Improved sensitivity to adrenocorticotrophin after purification and pre-incubation of isolated rat adrenal cells

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Abstract. A highly sensitive bioassay for adrenocorticotrophin (ACTH) has been developed, in which isolated adrenal cells, purified previously by passage through a 5% BSA layer were pre-incubated for 1 h. The detection limit for hACTH1-39 in this system is 0.19 ± 0.10 (sd) fmol/ml. As a result of the purification step the ED50 for hACTH1-39 decreased from 14.1 ± 4.8 to 6.9 ± 1.2 fmol/ml. Pre-incubation of the purified cells decreased this value significantly to 4.0 ± 1.5 fmol/ml. For ACTH1-24 the increase of sensitivity after purification was even higher, the ED50 changing from 7.1 ± 4.2 to 1.6 ± 0.2 fmol/ml. After pre-incubation of the purified cells this value amounted to 0.8 ± 0.3 fmol/ml. After purification and pre-incubation of the adrenal cells the changes observed for the ED50's of hACTH1-32 and of a highly purified human hypophyseal extract paralleled exactly the change observed for hACTH1-39.

The improved sensitivity of the purified cell suspension might in fact represent a decrease in ACTH degradation. Both purification and pre-incubation could lead to removal or inactivation of enzymes which attach the polypeptide hormone. This hypothesis is supported by experiments in which the cell suspension has been contaminated with an adrenal homogenate prior to the purification and pre-incubation steps.

The present results indicate that pre-incubation of purified isolated adrenal cells has increased the sensitivity and that the amino acid sequence 33–39 is not involved in protecting the molecule from proteolytic attack.

Isolated adrenal cells are much more sensitive to ACTH than adrenal quarters (Kloppenburg et al. 1968). Various bioassays for ACTH have been described which employ the corticosterone production in a suspension of adrenal cells as a measure for ACTH activity. The collagenase disruption technique of Kloppenburg et al. (1968) has proven to be a valuable method to produce an adrenal cell suspension (Haning et al. 1970; Rivkin & Chasin 1971; Richardson & Schulster 1972; Moyle et al. 1973; Mulder 1975; Falke et al. 1975). However, suspensions obtained by this technique generally appear to be less sensitive to ACTH than suspensions which can be produced by a trypsin treatment (Sayers et al. 1971). Nevertheless, assays with the trypsin technique (Kitabchi & Sharma 1971; Nakamura & Tanaka 1971; Lowry et al. 1973; Fehm et al. 1973; Gewirtz et al. 1974; Liotta & Krieger 1975; Finn et al. 1976) vary greatly in sensitivity to ACTH: the ED50 values range from about 10 fmol ACTH1-24/ml (Sayers et al. 1971) to about 400 fmol/ml (Nakamura & Tanaka 1971). Also, a rather broad range of potency values has been described for the various ACTH-peptides, particularly ACTH1-24 (Schwyzer et al. 1971; Seelig & Sayers 1971; Lowry et al. 1973).

In the present paper an isolated adrenal cell assay is described with a sensitivity high enough to measure plasma ACTH levels. The cells – disrupted by means of collagenase – were purified by passage at normal gravity through a 5% BSA solution, a modification of the procedure described by Bennett et al. (1974). To achieve a further gain in sensitivity a pre-incubation step was introduced to further decrease enzyme activity which attacks the ACTH molecule. The consequences of the
improvements are discussed and evaluated at the hand of the testing of different ACTH-peptides.

Parts of the work presented here has been published in abstract form (Goverde et al. 1977).

Materials and Methods

ACTH-peptides

ACTH₁⁻²⁴ and hACTH₁⁻³⁹ (revised sequence), hACTH₁⁻³₂, ACTH₁⁻¹⁰ and a human hypophyseal extract were generous gifts from Dr. W. Rittel and Dr. P. Desaulles (CIBA-Geigy, Basel), Dr. G. Fekete (Gedeon Richter, Budapest), Dr. Hondius Boldingh (Organon Oss), respectively.

Glassware

All glass surfaces were siliconized using dimethylidichlorosilane (BDH Chemicals Ltd.).

