Some Aspects of the Dietary Sodium Intake on the Regulation of Aldosterone Biosynthesis in Rat Adrenals

JEAN-GUY LEHOUX
Department d'Obstétrique et Gynécologie, Centre Hospitalier Universitaire,
Université de Sherbrooke, Sherbrooke, Québec J1H 5N4

AND
THOMAS SANDOR, I. W. HENDERSON, AND I. CHESTER JONES
Department of Zoology, The University, Sheffield, England

Received July 25, 1973

Experiments were performed to study the effect of dietary sodium deficiency on the in vitro biosynthesis of corticosteroids from tritiated cholesterol by homogenates of whole adrenal glands and on the properties of rat adrenocortical cytochrome P-450. Adrenal homogenates of rats kept on a sodium deficient diet for 17 days converted 3 times more tritiated cholesterol to corticosterone, 15 times more to 18-hydroxycorticosterone, and 6 times more to aldosterone than did homogenates from rats kept on the control diet. While adrenocortical cytochrome P-450 content did not differ significantly in the glands of experimental animals from that of control animals, changes could be observed in the cytochrome P-450–carbon monoxide association time constants ($K_{CO}$).


Nous avons poursuivi des expériences pour étudier l'effet d'une déficience en sodium alimentaire sur la biosynthèse in vitro de corticostéroïdes, à partir du cholestérol tritié, par des homogénats de glandes surrenales entières et sur les propriétés du cytochrome P-450 de la corticosurrénale de rat. Les homogénats surrenaux de rats privés de sodium durant 17 jours transforment 3 fois plus de cholestérol tritié en corticostéroné, 15 fois plus en 18-hydroxycortistéroné et 6 fois plus en aldostéroné que les homogénats de rats recevant la diète normale. La teneur en cytochrome P-450 de la corticosurrénale des animaux en expérience ne diffère pas de façon importante de celle des animaux témoins. Par contre, des changements sont observés dans les constantes du temps d'association du cytochrome P-450 avec le monoxyde de carbone ($K_{CO}$).


Nous avons poursuivi des expériences pour étudier l'effet d'une déficience en sodium alimentaire sur la biosynthèse in vitro de corticostéroïdes, à partir du cholestérol tritié, par des homogénats de glandes surrenales entières et sur les propriétés du cytochrome P-450 de la corticosurrénale de rat. Les homogénats surrenaux de rats privés de sodium durant 17 jours transforment 3 fois plus de cholestérol tritié en corticostéroné, 15 fois plus en 18-hydroxycorticostéroné et 6 fois plus en aldostéroné que les homogénats de rats recevant la diète normale. La teneur en cytochrome P-450 de la corticosurrénale des animaux en expérience ne diffère pas de façon importante de celle des animaux témoins. Par contre, des changements sont observés dans les constantes du temps d'association du cytochrome P-450 avec le monoxyde de carbone ($K_{CO}$).

Introduction
As recently reviewed by Müller (1) many factors are involved in the regulation of aldosterone biosynthesis. Among these factors, dietary sodium intake is particularly important, and the enzyme activity involved in the biosynthesis

of aldosterone was shown to be modified by this ion (2–6).

It is now generally accepted that cytochrome P-450 is involved in most if not all steroid hydroxylating reactions (7). Recently, we have also demonstrated that adrenal cell cytochrome P-450 concentration could be maintained at a normal level in the adrenals of hypophysectomized mice by ACTH administration (Stark, E., Sandor, T., Lehoux, J. G., Bacsy, E. & Rappay, G.; unpublished observations). Simpson (8) demonstrated that stress increased by about threefold the binding of pregnenolone to cytochrome P-450 in rat adrenal mitochondria as compared with cycloheximide treated animals. In a similar manner we have shown in preliminary communications (9, 10) that rats kept on a sodium deficient diet have abnormal cytochrome P-450 characteristics. The observations suggest that cytochrome P-450 is involved in the regulation of corticosteroidogenesis. In this paper we wish to report the results of in vitro studies on the conversion of 3H-cholesterol by whole rat-adrenal homogenates of animal fed on a low sodium diet. In addition the content and characteristics of the adrenocortical cytochrome P-450 are also discussed.

