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LOHRMAN, CHARLES RICHARD. The Early Effects of Cortisol on ^{3}H-Uridine and ^{14}C-Leucine Uptake and Incorporation in Cell Line I-407. (1977) Co-directed by: Dr. Richard C. Schauer and Dr. Laura G. Anderton. Pp. 71

The purpose of this study was to investigate the effect of cortisol on uridine and leucine metabolism in cell line I-407. Experiments were performed to determine the early effects of cortisol on the cells.

Monolayered cell cultures were exposed to cortisol, labelled precursors such as ^{3}H-uridine and ^{14}C-leucine and metabolic inhibitors. These cells were then homogenized and subjected to trichloroacetic acid (TCA) separation. Radioactive precursors in the TCA-soluble and insoluble fractions acted as a measure of precursor uptake and incorporation.

Preparatory experiments were performed to determine the fetal bovine serum (FBS) concentration for optimal cell growth. These experiments were also used to determine the cortisol concentration which produced the most consistent response. It was observed that cells grew best in 10% FBS. The lowest cortisol concentration which produced a consistently reproducible response in the cells was 0.05 µg/ml (1.7×10^{-7} M).

This study demonstrated, for the first time, the early and continuous parallel hormonal response to uridine uptake and incorporation in an in vitro cell system. Cortisol stimulated an early (15 minute) increase in both uptake and incorporation of ^{3}H-uridine followed by a rapid decrease at 30 minutes. The results of longer hormone incubations (2-4 hours) showed a decrease in specific activity of ^{3}H-nucleoside in both TCA-soluble and TCA-insoluble fractions.

Evidence is also presented showing early cortisol-induced inhibition of ^{14}C-leucine uptake (15 minute) and incorporation (30 minute) in an in vitro cell system. Cycloheximide's effect on leucine uptake was similar to that of cortisol in this time period. The protein inhibitor

also blocked cortisol effects on uridine and leucine incorporation. The possible biological implications of this inhibition of protein synthesis have been discussed in this study.

Preliminary experiments were performed to investigate early (15 minute) cortisol effects on different RNA species. High molecular weight RNA was extracted from whole cell homogenates and fractionated by polyacrylamide-agarose gel electrophoresis. Cortisol shifted the rapid incorporation of ^{3}H-uridine to a higher molecular weight RNA species. Synthesis of ribosomal (rRNA) was inhibited by low doses (0.1 μg/ml) of actinomycin D, blocking both ^{3}H-uridine uptake and incorporation. The simultaneous addition of cortisol and inhibitor produced increased nucleoside uptake and incorporation. The preliminary data from these experiments indicate high-weight, non-rRNA metabolism was preferentially affected by cortisol at 15 minutes.

It was concluded that: a) long term (2-4 hour) cortisol treatment decreased uridine uptake and incorporation while short term (15-60 minute) treatments produced a variable response; b) cortisol inhibited leucine uptake at 15 minutes and its incorporation at 30 minutes; c) cycloheximide effects on leucine uptake were similar to that of cortisol; d) preliminary data indicated cortisol stimulated ^{3}H-uridine incorporation into a high molecular non-rRNA species.

THE EARLY EFFECTS OF CORTISOL ON ^{3}H-URIDINE AND ^{14}C-LEUCINE UPTAKE AND INCORPORATION IN CELL LINE I-407

by

CHARLES RICHARD LOHRMAN

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Approved by

Richard C. Schauer
Co-Thesis Adviser

Laura G. Anderton
Co-Thesis Adviser

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

Co-Thesis Adviser Richard C. Shauer

Co-Thesis Adviser Laura G. Anderton

Committee Member Bruce M. Eberhart

December 20, 1977
Date of Acceptance by Committee

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INTRODUCTION

Hormones act as physiological modulators that alter the metabolism of various tissues in an organism. These alterations are believed to be initiated by the "primary" action of hormones on a target tissue. These primary activities are probably small in themselves but sufficient to cause changes in cell metabolism that occur later. Different types of primary actions have been proposed and all hormones are thought to initiate changes in target cells by one or more different mechanisms. These include alterations in cell and organelle membrane permeability, stimulation of the formation of secondary messengers such as cyclic AMP and cyclic GMP and stimulation or inhibition of specific gene activity (Martin, 1976).

Steroid hormones are a class of compounds that vary in their place of origin, target tissue, and the type of cellular activity. However, they all have the same basic four-ring structure and appear to have a similar mechanism of activity. This mechanism was first defined by the work of Jensen (Jensen and Jacobson, 1962) and Gorski (Gorski, et al., 1968) using radioactive estrogen. The hormone was found to bind to a specific cytoplasmic receptor protein which carried it from the cytoplasm to the nucleus. Figure 1 is a schematic drawing of this mechanism.

Later work by other investigators confirmed that a similar mechanism holds for the other classes of steroids: glucocorticoids (Beato et al., 1969, 1970; Gopalakrishnan and Sadgopal, 1972), mineralocorticoids and androgens (King and Mainwaring, 1974). Although the mode of action

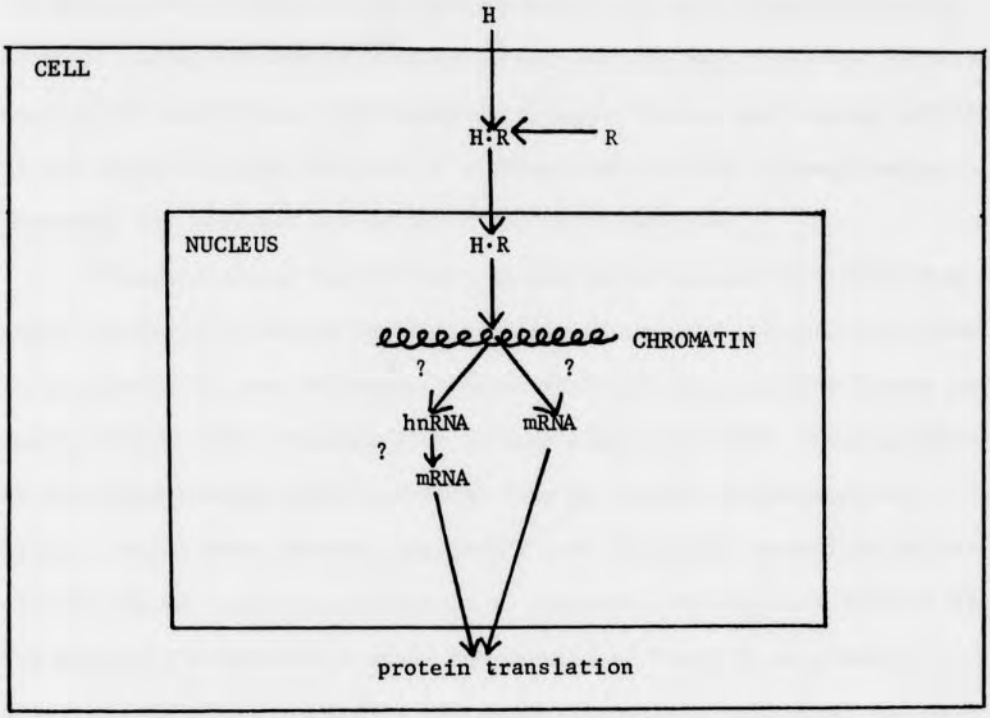

FIGURE 1

MODEL OF STEROID HORMONE ACTION

The model portrays the mechanism of action of most steroid hormones in a responsive cell type. The hormone (H) enters the cell and binds to a specific cytoplasmic receptor (R). The hormone-receptor complex enters the nucleus, where it binds to chromatin acceptors. Binding to chromatin initiates transcription of hnRNA and/or mRNA which alter cell metabolic processes. Modified from Gorski and Gannon (1976).

portrayed in Figure 1 applies to all steroids with some modifications, the cytoplasmic receptors are highly specific for each class of steroid hormone. Receptor-steroid binding affinities even vary with the different hormones in each class. For example, in human liver, the binding affinity of the glucocorticoid receptor is greatest for the drug dexamethasone, decreases for cortisol and is least for corticosterone.

