STUDIES ON THE BINDING OF CORTISOL-4-14C BY HUMAN LEUCOCYTES IN VITRO

By

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ABSTRACT

The amount of cortisol-4-14C bound by human leucocytes in a non-protein balanced salt solution was found to have a linear relationship to the concentration of cortisol-4-14C for a given number of leucocytes, and a linear relationship to the number of leucocytes for a given concentration of cortisol-4-14C. The binding was not affected by 10⁻³ M concentrations of arsenate, cyanide, fluoroacetate, dinitrophenol, fluoride, methylene blue, malonate, hydroxylamine or semicarbazide. The binding of cortisol-4-14C by leucocytes was found to be temperature dependent. At 0°C, no binding of cortisol-4-14C was detected. From 10°C to 80°C, each 10°C rise resulted in an approximately two fold increase in amount of cortisol-4-14C bound, but no further increase occurred between 80°C and 100°C. Experiments in which leucocytes were exposed to various specific temperatures without cortisol, and then incubated with cortisol-4-14C at 37°C revealed that exposure to lower temperatures had no effect on the uptake of cortisol-4-14C at 37°C, but that exposure to higher temperatures had increased the binding of cortisol-4-14C at 37°C. The addition of either human plasma or human serum albumin to the incubation medium markedly reduced the uptake of cortisol-4-14C by the leucocytes. Approximately 2/3 of the cortisol-4-14C (or metabolite) was removed upon incubation for 30 minutes at 37°C with either Hanks's solution (non-protein), plasma or serum albumin.


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Steroid hormones, after their release from the endocrine glands which produce them, are apparently reversibly bound to specific plasma proteins (for review see Daughaday 1959). The equilibrium between the free steroid hormone and the bound steroid hormone is such that a relatively small proportion of the hormone is in the unbound state (Daughaday 1956). Presumably only this relatively small unbound portion of the steroid in the blood is available to the cells (Daughaday 1958; Slaunwhite & Sandberg 1959).

The mechanism by which cells interact with steroids is not known. In vivo studies by Migeon et al. (1955) indicate that the liver takes up cortisol-4-14C. Erythrocytes also apparently bind cortisol (Sandberg et al. 1954; Peterson et al. 1956). In vitro studies by Daugherty & Schneebeli (1955) indicate that fibroblasts are able to take up cortisol, and Levin et al. (1955) have shown that liver, kidney, diaphragm, spleen and fat are all able to bind cortisol in vitro.

A previous report (Ketchel 1961 b) presented evidence which indicated that human leucocytes are able to bind cortisol in vitro. The cortisol bound by the leucocytes had the physiological effect of inhibiting the amoeboid migration of the leucocytes. Leucocytes were therefore considered to be an advantageous cell in which to study some aspects of the binding of cortisol.

MATERIALS AND METHODS

Leucocytes were obtained by mixing 10 ml of heparinized human blood with 2 ml of solution containing 4 g of dextran, 3 g of dextrose and 0.85 g of NaCl per 100 ml H2O. After the red cells had sedimented (30–35 minutes at room temperature), the leucocytes were recovered from the supernatant plasma, washed 2 times in Hanks's solution (Hanks & Wallace 1949), and suspended in 1 ml of Hanks's solution. The Hanks's solution used throughout these experiments was adjusted to pH 7.2.

The cortisol-4-14C used had a specific activity of 3.94 mc/mmol, and had been shown to have a purity greater than 97 0/o.

Tenth ml aliquots of the leucocyte suspension were mixed with 0.9 ml of Hanks's solution in which an amount of cortisol-4-14C had been dissolved to make a final concentration of 1 0/µg/ml. The tubes were then incubated in a water bath at 37°C for 20 minutes. At the end of the incubation period the cells were washed twice in 12 ml of Hanks's solution, lysed by the addition of 1 ml of H2O, transferred to a planchet, and dried. A blank containing no leucocytes but all of the other components of the system, was treated in the same manner as the tubes containing leucocytes. This blank showed how much radioactivity not bound to the leucocytes was being measured. Thus, the difference between the amount of 14C which was measured in the blank and the amount of 14C which was measured in the leucocytes was considered to be the amount of 14C taken up by the leucocytes.

RESULTS

In most experiments described in this report, 1/6 of the leucocytes recovered from 20 ml of human blood was used for each determination of uptake of
Amount of $^{14}\text{C}$ remaining in leucocytes after incubation for 20 minutes at $37^\circ \text{C}$ with cortisol-4-$^{14}\text{C}$.

