Glucocorticoid Action on Rat Thymus Cells

II. INTERRELATIONSHIPS BETWEEN RIBONUCLEIC ACID AND PROTEIN METABOLISM AND BETWEEN CORTISOL AND SUBSTRATE EFFECTS ON THESE METABOLIC PARAMETERS IN VITRO*

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SUMMARY

In rat thymus cells incubated with carbohydrate substrate the steady state rate of incorporation of uridine into RNA generally parallels the rate of incorporation of amino acids into protein. In both instances glucose is required for continued maximal incorporation rates, a requirement that is only partially satisfied by other readily metabolized substrates such as pyruvate, lactate, or β-hydroxybutyrate. The inhibitory effects of cortisol on the incorporation of uridine into RNA parallel those on the incorporation of amino acids into protein and appear to be largely on the carbohydrate-dependent portion.

Substantial differences in relative rates of incorporation of RNA and protein precursors are revealed, however, by a study of the rapid changes that occur after the delayed addition of carbohydrate; an immediate burst in the incorporation of uridine into RNA, maximal at 1 to 2 min, precedes the slower rise in the incorporation of amino acids into protein. Moreover, in cells previously treated for 2 hours with cortisol there emerges from the moment of carbohydrate addition a maximal cortisol inhibition on uridine incorporation, about 40% of the carbohydrate-induced rise, whereas cortisol inhibition of amino acid incorporation emerges gradually after a 10-min lag.

Studies with actinomycin and puromycin reveal that, for both the stimulatory effects of carbohydrates and the inhibitory effects of cortisol that emerge after delayed carbohydrate addition, the changes in RNA and protein metabolism are independent of each other since the influences of both carbohydrates and cortisol on the incorporation of precursors into either macromolecular species persist when the synthesis of the other species has been blocked by antibiotic.

It is concluded that the late, 2-hour, effects of cortisol on the labeling of both RNA and protein are secondary to early hormonal inhibition of carbohydrate metabolism, possibly mediated through alterations in adenine nucleotide metabolism.

A number of inhibitory effects of physiological levels of glucocorticoids in lymphoid tissues have been shown both in vivo and in vitro including a decrease in incorporation of precursors into macromolecules (1-6), an inhibition of RNA polymerase activity (7, 8), an inhibition of transport phenomena (4, 5, 9), and decrease in glucose metabolism (1, 10-14). This latter effect on glucose metabolism has been shown at times which clearly precede the other effects on macromolecular metabolism. In this paper we report the results of continuing studies whose aim is clarifying the interrelationships that may exist among hormone effects on those metabolic parameters.

In a previous report on the 2-hour glucocorticoid inhibition in vitro of the incorporation of amino acids into thymus cell protein (15), we concluded that the glucocorticoid inhibition of protein labeling seen at this time was secondary to prior hormonal inhibitory effects on carbohydrate metabolism, possibly mediated through demonstrated changes in ATP production. These conclusions were based on the observations that the inhibition by cortisol of the incorporation of amino acids into protein emerged only in the presence of added carbohydrate, that the inhibition was only on the carbohydrate-dependent portion of protein labeling, and that the inhibitory effects on protein metabolism were preceded by hormonal suppression of carbohydrate metabolism.

In view of the considerable evidence that in many endocrine systems effects on protein synthesis are mediated through changes in RNA synthesis (16) and in view of the inhibitory effects of cortisol on both thymus cell RNA labeling (3–6) and RNA polymerase activity (7, 8), we have extended our studies with regard to the hypotheses (a) that both the stimulatory effects of carbohydrates and the inhibitory effects of glucocorticoids on protein metabolism are mediated through changes in RNA metabolism, and (b) that the 2-hour glucocorticoid inhibitions on RNA metabolism are the result of prior hormonal effects on carbohydrate metabolism.