Glucocorticoids and cortisol in particular appear to differ from other classes of steroids in that specific glucocorticoid receptors seem to be present in many different tissues (Ballard et al., 1974; Lieung and Munck, 1975). These include such diverse tissue as liver, thymus, kidney (Feldman and Funder, 1972) and fetal lung in rabbits (Giannopoulous, 1972). In all these tissue, cortisol's mode of action appears to follow closely the pattern shown in Figure 1. However, the ultimate effects of the glucocorticoids differ among tissue types. There is an anabolic effect in the liver, including increased ribonucleic acid (RNA) and protein synthesis. Stimulation of enzyme systems such as RNA polymerase (Jacob et al., 1969; Bottoms et al., 1972) and enzymes of the gluconeogenic pathway (Martin, 1976) have also been reported. In the kidney, glucocorticoids increase Na^+-ion reabsorption from the distal tubules, although not to the extent of mineralocorticoids such as aldosterone (King and Mainwaring, 1974). In other tissue, cortisol produces a catabolic effect which may lead to eventual cell death. This is the case for the thymus, skeletal muscle, skin, fibroblasts and HeLa cells (Lieung and Munck, 1975).

No matter whether steroid hormones produce a catabolic or anabolic effect on a particular tissue, one of their primary effects has to be an

alteration of genetic activity. This effect is evident in rat uterine tissue and chick oviduct where estrogen produces large increases in RNA, protein and DNA synthesis (Munns and Katzman, 1971; O'Malley and Means, 1974; Katzenellenbogen and Gorski, 1975). A major problem to be solved in this field is how steroid hormones interact with the genome to produce changes in cellular metabolism. Since steroids appear to alter transcription, they must have an effect on the gene regulatory mechanism. Thus, in order to understand how steroids affect eukaryotic genome regulation, one has to know something about the regulatory system itself.

Eukaryotic Regulation

Genome regulation is the stimulation or repression of the transcription of particular structural genes. Structural genes are defined as genes that are coded for individual polypeptides or proteins. The final result is the control over synthesis of the diverse species of RNA. The mechanism of eukaryotic regulation is not well known, but it is thought that heterogeneous nuclear RNA (hnRNA) located in the nucleus is involved. Heterogeneous nuclear RNA has recently been the subject of extensive investigation to determine its function in gene regulation.

The work of Darnell and his colleagues, (Darnell et al., 1973; Puckett and Darnell, 1977) reflect one trend of thought concerning hnRNA function. These authors and other investigators view hnRNA as a possible precursor to cytoplasmic messenger RNA (mRNA). Lewin (1975a, b) has reviewed the evidence for this mRNA precursor role. Darnell and Lewin have presented the following evidence in favor of hnRNA acting as a possible precursor for mRNA. First, the vast majority of hnRNA and mRNA species have a poly-adenosine (poly (A)) tail approximately 200

nucleotides long at the 3' end of the molecule. The poly (A) tail appears to be added enzymatically following transcription of the hnRNA molecule. Thus, it has been argued that the hnRNA precursor is degraded from the 5' end until the mRNA molecule remains. Second, when cells are treated with antibiotic 3'-deoxyadenosine (Cordycepin), hnRNA transcription proceeds normally but no poly (A) tail is added to the molecule and no poly (A) mRNA appears in the cytoplasm (Darnell et al., 1973). This data would indicate that the poly (A) tail was added to the 3' end of hnRNA after its transcription and this addition may be necessary for the movement of the precursor mRNA into the cytoplasm. It should be mentioned however, that HeLa histone mRNAs (Adesnick and Darnell, 1972) and other species of eukaryotic messenger RNA (Sonenshein et al., 1976; Greenberg, 1976) have been found in the cytoplasm without poly (A) tails. Third, the use by Imaizumi et al. (1973) of a complementary DNA (cDNA) probe synthesized from duck globin mRNA has shown a similar message sequence in hnRNA molecules of several sizes. Although hnRNA has a large size distribution, its smallest sizes are comparable to those of mRNA (Lewin, 1975a). The evidence cited above has led many investigators to speculate that a large hnRNA precursor is transcribed from the genome with the ultimate mRNA sequence located toward the 3' end.

Georgiev (1969; 1974) has proposed a model of a eukaryotic operon based on the above evidence. He speculates that the transcriptional unit is composed of acceptor sites located in a proximal (5') position with the actual structural gene at the distal (3') end of this unit. The acceptor sites would be specific for various regulatory proteins that could turn on the operon and subsequent transcription. After transcription

the regulatory 5' end of the hnRNA is gradually degraded until only the mRNA portion with its newly attached poly (A) tail is left to enter the cytoplasm. This model has the advantage of providing an acceptor site for the binding of the steroid-protein receptor complex on the chromatin which could then activate transcription of the whole unit. It also has the additional advantage of having the structural gene adjacent to its regulators. Unfortunately, the nature of the processing steps and the necessity for a giant precursor molecule are questions that still remain unanswered.

An alternate regulatory scheme has been proposed by Britten and Davidson (Britten and Davidson, 1969; Davidson et al., 1977). Using data obtained from sea urchin embryos, the authors have proposed a model based on the organization of the chromatin in the eukaryotic genome. In most eukaryotes, the genome consists of "families" of short, repetitive DNA sequences several hundred nucleotides long which are interspersed among single copy sequences up to several thousand nucleotides long. They propose that a relatively small proportion of the single copy genome functions as the structural gene complement, while the great majority of these sequences act as a non-transcribed spacer. The repetitive sequences are proposed as the regulatory genes. In contrast to Georgiev's model these authors propose that regulatory hnRNA molecules are transcribed apart from the structural gene and move through the nucleus to a control region on the structural gene. A specific structural gene would be regulated by hnRNA transcribed from certain repetitive sequence "families" which would be the sites of regulatory changes caused by hormones, cAMP or other substances.

Evidence for this model is based primarily on studies of genome complexity. The authors have calculated that the single-copy DNA is much more abundant than necessary for the number of structural genes expressed. The non-transcribed portion of this single-copy DNA would act as spacers and binding sites for endonucleolytic RNase activity that remove the regulatory hnRNA transcribed from the repetitive DNA template. Hormonal evidence that indirectly supports this hypothesis has been reported by Szego (1972) who has shown that estrogen increased nuclear acid RNase activity. The regulatory RNA transcripts might then move to the structural gene(s) and alter transcription.

The major advantage of this mechanism is that it accounts for the expression of new genes during different stages of development or in response to environmental changes. A large over-abundance of hnRNA is made (Davidson et al., 1977) and its fast turnover would allow a positive regulatory control by a steady stream of regulatory transcripts. Shutting off this stream of stimulatory transcripts would allow differentiation or reaction to environmental change. However, this model tends to ignore experimental evidence showing similarities between hnRNA and mRNA including identical poly (A) tails, inhibitor studies and hybridization studies (see above).

Obviously, the origin and regulation of mRNA is still unknown. Intensive investigation still leads to equivocal results such as the kinetic studies performed by Perry and Kelly (1973a, b) and Puckett and Darnell (1975; 1977). The former failed to find evidence for a precursor relationship between hnRNA and mRNA while the latter have reported evidence supporting this precursor relationship. Obviously more work needs to be done in this area.