Amount of $^{14}\text{C}$ remaining in leucocytes after incubation for 20 minutes with various specific concentrations of cortisol-4-$^{14}\text{C}$.

cortisol-4-$^{14}\text{C}$. While the number of leucocytes varied from experiment to experiment, the number of leucocytes in all determinations within each experiment was carefully controlled by taking aliquots of a single leucocyte suspension. In Fig. 1 the amount of cortisol-4-$^{14}\text{C}$ taken up by a single aliquot of a leucocyte suspension is compared to the amount of cortisol-4-$^{14}\text{C}$ bound by 1/2, 2, 4 and 6 times that amount. As seen in Fig. 1, there is a linear relationship between the number of leucocytes and the amount of cortisol-4-$^{14}\text{C}$ bound to the cells. A previous report (Ketchel 1961 b) includes evidence that the red cells contaminating the leucocyte suspension do not account for the uptake of cortisol-4-$^{14}\text{C}$, and that it is cortisol-4-$^{14}\text{C}$, rather than a radioactive contaminant, that is being bound by the leucocytes.
Table 1.
Metabolic inhibitors used at a concentration of $10^{-3}$ M to attempt to inhibit the uptake of cortisol-4-$^{14}$C by leucocytes.

<table>
<thead>
<tr>
<th>Metabolic Inhibitor</th>
<th>Metabolic Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenate</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>Cyanide</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>Fluoracetate</td>
<td>Methylene blue</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>Malonate</td>
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<tr>
<td>Semicarbazide</td>
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</table>

Fig. 2 shows the results of a typical experiment in which the concentration of cortisol-4-$^{14}$C in a series of aliquots of a leucocyte suspension is varied. A linear relationship exists between the concentration of cortisol-4-$^{14}$C added and the amount of cortisol-4-$^{14}$C bound by the leucocytes.

Table 1 lists a series of metabolic inhibitors which were added to the leucocyte suspension in an attempt to determine whether or not the binding of cortisol by the leucocytes is dependent on enzyme systems affected by these inhibitors. None of the inhibitors listed in Table 1, at a concentration of $10^{-3}$ M, had any influence on the uptake of cortisol by the leucocytes.

The results of an experiment designed to determine the rate at which leucocytes bind cortisol-4-$^{14}$C are shown in Fig. 3. Equilibrium is established within 10–15 minutes, and 80 $\%$ or more of the total cortisol-4-$^{14}$C is taken up within the first 5 minutes.

In an attempt to gain further information concerning the nature of the chemical reaction between leucocytes and cortisol, a series of experiments was performed in which leucocytes were incubated with cortisol-4-$^{14}$C at various specific temperatures. The results, as shown in Fig. 4, show that virtually no
Amount of $^{14}$C remaining in leucocytes after incubation for 20 minutes with 10 $\mu$g/ml of cortisol-4-$^{14}$C at various specific temperatures.

Logarithm of amount of $^{14}$C remaining in leucocytes plotted against the reciprocal of the absolute temperature at which the cells were incubated with cortisol-4-$^{14}$C.

cortisol-4-$^{14}$C is bound by the leucocytes at $0^\circ$C. Each $10^\circ$ rise in incubation temperature, up to $80^\circ$, results in a proportional increase in binding of cortisol-4-$^{14}$C by the leucocytes. The reciprocal plot of the absolute temperature against the log$_{10}$ of the cortisol-4-$^{14}$C bound, as shown in Fig. 5, appears to satisfy the
Amount of $^{14}$C remaining in leucocytes incubated without cortisol at various specific temperatures, then incubated at 37° C with 1 μg/ml of cortisol-4-$^{14}$C.

Arrhenius equation. Thus, each 10 degree rise in temperature, from 0° to 80°, approximately doubles the uptake of cortisol-4-$^{14}$C by the leucocytes.

In the experimental results shown in Figs. 4 and 5, the temperatures indicated are those at which the leucocytes were incubated with cortisol-4-$^{14}$C. A further series of experiments were carried out in which leucocytes, subjected to various specific temperatures without cortisol, were then incubated with cortisol-4-$^{14}$C at 37°. The results are shown in Fig. 6. Leucocytes first subjected to a pre-incubation at lower temperatures without cortisol bind cortisol-4-$^{14}$C, when returned to 37°, to about the same degree as cells preincubated at 40°. However, leucocytes subjected to a preincubation at 100° without cortisol bind an increased amount of cortisol-4-$^{14}$C when returned to 37°.

A summary of the effect of temperature on the reaction between leucocytes and cortisol-4-$^{14}$C is shown in Table 2. Leucocytes incubated with cortisol-4-$^{14}$C at 0°, and then incubated with cortisol-4-$^{14}$C at 37°, bind cortisol-4-$^{14}$C to the same degree as cells which have not been subjected to 0°. Leucocytes which have been heated to 100°, when incubated with cortisol-4-$^{14}$C at either 37° or 100°, bind cortisol-4-$^{14}$C to an increased degree. Leucocytes which have been heated to 100°, when incubated with cortisol-4-$^{14}$C at 0°, bind more cortisol-4-$^{14}$C than leucocytes which have never been heated, but less than heated cells incubated with cortisol-4-$^{14}$C at 100°. It thus appears that the reaction between leucocytes and cortisol is a temperature dependent reaction, but that the denaturation of the cellular proteins irreversibly increases the amount of cortisol which the leucocytes bind.