From the data presented we are able to refute the hypothesis that the effects of both substrates and hormones on protein metabolism are the direct result of changes in RNA metabolism since, for both the carbohydrate stimulatory effects and the glucocorticoid inhibitory effects, changes in the rates of RNA and protein labeling occur independently. The data nevertheless do support our hypothesis that the glucocorticoid suppression of RNA labeling observed 2 hours after hormone administration is secondary to earlier inhibitory effects on carbohydrate metabolism, the inhibition possibly being mediated through effects on ATP generation. Some of these results have been reported briefly (9).
METHODS AND MATERIALS

Male Sprague-Dawley rats (Charles River Breeding Laboratories) were adrenalectomized 1 to 2 weeks prior to the experiment. After adrenalectomy, they were maintained on 1% NaCl and fed Labena (Ralston Purina Company). Suspensions of washed whole thymus cells were prepared as described previously (15). Pooled thymuses removed from five to 40 decapitated rats were rapidly minced in Krebs-Ringer bicarbonate buffer (pH 7.4 in equilibrium with 95% O$_2$-5% CO$_2$), homogenized gently by hand, filtered, and washed two times in 40 to 80 ml of buffer. Cells were resuspended in buffer and filtered, and 0.5- to 2.0-ml aliquots were added to the incubation flasks. Preparation of cell suspensions was done at room temperature; buffer containing cells was kept in equilibrium with gas throughout. The time that elapsed between killing the first rat and start of the incubations was 20 to 35 min.

Incubations were carried out in Neoprene-stoppered 10- or 25-ml Erlenmeyer flasks, shaken at 37-38° in a Dubnoff metabolic incubator at about 100 cycles per min. Flasks were gassed at 30-min intervals during the incubation. Packed cell volume was determined by a standard microhematocrit procedure on small aliquots taken directly from the incubation flasks.

Carbohydrate substrates were generally added as a 5% aqueous solution. Cortisol was added as a 10$^{-4}$ M aqueous solution (11) to give a final concentration slightly lower than 10$^{-4}$ M. Controls received water instead of substrates or cortisol. Solutions of labeled uridine or valine were neutralized and diluted in buffer, carrier was added, and 100-μl aliquots were added to flasks with a microliter syringe.

To determine radioactivity from $^{3}$H-uridine incorporated into RNA and $^{14}$C-valine into protein 50- or 100-μl samples of cells were removed from flasks during incubation and plated on 24-mm filter paper discs which 10 sec later were floated onto cold 10% trichloroacetic acid. Dishes were then thoroughly washed (three or four 1-hour washes) with cold 5% trichloroacetic acid, washed with alcohol-ether and ether, dried, placed in vials with 5 ml of 0.4% 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]thiophene (BBOT) in toluene, and counted in a dual channel liquid scintillation counter to determine, after appropriate corrections were made for cross-channel contamination, cold trichloroacetic acid-insoluble $^{14}$C and $^{3}$H counts. The $^{3}$H counts are reported as “counts in RNA.”

After removal of the scintillator with toluene, toluene-ethanol, and ethanol-ether washes the discs were subjected to a 30-min 75% trichloroacetic acid wash, and, after subsequent trichloroacetic acid, alcohol-ether, and ether washes replaced in their original vials and recounted to give hot trichloroacetic acid-insoluble counts, which are reported as “counts in protein.” In these experiments a hot trichloroacetic acid wash of the discs after counting quantitatively removes all $^{3}$H counts, but only a very small fraction of the $^{14}$C counts, indicating that the “cold trichloroacetic acid-insoluble” $^{3}$H from uridine is in RNA and that the $^{14}$C (from valine) is almost all in protein.

Quenching variations under these counting conditions were shown to be negligible and no corrections were made. Counts on control discs in which known radioactive material was added to cell suspensions previously cooled in ice, and which were immediately plated and washed along with experimental discs, were subtracted from experimental discs, although the correction was slight.

ATP assays were done as described previously from neutralized perchloric acid extracts (15). When assays on whole cell supernatants are compared with assays on cell pellets, ATP is confined to the cells. We report levels of ATP as micromoles per ml of packed cells (micromoles per ml of cell volume) which equals about 5 × 10$^9$ cells.

L(+)-Lactic acid was purchased from Sigma and titrated with NaOH to pH 7.4 on the day of the experiment. Enzymes and cofactors were purchased from Boehringer, uniformly labeled $^{14}$C-L-valine and 6-$^{3}$H-uridine from New England Nuclear, cortisol from Calbiochem, $^{14}$C-L-amino acids from Sigma, and puromycin from Worthington. Actinomycin D was the generous gift of Merck, Sharpe, and Dohme.

