 × 10⁻⁴ M.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Homogenates prepared from control slices</th>
<th>Homogenates prepared from ACTH-treated slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Glucose-6-PO₄ (10⁻² M)</td>
<td>110</td>
<td>102</td>
</tr>
<tr>
<td>Glucose-1-PO₄ (10⁻⁸ &quot;&quot;&quot;)</td>
<td>91</td>
<td>67</td>
</tr>
<tr>
<td>Glycogen (10 mg. per ml.)</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>&quot; + liver phosphorylase</td>
<td>69</td>
<td>62</td>
</tr>
</tbody>
</table>
this enzyme forms reduced TPN in the process of dehydrogenating glucose-6-phosphate, it was thought that, if reduced TPN were indeed the critical stimulating factor, then the addition of glucose-6-phosphate to the homogenates should increase corticosteroidogenesis. Table III shows that the addition of glucose-6-phosphate was effective. In other experiments it was found that fumarate plus glucose-6-phosphate was no more effective than glucose-6-phosphate alone.

It was found that glucose-1-phosphate was nearly as effective in stimulating corticosteroid synthesis as was glucose-6-phosphate. This can be seen from the data of Table III. These findings suggested that the phosphoglucomutase of the adrenal cortex is highly active, and consequently glucose-1-phosphate can be converted rapidly to glucose-6-phosphate.

**Relationship of Phosphorylase to Steroidogenesis**—The addition of glycogen failed to produce the strong stimulus to corticosteroid synthesis that the two glucose phosphate esters elicited, indicating that phosphorylase is relatively inactive in the adrenal cortex and thus constitutes a rate-limiting step which might be a point of hormonal control. Such a situation has been shown to exist in liver, where phosphorylase activity limits the rate of glucose production from glycogen, and the level of phosphorylase activity is regulated by epinephrine and glucagon (13). That the concentration of phosphorylase has a profound effect on the production of corticosteroids in these homogenates can be seen from the data of Table III, last line. A purified preparation of liver phosphorylase was added (the final concentration was 100 γ per ml.) to homogenates fortified with glycogen, and a marked stimulation of corticosteroidogenesis occurred. Paper chromatography indicated that this increase in corticosteroids occurred in compounds more polar than cortisol.

In experiments in which homogenates were fortified with glycogen it was found that homogenates prepared from ACTH-treated slices produced several times more corticosteroids than did homogenates prepared from control slices although the absolute output of corticosteroids was small with both types of homogenates. The same difference in steroid production was seen occasionally without added glycogen, but could not be reproduced consistently. It may be that homogenates responding without added glycogen had a relatively high intrinsic content of glycogen. The results of three experiments in which glycogen was added are presented in Table IV. The difference in rates of corticosteroidogenesis between "control" and "ACTH" homogenates suggested that there was a higher phosphorylase level present in homogenates prepared from ACTH-treated slices.

The effect of ACTH on phosphorylase activity of adrenal slices was then investigated. It was found that, if adrenal slices were incubated with
ACTH, then chilled, homogenized, and the homogenates assayed for phosphorylase activity, there was a definite increase in phosphorylase activity ranging from 30 to several hundred per cent greater than the phosphorylase activity of similarly treated control slices to which no ACTH had been added. Table V shows the results of two such experiments; the phosphorylase activity of homogenates made in sucrose solutions is reported, together with assays of homogenates prepared in 0.1 M sodium fluoride solution. Comparable results were obtained when the results of the phos-

3 Fluoride solutions were used because of the possibility that the inactivation of adrenal phosphorylase may be inhibited by fluoride as is the inactivation of liver phosphorylase (14).

### Table IV

**Effect of Added Glycogen on Corticosteroid Production of Homogenates**

The values are expressed as micrograms of corticosteroids produced per gm. of tissue (wet weight) during a 30 minute incubation of the homogenates. All the homogenates were fortified with TPN, $4 \times 10^{-4}$ M.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No addition</th>
<th>Glycogen added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenates from control slices</td>
<td>Homogenates from ACTH-treated slices</td>
</tr>
<tr>
<td>1</td>
<td>$-4, -4$</td>
<td>$-4, -3$</td>
</tr>
<tr>
<td>2</td>
<td>$1, 3$</td>
<td>$3, 2$</td>
</tr>
<tr>
<td>3</td>
<td>$-3, -2$</td>
<td>$0, -1$</td>
</tr>
</tbody>
</table>

* Ascorbic acid $10^{-3}$ M added.
† Incubation time, 120 minutes.

### Table V

**Phosphorylase Activity of ACTH-Treated Tissue Slices**

The values are in micromoles of phosphate released per gm. of tissue (wet weight) during the 30 minute incubation of a standard phosphorylase assay.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Phosphorylase activity in control slices</th>
<th>Phosphorylase activity in ACTH-treated slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Assay of sucrose solution homogenate</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; NaF solution homogenate</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>Assay of sucrose solution homogenate</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; NaF solution homogenates</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>
phorylase assays were determined by a quantitative glycogen-iodine color technique. When tissue extracts were incubated under the conditions of the phosphorylase assay, but no primer glycogen was added to the system, the liberation of inorganic phosphate decreased to approximately 12 per cent of the amount liberated in the presence of primer glycogen. This finding, coupled with the demonstration of increases in the iodine-glycogen color during phosphorylase assay, is good evidence that the liberation of inorganic phosphate from glucose-1-phosphate was truly the result of phosphorylase activity and not the result of phosphatase action. The differ-

Fig. 1. Effect of ACTH on phosphorylase activity of adrenal tissue slices. Phosphorylase activity is given in micromoles of inorganic phosphate liberated from glucose-1-phosphate per gm. (wet weight) of adrenal tissue during the phosphorylase assay. Each value represents the mean of duplicate slice incubations. X, phosphorylase activity of slices to which ACTH (approximately 1 unit per gm. of tissue) had been added at zero time. , phosphorylase activity of slices to which saline had been added at zero time.

ence in phosphorylase activity between extracts prepared from ACTH-treated slices and control slices was maintained when the phosphorylase was precipitated with ammonium sulfate. This suggested that the activation of phosphorylase brought about by ACTH represents a change in or on the phosphorylase molecule itself.