Steroid-Genome Interaction

Since the regulatory system of the eukaryotic genome is not well understood, the mechanism of steroid-genome interaction also remains relatively unexplained. Investigators of steroid action often compare data resulting from work with several different hormones. This is especially true when comparing the similarity of the early action of all steroid hormones (see Figure 1).

The apparent initial genome effect of all steroids is the binding of the hormone-receptor complex to the nuclear chromatin. This step in the mechanism of steroid hormone action has been found to occur for estrogen (Gorski et al., 1968; Yamamoto and Alberts, 1975 and others), glucocorticoids (Beato et al., 1970; Dástague et al., 1971; Gopalakrishnan and Sadgopal, 1972) and mineralocorticoids (Wilce et al., 1976). Davidson et al. (1977) have presented evidence that the structure of eukaryotic chromatin is similar in all systems they have studied. Therefore, it is possible that each class of steroids might cause similar initial changes in the genome, although the ultimate effects to the cells could be quite different.

O'Malley and his collaborators have intensively studied the effect of estrogen on the genome of the chick oviduct. Using rifampicin, an inhibitor of reinitiation of E. coli RNA polymerase, they have found that an 8 hour estrogen treatment increased the number of initiation-sites where E. coli RNA polymerase starts transcription of the genome (Schwartz et al., 1975). They calculated the rate of chain elongation and ultimate chain length of the RNA synthesized to be similar to controls. Thus, according to these investigators, the estrogen made more sites available

for initiation, but did not appear to change enzyme activity nor the type of chain that was formed. One objection to this assay is that rifampicin only inhibits E. coli RNA polymerase. Thus, in conducting this investigation, the authors did not use the native oviduct RNA polymerase. Although comparison tests by the O'Malley group showed little difference between chick oviduct polymerase and E. coli polymerase using oviduct chromatin template (Tsai et al., 1976), other investigators have found that E. coli polymerase has many more initiation sites on a calf thymus chromatin template than the natural thymus polymerase (Cedar, 1975). Also, Barry and Gorski (1971) have reported that estrogen stimulated an increase in rate of RNA chain lengthening in rat uterus. Thus, it is still uncertain if the increase in number of initiation-sites is caused by the estrogen, or the type of RNA polymerase used, or if estrogen stimulates RNA synthesis in a different manner.

Evidence for increased RNA synthesis has been found by measuring steroid effects on RNA polymerase activity. In contrast to prokaryotes, whose RNA polymerase enzymes synthesize all types of RNA, there are three different classes of RNA polymerase found in eukaryotes. Excellent reviews by Chambon (1974, 1975) and Roeder (1976) summarize the current knowledge concerning these enzymes. Originally named RNA polymerase I, II, and III, they are often classified A, B, and C respectively. This latter system is based on their sensitivity to the toxin, α-amanitin. RNA polymerase A, located primarily in the nucleolus, synthesizes ribosomal RNA (rRNA) and is resistant to extremely high concentrations of α-amanitin. RNA polymerase B, located in the extranucleolar regions of the nucleus, synthesizes hnRNA and/or mRNA. This enzyme is sensitive to

low concentrations of α-amanitin. Finally, RNA polymerase C, which is believed to synthesize both 5S RNA and transfer RNA (tRNA), is also found in the nucleus. Polymerase C is sensitive to intermediate amounts of α-amanitin.

Measurements of steroid effects on RNA polymerase have been reported using both estrogen (Gorski, 1964; Knowler and Smellie, 1971; Barry and Gorski, 1971; Glasser et al., 1972; Borthwick and Smellie, 1975; Courvalin et al., 1976) and cortisol (Jacob et al., 1969; Sajdel and Jacob, 1971; Schmid and Sekeris, 1972; Borthwick and Bell, 1975). This work was based primarily on assays of RNA polymerase activity. Assays of this type are usually made by measuring incorporation of a labelled nucleotide (usually ^{3}H-uridine triphosphate) into RNA in a reaction mixture with nuclei isolated from pre-treated tissue. Although steroid-induced alterations in RNA polymerase activity have been reported, this technique does not determine their nature. It is possible the changes were due to changes in the number of initiation sites, but it is also conceivable the hormones alter the activity of polymerase molecules already present or the number of RNA polymerase molecules available for transcription processes.

Investigations of the various RNA polymerases using either estrogen (Glasser et al., 1972; Borthwick and Smellie, 1975) or cortisol (Schmid and Sekeris, 1972; Borthwick and Bell, 1975) have shown an interesting sequence of events apparently stimulated by these steroids. Both hormones stimulate an early (15-30 min.) increase in RNA polymerase B activity which would indicate the early synthesis of hnRNA (mRNA). Polymerase B activity drops almost to control levels by 30-60 minutes. Although this

early increase in enzyme activity is found in all the investigations cited, changes in the later activity of RNA polymerase B and that of RNA polymerase A are dependent upon the type tissue studied. In the case of tissue with anabolic response to hormone, such as the estrogen-uterus system, (Glasser et al., 1972; Borthwick and Smellie, 1975) and cortisol-liver system (Schmid and Sekeris, 1972), there is a sustained increase in RNA polymerase A activity about 30 minutes after hormone treatment. This is coupled with a second rise in RNA polymerase B activity. On the other hand, in the case of a catabolic response, such as the cortisol-thymus system (Borthwick and Bell, 1975), the small early peak in RNA polymerase B activity is followed by a sustained decrease in both RNA polymerase B and RNA polymerase A activity.

These studies implied the early action of steroid hormones on the genome appeared to stimulate the early synthesis of a small number of mRNAs or hnRNAs. One would expect a commensurate number of early proteins to be translated from these mRNAs. The new proteins might then produce either an increase (anabolic response) or decrease (catabolic response) in subsequent RNA polymerase activity. An alternative possibility is the newly synthesized proteins might stimulate (anabolic) or repress (catabolic) the transport of new mRNAs from the nucleus. The former would enable RNA synthesis to continue while the latter would stop RNA synthesis through feedback inhibition (Lichtenstein and Shapot, 1976). It is also conceivable the new synthesized hnRNA acts to directly control the later increases or decreases in rRNA and mRNA synthesis.

There is some evidence to support the concept of protein influenced steroid action. Estrogen in the uterus has been found to induce the early

(20-60 min.) synthesis of a protein or small group of proteins (Gorski and DeAngelo, 1970; Gorski and Barnea, 1970; Katzenellenbogen and Gorski, 1975; Cohen and Hamilton, 1975). Induced uterine proteins appear several hours prior to the larger increase in protein synthesis found in later stages of estrogen response. Although a search of the literature has failed to show direct evidence of a similar protein induced by cortisol, there is some indirect evidence of the importance of early protein synthesis. Munck et al. (1972) found that cycloheximide blocked the normal cortisol induced inhibition of glucose uptake in thymus cells only if given 15-25 minutes after the hormone. This implied that ultimate thymic response to cortisol was dependent upon early protein synthesis.

Evidence that this pattern of RNA synthesis is not confined to cortisol and estrogen has been reported by I.S. Edelman and his colleagues (Wilce et al., 1976a, b). The mineralocorticoid, aldosterone, increased Na^+ transport in toad bladder after a lag period of 60-90 minutes. During this lag period a rise in poly (A) RNA synthesis (presumably mRNA - see above) was detected at 30 minutes followed by a later rise in rRNA synthesis and increased Na^+ transport at 90 minutes. This would imply a similar anabolic pattern occurs with early aldosterone induction of mRNA transcription followed by increase in rRNA as was seen above with estrogen in the uterus.