Materials and Methods

Animals and Diet

Four-month-old rats of the Lister-Hooded strain weighing about 350 g were used. They were divided into groups and fed on various diets for periods of time varying between 1 and 17 days as specified in the text. (1) Control diet was Purina rat chow plus tap water (tap water contained virtually no sodium as measured by flame photometry). (2) Sodium deficient diet was sodium free diet (Hartroft's formula (11) plus de-mineralized water).

Tissue Preparations

All rats were anesthetized with ether. The adrenal glands were removed and chilled on ice. Adrenals of each particular group of rats were pooled and homogenized in a Krebs–Ringer bicarbonate buffer (KRB) (pH 7.4) containing 0.25 M sucrose (1 g tissue/5 ml). The KRB was gassed with a 95% O2–5% CO2 gas mixture for 30 min before use. Subcellular fractions were obtained by centrifugation of adrenal homogenates in an MSE “Superspeed 65” preparative ultracentrifuge as previously described (12).

Substrate and Incubation

Cholesterol-1,2,4,6H (specific activity (S.A.) 46 Ci/mmole) was used as substrate (obtained from the Radiochemical Center, Amersham, England). Samples of the adrenal whole homogenates corresponding to 50 mg of tissue were incubated with 25 μCi of the substrate (dissolved in 2 drops of Tween-80) in the presence of a NADPH generating system at 37 °C (13).

Isolation and Identification of 3H-cholesterol Metabolites

Incubation media were extracted with a 1:1 mixture of ethyl acetate–chloroform. The extracts were fractionated by paper partition chromatography (P.P.C.) and radioactive fractions corresponding to the expected metabolites were characterized by derivative formation and when possible by crystallization to constant isotope ratio as previously described by Sandor et al. (14–16). Losses due to manipulations were monitored by the addition of known amounts of 3H reference steroids to the incubation media before extraction.

Spectrophotometric Determinations

As previously described in detail (12) spectral changes of mitochondrial and microsomal suspensions were measured on a UNICAM SP-8000 automatic spectrophotometer. The instrument was equipped with a temperature controlled cell holder and all readings were made at 0 °C. Protein determinations were performed by the technique of Lowry et al. (17), using bovine serum albumin as standard.

Results

Metabolism of 3H-cholesterol by Adrenal Gland Whole Homogenate of Rats Kept on a Low Sodium Diet for 2, 14, and 17 Days

The effect of sodium deficient diet upon the metabolism of 543 pmol of tritiated cholesterol by adrenal gland whole homogenate of rats kept on a low sodium diet for 17 days was studied. Incubation times used were 10, 20, and 60 min, respectively. The following cholesterol metabolites were isolated and identified: tritiated progesterone, deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, and aldosterone. Fig. 1 shows the transformation of tritiated cholesterol to tritiated corticosterone by the adrenal homogenates of the control and experimental group as a function of the time of incubation. After 20 min of incubation, there was 3 times more tritiated corticosterone formed by the experimental group as compared with control group. Data obtained on the yield of the other

