Inhibitory Effects of Cortisol and Antibiotics on Substrate Entry and Ribonucleic Acid Synthesis in Rat Thymocytes in Vitro*

(Received for publication, October 21, 1969)

Maynard H. Makman,† Sachiko Nakagawa, B. Dvorin, and Abraham White

From the Departments of Biochemistry and Pharmacology, Albert Einstein College of Medicine, Yeshiva University, New York, New York 10461

SUMMARY

Incubation of rat thymocytes with either puromycin, cycloheximide, or actinomycin D resulted in a progressive inhibition of the accumulation of radioactivity derived from amino acids and uridine in the trichloracetic acid-soluble fraction. No further inhibitory effects on incorporation into either the trichloracetic acid-soluble or trichloracetic acid-insoluble fraction were produced by $1 \times 10^{-9} \text{M}$ cortisol when incubations were carried out for 3 hours in the presence of the antibiotic. However, with antibiotic added late in the incubation period, inhibitory effects of steroid on incorporation into both trichloracetic acid fractions, similar to those obtained without antibiotic, were still evident. Cortisol and puromycin inhibited incorporation of orotic acid into the trichloracetic acid-insoluble fraction, with no corresponding effects on the trichloracetic acid-soluble fraction. Moreover, in the presence of puromycin, an additional inhibitory effect of cortisol on orotic acid incorporation into RNA was obtained. An inhibitory effect of cortisol on incorporation of uridine into the trichloracetic acid-insoluble fraction occurred in the absence of significant effects on the trichloracetic acid-soluble fraction when cells were incubated in the presence of puromycin for 6 hours.

Fractionation by sucrose gradient centrifugation of the total RNA obtained from thymocytes exposed to cortisol and labeled with uridine revealed no differential effects of the steroid on a specific species of RNA.

Incubation of thymocytes with cortisol resulted in a progressive decrease of RNA polymerase activity as measured directly with either a nuclear or aggregate enzyme preparation. This inhibitory effect on polymerase activity was also produced by incubation of thymocytes with $6\alpha,9\alpha$-difluoro-11α,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide (fluocinolone acetonide) ($1 \times 10^{-9} \text{M}$) but not by incubation with progesterone ($1 \times 10^{-6} \text{M}$). Incubation of the cells with puromycin also resulted in decreased polymerase activity; however, a further inhibitory effect of cortisol was evident in the presence of the antibiotic.

It is concluded that continuing protein synthesis throughout the incubation period is required for amino acid transport, for uridine transport or conversion to UMP (or both), and for the manifestation of the steroid effects on these processes. In contrast, continuing protein synthesis is not required for the inhibitory effect of cortisol on RNA synthesis.

In previous publications from this laboratory it was shown that addition of physiological concentrations ($10^{-8}$ to $10^{-4}$ M) of a thymolytic steroid, e.g., cortisol, to lymphoid cells in vitro inhibited significantly the transport and further utilization of amino acids and of precursors of RNA and DNA (2-4). These inhibitory effects appeared to be dependent both upon an energy source (3, 4) and upon the synthesis of RNA or protein (or both) (1, 3). In additional studies, the activity of thymic DNA-dependent RNA polymerase has also been shown to decrease following injection of cortisol (3, 5-8). The studies in the present paper permit a more definitive distinction between two proposed sites of cortisol action (3), inhibition of transport processes, and decreased rate of RNA synthesis, as well as partial clarification of the role of protein synthesis in these processes.

EXPERIMENTAL PROCEDURE

Cortisol was obtained from Steraloids, Inc., Pawling, New York. Fluocinolone acetonide† was a gift of Syntex Laboratories, Palo Alto, California. Uridine-6-3H (9.3 Ci per mmole), uridine-2-14C (50 mCi per mmole), deoxyuridine-2-14C (9.3 mCi per mmole), glycin-3-14C (74 mCi per mmole), L-leucine-14C (233 mCi per mmole), and α-aminoisobutyric-3-14C acid (4.7 mCi per mmole) were purchased from New England Nuclear. Thymidine-3H (3.0 Ci per mmole) and UTP-3H (2 Ci per mmole)

† The trivial name used is: fluocinolone acetonide, $6\alpha,9\alpha$-difluoro-11α,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide.

† Career Development Awardee 5-K3-AM-28,889-04, National Institutes of Health, United States Public Health Service.
were purchased from Schwarz BioResearch. Orotic acid-6-^C
(60.8 mCi per mmole) was purchased from Amersham-Searle.
ATP, GTP, CTP, UTP, phosphoenolpyruvate, pyruvate kinase
(10 mg per ml in 2.1 M (NH₄)₂SO₄), and progesterone were pur-
chased from either Sigma or Calbiochem. Eagle’s minimum
essential medium for spinner culture (without serum) (8) was pur-
chased from General Biochemicals as a dry powder; NaH₂PO₄,
was added to reconstitute the complete medium. Phenol
was purchased from Mallinckrodt and was redistilled and stored
prior to use. Sucrose was purchased from Merck Sharp
and Dohme, and puromycin and cycloheximide were from Nutritional
Biochemicals. Actinomycin D was kindly provided by Merck
Sharp and Dohme. Other reagents were analytical grade and
obtained from commercial sources.

Male rats of the Sprague-Dawley strain, 7 to 10 weeks of age,
were purchased from Carworth Farms and maintained on Rock-
land rat chow and water ad libitum for at least 1 week prior to use.
When indicated, rats were injected intraperitoneally either with
5 mg of cortisol (suspended in 0.85% NaCl solution, 15 mg per
ml) per 100 g of body weight or with an equal volume of NaCl
solution at the low speed setting for 30 sec. The cell lysate was centrifuged
precipitates resuspended and collected on Millipore filters for
RNA polymerase assay.