There appears to be an emerging pattern of early steroid action in the nuclei of responsive cells. Steroid hormones act to stimulate the early transcription of a small number of messenger RNAs which induce the production of a limited number of early proteins. Some early proteins may enter the nucleus and, depending on the hormone and tissue, either

stimulate or inhibit first RNA polymerase A and then RNA polymerase B. Indirect evidence for such early protein action was presented by Borthwick and Smellie (1975). They demonstrated that cytosol from rat uteri treated for 15 minutes with estrogen stimulated RNA polymerase A activity in untreated uterine nuclei. In a separate series of experiments, they found that uteri treated with cycloheximide 30 minutes before estrogen was administered prevented the rise in RNA polymerase A activity.

Many questions still remain unanswered concerning steroid-genome interaction. Since the general eukaryotic regulatory mechanism is virtually unknown, it is not known how the steroids act to initiate or alter the rate of transcription of certain genes. One possible model has been presented by Yamamoto and Alberts (1976). They have found that most binding of estrogen-receptor complex to chromatin is non-specific (Yamamoto and Alberts, 1975). They hypothesize that this non-specific binding masks specific binding of steroid-receptor complexes to clusters of specific chromatin-acceptors on the regulatory portion of the transcription unit. This cluster would act to open the gene to RNA polymerase B activity.

A second model has been proposed by O'Malley and Means (1974). They have developed evidence that the progesterone cytoplasmic receptor consists of two entities. Part B of the receptor binds the progesterone-receptor complex to gene-specific non-histone chromatin proteins. Part A binds to DNA opening the gene(s) to RNA polymerase activity. Unfortunately, this type of cytoplasmic receptor has been found only for progesterone and this model might not hold for other steroids. Further research is necessary to determine what is the actual mechanism of steroid action.

Many of the recent investigations into early cortisol effects on RNA have used cell-free RNA polymerase assays (see above). These assays were performed on isolated nuclei removed from target tissue after hormone treatment. The general purpose of the present study was to investigate cortisol's action on RNA and protein precursor uptake and incorporation into whole cells. The use of intact cells from a known cell type confers certain benefits over either cell-free or whole organ studies. First, we were able to observe changes in total uridine metabolism rather than only effects on RNA synthesis. This included measurements of steroid effects on nucleoside transport, an area that has undergone comparatively little investigation (Oliver, 1971; Munns and Katzman, 1971; Wilce et al., 1976). The use of cell-free systems can result in artifactual changes that could possibly distort the actual hormone effects (Gorski and Gannon, 1976). Second, this system takes into account possible nuclear-cytoplasmic interactions that might be affecting RNA metabolism. Finally, using an established tissue culture line grown in a consistent, controlled environment enabled us to study cortisol's effect without the interference of other tissue types and their metabolites.

Culture of a single cell type also conferred some methodological advantages over _in vivo_ studies. For instance, growth of cells in flasks allowed quick addition of compounds such as hormones, nucleosides, amino acids and inhibitors to the cells. It also permitted fast termination of experiments (see Methods). Thus, it was possible to perform short-term incubation studies that would not have been feasible with _in vivo_ techniques.

Another technical advantage was the fact that the monolayered cells all had equal exposure to the cortisol, nucleosides, amino acids and inhibitors. The use of cell culturing technique also eliminated the possible artifactual effect that extracellular spaces may have on uptake studies.

The present study of cell line I-407 was undertaken to:

1. determine cortisol's early effect on RNA synthesis by measuring ^{3}H-uridine uptake and incorporation;
2. discover if cortisol affected facilitated nucleoside transport through the cell membrane;
3. test the effect of cortisol on early amino acid uptake and incorporation;
4. determine if inhibition of protein synthesis changed cortisol's effect on RNA metabolism;
5. attempt the separation of different RNA species from the total RNA of hormone-treated and control cells using polyacrylamide-agarose gel electrophoresis techniques.

METHODS AND MATERIALS

Sterile Techniques

The growth of a cell line such as I-407 requires that the utmost attention be given to the maintenance of sterile conditions. This involves all phases of work in the laboratory including the use and care of equipment. This section of Chapter II illustrates the procedures used to maintain sterile conditions in the laboratory.

Preparation of Tissue Culture Glassware

Glassware used in the tissue culture laboratory can be divided into two categories: a) serological and pasteur pipets, and b) glass bottles. These two classes were treated somewhat differently to obtain the desired sterile condition.

All used pipets were placed in a 1% 7x Solution (Microbiological Products, Bethesda, Maryland) until ready for sterilization. One Alconox detergent tablet (Alconox Detergent, Inc., New York, N.Y.) was added, and pipets were washed in a Nalgene pipet washer for one hour in continuous running warm water followed by a second hour in running cold water. The pipets were rinsed three times in a distilled water tank and dried in an oven at 250°C. Pipets were allowed to cool, plugged with cotton, and autoclaved under 15 lbs./in.^{2} pressure at 121°C for 30-35 minutes.

Used glass bottles were rinsed four times in tap water, filled with tap water and stored until washed. Filling bottles with water acts to prevent the build up of dried media, salts or other types of film.

The first step of sterilization consisted of completely submerging bottles in a boiling 1% 7x solution for five minutes. Care was taken to

prevent the formation of any bubbles on insides of the bottles. This insured that the entire inner surface of the bottle was in contact with the detergent solution while it was boiling. After boiling, the bottles and caps were scrubbed in the 7x solution with brushes set aside exclusively for this purpose. The bottles were rinsed ten times in tap water, twice in distilled water and dried at 250°C.

Prior to autoclaving the caps were loosely applied to the bottles to allow venting of vapor. All glassware was autoclaved at 121°C for 30-35 minutes, then removed to a 150°C drying oven, and gradually brought to room temperature.

Media

Cells were maintained in a medium based on sterile Eagle's Minimal Essential Medium (MEM). All medium components, with the exception of those purchased as sterile, were autoclaved at 121°C for 30-35 minutes at 15 lbs./in.^{2} pressure prior to mixing them into the medium solution. Fifty milliliters of sterile 10x MEM was added to 450 ml of autoclaved glass distilled water. A 25-50 ml portion of medium was removed, mixed with 0.77 grams of autoclaved sodium bicarbonate until dissolved, and returned to the remainder of the medium. Sodium bicarbonate in a 5% CO_2 atmosphere acts to buffer the medium at pH 7.4.

The medium volume was then reduced to 425 ml. Five ml each of L-glutamine (146 mg), penicillin (50,000 units) and streptomycin (50,000 ug) added together, fungizone (1250 ug), sodium pyruvate (25 mg) and Non-Essential Amino Acid solution (NEAA) were added in order. Fifty ml of virus screened Fetal Bovine Serum (FBS) was added to make 500 ml of a 10% (v/v) FBS solution. Finally, 2 ml of autoclaved 1N HCl was added to

maintain an approximate pH of 7.4. Later, it was found that two drops of the 1N HCl was sufficient to maintain pH. The 10% FBS medium was stored at 4°C and warmed to 37°C when added to the cells. In later experiments, gentamicin sulfate (Schering Corp., Kenilworth, N.J.) (25 mg) replaced penicillin and streptomycin. Except for gentamicin sulfate, all medium components were purchased from Grand Island Biological Company, Buffalo, New York.

Cell Culture Procedure

The cell line Intestine 407 (I-407, CCL6, Henle) was obtained from the American Type Tissue Culture Collection (ATTC) Baltimore, Maryland. These cells were originally cultured from human embryonic intestine. Recent evidence indicates that these cells probably have been contaminated with HeLa cells (Garther, 1968; Nelson-Rees and Flandermeyer, 1976). Cells sealed in glass ampules arrived from ATTC frozen in dry ice. Prior to thawing the cells, a triangular file, pasteur pipet, and a clean cloth were autoclaved at 121°C for 30-35 minutes then dried in an oven as described for autoclaved glassware. The ampule was thawed in a 37°C water bath, scored with the sterile file and wrapped in the cloth. The ampule was opened and the cells were pipeted into 25 cm^2 sterile plastic tissue culture flasks to which 5 ml of 20% FBS in MEM was added. The flasks were then placed in a 5% CO_2 atmosphere incubator at 37°C. Twenty-four hours later the culture media was then removed and 3 ml fresh media added to the cells.