Preparation of isolated adrenal cells

For each experiment 5 male Wistar rats (180–220 g) were killed by decapitation. The adrenals were removed, freed of fat, cut in about 10 pieces and transferred to a cold (4°C) 25 ml flask containing 0.2% glucose in 10 ml Krebs-Ringer bicarbonate buffer (KRBG), 32 mg crude collagenase (Sigma, type I) and 400 mg bovine serum albumin (BSA, OHRD, Hoechst). The tissue was incubated in a Lab-Line metabolic shaker at 100 oscillations/min at 37°C in an atmosphere of 95% O₂, 5% CO₂. After 50 min of incubation the tissue was disrupted by pipetting up and expelling the adrenal tissue with a siliconized Pasteur pipette for about 50 times (Haning et al. 1970). The large material was allowed to settle before the suspension was transferred to a cold 100 ml polyethylene tube. Two ml of buffer containing 0.5% BSA and 7.65 mM Ca (KRBGCa) were added to the residue and the material was disrupted again. Both supernatants were combined and centrifuged at 100 g for 10 min (4°C). The supernatant was discarded, the pellet re-suspended in 10 ml KRBGCa and the suspension was centrifuged again. This procedure was repeated once more and after the final centrifugation the cells were re-suspended in 4 ml KRBGCa (non-purified suspension) and the resulting suspension was purified following a modification of the method of Bennett et al. (1974). One ml was layered upon 8 ml 5% BSA in KRBGCa in a polystyrene tube (Ø: 14 mm). After 30 min the upper layer was removed by suction and the BSA layer, which contained the purified cells, was diluted appropriately.

Incubation procedure

Incubation of the non-purified suspension was performed by adding 0.9 ml to a polyethylene tube which contained 0.1 ml ACTH-solution (0.9% NaCl, 0.5% BSA, pH adjusted to 3.5 with 1 N HCl). The tubes were placed in a Lab-Line metabolic shaker and the cells were incubated at 37°C under an atmosphere of 95% O₂, 5% CO₂. When the purified suspension was used the cells were pre-incubated in the tubes for 60 min, prior to addition of the ACTH-solution. All observations were made in duplicate except for the observations listed in Table 2. In a number of experiments the purified cells were not pre-incubated in order to detect the effect of pre-incubation. Incubation time was 120 min. In some experiments 0.1 mg NADPH (Boehringer) was added to check the quality of the suspension.

Corticosterone measurement

To each incubation tube 6.5 ml methylene chloride was added. After extraction for 2 min the tubes were centrifuged at 200 g for 10 min. The aqueous phase was removed by suction and 1 ml of reagent (sulphuric acid: ethanol, 70:30 v/v) was added to 5 ml of the methylene chloride phase. After a contact time of 1 min the organic phase was removed and the fluorescence was measured after 50 min, using an Amino-Bowman spectrophotofluorometer at 470 nm (exciting wavelength) and 530 nm (emitting wavelength). Extracted corticosterone was used as standard.

Potency analysis

The potencies of the polypeptides were expressed as reciprocals of their molar ED₅₀ and were related to the

corticosterone (µg/2h) vs hACTH₁⁻³⁹ fmol/ml

Fig 1.

Log dose-response curves obtained both with non-purified (●●●●) and pre-incubated, purified cells (○○○○). Each point is the mean of duplicate incubations.
molar ED50 of hACTH$^{1-39}$. The potency of hACTH$^{1-39}$ is assigned a value of 100 in the non-purified suspension as well as in the pre-incubated, purified suspension.

**Cell viability**

Cell viability was tested by mixing an aliquot of the suspension with an equal volume of trypan blue (BDH; 100 mg in 100 ml 0.9% NaCl). Cells which excluded the stain were scored as viable. The cell content of the incubation tubes in the different experiments varied from 25000 to 50000 cells (mean: about 40000 cells/ml) for both types of suspensions.

**Statistical evaluation**

In the tables the mean values are presented together with the standard deviation. Student's t-test was used as a test of significant difference between groups.