For preparation of thymic nuclei for RNA polymerase assay,
the cell pellets were resuspended and precipitated in cold 5
% trichloroacetic acid, and the precipitates were washed and col-
lected on Millipore filters (0.45 μm pore size). For separation and
recovery of both intracellular trichloroacetic acid-soluble and tri-
chloroacetic acid-insoluble fractions, cell pellets were washed in
cold incubation medium, resuspended in trichloroacetic acid,
and centrifuged; aliquots of the supernatant fluids were removed for
measurement of trichloroacetic acid-soluble radioactivity and the
precipitates resuspended and collected on Millipore filters for
measurement of trichloroacetic acid-insoluble radioactivity. Ra-
dioactivity was determined by liquid scintillation spectrometry as
described previously (4).

For preparation of thymic nuclei for RNA polymerase assay,
20-ml aliquots of the cell suspensions were centrifuged at 120 × g
for 4 min and washed once with 10 ml of ice-chilled spinner me-
dium. Washed cells were resuspended in 2 ml of medium con-
taining 0.25 M sucrose, 0.075 M KCl, 0.01 M MgCl₂, and 0.035 M
Tris-Cl (pH 7.8). Nuclei were isolated by sonic oscillation of the
cells in a Branson Sonifier for 10 set (20 kcycles per set, 3 amps
d.c.) and then by centrifugation at 700 × g for 5 min. The
nuclear sediment was washed once with the above medium and resuspended in the same medium for RNA polymerase assay.

The aggregate polymerase preparation was obtained by lysis of
the cells at a concentration of 1.6 × 10⁹ per ml in 0.05 M Tris-Cl
buffer, pH 7.8. Following lysis, the MgCl₂ concentration of the
mixture was adjusted to 0.005 M and the nuclear gel was dispersed by
homogenizing in a Waring Blender with a microcontainer at
the low speed setting for 30 sec. The cell lysate was centrifuged
at 15,000 × g for 10 min. The supernatant fluid was discarded and the sediment was washed once with a 0.05 M Tris-Cl (pH 7.8)
-0.005 M MgCl₂ medium. The washed pellet was resuspended in a

The 0.5-ml (final volume) incubation medium for RNA poly-
merase assay included 0.25 ml of either the nuclear suspension or the
aggregate enzyme preparation, each containing approximately
0.5 mg of DNA. In addition, the following were present: 1
μmole each of ATP, GTP, CTP, and phosphoenolpyruvate, 2.5
μmole of UTP, 10 μg of pyruvate kinase, 50 μmole of
Tris-Cl (pH 7.8), 10 μmole of MgCl₂, and 6 μmole of 2-
mercaptoethanol. Incubation was carried out at 37° for 10 min,
during which period the reaction rate was linear. Other details
were as described previously (7, 8). Concentrations of UTP and
of other nucleoside triphosphates in the assay system were at
saturation (7, 8).

For sucrose gradient studies, separate aliquots of control and
cortisol-treated cell suspensions were labeled either with uridine-
6-3H or with uridine-2-^C. In some experiments, the cells were
collected with unlabeled uridine at a suitable time after addition of labeled uridine. The H-labeled cells exposed to cortisol were combined with u⁴C-labeled control cells, or vice versa, and the pooled cell suspensions were then extracted with
phenol by the method of Warner et al. (10), with the following
modifications. Extraction of lysed cells with phenol was con-
ducted at 50°; following phenol extraction, RNA was precipitated by adding 3.5 volumes of cold absolute ethanol (−20°C).
The mixture was kept at −20°C overnight for complete precipitation of
RNA. After centrifugation, the RNA was redissolved at room temperature in 0.01 M Tris-Cl buffer (pH 7.5) containing
0.01 M EDTA, and 0.2% sodium dodecyl sulfate. An aliquot of 0.1 to 0.6 ml of RNA solution was layered on a continuous
gradient of 15 to 30% sucrose containing 0.1 M NaCl, 0.01 M
Tris-Cl (pH 7.5), 0.001 M EDTA, and 0.5% sodium dodecyl
sulfate. Centrifugation was performed at 25° in a Spincen-
centrifuge, model L2-65B, with either a three- or six-place rotor as indicated in the results. After centrifugation, successive gra-
dient fractions were collected with continuous monitoring of optical
density at 260 nm. To each fraction was added trichloro-
acetic acid (final concentration 5%) plus 3.5 μl of calf serum,
and the precipitate was collected on a Millipore filter (0.9 μm pore
size) for determination of trichloroacetic acid-insoluble radioac-
tivity.

RESULTS

In order to examine the possible influence of antibiotics on the
response of thymocytes to cortisol, initial studies were con-
ducted with antibiotics alone. The data in Table I show the
influence of length of incubation and concentration of antibiotics
on incorporation of precursors into protein and nucleic acids.
Actinomycin D exerted an early inhibitory influence on RNA
synthesis and a later action on incorporation of precursors into
protein and DNA, in agreement with the generally accepted
causal relationship of protein synthesis to continuing RNA
formation. Moreover, the incorporation of uridine-2^C into
RNA was completely inhibited by the higher concentration of
actinomycin D. We have shown previously that under these
experimental conditions 80 to 90% of the labeled uridine incor-
porated into the acid-insoluble fraction is in RNA (4). The
effects of cycloheximide and puromycin on leucine-2^C incor-
poration are also evident after relatively short incubation periods
Influence of antibiotics and analogues on incorporation of precursors into protein, RNA, and DNA of thymocytes in vitro