In the experiments so far described, the interaction between the leucocytes and cortisol-4-$^{14}$C took place in Hank's solution, a non-protein medium. Under such conditions, all of the cortisol-4-$^{14}$C was available to the cells. However,
Table 2.
Amount of $^{14}$C in leucocytes subjected to a preincubation at specific temperatures without cortisol, followed by an incubation with cortisol-4-$^{14}$C.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Preincubation without cortisol</th>
<th>Incubation with cortisol-4-$^{14}$C</th>
<th>Counts/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>37</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0</td>
<td>0</td>
<td>114</td>
</tr>
<tr>
<td>37</td>
<td>37</td>
<td>432</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>37</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

Fig. 7.
Comparison of binding of cortisol-4-$^{14}$C by leucocytes suspended in Hanks's solution (non-protein), plasma and serum albumin.

if the cells and the cortisol-4-$^{14}$C were in plasma, presumably much of the cortisol-4-$^{14}$C would be bound to plasma proteins, and there would be less cortisol-4-$^{14}$C available to the cells. The following experiment was performed to test this reasoning. A suspension of leucocytes was prepared in the usual manner. One 0.1 ml aliquot was resuspended in 0.8 ml of Hank's solution, another 0.1 ml aliquot was suspended in plasma, and a third 0.1 ml aliquot was suspended in 4% human serum albumin. Each of these suspensions was added to 0.1 ml of Hanks's solution containing 1 μg of cortisol-4-$^{14}$C. After incubation the amount of $^{14}$C in the leucocytes was determined. As seen in
Comparison of ability of Hanks's solution, plasma and serum albumin to remove cortisol-4-\(^{14}\)C bound by leucocytes. For explanation see text.

Fig. 7, less \(^{14}\)C was measured in the leucocytes incubated with cortisol-4-\(^{14}\)C in the presence of plasma or albumin than was measured in leucocytes incubated with cortisol-4-\(^{14}\)C in Hanks's solution. Presumably the protein and the leucocytes compete for the steroid which is to be bound.

A further series of experiments was performed to determine the degree to which cortisol-4-\(^{14}\)C bound to the leucocytes may be removed by plasma, serum albumin, and Hanks's solution. In these experiments a series of aliquots of leucocytes were incubated in the usual manner with cortisol-4-\(^{14}\)C in Hanks's solution, and then washed free of unbound cortisol-4-\(^{14}\)C. A control aliquot was planchotted at this time to determine the amount of \(^{14}\)C contained in the leucocytes. To each remaining aliquot was added 1 ml of either plasma, 4 % serum albumin, or Hanks's solution. After twenty minutes the cells were washed twice in Hanks's solution and transferred to planchets. Thus the difference in the amount of \(^{14}\)C measured in the control aliquot and in those aliquots which received a second incubation with either plasma, serum albumin, or Hanks's solution indicated the amount of cortisol-4-\(^{14}\)C (or metabolite) which was removed from the leucocytes by these agents. The results, as seen in Fig. 8, show that about 2/3 of the radioactivity was removed. However, plasma and serum albumin were only slightly more effective than Hanks's solution in removing bound cortisol-4-\(^{14}\)C (or metabolite) from the leucocytes. This is in contrast to the ability of plasma and serum albumin, as compared with Hanks's solution, to compete with the leucocytes for unbound cortisol-4-\(^{14}\)C.

**DISCUSSION**

In an attempt to establish some characteristics of the reaction between a cell and a steroid hormone, attention has been focused on human leucocytes and
cortisol-4-14C. Leucocytes were chosen for this study in spite of certain obvious disadvantages because (1) discrete cells, unlike solid tissue cells, are all equally available to the steroid; and (2) evidence exists (Ketchel 1961 b) that cortisol bound by the leucocytes under similar in vitro conditions has a physiological effect on the cells.

Leucocytes incubated with cortisol-4-14C in the presence of either plasma protein or serum albumin bound less cortisol-4-14C than those incubated with cortisol-4-14C in a non-protein medium. Thus, the plasma proteins appear to be in competition with leucocytes, and presumably other cells, for the available cortisol. Evidence from previous experiments (Ketchel et al. 1958) indicates that the amoeboid migration of leucocytes is inhibited in plasma to which cortisol has been added. Further work will be necessary to determine whether or not the relatively large amounts of cortisol used in these experiments has a normal physiological action, and whether or not the binding of small amounts of cortisol which occurs in vivo follows the same principles elucidated in this in vitro study.

REFERENCES

Ketchel M. M.: Endocrinology 69 (1961 b) 60.

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