\[ \text{3Progesterone-4,11C} \quad \text{(S.A. 60 mCi/mmol), deoxycorticosterone-4,11C} \quad \text{(S.A. 44 mCi/mmol), corticosterone-4,11C} \quad \text{(S.A. 56.7 mCi/mmol), and aldosterone-4,11C} \quad \text{(S.A. 56.7 mCi/mmol) were obtained from the Radiochemical Center, Amersham, England, and purified before use; 18-hydroxycorticosterone-4,11C} \]
cholesterol metabolites are shown in Table 1. After 60 min of incubation there was 6 times more aldosterone and 15 times more 18-hydroxycorticosterone formed by the adrenals of the experimental animals than by those of the control group. The amount of progesterone was similar for both groups while the experimental tissue formed less deoxycorticosterone than did the control tissue. This latter finding could possibly be accounted for by a more rapid turnover of deoxycorticosterone in the low sodium experiment. A second series of experiments was performed to verify if the increased transformation of tritiated cholesterol to aldosterone, 18-hydroxycorticosterone, and corticosterone could also be found after a longer incubation time. The adrenal homogenates of sodium depleted rats for 17 days and control group were incubated for 2 h under similar conditions as mentioned above. After 2 h, the adrenal homogenate of rats kept on a low sodium diet synthesized more than twice as much corticosterone, aldosterone, and 18-hydroxycorticosterone from tritiated cholesterol than did the adrenals of the controls (results not shown). The results are therefore in general agreement with the results of the first series of experiments.

In a third series of experiments with rats maintained on a sodium deficient diet for 2 and 14 days, respectively, adrenal homogenates were incubated in the presence of 50 µCi (1086 pmol) of tritiated cholesterol for 20 min in a KRB medium containing a NADPH generating system. The conversion of tritiated cholesterol to tritiated aldosterone was found to be 10.6 pmol/100 mg of adrenal tissue for the low sodium group after 2 days of diet and 11.8 pmol after 14 days of diet while it was 2.9 pmol for the control. Homogenates of rats maintained on a low sodium diet for 2 days converted already 3.6 times more tritiated cholesterol to aldosterone than control.

**Investigation of Adrenal Mitochondrial and Microsomal Cytochrome P-450 of Sodium Depleted Rats**

To determine whether the increased capability of sodium depleted rat adrenals to synthesize corticosteroids was due to an increase in adrenal cortical cytochrome P-450 content, the hemoprotein concentration of adrenal mitochondria and microsomes of both normal and sodium depleted rats for 1, 4, 8, and 17 days, respectively,
FIG. 2. Double reciprocal plots of microsomal cytochrome P-450 optical density changes (Δ O.D. 450–490 nm) in relation to time (t). Adrenocortical microsomal preparations of rats kept on a low sodium diet (0.51 mg protein/ml) (● - - - ●) and microsomal preparations of rats kept on a normal diet (0.53 mg protein/ml) (● - ●).

Table 2. Rat adrenal mitochondrial and microsomal cytochrome P-450 content

<table>
<thead>
<tr>
<th>Group</th>
<th>Time under diet (day)</th>
<th>Mitochondria</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Na+</td>
<td>1</td>
<td>1.79</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.69</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.17</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.19</td>
<td>1.50</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1.91</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.29</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.99</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.22</td>
<td>1.82</td>
</tr>
</tbody>
</table>

Note: Mitochondria or microsomes were suspended in a 0.25 M sucrose solution containing 10 mM of β-mercaptoethlyamine and buffered to pH 7.4 with 0.1 M phosphate buffer. Each value is the mean of two readings and variation between two readings for the same preparation was within 10%.

The molar extinction coefficient used was 91 mM⁻¹ cm⁻¹ (18). Table 2 presents the results of these determinations expressed as nanomoles of mitochondrial and microsomal cytochrome P-450/100 mg of adrenal tissue. It can be seen that the adrenal mitochondrial and microsomal cytochrome P-450 concentrations of the sodium deficient group did not differ significantly from that of the control group for any stage of the treatment.