Cell suspensions (2 \times 10^6 cells per ml in complete medium) were prepared from thymi of normal rats and equilibrated for 30 min at 37°. Separate aliquots were then incubated with or without antibiotic for either 10 min or 3 hours prior to labeling. For pulse labeling three 0.5-mM aliquots of each suspension were incubated for an additional hour with 0.3 \mu Ci (1.3 \text{ pmol}) of L-leucine-U-^{14}C (additional L-leucine (0.4 mM) was present as a normal constituent of the medium), 0.20 \mu Ci (6.7 \text{ pmol}) of uridine-2-^{14}C, 0.5 \mu Ci (0.28 \text{ pmol}) of uridine-3H, or 0.25 \mu Ci (29 \text{ pmol}) of deoxyuridine-2-^{14}C. Other conditions and determination of radioactivity in the total trichloracetic acid-soluble and -insoluble fractions were as described under “Experimental Procedure.” Average control values for incorporation of L-leucine, uridine-^{14}C, and deoxyuridine into the trichloracetic acid-insoluble fractions were, respectively, 38.5, 59.9, and 13.4 dpm per \mu g after the 10-min preliminary incubation period and 30.4, 51.2, and 15.3 dpm per \mu g after the 3-hour preliminary incubation period. Average control values for incorporation of uridine-3H and deoxyuridine into the acid-soluble fraction were respectively, 3.5, 95.2, and 3.8 dpm per \mu g.

Values for percentage change were calculated from the means for the three determinations; values greater than 15 are significant at $p < 0.01$ (for paired comparison).

| Antibiotic or analogue added | Trichloracetic acid fraction | Change of incorporation of radioactivity due to antibiotic |
|-----------------------------|-----------------------------|--------------------------------------------------------|
|                             | Leucine                     | Uridine       | Deoxy-
|                             | 10 min | 3 hrs | 10 min | 3 hrs | 10 min | 3 hrs |
| Cycloheximide, 5 $\times 10^{-4}$ M | Insoluble | $-39 - 84$ | $-85 - 84$ | $-97 - 84$ |
| Cycloheximide, 2.5 $\times 10^{-5}$ M | Insoluble | $-95 - 86$ | $-95 - 86$ | $-97 - 86$ |
| Cycloheximide, 2.5 $\times 10^{-4}$ M | Soluble | $-95 - 86$ | $-85 - 86$ | $-84 - 86$ |
| Puromycin, 5 $\times 10^{-7}$ M | Insoluble | $-80 - 85$ | $-85 - 85$ | $-86 - 85$ |
| Puromycin, 2.5 $\times 10^{-4}$ M | Insoluble | $-95 - 95$ | $-95 - 95$ | $-97 - 95$ |
| 6-Dimethylaminopurine, 2.5 $\times 10^{-4}$ M | Insoluble | $-95 - 95$ | $-85 - 85$ | $-86 - 85$ |
| Actinomycin D, 0.4 mg/ ml | Insoluble | $-95 - 95$ | $-85 - 85$ | $-86 - 85$ |
| Actinomycin D, 4.0 mg/ ml | Insoluble | $-95 - 95$ | $-85 - 85$ | $-86 - 85$ |

and are maintained as the period of incubation is extended. These inhibitors produced a practically complete inhibition of protein synthesis at the higher concentrations. In contrast, 6-dimethylaminopurine, a substance without effects on protein metabolism but mimicking the action of puromycin on carbohydrate metabolism (11), had no influence on incorporation of any of the precursors studied.

Cycloheximide and puromycin, but not actinomycin D, exert early inhibitory effects on the incorporation of deoxyuridine-^{14}C into an acid-insoluble form (Table I). Under these conditions of incubation, deoxyuridine is incorporated exclusively into DNA (2, 4). The early inhibition of deoxyuridine incorporation into DNA produced by cycloheximide was not associated with any change in the acid-soluble fraction; incorporation of thymidine was influenced similarly (data not presented). The effects of cycloheximide and puromycin on deoxyuridine incorporation into DNA at the earlier time period were appreciably greater than those on uridine incorporation into RNA. The data suggest a close relationship between continuing protein synthesis and DNA formation. Sensitivity of DNA synthesis to puromycin (12, 13) and to cycloheximide (14) in other mammalian cells in vitro has been reported.

Inhibitory effects of cortisol on the accumulation of radioactive uridine, glycine, and AIB in the trichloracetic acid-soluble and -insoluble fractions of thymocytes incubated with and without inhibitors of protein and RNA synthesis are summarized in Tables II and III. Incubation of thymocytes with either puromycin, cycloheximide, or actinomycin D throughout the entire period of incubation resulted in decreased accumulation of uridine-3H in both the trichloracetic acid-soluble and -insoluble fractions of the cells (Table II). The magnitude of the inhibition by these antibiotics increased with time of exposure of the cells. The data for the acid-soluble fraction suggest that each of the antibiotics inhibits transport or phosphorylation of nucleoside (or both). However, the antibiotics did not inhibit appreciably uridine transport or phosphorylation (or both) when added at a later time (2.5 hours) even though the antibiotics depressed incorporation into the trichloracetic acid-insoluble fraction (Table II). When an antibiotic was present from the beginning of the incubation, no additional inhibitory influence of cortisol on uridine-3H accumulation was apparent. In contrast, when protein or nucleic acid synthesis was depressed by late addition of antibiotic shortly before pulse labeling, the inhibitory influence of steroid on the accumulation of precursors into both acid-soluble and acid-insoluble fractions was still evident.