### Results

**Effect of purification and pre-incubation on isolated adrenal cells**

When the adrenal cell suspension was not purified a considerable amount of non-viable cells and numerous free-moving cell fragments were seen at microscopic examination. After purification only a very small quantity of the cell fragments remained. Recovery of the viable cells after purification varied in 10 experiments from 38 to 89% with a mean of 64%.

Log dose-response curves obtained for hACTH$^{1-39}$, characteristic for untreated and pre-incubated purified cell suspensions are presented in Fig. 1. Purification and pre-incubation produced a marked increase in sensitivity for ACTH. The standard curve was linear between 1 and 50 fmol when a pre-incubated purified suspension was employed and between 5 and 50 fmol when the non-purified suspension was used.

The improved sensitivity of the purified cells is also reflected in the ED50 values (Table 1). These values were calculated from the log dose-response curves obtained in experiments which were performed on separate days. The ED50's obtained with a non-purified suspension were significantly higher than those obtained with a purified non-pre-incubated cell suspension. In addition the effect on pre-incubation of the purified cells was studied. After this procedure the ED50 decreased further to the significantly lower values of 4 fmol for hACTH$^{1-39}$ and 0.8 fmol for ACTH$^{1-24}$.

In three separate experiments the effect of pre-incubation on the sensitivity level was studied.

#### Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Cells non-purified non-pre-incubated</th>
<th>Cells purified non-pre-incubated</th>
<th>Cells purified pre-incubated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ED50 in fmol/ml</td>
<td>n</td>
<td>ED50 in fmol/ml</td>
</tr>
<tr>
<td>hACTH$^{1-39}$ mean ± SD</td>
<td>14.1 ± 4.8</td>
<td>10</td>
<td>6.9 ± 1.2*</td>
</tr>
<tr>
<td>ACTH$^{1-24}$ mean ± SD</td>
<td>7.1 ± 4.2</td>
<td>10</td>
<td>1.6 ± 0.2*</td>
</tr>
</tbody>
</table>

Netto corticosterone production in ng.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>n</th>
<th>Mean ± SD</th>
<th>n</th>
<th>Mean ± SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mg NADPH</td>
<td>316 ± 206</td>
<td>10</td>
<td>29 ± 24*</td>
<td>12</td>
<td>13 ± 12*</td>
<td>18</td>
</tr>
</tbody>
</table>

* Significant difference between this value and the value of non-purified non-pre-incubated cells ($P < 0.001$).

** Significant difference between this value and the value of purified non-pre-incubated cells ($P < 0.001$).
Effective sensitivity

Although the sensitivity was significantly enhanced by solely pre-incubating the cell suspension (mean ED50 10.5 fmol/ml vs. mean ED50 16.3 fmol/ml $P < 0.001$) in each of these experiments a higher sensitivity was reached when purified cells were pre-incubated (mean ED50 6.5 fmol/ml vs. mean ED50 10.5 fmol/ml).

Fig. 2 shows that the optimal pre-incubation time was between 60 and 120 min.

In the presence of NADPH the non-purified suspension produced 316 ng of corticosterone (Table 1). After purification with or without pre-incubation the steroidogenesis induced by NADPH appeared to be minimal.

Sensitivity and accuracy with purified pre-incubated suspensions

In order to detect the lowest effective dose of ACTH in the improved bioassay, the responsiveness of the adrenal cells was analysed in 10 experiments performed on separate days. In each experiment the corticosterone response to a range of hACTH1-39 doses from 0.1-1.11 fmol was measured. Each dose was analysed 5-fold (Table 2). In 5 out of 9 experiments the addition of 0.11 fmol ACTH resulted in a significant increase in corticosterone production as compared to the control values. The addition of 0.22 fmol of ACTH resulted in a significant increase in corticosterone production in 9 out of 10 experiments. The mean lowest dose which resulted in a significant response was $0.19 \pm 0.10$ fmol/ml (i.e. $0.85 \pm 0.47$ pg/ml).

In 10 consecutive experiments the mean index of precision $\lambda$ (Gaddum 1953) for the dose range where response is linearly related to the log of the dose, amounted to $0.050 \pm 0.019$.

Table 2.