*RESULTS*

The dual labeled experiment in Fig. 1 compares both the stimulatory effects of substrates and the inhibitory effects of cortisol on RNA labeling with those on protein labeling. Considering first the effects of substrates, in most respects the influences on the labeling of both types of macromolecules are parallel: glucose is essential for continued maximal rates of precursor incorporation, whatever glucose provides is not missed until after 20 to 30 min of incubation, and other substrates can only partially replace glucose. In one particular, however, we consistently find that the substrate influence on labeling rates of RNA and protein are not entirely in accord: in the pres-
trace-dependent portion of macromolecular labeling, then this effect is not appreciably altered by cortisol, but without adduced substrate. At 110 min each flask received a mixture of 4H-uridine (0.014 mg per ml; specific activity, 195 mCi per mmole) and 14C-valine (0.44 mg per ml; specific activity, 33 mCi per g). At 130 min glucose (circles) or lactate (triangles), each at a final concentration of 1 mg per ml, or H2O (squares) was added. Aliquots, 100 μl, were removed at indicated times for determination of counts in RNA and protein. Symbols represent the mean value for six flasks. Standard errors were usually within the size of the symbols. When drawn as separate lines the differences between cortisol and control flasks were significant at the p < 0.01 level.

ence of pyruvate the absence of glucose is apparent sooner in the case of protein labeling than in the case of RNA labeling. Comparing the relative efficacy of several substrates, the similarity between the effects of β-hydroxybutyrate and pyruvate suggests that the influence of both is through the provision of a common metabolite, perhaps acetyl-CoA. In other experiments we find (as is the case with protein labeling) that the effects of lactate and pyruvate on RNA labeling are equivalent and that succinate and α-ketoglutarate have no stimulatory effect. None of these substrates at the concentration used is inhibitory per se, nor is its relative efficacy appreciably increased by increasing its concentration in the incubation flasks. So far we have not found a combination of substrates at any concentration that can completely replace the effect of glucose on either RNA or protein labeling.

Turning to the effects of cortisol, with some exceptions, the inhibitory effects on RNA labeling are similar to those on protein labeling, the exceptions being that inhibitory effects on RNA labeling are larger on a percentage basis, that they often emerge somewhat earlier (this is not apparent in the experiment in Fig. 1), and that significant inhibition of RNA labeling is sometimes seen after 2 hours in the absence of added substrate. The earlier emergence of effects on RNA metabolism seems explicable on the basis of the larger inhibition, larger effects being detectable sooner. The small cortisol inhibition of RNA labeling seen without added carbohydrate do, however, reflect a difference between RNA and protein labeling. If the major inhibitory effects of cortisol are indeed on the carbohydrate-dependent portion of macromolecular labeling, then this small difference in the case of RNA labeling indicates either that there is a small cortisol effect on RNA labeling independent of effects on carbohydrate metabolism or, alternatively, that the hormone effect is on the metabolism of endogenous substrate, or substrates such as lactate and pyruvate that leak out of cells during prolonged incubation.

Experiments in which carbohydrate is added to previously labeled cells 2 hours after cortisol exposure (Fig. 2) and in which instantaneous incorporation rates are examined by pulse labeling before and after delayed carbohydrate addition (Fig. 3) allow a more detailed investigation of the factors which influence RNA and protein-labeling rates. In both types of experiments cortisol inhibitions on RNA and protein labeling are not seen in the absence of added substrate, whereas upon substrate addition there is an immediate increase in both RNA and protein labeling, as well as the rapid emergence of cortisol inhibition. In such experiments certain differences in the changes in rates of RNA and protein labeling and in the emergence of the cortisol inhibitions become apparent. In the case of protein a small cortisol inhibition begins about 10 min after carbohydrate addition, as contrasted to the case of RNA in which an immediate maximal cortisol inhibition (40 to 50% of the carbohydrate-induced rise) is seen on the burst in RNA labeling that occurs following carbohydrate addition. The results of a number of experiments similar to that in Fig. 2 in which cells are previously labeled with large (as compared to trace) amounts of uridine have indicated that in this type of experiment (as well as in the type of experiment in Fig. 3) the cortisol inhibition of RNA labeling is maximal from the moment of carbohydrate addition. Although the pulse experiment in Fig. 3 was done with trace amounts of uridine and valine, we find that varying the precursor concentrations over a wide range and increasing the pulse duration from 1 to 5 min does not appreciably alter these results.