In the presence of concentrations of actinomycin D which produced only partial inhibition of RNA synthesis (0.1 or 0.4 \mu g per ml), effects of cortisol on the incorporation of uridine-3H into the acid-soluble and -insoluble fractions were still evident (Table II). The data indicate that steroid action is not selective for one or more species of RNA whose synthesis is either more or less sensitive to actinomycin D (see below).

The presence of puromycin at the beginning of the incubation depressed markedly the incorporation of glycine-^{14}C into the acid-insoluble fraction and to a lesser degree into the acid-soluble fraction (Table III). Addition of cortisol had no further inhibitory influence on the incorporation of glycine into either fraction. We have previously shown that essentially all of the glycine incorporated into an acid-insoluble form under these conditions is present in the protein fraction of the cell (4). Essentially similar data were obtained when actinomycin D was present from the beginning of the incubation. In contrast, addition of either puromycin or actinomycin D 2.5 hours after initiation of the incubation, although significantly inhibiting glycine incorporation, did not block the steroid effects.

The previously observed inhibitory effect of cortisol on the entry of AIB (3, 4) was not evident in the presence of either puromycin or actinomycin D (Table III). The steroid and puromycin appeared to exert a similar influence on transport process. The failure of actinomycin D to depress AIB entry

\footnote{The abbreviation used is: AIB, α-aminoisobutyric acid.}

\footnote{As previously reported, since no free uridine can be detected in the cells, these data do not permit the distinction between transport and phosphorylation of uridine (4).}
into the cells, in contrast to its effect on uridine and glycine accumulation, is not understood at this time. This result was unexpected, particularly in view of the absence of cortisol effects on AIB accumulation in the presence of actinomycin D. Except for this last point, the influence of the antibiotics on the accumulation of radioactivity in the acid-soluble fraction resembled that produced by cortisol.

Cortisol inhibited the incorporation of orotic acid-14C into the acid-insoluble fraction but had no effect on the accumulation of radioactivity in the acid-soluble fraction (Table IV). These results are in contrast to the above-described actions of the steroid on the incorporation of uridine into the acid-soluble and -insoluble fractions. Moreover, inhibition of uridine incorporation into the acid-insoluble fraction was significantly greater than that for orotic acid. These results support the conclusion that cortisol exerts an action on RNA synthesis by thymocytes in vitro independent of possible steroid effects on transport or phosphorylation of nucleoside (or both). The data in Table IV also indicate that the effect of cortisol on uridine incorporation into the acid-soluble fraction must be localized at a step involving transport of uridine or formation of UMP from uridine, since the reactions leading to the conversion of UMP to UTP are identical with either uridine or orotic acid as precursor.

Experiments in which thymocytes were incubated with cortisol for longer periods of time are summarized in Table V. After 6 hours of incubation in the presence of puromycin, no steroid effects were evident on the incorporation of glycine into either the trichloracetic acid-soluble or -insoluble fraction. This finding is in agreement with data presented above for 3-hour incubation periods (Table III). However, after 6-hour incubation in the presence of puromycin, inhibitory effects of steroid were evident on the incorporation of uridine-3H into the acid-insoluble but not into the acid-soluble fraction (Table V). With orotic acid-14C as precursor, the inhibitory effect of steroid on accumulation of radioactivity into the acid-insoluble fraction was greater when the incubation was extended from 3 hours (Table IV) to 6 hours (Table V). Even after 6-hour incubation with steroid, the accumulation of orotic acid in the acid-soluble fraction of the cells was only slightly decreased. Furthermore, in the presence of puromycin, a significant action of cortisol on accumulation of orotic acid in the acid-insoluble fraction was still evident. Also to be noted are the similar magnitudes of the inhibitory effects of cortisol on the incorporation of either uridine or orotic acid into the acid-insoluble fraction in the presence of puromycin. This supports our earlier suggestion (1) of a direct effect of cortisol on the RNA-polymerase system (also see below).

Thymic nuclei obtained by sonic oscillation, as well as the aggregate enzyme fraction, both described under “Experimental Procedure,” were found to be satisfactory for measurement of RNA polymerase activity. The polymerase activities of these preparations were similar to one another and to those of intact nuclei and of the nuclear aggregate fractions prepared from whole thymus (data not presented). The sonic disruption technique was developed because of difficulties encountered in homogenizing cell suspensions by the procedure previously described for whole

## Table II

**Influence of antibiotics and cortisol on incorporation of uridine into acid-soluble and acid-insoluble fractions of thymocytes incubated in vitro**

| Antibiotic addition | Incorporation into trichloracetic acid-soluble fraction | Incorporation into trichloracetic acid-insoluble fraction |
|---------------------|------------------------------------------------------|--------------------------------------------------------|
|                     | Control          | Cortisol   | Inhibition produced by cortisol | Control          | Cortisol   | Inhibition produced by cortisol |
|---------------------|-----------------|-----------|---------------------------------|-----------------|-----------|---------------------------------|
| None                | 414             | 244       | 42 ± 2 (2)*                      | 873             | 586       | 34 ± 2 (5)*                      |
| Puromycin, initially| 121             | 120       | 0 ± 8 (2)                        | 80              | 77        | 6 ± 5 (3)                        |
|                  | 635             | 380       | 40 (1)                           | 980             | 645       | 35 ± 4 (2)*                      |
| Puromycin at 2.5 hrs| 555             | 290       | 46 (1)                           | 490             | 258       | 45 ± 15 (2)*                     |
|                  | 75.4            | 46.3      | 38 ± 3 (2)*                      | 69.3            | 38.4      | 43 ± 2 (3)*                      |
|                  | 18.1            | 17.4      | 6 ± 6 (2)                        | 9.1             | 8.7       | 5 ± 9 (2)                        |
|                  | 77.9            | 50.1      | 36 ± 3 (4)*                      | 54.0            | 35.6      | 33 ± 6 (4)*                      |
|                  | 62.8            | 43.7      | 29 ± 3 (4)*                      | 26.5            | 16.5      | 36 ± 10 (4)*                     |
|                  | 54.7            | 43.5      | 23 ± 3 (4)*                      | 6.1             | 4.6       | 21 ± 9 (4)*                      |
|                  | 42.6            | 39.4      | 7 ± 5 (4)                        | 0.1             | 0         | 4 (4)                           |
|                  | 100             | 100       | 34 (1)                           | 147             | 111       | 25 (1)                           |
|                  | 132             | 83.5      | 36 (1)                           | 8.6             | 5.5       | 36 (1)                           |