Effect of low doses of ACTH on corticosterone production (ng/tube) in pre-incubated purified suspensions.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Dose hACTH1-39 fmol/ml (n = 5)</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>25 ± 3</td>
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<tr>
<td>3</td>
<td>17 ± 3</td>
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<tr>
<td>4</td>
<td>11 ± 1</td>
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<tr>
<td>5</td>
<td>8 ± 1</td>
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<tr>
<td>6</td>
<td>22 ± 3</td>
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<tr>
<td>7</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>8</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>9</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

* Lowest dose at which the value differs significantly ($P < 0.001$) from the control value.
** Lowest dose at which the value differs significantly ($P < 0.01$) from the control value.
Activities of different ACTH-peptides

In the non-purified suspension as well as in the pre-incubated, purified suspension the tetracosapeptide ACTH₁⁻²⁴ appeared to be more potent than hACTH₁⁻³⁹ (Fig. 3). The increase in sensitivity, which resulted from the purification and pre-incubation procedure was more pronounced for ACTH₁⁻²⁴ than for hACTH₁⁻³⁹. The ED₅₀ for ACTH₁⁻²⁴ in the pre-incubated purified suspension was about 8.5 times lower than in the non-purified suspension whereas for hACTH₁⁻³⁹ the ED₅₀ decreased only with a factor 3.5 (Table 1).

Table 3 shows that no change in molar potency of hACTH₁⁻³² and of human hypophyseal extract was observed when the cells were purified and pre-incubated. ACTH₁⁻¹⁰ has a very low potency as compared to hACTH₁⁻³⁹. Nevertheless, an increase in sensitivity of the cells to the decapeptide was observed when the cell suspension was purified and pre-incubated.

Removal of ACTH-inactivating substances by purification and pre-incubation

The following experiment was performed in order to assess the capacity of the purification and pre-incubation process to remove ACTH-inactivating cell fragments and substances from the suspension. A concentrated crude adrenal cell suspension (7 ml) was divided in two equal parts. To one part 0.5 ml of an adrenal homogenate (2 adrenals homogenized in 0.5 ml buffer) was added and to the other part 0.5 ml of buffer. Each suspension was tested for steroidogenic response to hACTH₁⁻³⁹ and to ACTH₁⁻²⁴, both without further purification and after purification and pre-incubation (Fig. 4). The log dose-response curves for hACTH₁⁻³⁹ show clearly that the non-purified suspension to which homogenate has been added is far less sensitive than the non-purified suspension without homogenate added. In fact, addition of homogenate lowers the sensitivity with a factor 2 (Fig. 4a). However, the sensitivities of both the suspension with added homogenate and the suspension without added homogenate were equal if measured after purification and pre-incubation. Fig. 4b shows that the addition of homogenate lowered the sensitivity for ACTH₁⁻²⁴ to a greater extent than for hACTH₁⁻³⁹. As with hACTH₁⁻³⁹, the purifica-

![Graph](image-url)
tion and pre-incubation procedure appeared to remove the ACTH\textsuperscript{1-24} inactivating factors completely from the suspension.

**Discussion**

Isolated adrenal cells should offer the means for a sensitive bioassay for ACTH since they are much more responsive to ACTH than adrenal quarters (Kloppenborg et al. 1968). Such a system would eliminate the problem regarding diffusion barriers which are present in adrenal quarters. Yet the isolated cells present difficulties. Even after careful washing of the suspension, in order to remove collagenase and cell fragments, non-viable cells and cell fragments remain. This might affect the sensitivity of the assay for two reasons. Firstly, ACTH may be bound to the receptors of damaged cells, which will not result in corticosteroiogenesis (Halkerston 1968). Secondly, enzymes derived from the broken cells may destroy ACTH and its derivatives (Bennett et al. 1974).