Since we have previously demonstrated (15) in experiments in which carbohydrate was either added at the start of incubation
flasks depicted by nomycin D. Cell suspensions (1 ml) were incubated in 35 flasks with or without 10^{-4} M cortisol from zero time. At 120 min glucose or lactate to make final concentration of 1 mg per ml or H_{2}O was added to each flask. At 121.5 min 0.2 ml of 60% perchloric acid was added directly to the incubation flasks. Duplicate ATP assays were done on each flask. Each bar represents the mean value for the averaged determinations on six flasks. Cortisol effects with lactate and glucose are significant at the p < 0.01 level. Vertical bars depict ± standard error.

![ATP levels](image)

FIG. 4. Cortisol effects on ATP levels at 1.5 min after the delayed addition of glucose or lactate. Suspensions of thymus cells, 2 ml, were incubated in each of 36 flasks with or without 10^{-4} M cortisol from zero time. At 120 min glucose or lactate to make final concentration of 1 mg per ml or H_{2}O was added to each flask. At 121.5 min 0.2 ml of 60% perchloric acid was added directly to the incubation flasks. Duplicate ATP assays were done on each flask. Each bar represents the mean value for the averaged determinations on six flasks. Cortisol effects with lactate and glucose are significant at the p < 0.01 level. Vertical bars depict ± standard error.

![protein labeling](image)

FIG. 5. Substrate and cortisol effects on the incorporation of precursors into protein (A) and RNA (B) in the presence of actinomycin D. Cell suspensions (1 ml) were incubated in 35 flasks from zero time, with (filled symbols) or without (open symbols) 10^{-4} M cortisol. Actinomycin D (A), 100 µg per ml, was added to flasks depicted by triangles at 101.5 min: glucose (G), 1 mg per ml, or H_{2}O (no substrate) was added at 120 min along with 4.2 µCi [3H]-uridine (specific activity, 9.43 Ci per mmole), and 0.31 µCi of [14C]-valine (specific activity, 195 mCi per mmole). At indicated times 100-µl aliquots of cell suspension were removed and placed on filter paper discs for determination of counts in RNA and protein. Each point represents the mean of five flasks. The standard errors were almost all within the size of the symbols. Shaded areas represent significant inhibition (p < 0.01) by cortisol.

or delayed for 2 hours that both carbohydrate and cortisol influences on protein labeling were preceded by changes in adenine nucleotide levels, we, accordingly, under conditions identical with those in Fig. 3, looked for changes in adenine nucleotides 1.5 min after carbohydrate addition. The results in Fig. 4 indicate that, although there is no cortisol effect without carbohydrate, in those flasks with carbohydrate small but highly significant changes in ATP are already present at this time. Moreover, after the delayed addition of carbohydrate, both the stimulatory effects of glucose and lactate on RNA labeling as well as the inhibitory effects of cortisol may be correlated with these small changes in ATP levels. As in the case of the adenine nucleotide changes observed at 8 min after carbohydrate addition reported previously (15), there are at 1.5 min inverse changes in AMP and ADP which correspond in amount to the ATP changes. These carbohydrate and cortisol effects on adenine nucleotides, also present when carbohydrates are present from the start of incubation, persist for at least 1 hour after delayed carbohydrate addition, the longest time studied.

When contrasted with the rapid substrate and cortisol effects on ATP levels and on RNA labeling, seen in Figs 3 and 4, the slower rise in rates of protein labeling and the 10-min delay and gradual onset of the cortisol inhibition of the latter suggest that changes in protein labeling are mediated via some intervening process with an inherent time lag. The experiments presented in Fig. 5 show that the intervening process is not actinomycin D-sensitive RNA synthesis since with these large doses of actinomycin, which eliminate almost all precursor incorporation into RNA, both the substrate and cortisol influences on amino acid incorporation are still essentially intact.