* Significant at p < 0.01 (paired comparisons).
and of low ionic strength (4 x 10^{-3} M) presented). The measurements of polymerase activity described did not incorporate nucleoside triphosphates into RNA (data not shown). In the polymerase assay system, intact cells fraction prepared by sonic disruption contained approximately 50% of the labeled protein, 85% of the labeled RNA, and 98% of thymus (5). In studies involving incubation of thymocytes to a thymolytic steroid added in vitro, the influence of time of exposure of thymocytes to cortisol and to puromycin in vitro on nuclear RNA polymerase activity is summarized in Fig. 1. After 1.5 hours of incubation of cells with cortisol, no significant effect on polymerase activity was apparent. After 3 hours of incubation, however, the inhibitory action of the steroid was evident and was approximately twice as great after 6 hours. Incubation of cells with puromycin also resulted in a decreased polymerase activity. Moreover, even in the presence of puromycin, an inhibitory effect of cortisol on RNA polymerase activity was still evident. Thus,继续的合成 or synthesis of the antibiotic was not required for this action of cortisol in vitro. This observation is in agreement with the results presented above (Tables IV and V). Since the effect of steroid on polymerase activity was somewhat reduced in magnitude when antibiotic was present, it is possible that cortisol may have more than one action on the polymerase system (see “Discussion”).

The specificity of the response of the RNA polymerase activity of thymocytes to a thymolytic steroid added in vitro is illustrated by the data in Table VI. Incubation of thymocytes with either cortisol or another potent thymolytic steroid, flucinolone acetonide, lowered the RNA polymerase activity. In contrast, a nonthymolytic steroid, progesterone, was without significant effect on this enzymic activity under the same experimental conditions.

### Table III

Influence of antibiotics and cortisol on incorporation of glycine-^{14}C and AIB-^{14}C by thymocytes in vitro

| Antibiotic addition | Incorporation into trichloroacetic acid-insoluble fraction | Incorporation into trichloroacetic acid-soluble fraction |
|---------------------|----------------------------------------------------------|-------------------------------------------------------|
|                     | Control | Cortisol | Change produced by cortisol | Control | Cortisol | Change produced by cortisol |
| Glycine-^{14}C      |         |          | dpm/μg protein | %                  | dpm/μg protein | %                  |
| None*               | 136.5   | 115.5    | -15 (1)         | 44.4               | 30.8          | -30 (1)          |
| Puromycin, initially* | 116.2   | 121.2    | +4 (1)          | 7.2                | 7.9           | +9 (1)            |
| None               | 42.9    | 27.7     | -35 (1)         | 24.7               | 12.8          | -48 ± 2 (3)*     |
| Puromycin, initially | 52.0    | 48.2     | -7 (1)          | 4.1                | 3.5           | -11 ± 6 (3)      |
| None               | 42.9    | 27.7     | -35 (1)         | 34.5               | 17.8          | -49 ± 3 (2)*     |
| Puromycin, at 2.5 hrs | 38.9    | 31.7     | -18 (1)         | 6.2                | 3.5           | -44 ± 4 (2)*     |
| None               | 28.5    | (1)      |                 | 30.0               | 17.9          | -42 ± 4 (3)*     |
| Actinomycin D, initially | 21.0    | (1)      |                 | 5.8                | 5.4           | -11 ± 3 (3)*     |
| None               | 28.5    | (1)      |                 | 43.4               | 28.5          | -34 (1)          |
| Actinomycin D, at 2.5 hrs | 25.7    | (1)      |                 | 23.6               | 15.5          | -34 (1)          |

| AIB-^{14}C         |         |          | dpm/μg protein | %                  | dpm/μg protein | %                  |
|-------------------|---------|----------|----------------|--------------------|----------------|--------------------|
| None*             | 125.5   | 88.1     | -28 ± 4 (3)*   | 97.8               | 98.0           | +3 ± 5 (3)        |
| Puromycin, initially* | 36.9    | 29.3     | -19 (1)        | 41.6               | 29.9           | -28 (1)          |
| None              | 111.0   | 79.3     | -29 ± 4 (2)*   | 122.1              | 111.6         | -8 ± 4 (2)       |
| Actinomycin D, initially | 39.3    | (1)      |                 | 30.8               | 17.9          | -42 ± 4 (3)      |

*Pulse labeling carried out after 1 hour of incubation of cells with and without cortisol (1 x 10^{-6} M) and with and without puromycin.