Fig. 1 shows the rate at which phosphorylase activation occurs in tissue slices. It can be seen that 1 minute after ACTH addition there was a definite increase in the level of phosphorylase activity.

Specificity of Response of Phosphorylase to ACTH—A number of substances have been tested for the ability to activate adrenal phosphorylase. No compound other than ACTH has shown an effect on adrenal phosphorylase under the experimental conditions employed. Substances tested in-
cluded epinephrine, glucagon, insulin, bovine albumin, bovine globulin, and sodium salicylate. On the other hand, ACTH was tested in a disrupted cell preparation of liver which consistently responds to glucagon and epinephrine with an increased phosphorylase concentration (15). ACTH had no effect on this system. Thus it would appear that the adrenal phosphorylase activation is highly specific for ACTH, and ACTH is not capable of activating phosphorylase in one other tissue tested, the liver.

In one experiment, two other enzymes of the adrenal cortex were assayed simultaneously with phosphorylase; these enzymes were phosphoglucomutase and glucose-6-phosphate dehydrogenase. It was found that, whereas ACTH caused an activation of phosphorylase under the conditions of the experiment, no effects on phosphoglucomutase and glucose-6-phosphate dehydrogenase were noted. Thus it would appear that the augmentation of phosphorylase activity by ACTH is not merely the result of a general enzymatic activation, but is a specific effect of ACTH.

**DISCUSSION**

Because the intracellular content of corticosteroids was increased after ACTH stimulation, it was concluded that ACTH acts to stimulate corticosteroid synthesis rather than release, and it was possible to proceed experimentally on this basis.

The discovery of Sweat and Lipscomb (12) of the importance of reduced TPN in certain oxidations performed by the adrenal cortex made possible a coherent explanation of the stimulation noted when TPN and fumarate or TPN and a glucose phosphate ester were added to adrenal homogenates. Further studies with homogenates suggested that ACTH might act to regulate the level of glucose-6-phosphate in the adrenal cortex by stimulating phosphorylase activity. The finding that ACTH does indeed increase phosphorylase activity of adrenal tissue slices lends strong support to the concept that ACTH accelerates corticosteroid synthesis by activating phosphorylase. This activation presumably causes larger amounts of glucose-1-phosphate to be formed from glycogen. The glucose-1-phosphate is rapidly converted to glucose-6-phosphate, which is oxidized largely by the dehydrogenase system (8). The dehydrogenase reactions generate reduced TPN which appears to stimulate corticosteroid synthesis.

This concept of action of ACTH is consistent with the findings of Noble and Papageorge (16), who found that stimulation of the adrenal cortex is accompanied by a fall in glycogen content of the gland.

The activation of phosphorylase by ACTH is rapid and specific in terms of both the activator and enzyme. For these reasons, plus the fact that phosphorylase activity appears to be rate-limiting in adrenal homogenates

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4 Personal communication from Dr. Jacques Berthet.
fortified with glycogen and TPN, it is felt that the activation of phospho-
rylase by ACTH is of major importance in the stimulation of the adrenal
cortex to synthesize and release increased quantities of corticosteroids.

Stone and Hechter (17) have presented evidence that the rate-limiting
steps in corticosteroid synthesis occur between cholesterol and pregnen-
alone. It will be of interest to learn whether these enzymatic reactions
require reduced TPN. The concepts proposed in this paper would imply
that this was so.

SUMMARY

1. Adrenocorticotropic hormone (ACTH) acts to increase the concen-
tration of corticosteroids within adrenal cells; thus the primary action of
ACTH is on synthesis rather than release of the corticosteroids.

2. The rate of synthesis of corticosteroids in beef adrenal homogenates
appears to depend upon the rate of production of reduced triphosho-
pyridine nucleotide (TPN). Because of the high level of activity of the
TPN-dependent enzyme, glucose-6-phosphate dehydrogenase, in adrenal
tissue, glucose-6-phosphate added to homogenates can serve as a generator
of reduced TPN.

3. In beef adrenal homogenates to which glycogen has been added, the
concentration of glucose-6-phosphate appears to depend primarily upon
the phosphorylase activity of the preparation. Addition of phosphorylase
enzyme to such a preparation stimulates corticosteroid synthesis.

4. ACTH causes a rapid and specific activation of phosphorylase in ad-
renal tissue slices.

5. It is thought that one major mechanism by which ACTH stimulates
corticosteroid synthesis is the following: ACTH increases phosphorylase
activity; this, in turn, converts glycogen to glucose-1-phosphate at an in-
creased rate. Glucose-1-phosphate is rapidly converted to glucose-6-
phosphate, which is metabolized primarily by dehydrogenation. In the
process of dehydrogenation reduced TPN is generated; reduced TPN then
stimulates the processes of corticosteroid synthesis.

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ing problems of paper chromatography.

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