The quality of a cell suspension can be assessed by the effect of exogenous NADPH. NADPH is not able to cross cell membranes and therefore it only stimulates the steroidogenesis of an adrenal homogenate and not the steroidogenesis of intact viable cells (Halkerston et al. 1968; Tsang & Stachenko 1968). In our study NADPH shows a clear effect upon steroidogenesis in a non-purified cell suspension, but it is hardly effective when the cell suspension is purified (Table 1). It can be concluded that purification excludes interfering cell debris to a great extent. This is in accordance with the microscopic evidence that only a very small quantity of cell fragments enters the BSA medium. It is known from the work of Bennett et al. (1974) that purification of the adrenal cells removes substances which attack ACTH. However, non-viable cells – those which cannot exclude the trypan blue dye – are still present in the purified suspension. In order to inactivate ACTH-damaging enzymes from these broken cells, a pre-incubation step has been introduced. This procedure results in a lowering of the ED\textsubscript{50} with a factor two (Table 1). This combination of purification and pre-incubation appears very effective in eliminating interfering substances and makes adrenal cell suspensions hypersensitive to ACTH and its derivatives.
Both the ED50 and the lowest detectable dose were used to examine the sensitivity of the assays. Our final procedure offers an ED50 for ACTH1-39 of 4 fmol/ml. For ACTH1-24 this value is 0.8 fmol/ml. A wide range of ED50 values for the complete ACTH molecule has been described in the literature. The values ranged from about 10 fmol/ml (Lowry et al. 1973; Sayers et al. 1971) to far more than 200 fmol/ml (Richardson & Schuster 1972; Nakamura & Tanaka 1971; Moyle et al. 1973). Values reported for the ED50 of ACTH1-24 also vary rather dramatically, ranging from 2.4 fmol/ml (Lowry et al. 1973) to far more than 100 fmol/ml (Finn et al. 1976). Adrenal cell suspension from rats, hypophysectomized two days prior to collection of the adrenals, appeared to be much more sensitive for ACTH1-24 as judged from the ED50 of 1.1 fmol/ml (Sayers & Beall 1973).

The lowest detectable dose of ACTH1-39 which can be measured in our system is 0.19 fmol/ml. It is rather difficult to compare this value with values found elsewhere, because they are seldom reported. Liotta & Krieger (1977) developed a culture technique for adrenal cells, yielding a very sensitive assay for which the lowest detectable dose was 0.22 fmol hACTH1-39/ml.

The capacity of the present method to remove interfering substances was examined by adding a certain amount of an adrenal homogenate to the cell suspension. The addition of the homogenate resulted in a steep fall in sensitivity to hACTH1-39. However, when after the addition of the homogenate the crude cell suspension was purified and pre-incubated, no change in sensitivity was observed as compared to a suspension without added homogenate. The deteriorating effect of homogenate addition was even more pronounced when the dose-response of ACTH1-24 was analysed. This implies that the homogenate contained substances which damage ACTH1-24 to a greater extent than the complete hormone. An analogous finding was that the gain in sensitivity upon purification was more pronounced for ACTH1-24 than for hACTH1-39 (Table 1). These results are in agreement with those of Bennett et al. (1974). They showed that in adrenal cell suspensions ACTH1-24 is inactivated more extensively than hACTH1-39. This different rate in destruction may offer an explanation for the discrepancy between the results of Schwyzter et al. (1971) who found a potency for ACTH1-24 of 140 as compared to hACTH1-39 and those of Lowry et al. (1973) who found the potency to be considerably higher.

The finding that the potency of ACTH1-10 increases as well when the ACTH-destroying enzymes are removed or inactivated, confirms the work of Imura et al. (1967) who found an inactivation of short chain ACTH peptides in plasma. They suggested that part of the C-terminal chain of the ACTH molecule is important in protecting ACTH from inactivation in plasma. It appears that in crude adrenal cell suspensions the C-terminal end of ACTH also protects, to a certain extent, the molecule from inactivation. It is noteworthy from this study that hACTH1-32 shows the same potency as hACTH1-39 in the non-purified cell suspension as well as in the pre-incubated, purified suspension which suggests that the chain 33-39 is not involved in the protection of the molecule.

As appears from this study pre-incubation of isolated adrenal cell decreases deteriorating effects which are generally inherent in cell suspensions. It is very well possible that also in other systems for instance pituitary cells the introduction of a pre-incubation step may be of value.

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References


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