*Significant at p < 0.01 (paired comparisons).

thymus (5). In studies involving incubation of thymocytes with labeled precursors for 30 min, it was found that the nuclear fraction prepared by sonic disruption contained approximately 50% of the labeled protein, 85% of the labeled RNA, and 98% of labeled DNA. In the polymerase assay system, intact cells did not incorporate nucleoside triphosphates into RNA (data not presented). The measurements of polymerase activity described in this paper were conducted with a medium containing Mg^{2+} and of low ionic strength (4 x 10^{-4} M (NH_{4})_{2}SO_{4}). The influence of Mg^{2+}, Mn^{2+}, and (NH_{4})_{2}SO_{4} on thymus RNA polymerase activity of thymocytes exposed to cortisol have been described (8). Those studies showed that the lower RNA polymerase activity of thymocytes exposed to cortisol in vitro was not influenced by changes in the divalent cation or the ionic strength of the assay medium.

The specificity of the response of the RNA polymerase activity of thymocytes to a thymolytic steroid added in vitro is illustrated by the data in Table VI. Incubation of thymocytes with either cortisol or another potent thymolytic steroid, flucinolone acetonide, lowered the RNA polymerase activity. In contrast, a nonthymolytic steroid, progesterone, was without significant effect on this enzymic activity under the same experimental conditions.
Influence of cortisol added in vitro on incorporation by thymocytes of orotic acid-\(^{14}\)C and uridine-\(^{3}\)H into the trichloracetic acid-soluble and trichloracetic acid-insoluble fractions of rat thymocytes

Preparation, equilibration, incubation, and pulse labeling of cell suspensions were carried out as in Table II. Pulse labeling was with 0.75 \(\mu\)Ci (12.3 mmoles) of orotic acid-\(^{14}\)C and 1.0 \(\mu\)Ci (0.56 m mole) of uridine-\(^{3}\)H. In four of the eight experiments in the table, pulse labeling was conducted by adding the two precursors to the same aliquot of cells. Other details and designations are as in Table II.

### Table IV

| Radioactive precursor | Fraction                        | Control | Cortisol, \(1 \times 10^{-6}\) M | Inhibition produced by cortisol |
|-----------------------|---------------------------------|---------|----------------------------------|---------------------------------|
| Orotic acid-\(^{14}\)C | Trichloracetic acid-soluble     | 4.6     | 4.5                              | 3 \(\pm\) 4 (8)                  |
|                       |                                  | 98      | 55                              | 44 \(\pm\) 3 (8)*                |
| Uridine-\(^{3}\)H      | Trichloracetic acid-soluble     | 3.0     | 2.3                              | 25 \(\pm\) 3 (8)*                |
| Orotic acid-\(^{14}\)C | Trichloracetic acid-insoluble   | 107     | 65                              | 38 \(\pm\) 3 (8)*                |

*Significant at \(p < 0.01\) (paired comparisons).

### Table V

Incorporation of precursors into thymocytes after incubation for 6 hours with and without cortisol and puromycin

Preparation, equilibration, incubation, and pulse labeling of cell suspensions were carried out as in Table II. Puromycin (2.5 \(\times\) 10\(^{-5}\) M) and cortisol (1 \(\times\) 10\(^{-6}\) M) were added initially. Pulse labeling was begun after 6 hours of incubation and carried out for 30 min with 1.5 \(\mu\)Ci (13 mmoles) of glycine-\(^{14}\)C, 1.0 \(\mu\)Ci (0.56 m mole) of uridine-\(^{3}\)H, and 0.75 \(\mu\)Ci (12.3 m moles) of orotic acid-\(^{14}\)C. Other details and designations are as in Table II.

| Precursor and fraction | Additions | Control | Cortisol | Change produced by cortisol |
|-----------------------|-----------|---------|----------|----------------------------|
| Glycine-\(^{14}\)C      | None      | 42      | 23       | -45 \(\pm\) 1 (2)*          |
|                       | Puromycin | 26      | 27       | +4 \(\pm\) 4 (2)             |
| Acid-insoluble        | None      | 19.2    | 5.4      | -72 \(\pm\) 3 (5)*          |
|                       | Puromycin | 1.4     | 1.5      | +7 \(\pm\) 10 (3)            |
| Uridine-\(^{3}\)H      | None      | 120     | 44       | -64 \(\pm\) 2 (2)*          |
|                       | Puromycin | 37      | 38       | +2 \(\pm\) 10 (2)            |
| Acid-insoluble        | None      | 92      | 24       | -74 \(\pm\) 3 (3)*          |
|                       | Puromycin | 7.4     | 4.7      | -37 \(\pm\) 11 (3)*         |
| Orotic acid-\(^{14}\)C | None      | 3.9     | 3.3      | -16 \(\pm\) 1 (2)*          |
|                       | Puromycin | 3.4     | 3.6      | +1 \(\pm\) 5 (2)             |
| Acid-insoluble        | None      | 2.6     | 1.0      | -60 \(\pm\) 3 (3)*          |
|                       | Puromycin | 1.0     | 0.6      | -39 \(\pm\) 11 (3)*         |

*Significant at \(p < 0.01\) (paired comparisons).

### Table VI

Specificity of steroid influence in vitro on RNA polymerase activity of thymic nuclei

Thymocytes were incubated in the presence or absence of steroids for 3 hours. Nuclei were then prepared by sonic oscillation of the incubated cells and RNA polymerase activity was assayed in triplicate as described under "Experimental Procedure." Values for percentage change due to steroid are mean \(\pm\) standard error. Number of experiments is given in parentheses.

| Steroid present during cell incubation | UTP-\(^{3}\)H incorporation | Change due to steroid |
|---------------------------------------|----------------------------|----------------------|
| Cortisol, \(10^{-6}\) M ; \(- - -\)     | 26.8                       | 3.2                  |
| Fluocinolone acetoneide, \(10^{-5}\) M | 35.5                       | 5.0                  |
| Progesterone, \(10^{-6}\) M            | 30.0                       | 5.2                  |

*Significant at \(p < 0.01\) (paired comparisons).