Subculturing and Maintenance of Cell Cultures

Subculturing of cells is the removal of attached cells from the tissue culture flask and the subsequent transfer of these cells to new

flasks. This procedure enables the investigator to increase the number of tissue-containing flasks by dividing the contents of one monolayered flask between two or more new flasks.

At least 8 hours and preferably the night before subculturing, loosely capped flasks were incubated at 37°C, 5% CO_2 atmosphere and constant humidity. Preincubation allowed the flasks to equilibrate to the same 5% CO_2 atmosphere in which the cells were maintained. This procedure prevented the need for additional gassing of newly subcultured cells in order to maintain pH 7.4.

To initiate subculturing, the medium was first removed and the cells washed three times in sterile Ca^{++} and Mg^{++} free phosphate buffered saline pH 7.4 (PBS). Following the PBS washes, the cells were incubated at 37°C for 3-8 minutes in a 0.25% trypsin solution. Trypsin acts to loosen the cells from the bottom of the culture flask by digesting extracellular proteins holding cells to the flask and each other. Shaking of flask completed the dissociation and left the cells floating in the trypsin solution. The cell solutions from all the flasks were pooled in one flask and an equal volume plus 0.5 ml of MEM was added.

A 0.5 ml aliquot of the cell-medium mixture was pipetted into a 1.0 ml solution of 0.5% trypan blue, a vital stain which diffuses into dead cells but is excluded from live cells (Yip and Auersperg, 1972). The cells were counted using a white blood cell hemocytometer and the number of live cells per ml was calculated according to the formula:

$$\text{cells/ml} = \frac{\text{no. of live cells counted}}{5} \times 3 \times 10^4$$

where 5 is the number of large squares counted, three is the dilution factor and 10^4 is the multiplier that converts area of counted space into

volume. Percent viability of the cells was determined by keeping separate tallies of live and dead cells and dividing the number of live cells by the total number of cells counted.

After calculating the number of cells/ml, equal volumes of cells were pipeted into new flasks. The number of cells planted varied depending on the size of the flasks used. Cells were maintained by changing the growth medium every two or three days until a monolayer had formed, at which point they were either subcultured or used in experiments. Table I shows the number of cells planted and the amount of medium added for each size flask utilized throughout this study.

TABLE I

Cell Culture Inoculation

Flask Size in Area	Avg. No. Cells Planted	Amt. Medium Added
25 cm^2	1.5×10^6	2.5- 3.0 ml
75 cm^2	4.5×10^6	9.0-10.0 ml
150 cm^2	5.0×10^6	17.0-22.0 ml

Different size flasks were used for growing I-407 cells in culture. Table I shows the average number of cells planted in each size flask. Procedures were identical for all size flasks.

Sterile Technique

Since cultured cells must be maintained under very rigorous sterile conditions, several techniques were used to reduce the chance of contamination. Prior to any work with the cells, the floor of the tissue culture hood was washed with 47.5% ethanol (one volume 95% ethanol plus one volume of distilled water). After all materials had been assembled

in the hood, the investigator washed his hands in a germicidal soap (if available) or ordinary hand soap, returned to hood and waited from 30-60 seconds with hands in the hood before starting any operation. This wait allowed the cessation of air currents created by the investigator. It has been demonstrated that various biological contaminants such as bacteria, fungi, viruses and other cell types can be carried from one culture to another by these air currents (Chatigny, 1975).

The neck of each glass bottle was passed through a low bunsen burner flame just after opening and just before closing. The tips of pipets were also "flamed" and each pipet was used only once. Care was taken to insure that the pipets touched neither the hood nor any bottles or other materials. If such an accident did happen, the pipet plus its contents was immediately discarded. Bottle caps were never dropped or placed on the hood counter but were hand-held as long as the bottle was open. After all operations were completed, the hood floor was again wiped with the above ethanol solution.

These techniques were developed over a two to three year period and were vital to the consistent culturing of these cells. It was imperative that they be used as any mistakes invariably resulted in contaminated cultures.

Experimental Procedures

TCA Fractionation

Exposure of tissue or cells to trichloroacetic acid (TCA) causes the precipitation of macromolecules such as RNA, DNA and protein. This facilitates the separation of these macromolecules from their acid-soluble precursors. By measuring radioactivity in TCA-soluble and insoluble

fractions, it is possible to determine labelled precursor uptake and incorporation in the cell. Various hormonal experiments were performed to measure uptake and incorporation of ^{3}H-uridine and ^{14}C-leucine after exposure to cortisol and/or inhibitors.

Upon formation of a cellular monolayer the medium was changed 12-24 hours before hormonal treatment. Cortisol (3 mg/ml 95% ethanol stock solution) was added to the cell medium in varying concentrations and incubated for predetermined amounts of time. Either 15 or 30 minutes before the end of hormone incubation 1.25 μCi of ^{3}H-uridine (25.7-29.6 Ci/mmol) (New England Nuclear, Albany, Mass.) was added to the flasks bringing total medium concentration to 3 ml. In later experiments designed to study early effécts of cortisol, the labelled uridine was added 15 minutes before the experiment was terminated. Cycloheximide experiments required the addition of 0.95 μCi ^{14}C-leucine (320 mCi/mmol, New England Nuclear) to each flask 15 minutes before termination.

Upon termination of the experiment the treatment medium was removed and the cell monolayers washed three times with ice cold PBS. In earlier experiments, cells were incubated for 2-5 minutes at 37^{o}C in 0.25% trypsin solution to loosen the cells from the flask surface (see above). Cells were then pipetted into cold centrifuge tubes which were placed immediately on ice. In later experiments which did not use trypsin, the cellular removal procedure was altered to scraping the cell monolayer from the flask surface by use of a rubber policeman. This procedure reduced exogenous RNase contamination found in trypsin. The flasks were placed on an ice-filled tray in order to rapidly lower the cellular temperature and slow metabolic activity that might have continued after

removal of the medium. This alteration in cell removal technique had an added advantage in that the cells were removed from flasks to the centrifuge tubes more rapidly. This helped to decrease variability in results between individual experiments.

Free cells were washed in 3 ml ice-cold PBS, vortexed 5-10 seconds and centrifuged at 1500 x g at 4°C using an IEC Model B20A centrifuge with No. 870 head. This procedure was repeated 3 more times to remove exogenous labelled material from the cells. Following the fourth centrifugation, a 100 µl aliquot of wash was removed, solubilized in 0.5 ml Nuclear Chicago Solubizer (NCS) (Amersham/Searle, Chicago, Ill.) and counted in a Beckman Scintillation Counter, Model LS-100 in order to monitor the intercellular precursor radioactivity. When studying facilitated diffusion of uridine into or out of the cell, 100 µl aliquots were removed from each wash and counted in a similar fashion.

One milliliter of PBS was added to above cell pellet, vortexed, transferred to a glass Duall homogenizer size 21 (Kontes, Vineland, N.J.), and ground with 20 strokes of the homogenizer. In later experiments, homogenizations were done on ice to lower any possible RNase activity. Upon removal of homogenate, the homogenizer was rinsed in 1 ml PBS for 10 additional strokes. An equal volume of 10% TCA was added to the combined cell homogenate and rinse, making 4 ml of a 5% TCA solution. The homogenate was centrifuged at 1440 x g for 8 minutes at room temperature using an IEC clinical centrifuge with No. 221 head. A 100 µl acid soluble aliquot was removed and mixed with 0.5 ml NCS to determine labelled uridine present in the TCA soluble fraction. NCS allowed homogeneous mixing of the polar aliquot with non-polar counting cocktail.