Comparable experiments with puromycin (Fig. 6) indicate that protein synthesis is neither required for the emergence of the carbohydrate-induced rise in RNA labeling, nor for the cortisol inhibition of that rise.

**DISCUSSION**

The similarity between carbohydrate requirements for RNA and protein labeling suggests a common mechanism for the stimulation of both pathways. In both instances the stimulatory effects correlate with the ability of added carbohydrate to restore in the case of glucose, or to partially restore in the case of lactate, ATP levels, which after 2 hours of incubation without substrates have declined 10 to 25%. If, in the experiments with delayed carbohydrate addition, these relatively small changes in ATP levels, which after 2 hours of incubation without substrates' inhibition of that rise.

1 Unpublished experiments by Giddings and Young which show that in the absence of carbohydrate 10 min of anoxia depletes ATP to one-fourth of original levels and that oxygen addition restores ATP within 5 min indicate that ATP turnover is rapid and that there is abundant endogenous substrate available in those cells incubated without added carbohydrate.
levels do provide the stimulus for resumption of RNA or protein labeling, since the labeling rates vary many-fold in response to only a small percentage change in ATP, it would seem likely that either ADP or AMP whose changes are larger on a percentage basis may be inhibitory or, alternatively, that larger ATP changes occur within specialized compartments of the cell that are less able to generate ATP from endogenous substrates, perhaps within the nucleus as suggested by us previously (15).

The dramatic onset of a 40 to 50% cortisol inhibition of the carbohydrate-induced rise in RNA labeling within 1.5 min of carbohydrate addition (Fig. 3), as well as our results with previously labeled cells (Fig. 2), strongly suggest that, as with protein labeling, the cortisol inhibition of RNA labeling at 2 hours after hormone addition is the result of hormone effects on carbohydrate metabolism, since in both types of experiment cortisol inhibition appears only on the carbohydrate-dependent portion of RNA labeling. Moreover, as they emerge, the cortisol inhibitory effects on RNA labeling are preceded by inhibitory effects on ATP generation, here seen at 1.5 min after carbohydrate addition.

Since sequence is of interest when one looks for possible causal relationships, in other experiments (not presented) we have, in collaboration with S. Giddings, compared the onset time for the emergence of cortisol inhibitory effects on glucose metabolism to glucose-6-P (by adding glucose at various times after the start of incubation) with the onset time for the emergence of the cortisol inhibition of RNA labeling and find in accord with Munek (14) a large (20 to 30%) cortisol inhibition of glucose metabolism to glucose-6-P at 30 min, when measurable cortisol inhibitory effects on total cellular RNA labeling are just beginning to appear. Since glucose is required for maximal RNA labeling it is possible, if not likely, that when glucose is present throughout the incubation the cortisol inhibition of total RNA labeling is at least in part secondary to the prior inhibitory effects of the hormone on glucose metabolism. That a cortisol inhibition located either at the glucose transport or phosphorylation step (14) is the only metabolic site of cortisol action does, however, seem to be ruled out by our demonstration of inhibitory effects on both RNA and protein metabolism when lactate, pyruvate, or β-hydroxybutyrate replaces glucose.

The experiments presented here were those chosen to emphasize the relationship seen in our investigations between carbohydrate metabolism and RNA-labeling rates, and the necessity of added carbohydrate for the emergence of cortisol inhibitory effects. The results of Makman, Dvorkin, and White (5), however, may be also interpreted within this framework since added carbohydrate is required in their experiments both for maximal macromolecular labeling rates and for the emergence of cortisol inhibitory effects. It also seems that the reported glucocorticoid action on RNA polymerase activity may also be related to effects on carbohydrate metabolism. Nakagawa and White (7) found that the effects of fasting on RNA polymerase were larger than the effects of cortisol; they attributed the effects of fasting to enhanced adrenal secretion. Another interpretation is that fasting as well as glucocorticoids influence carbohydrate metabolism which in turn has regulatory effects on RNA polymerase activity. These possible alternative interpretations could be resolved by a study of the effects of fasting on thymus RNA polymerase in adrenalectomized animals and by studies of the effects of added carbohydrate on the activity of RNA polymerase isolated from thymus cells incubated in vitro.