FIG. 1. Influence of time of exposure of thymocytes to cortisol and puromycin in vitro on RNA polymerase activity. Preparation and incubation of cell suspensions, isolation of nuclei from cells, and RNA polymerase assay were as described under "Experimental Procedure." The upper portion of the figure shows the RNA polymerase activity of nuclei prepared from control cells, \(\bullet - - -\); cells incubated with cortisol (1 \(\times\) 10\(^{-6}\) M), \(\bullet ---\); with puromycin (2.5 \(\times\) 10\(^{-5}\) M) \(\circ - - -\); with cortisol plus puromycin, \(\circ ---\). The lower portion of the figure shows the percentage inhibition of RNA polymerase activity due to incubation of cells with cortisol in the absence (\(\bullet - - -\)) and in the presence (\(\bullet ---\)) of puromycin. Number of experiments is given in parentheses. Points represent mean values, and vertical lines at each point indicate standard errors (based upon the method of paired comparisons). Duplicate determinations were carried out in each experiment.

Thymocyte is a reflection of an increased rate of degradation of RNA and protein. Thymocytes were first incubated with and without cortisol for 3 hours and then pulse-labeled with either phenylalanine-\(^{14}\)C or uridine-\(^{3}\)H during the 4th hour. The cells...
Thymocytes were incubated for 3 or 6 hours with or without 1 X 10^-4 M cortisol. Aggregate enzyme fractions were prepared from these cells and assayed in duplicate for RNA polymerase activity as described under “Experimental Procedure.” Other details and designations are as in Table VI.

| Time of cell incubation | UTP-3H Incorporation | Inhibition due to cortisol |
|------------------------|----------------------|---------------------------|
|                        | dpm/μg DNA           |                           |
|                        | Control cells | Cells with cortisol | %  |
| 3                      | 18.5          | 15.7          | 15 ± 1.7 (7)* |
| 6                      | 10.2          | 8.0          | 22 ± 6.5 (2)* |

*Significant at p < 0.01 (paired comparisons).

Fig. 2. Sucrose density gradient fractionation of thymocyte RNA following exposure of cells to cortisol in vitro (A) or in vivo (B and C) and labeling of cells in vitro. Suspensions of thymocytes were either incubated with or without 1 X 10^-4 M cortisol in vitro for 30 min (A), or were derived from control rats or rats injected with cortisol 3 hours prior to death (B and C). Aliquots of 10-ml each of the suspensions were then labeled with 1 μCi of uridine-3H or 50 μCi (A and B) or 100 μCi (C) of uridine-3H for 15 min followed by a 30-min “chase” (A), no “chase” (B), or a 40-min “chase” (C) with 6.6 μmoles of unlabeled uridine. Other details concerning double labeling of cells and extractions of RNA were as described under “Experimental Procedure.” Samples of RNA layered on the gradients contained an average of 2,000 cpm (A), 2,414 cpm (B), or 8,700 cpm (C) of uridine-3H and 2,100 cpm (A), 954 cpm (B), or 2,200 cpm (C) of uridine-3H. Centrifugation was either at 20,000 rpm for 15 hours (A) or 21,000 rpm for 16.5 hours (C) in a six-place SW 25.3 rotor or at 18,000 rpm for 16.6 hours in a SW 25.1 rotor (B). O---O, control; •--•, cortisol.

These findings are in agreement with our previous studies (4) in which antibiotics were not utilized.

The possibility that cortisol might influence the synthesis of a specific species of RNA was examined by exposing thymocytes to cortisol either in vitro or in vivo and utilizing double labeling techniques with RNA precursors in vitro. These studies included gradient centrifugation of total cellular RNA obtained with and without a “chase” with nonradioactive precursor. Representative experiments are summarized in Fig. 2. In studies involving exposure of thymocytes to cortisol in vitro for either 30 min (A) or 3 hours (data not shown) prior to labeling of RNA, the distribution patterns were not altered. Also, the distribution patterns of labeled RNA from thymocytes obtained from control animals and from animals injected with cortisol 3 hours prior to death were identical (B and C). Alterations in the RNA pattern attributable to cortisol were not seen either without (B) or with (C) a 40-min chase. It is possible that small changes in messenger RNA might have been obscured by the larger amount of 45 S RNA present (also see “Discussion”).

DISCUSSION

We have found that inhibitors of protein and RNA synthesis added to rat thymocytes incubated in vitro produce a progressive inhibition of amino acid transport and nucleoside accumulation. Thus, processes involving substrate accumulation by the cells are markedly dependent upon continuing protein synthesis. Also, such inhibitory effects on substrate entry or accumulation must be considered in evaluation of the actual net inhibition of macromolecular synthesis caused by antibiotics as well as by other agents, including steroids. We have found that puromycin does not influence the rate of entry of cortisol into rat thymocytes, even though this antibiotic does inhibit the entry of other small molecules as described in this paper. The data obtained do not, however, permit distinction between a direct effect of cortisol on a rate-limiting step in transport and an effect on the synthesis of macromolecules which influences the rate of transport. Inhibitory effects of adrenal steroids on amino acid transport in rat diaphragm (15) and on uridine accumulation in bone cells (16) have been found to be dependent upon continuing protein synthesis. In other studies, we have found that cortisol has an inhibitory influence on the rate of uptake not only of glucose (17, 18), but also of 3-O-methylglucose, an analogue of glucose which is actively transported into the cell, but which is not phosphorylated. In addition, these effects of cortisol on hexose uptake were not evident in the presence of cycloheximide, puromycin, or actinomycin D.