The acid insoluble pellet was then washed in fresh cold 5% TCA, vortexed, and centrifuged at 1440 x g for 3 minutes. This process was repeated two more times to remove any labelled TCA-soluble contamination from TCA-insoluble fraction. Aliquots taken from the final wash showed counts to be at background levels.

Following the final TCA wash, the acid insoluble fraction was washed once in absolute ethanol to remove phospholipids. Aliquots counted from this wash were at background levels. After the ethanol wash, the TCA-insoluble pellets were dried at 37°C overnight.

The dried pellets were divided into two portions and weighed. One portion placed in scintillation vial was moistened with 0.1 ml distilled water for 24 hours. The moisture accelerates the solubilizing action of NCS. NCS (0.5 ml) was then added to the pellet and incubated at 37°C for 24 hours to digest the pellet. Ten milliliters of a liquid scintillation cocktail containing 5.0 g of 2,5-diphenyloxazole (POP) and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenoxazolyl)]-benzene (M_2POP) per liter of toluene was added to each vial. Simultaneously 10 ml of the cocktail was added to each scintillation vial containing aliquots of either the final PBS wash or the TCA-soluble fraction. The samples were counted at 2% error with a maximum counting time of 10 minutes.

The second portion of the dried TCA-insoluble fraction was colorimetrically assayed for deoxyribonucleic acid (DNA) by a modification of the Burton assay (Burton, 1956). The pellet was washed and centrifuged at 1440 x g three times in a 1-3 ml alcohol and ether (3:1) solution to remove lipids. Pellets were allowed to drain, one ml of 0.86 M perchloric acid was added to each sample and placed in a 90°C water bath for 15

minutes to hydrolyze the DNA. Simultaneously two 1 ml blank aliquots of perchloric acid and a DNA standard solution (200 µg/ml) were also incubated in the same 90°C water bath.

The hydrolized nucleic acid solutions were pipeted into clean graduated centrifuge tubes and adjusted to 1.0 ml volume if necessary with perchloric acid. Two milliliters of diphenylamine reagent [0.1 ml of 16 mg/ml acetaldahyde to 20 ml diphenylamine solution (1.5 g diphenylamine completely dissolved in 100 ml glacial acetic acid plus 1.5 ml concentrated sulfuric acid)] was added to each of the samples. These were covered with aluminum foil, incubated with blanks and DNA standards in a 37°C water bath for 18 hours. Optical absorbance was determined at 600 nm by a Spectronic 20 spectrophotometer (Bauch and Lomb, Rochester, New York) equipped with red filter and red sensitive phototube.

Calculations and Statistics

In presenting the data, one problem that had to be solved was the adjustment of counts per minute (CPM) to account for differences in cell number in each flask. Thus, the number of counts for each TCA-soluble fraction was divided by the amount of DNA that would have been present in 0.5 mg of TCA-insoluble pellet (CPM/µg DNA/0.5 mg). This latter calculation created an artificial number, but also standardized the raw counts of the TCA-soluble fraction to the approximate number of cells in each pellet. TCA-insoluble pellets were standardized by dividing the recorded CPM by the amount of DNA in the vial as determined by the DNA assay described above. Basic to the rationale for these calculations was the assumption that the range of DNA/cell is approximately the same throughout the cell population of each flask.

The Student t-test was used to evaluate the significance of variability in the results of these experiments. It measures whether the data varies because of chance alone or because of variation in treatment during the experiment. Thus, this procedure helped determine the significance of variations in cell metabolism caused by cortisol. A significance of 5% would mean there was a less than 5% probability ($p<0.05$) that variability in data was due to chance alone and greater than 95% probability it was due to the variations in the experiment.

Procedures for RNA Extraction and Isolation

Glassware

During RNA extraction extra care must be taken to prevent glassware contamination by exogenous RNase. This enzyme is apparently ubiquitous and can easily degrade the RNA extracted regardless of the procedure used. All glassware used in these experiments was especially treated according to the method of Rosen and Monahan (1977) to reduce RNase activity.

All clean glassware was washed once in a 1N NaOH followed by six rinses in distilled water. The glassware was dried at 200-250^{o}C for at least one hour to denature any remaining exogenous RNase activity.

Treatment of Cell Cultures

Cells cultured for early RNA extraction experiments (see above method) were grown either in 75 cm^2 flasks (Falcon, Oxnard, California) or later 150 cm^2 flasks (Corning, Corning, New York) in order to obtain a greater number of cells. Medium was changed 12-24 hours before the experiment was started. Hormone and inhibitors were added as required and double labelled uridine, (5,6-^{3}H)-uridine (30.7 µCi/mmol) (ICN

Pharmaceuticals, Irvine, California) was added 15 or 30 minutes before the experiment was terminated. At termination of experiment, medium, labelled uridine, hormone and inhibitor (if present) totaled 20 ml in the Corning flasks and 10 ml in Falcon flasks. The RNA extraction experiments were terminated in a similar manner to the TCA-fractionation experiments as described above.

RNA Extraction Procedures

RNA was extracted at 55°C using a modified phenol extraction procedure of Kirby (1968). Extraction at this temperature retains a greater proportion of A-U rich (non-ribosomal) RNA while most DNA is discarded in the phenol (Georgiev et al., 1974). The cells were washed and centrifuged in 3 ml PBS similar to cells used in TCA experiments (see above). Cells from each treatment were pooled into appropriate centrifuge tubes.

Following the last PBS wash, 1 ml ice cold homogenizing medium [1 volume 6% p-aminosalisylic acid and polyvinylsulfate (PVS) (10 µg/ml) + 1 volume 0.9% NaCl] was added to the pooled cells. PVS was used to inhibit RNase activity (Cheng et al., 1974; Catelli and Baulieu, 1976). Cells were homogenized on ice in an identical manner to TCA fractionation procedure. The homogenate was immediately added to 3 ml stirring phenol solution, (250 grams phenol, 0.25 grams 8-hydroxyquinoline, 35 ml m-cresol, 27.5 ml water). The glass homogenizer was rinsed with 1 ml ice cold homogenizing medium and this plus an additional 4 ml of the medium added to the stirring phenol. The combined emulsion was stirred constantly in a water bath at 55°C for 20 minutes.

The phenol-homogenate mixture was centrifuged at 800 x g, -5°C for 30 minutes. The aqueous top phase and interphase were removed and 3 grams

NaCl was added to each 100 ml of these phases to precipitate unwanted proteins. The top phase was then added to one-half volume stirring phenol-solution. This mixture continued stirring for 10 minutes at 55°C.

The emulsion mixture was centrifuged at 13,000 x g, -5°C for 20 minutes. Only the top aqueous phase was removed into 2 volumes ethanol m-cresol (9:1 v/v) and stored overnight at -20°C to precipitate the RNA.

After overnight storage, the ethanol m-cresol RNA solution was centrifuged at 13,000 x g for 20 minutes at -5°C, the top phase was discarded and the bottom phase, which was identified by differences in light refraction, contained the RNA. This phase was transferred to 5 ml ethanol-salt solution (75 ml absolute ethanol, 25 ml H_2O, 1 gram NaCl) stirred by microspatula and centrifuged 1550 x g at -5°C for 15 minutes. The ethanol acts to precipitate the RNA while the NaCl stabilizes the RNA structure.

After centrifugation, the RNA was washed first in 75% ethanol, then two successive washes in absolute ethanol. Following each wash, the RNA was stirred and centrifuged identically as in the ethanol-salt wash. The last three washes acted to remove the excess NaCl and water from the precipitated RNA. The final precipitate was dried under vacuum and stored at -20°C.