In a number of instances, early changes in RNA labeling from the nucleoside precursors of RNA such as uridine have been interpreted as changes in rates of cellular RNA synthesis. Justification for the assumption that the observed changes in RNA labeling are the result of changes in RNA synthetlc rates (rates of nucleotide polymerization) has been based on concomitant demonstrations of changes in the activity of RNA polymerase preparations isolated from the cells (7, 8) and upon the persistence of the hormonal effects on RNA labeling in experiments with large (as contrasted to trace) amounts of labeled nucleosides (6). There is, however, other data suggesting that this assumption is incorrect. Makman et al. (5) demonstrated a glucocorticoid inhibition of the incorporation of RNA precursors into the trichloroacetic acid-soluble as well as the trichloroacetic acid-insoluble fraction of thymus cells. Moreover, Swonger and Young have demonstrated that the 2-hour cortisol-induced changes in thymus cell RNA labeling from 3H-uridine are secondary to changes in labeling of UTP when either large or small amounts of 3H-uridine are used, and that such endocrine effects on intracellular UTP labeling persist when RNA synthesis is abolished by actinomycin D (17). This would seem to fix the metabolic site of early hormone action on RNA labeling at the level of nucleoside transport or phosphorylation. In our opinion these studies cast considerable doubt on whether there are 2-hour hormone-induced effects at the level of nucleotide polymerization that are detectable by a study of total cellular RNA labeling. If the differences observed here in RNA labeling are the result of changes in the specific activity of intracellular UTP it seems likely that altered ratios of adenine nucleotides could account for the effects of both substrates and cortisol since nucleotide ratios are reported to be of regulatory significance in the phosphorylation of nucleosides (18).

The results in our previous report (15), as well as other unpublished studies which show rapid equilibrium between intracellular and extracellular valine, have led us to the conclusion that the carbohydrate and hormone effects seen in these experiments are on the incorporation of intracellular amino acids into protein. Regulatory effects of adenine nucleotide levels or ratios on protein synthesis have yet to be clearly defined. ATP influences might be postulated at the amino acid activation step or through the provision of GTP. Since inhibitory effects of ADP on the incorporation of amino acid from aminoacyl transfer RNA into protein have been seen in cell-free systems; it seems at least possible that adenine nucleotide levels or ratios may have as yet unappreciated significance in the regulation of protein synthesis in mammalian systems. Our demonstration of an immediate carbohydrate-induced increase in protein labeling when RNA synthesis is blocked by actinomycin (Fig. 5) strongly suggests that such regulatory effects exist.

Studies showing that hormone effects on protein labeling are preceded by effects on RNA labeling have in many instances (16) provided a measure of support for the theory first proposed by Karlson (19) and Zalokar (20) that hormone effects on protein synthesis are the result of earlier hormone actions on RNA synthesis. While the results in Figs. 2 and 3 taken alone fit this picture very nicely, the experiments in Figs. 5 and 6 indicate that, at least for the emergence of substrate and cortisol effects on whole cell labeling in thymus cells, the changes in RNA and protein labeling are independent of each other and occur in the absence of the synthesis of the other macromolecular species.

2 M. Hosglund, personal communication.
The results presented here, when taken together with those of our previous report (15), support a working hypothesis suggested by Morita and Munck (1) as an extension of the more general hypothesis of Drury and Ingle that the ultimate catabolic actions of the glucocorticoids on macromolecular metabolism that emerge within several hours after hormone administration are the consequence of early inhibitory hormone actions on carbohydrate metabolism. In view of the special effects of glucose on macromolecular metabolism and on ATP levels, it seems likely that the late hormone actions on macromolecular metabolism are largely, if not entirely, the result of the large early hormone actions on glucose uptake. We have, however, not yet ruled out the possibility that the early action on glucose uptake is the result of still earlier actions on macromolecular metabolism. Such effects should occur without substrate, may be small in terms of total cell labeling, and would have to occur within minutes of hormone binding and so may not be measurable in the experiments presented here. Studies aimed at the detection of such very early glucocorticoid effects on macromolecular metabolism are now in progress.

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