During the experiments described above by scanning between 450 and 480 nm at different time intervals, it was possible to follow the temporal change in the height of the Soret band at 450 nm produced by the addition of CO to reduced mitochondrial and microsomal suspensions. From these measurements the cytochrome P-450–carbon monoxide association time constants could be established. Fig. 2 is an example of the type of curves obtained represented in the form of a double reciprocal plot (reciprocal of optical density (O.D.450–490) plotted against minutes⁻¹). The curves shown in Fig. 2 were drawn from data obtained on the adrenal microsomal suspensions of control rats and of rats maintained on a sodium deficient diet for 17 days. From the abscissa intercept, a constant was

was quantitated spectrophotometrically. These determinations were done at 0 °C on sodium hydrosulfite reduced mitochondrial and microsomal preparations. The concentration of P-450 was calculated using differences in absorbancy between 450 and 490 nm, obtained 60 min after gassing the sample cell with carbon monoxide.
calculated. This constant ($K_{CO}$) has been defined as being the time necessary for the carbon monoxide to saturate one-half of the P-450–CO binding sites (12). In all experiments but those with microsomal suspension from rats on sodium deficient diet two association constants ($K_{CO \text{ fast}}, K_{CO \text{ slow}}$) could be calculated as the double reciprocal curves were found to be biphasic. In the case of microsomal suspension from rats kept on sodium deficient diet the fast component of the association constant of carbon monoxide had disappeared. A summary of the constants obtained are shown in Table 3.

### Discussion

We have shown that whole homogenate of adrenals of rats kept on a sodium deficient regimen converted more exogenous cholesterol to corticosterone, aldosterone, and 18-hydroxycorticosterone than tissue from control animals. This elevated aldosterone formation confirms the findings of Müller and Huber (3). However, these authors reported that adrenal quarters from sodium deficient rats produced less corticosterone than adrenal from sodium replete rats. From Table 2 it can be seen that the adrenal mitochondrial and microsomal cytochrome P-450 concentrations of the sodium deficient group did not differ significantly from those of the control group. As sodium depletion is thought to affect the adrenocortical zona glomerulosa only, changes in adrenal cytochrome P-450 concentration provoked by a sodium deficient diet, if any, should presumably be localized in this histological zone. Since the zona glomerulosa is very small compared with the rest of the gland, even large changes in the cytochrome P-450 content of this zone could be masked when analyzing whole gland preparations. Work is presently in progress to study this aspect on isolated zona glomerulosa preparations.

The cytochrome P-450 – CO association time constants (Table 3) confirm the previously reported existence of two types of CO binding sites (12): one type would be rapidly saturated with carbon monoxide while binding to the other site would occur more slowly. The intake of dietary sodium altered the association constants as shown in Table 3. In adrenal microsomes of rats kept on a low sodium diet, the time-association curves changed completely and only one association constant could be obtained. Changes in the association constants produced by the low sodium diet in adrenocortical mitochondria were less striking than in those of microsomes. The fact that mitochondria are much more complex than microsomes as far as hydroxylation reactions are concerned may possibly explain the less defined overall changes in the association constants of CO to cytochrome P-450 in the mitochondria. At present, work is in progress to assign the biochemical meaning to these association constants in terms of the hydroxylation of steroidal substrates. Data reported here suggest that low sodium intake may provoke changes in adrenocortical cytochrome P-450, at least as far as its affinity to CO and presumably to molecular oxygen is concerned. This may represent part of

---

**Table 3. Rat adrenal mitochondrial and microsomal cytochrome P-450–CO time constants**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time under diet (days)</th>
<th>Microsomes</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_{CO \text{ fast}}$</td>
<td>$K_{CO \text{ slow}}$</td>
</tr>
<tr>
<td>Low Na+</td>
<td>1</td>
<td>*</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>*</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>*</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>*</td>
<td>2.1</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Only one constant was obtained.

**Note:** Each value is the mean of two readings performed on the same pool of mitochondria or microsomes.
the mechanism by which the biosynthesis of aldosterone is regulated by sodium.

These studies were supported by the Medical Research Council of Canada, by the "Conseil de la Recherche Médicale du Québec," and the Science Research Council of Great Britain. One of us (T.S.) participated in these studies while on an extended study leave (1970, 1971) from the Département de Médecine, Université de Montréal, and holding a Visiting Professorship at the University of Sheffield. Thanks are due to Dr. J. C. Forest for his comments and suggestions.