RNA Separation by Electrophoresis

RNA extracted at 55°C was separated on 2.4% polyacrylamide - 0.5% agarose gels using a modified method of Cattolico and Jones (1975). This method involves the casting of flat bed gels with the polyacrylamide-agarose mixture. This procedure eliminates the necessity of pre-gel formation. The flat bed aids in dissipation of heat from the electrophoresis gel.

Preparation of Ribosomal RNA Markers

Ribosomal RNA (18S and 28S) markers were prepared from rat liver ribosomes according to the method of Wettstein et al. (1963). These ribosomal RNA (rRNA) markers along with 4S soluble brewer's yeast (tRNA) (Schwarz/Mann, Orangeburg, N.Y.) were used to calibrate gel electrophoresis analysis. In order to reduce possible liver glycogen contamination of rRNA, all rats were starved 24 hours prior to extraction.

Animals were decapitated, livers were immediately excised and placed in ice-cold isotonic saline. The tissue was blotted to remove excess fluids and placed in 15 ml ice cold extraction medium contained in the pre-weighed beaker (0.25M RNase-free sucrose (Sigma), 0.025M KCl, 0.001M $MgCl_2$, 0.05M tris-HCl pH 7.6 to which was added 10 µg/ml PVS just prior to extraction). The livers were weighed and extraction medium was added to a final concentration of 2.5 ml/gm liver tissue. The tissue was homogenized on ice with a Virtis 23 homogenizer at 23,000 rpm for 1 minute. The homogenate volume was measured with a graduated cylinder, pipetted in equal volumes into centrifuged tubes, and centrifuged at 12,000 x g for 15 minutes at $4^{o}C$.

The supernatant was filtered through four layers of gauze to remove glycogen and fat and the filtrate again centrifuged at 12,000 x g for 15 minutes at $4^{o}C$. Finally the supernatant was centrifuged at 100,000 x g for one hour using a Beckman Model L-2 ultracentrifuge equipped with a model 40 head. The pellet consisted of liver polysomes containing the desired rRNA markers. Polysome fraction was subjected to a 24-$26^{o}C$ (room temperature) phenol extraction otherwise identical to the procedure described in above methods. Extraction at room temperature allows preferential extraction of rRNA (Georgiev et al., 1974).

Electrophoresis Reagents

Acrylamide stock solution was prepared by mixing 4.8 g acrylamide and 0.25 g bisacrylamide (Eastman Kodak, Rochester, N.Y.) in 58.6 ml 1M tris-HCl pH 8.7. This solution was then diluted up to 86 ml with distilled water and stored at $4^{o}C$ for a maximum of seven days. Tank buffer consisted of a 0.036M tris-0.03M NaH_2PO_4 buffer pH 7.9, 0.001M Na_4EDTA. This was stored at $4^{o}C$ and used no more than six times. Buffer used in the upper tank was discarded after one use.

Preparation of Gel

The acrylamide solution for a single cell was prepared by mixing 17.2 ml stock acrylamide with 2.5 ml 3.2% N,N,N',N'-tetra-methylethylene-diamine (TEMED) (Eastman) and degassing by heating in a $54^{o}C$ water bath for 15 minutes. Twenty milliliters of 1% agarose (Nutritional Biochem., Cleveland, Ohio) which had been refluxed to degas and cooled to $54^{o}C$ were added to degassed acrylamide solution. One ml of 1.4% ammonium persulfate (Eastman) was added to acrylamide-agarose solution in order to initiate gel polymerization.

The acrylamide-agarose solution was poured into the preleveled Ortec electrophoresis cells. A well former was placed in the gel immediately after pouring. The gel was allowed to stand at room temperature for 20 minutes, $4^{o}C$ for 15 minutes, and again at room temperature for 40 minutes. The well former was removed after the refrigeration period. Removal was facilitated by applying distilled water around the well former before refrigeration.

Electrophoresis Procedure

Electrophoretic separation of the RNA was performed using an ORTEC 4100 Constant Pulse Power Supply System (Oak Ridge, Tenn.). RNA was dissolved in buffer (0.01M Tris-HCl pH 7.6, 20% Sucrose) and 50 µl aliquots were added to each well in the gel. Ribosomal RNA and transfer RNA markers or a combined marker solution were added to at least one well of each gel. Prior to starting the electrophoresis, frozen tank buffer in form of ice cubes was added to upper and lower tanks followed by the buffer solution. The frozen buffer helped maintain low temperatures throughout the electrophoretic run allowing the heat of electrophoresis to dissipate from the gel. Upon termination of electrophoresis tank buffer temperatures ranged from 3-12^{o}C.

Running times and voltage varied depending on whether one or two cells were being used. Table II shows the time regimes used.

Immediately after electrophoresis, the gel(s) was removed from the cell and fixed in 1M acetic acid for 15 minutes. If it was to be stained, the gel was placed in toluidine blue stain for one hour then washed and stored in tap water. Several changes with tap water were required over a 2 day period to remove the stain from the gel.

If the radioactive RNA was to be counted, the gel slab was sliced on a special microtome into 1 mm sections, placed in 0.5 ml NCS and stored overnight at 37^{o}C. The NCS solubilized RNA from each gel section. Ten ml of scintillation cocktail was added to each gel section and counted in scintillation counter.

TABLE II

Electrophoresis Time Regime

Cell No.	Time	Pulse/Min	Capac.	Voltage	mAmp
1	5 min	50	1.0 mfd	140	13
	60 min	75	1.0 mfd	140	20
	120 min	150	1.0 mfd	140	37.5
2	5 min	50	1.0 mfd	170	16
	60 min	75	1.0 mfd	170	25
	150 min	150	1.0 mfd	170	47

Table II depicts the settings for each variable in the ORTEC 4100 Constant Power Supply. All settings were kept constant except mAmp. A record of changing mAmp values was made and never varied more than ± 1 from the average figures.

RESULTS

Facilitated Diffusion of ^{3}H-Uridine

Uridine is believed to enter cells by facilitated diffusion (Plageman and Richey, 1974). Although the exact nature of the carrier molecule has yet to be determined, some investigators believe its structure and binding affinity with a substrate, such as uridine, can be altered by hormones, amino acids and various chemicals (Eilam and Cabantchick, 1977).

The possibility arose that the radioactivity measured in the series of phosphate-buffered saline (PBS) washes employed to remove excess exogenous labelled uridine from the extracellular environment might also reflect the effect of hormonal treatment on the outward facilitated diffusion of nucleoside already taken up by the cells. Experiments were performed to determine first, if there was a major outward diffusion of ^{3}H-uridine from the cells and second, did hormone treatment alter this diffusion rate? One hundred microliter aliquots were taken from each of the four PBS washes, and labelled uridine levels monitored with the scintillation counter (see Methods).

Results of these studies (Table III) showed ^{3}H-uridine from the TCA-soluble fractions was in much higher concentration than the labelled uridine from the final PBS wash. This was the case for both control and hormone-treated cells up to 60 minutes. Due to the large differences between the TCA-soluble counts and the corresponding final PBS wash, it appeared the former was not greatly decreased by the loss of intracellular

TABLE III

Comparison of Final PBS Wash With
TCA-Soluble Aliquots

Time	Treatment	N	% Difference
15 min	+	12	665±89
	-	11	627±76
30 min	+	7	782±65
	-	6	785±52
60 min	+	2	812±121
	-	2	972±37

Cells were pulsed with ^{3}H-uridine for 15 or 30 minute time periods. One hundred µl aliquots of the final PBS cellular wash were compared to equal aliquots of the corresponding TCA-soluble fraction from each flask. The percent difference reflects the ^{3}H-uridine radioactivity in the TCA-soluble fraction as compared to the final PBS wash. Both aliquots were measured in CPM. N equals the number of flasks measured. Figures are mean ± SEM except for 60 minutes which is mean ± range. (+) signifies 0.05 µg/ml cortisol-acetate added to medium. (-) signifies only hormone vehicle was added to medium.