In the presence of puromycin the inhibitory effect of cortisol added in vitro on RNA polymerase activity was less but still evident. A similar result was obtained when aggregate enzyme was prepared from thymocytes incubated with cortisol and puromycin or cycloheximide. Calculations from the data in Fig. 1 indicate that the fraction of RNA polymerase activity which is sensitive to puromycin is also highly sensitive to cortisol. Thus, it may be calculated that, because of the presence of cortisol, the puromycin-sensitive activity is decreased from 7.6 to 5.2 dpm per μg of DNA (32%) after 3-hour incubation and from 7.3 to 4.2 dpm per μg of DNA (42%) after 6 hours. These results suggest that cortisol may exert more than one effect on the RNA polymerase system. These effects may perhaps be

M. H. Makman, B. Dvorkin, and A. White, manuscript in preparation.
differentiated on the basis of their dependence upon continuing protein synthesis. In this regard, it is of interest that the estrogen-stimulated portion of RNA polymerase activity in rat uterus has been reported to be extremely sensitive to treatment with either puromycin or cycloheximide, both in vivo and in vitro (19, 20). Inhibition of protein synthesis not only prevented initiation of the stimulatory effect of estrogen on RNA polymerase activity, but also reversed the hormone effect after it had been initiated. Therefore, synthesis of a rapidly turning over protein has been suggested to be involved in the action of estrogen on the uterine RNA polymerase activity.

Our data from sucrose density gradient studies indicate that the decrease in the synthesis of RNA due to steroid was not selective. Although the techniques used for detecting alterations in specific RNA species may not have been adequately sensitive, our data are in agreement with those of Drews (21) which appeared after our studies had been completed. Utilizing hybridization techniques, this investigator could not detect the loss of specific species of RNA in rat thymic cells exposed to prednisolone in vivo. Our results and those of Drews are in contrast to the data of Kidson (22, 23), who reported that incubation of lymphoid cells in vitro with cortisol resulted in an early inhibition of synthesis of a rapidly turning over RNA species. Alterations in specific RNA species have also been reported in studies of cortisol action on rat hepatoma cells (24), and estrogen and progesterone action on the chick oviduct (25). In contrast, recent studies of McGregor and Mahler (26) failed to reveal preferential effects of a glucocorticoid on a rapidly turning over RNA species in rat liver.

REFERENCES

1. Makman, M. H., Nakagawa, S., and Dvorkin, B., Fed. Proc., 26, 483 (1967).
2. Makman, M. H., Dvorkin, B., and White, A., J. Biol. Chem., 241, 1646 (1966).
3. Makman, M. H., Nakagawa, S., and White, A., Recent Progr. Hormone Res., 23, 195 (1967).
4. Makman, M. H., Dvorkin, B., and White, A., J. Biol. Chem., 243, 1485 (1968).
5. Nakagawa, S., and White, A., Proc. Nat. Acad. Sci. U. S. A., 55, 900 (1966).
6. Fox, E. K., and Gaborel, J. D., Mol. Pharmacol., 3, 479 (1967).
7. Nakagawa, S., and White, A., Endocrinology, 81, 861 (1967).
8. Nakagawa, S., and White, A., J. Biol. Chem., 246, 1448 (1970).
9. Eagle, H., Science, 130, 432 (1959).
10. Warner, J. R., Sorio, R., Birnboim, H. C., Girard, M., and Darnell, J. E., J. Mol. Biol., 19, 349 (1966).
11. Hofert, J., and Boutilier, R. K., Arch. Biochem. Biophys., 103, 338 (1963).
12. Mueller, G. C., Kajiwara, K., Stubblefield, E., and Rueckert, R. R., Cancer Res., 22, 1084 (1962).
13. Lederer, I., Abrams, R., Hunt, N., and Oye, P., J. Biol. Chem., 238, 3955 (1963).
14. Bennett, L. L., Jr., Smith, D., and Ward, C. T., Biochim. Biophys. Acta, 87, 60 (1964).
15. Koslowski, J. A., and Redmond, A. F., Endocrinology, 79, 531 (1966).
16. Peck, W. A., Messinger, K., Brandt, J., and Carpenter, J., J. Biol. Chem., 244, 4174 (1969).
17. Munson, A., Endocrinology, 77, 356 (1965).
18. Young, D. A., J. Biol. Chem., 244, 2210 (1969).
19. Gorski, J., and Morgan, M. S., Biochim. Biophys. Acta, 149, 268 (1967).
20. Nocolite, J. A., and Mueller, G. C., Biochim. Biophys. Res. Commun., 34, 851 (1966).
21. Drews, J., Eur. J. Biochem., 7, 200 (1969).
22. Kidson, C., Biochem. Biophys. Res. Commun., 21, 283 (1965).
23. Kidson, C., Nature, 213, 779 (1967).
24. Gelehrter, T. D., and Tomkins, G. M., J. Mol. Biol., 29, 59 (1967).
25. O'Malley, B. W., and McGuire, W. L., Endocrinology, 84, 63 (1969).
26. McGregor, R. R., and Mahler, H. R., Biochemistry, 8, 3036 (1969).
Inhibitory Effects of Cortisol and Antibiotics on Substrate Entry and Ribonucleic Acid Synthesis in Rat Thymocytes in Vitro
Maynard H. Makman, Sachiko Nakagawa, B. Dvorkin and Abraham White

J. Biol. Chem. 1970, 245:2556-2563.

Access the most updated version of this article at http://www.jbc.org/content/245/10/2556

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/10/2556.full.html#ref-list-1