^{3}H-uridine through facilitated diffusion during PBS washing. The "outward" diffusion is probably prevented by the rapid conversion of the nucleoside into the nucleotide UTP. These data also indicated cortisol had no noticeable effect on the outward diffusion of labelled uridine. This is shown in Table III by the similarity between hormone-treated and control flasks in each time period.

Table IV shows the ^{3}H-uridine specific activity measured in the PBS washes. In this Table, wash one represents the PBS solution used to remove the cells from the flask (see Methods). A sharp drop was demonstrated between washes one and two with a smaller decrease between two and three caused by the continued removal of the exogenous ^{3}H-uridine. The radioactive nucleoside in the succeeding washes leveled off but stayed above background levels at 100-300 CPM which might reflect a slight, but continuous outward facilitated diffusion of labelled uridine from the cell. This pattern was consistent for each of the six experiments following this protocol.

The combined results from Tables III and IV would indicate that much of the ^{3}H-uridine taken up by the cells was retained after the first PBS wash. As seen in Table IV, the addition of cortisol for either 15 or 30 minutes to the flasks did not appreciably alter the ^{3}H-uridine profile in relation to control flasks. Thus, comparisons of nucleoside uptake and incorporation, as measured by TCA fractionation, did not seem to be affected by differences in outward facilitated diffusion of ^{3}H-uridine caused by the hormone.

TABLE IV

Radioactivity in PBS Washes

Wash	Treatment	Time	N	CPM/µg DNA/ 0.5 mg
1	+	15	13	93.1±14.3
	+	30	5	97.4±12.7
	-	15	7	106.1±36.7
	-	30	4	93.9±11.5
2	+	15	13	16.0±2.6
	+	30	5	15.8±3.2
	-	15	8	23.5±7.4
	-	30	4	20.1±5.6
3	+	15	13	8.6±2.4
	+	30	5	12.2±4.0
	-	15	8	11.0±2.9
	-	30	4	17.4±7.0
4	+	15	13	8.0±2.2
	+	30	5	14.2±5.6
	-	15	8	8.3±2.3
	-	30	4	16.4±6.7
5	+	15	13	7.5±1.9
	+	30	5	12.9±4.1
	-	15	8	8.0±2.6
	-	30	4	13.3±4.8

One hundred µl aliquots were removed from each PBS wash, digested and counted as described in Methods. Counts are expressed as CPM/µg DNA/0.5 mg in each flask to adjust for the varying number of cells in each flask. Figures are mean ± SEM. N equals the number of flasks measured.
(+) signifies 0.05 µg/ml cortisol-acetate added to medium.
(-) signifies only hormone vehicle was added to medium.

FBS Concentrations

During early stages of this investigation, the question arose as to the optimal fetal bovine serum (FBS) concentration needed to both grow the cells and perform experiments. FBS is vital to maintenance of the cell line, but the serum is also known to contain cortico-binding globin (CBG), a protein known to bind corticosteroids of which cortisol is the most abundant (Catt, 1970; Amaral et al., 1974). Therefore, the problem was to determine an FBS concentration low enough to prevent excessive steroid binding, but high enough to allow long-term maintenance of the cell line. Trichloroacetic acid (TCA) fractionation experiments were carried out with various cortisol doses at both 5 and 10% FBS concentrations for a treatment period of 2 hours. Table V shows results obtained for each dose in TCA-soluble and TCA-insoluble fractions at both 5 and 10% FBS concentrations. There seemed to be no difference in TCA-insoluble radioactivity caused by changes in FBS concentration. However, uridine uptake, as measured by TCA-soluble fractions, although not significant, ranged up to 20% higher in 5% FBS medium. A review of the literature has failed to show studies comparing the effects of different serum concentrations on uridine metabolism.

Throughout the remainder of this study, the cells were grown in 10% FBS medium as described under Methods and Materials. Over a three year period, it was found that cell cultures were consistently sustained at this serum concentration, possibly because of better adherence to the plastic flask surface (Temin et al., 1972). Using a radioimmunoassay technique, an independent laboratory analyzed our 10% FBS medium and found no trace of cortisol to a 0.1 µg/ml sensitivity (R. Patty, Personal Communication).

TABLE V

Combined Effect of FBS Concentration and Cortisol Dose Response on ^{3}H-Uridine Uptake and Incorporation

Hormone Dose (µg/ml)	FBS Concentration	N	CPM of ^{3}H-uridine/ µg DNA/0.5 mg as % of Control	CPM of ^{3}H-uridine/ 100 µg DNA as % of Control
			TCA-SOLUBLE	TCA-INSOLUBLE
0.005	5%	5	87.2±12.2	77.9±11.1
	10%	4	86.8±21.9	79.4±16.5
0.01	5%	6	103.2±10.1	90.1±13.1
	10%	4	84.5±12.1	89.6±11.3
0.05	5%	5	85.5± 5.0	69.9± 3.8
	10%	4	72.6± 3.9	70.2± 4.8

Cells were pulsed with 1.25 µCi ^{3}H-uridine/flask for 30 min. ^{3}H-uridine uptake (TCA-soluble) and incorporation (TCA-insoluble) were measured for both 5 and 10% FBS concentration as described in Methods. Hormone dose was expressed in µg/ml. N is the number of duplicate experiments. Results are expressed as percent control (mean ± SEM).

Dose Response

Since only limited information was available on the effect of cortisol on cell line I-407 (Melnykovich and Bishop, 1967), it was necessary to perform a series of dose response experiments to determine the concentration of cortisol which would have a consistent effect on uridine uptake and incorporation. Table V shows the results of these experiments. There was no significant difference between the low 0.005 µg/ml (1.7×10^{-8}M) and 0.01 µg/ml (3.4×10^{-8}M) cortisol doses in either TCA-soluble or TCA-insoluble fractions. The higher 0.05 µg/ml (1.7×10^{-7}M) hormone dose inhibited both uridine uptake and incorporation by approximately 30%. As measured by Student t-test, the difference between 0.05 µg/ml and lower doses appeared to be due to the difference in dose. This dose has been reported as the minimum level found in the human peripheral blood (Mountcastle, 1974; Schulster et al., 1976) and thus seemed to be an appropriate concentration to use for in vitro experiments. This in vitro hormone dose is lower by a factor of ten than those reported by some other recent investigators (Munck et al., 1972; Borthwick and Bell, 1975). This finding in itself is interesting, but more importantly assures that cortisol's effects on the cell are caused by metabolic alterations rather than a possible steroid detergent effect found at higher doses (Munck, 1965).

Time Course

Experiments were performed to determine the effects of cortisol over a 4 hour period. Hormone was added to each of the flasks and incubated with the cells for the appropriate time period. Either fifteen minutes or thirty minutes before the end of incubation, ^{3}H-uridine was

added to the medium. At the end of the treatment period, the hormone and labelled nucleoside were removed by repeated washing, cells homogenized, and separated into TCA acid soluble and insoluble fractions as described in the Methods.

It should be mentioned that Munns and Katzman (1971) have reported that differences in ^{3}H-uridine incubation times can result in changes in TCA-soluble and TCA-insoluble radioactivity. Shorter pulse times were shown to produce greater uptake and incorporation of labelled precursor. However, in this study, there was no difference in uridine uptake and incorporation observed between 15 and 30 minute ^{3}H-uridine pulse times.

Cortisol's initial effect appears to be a small, but definite increase in both uridine uptake and incorporation at 15 minutes followed by a decrease to below control values at 30 minutes (Figures 2 and 3). Uridine incorporation returned to control values at 60 minutes while uptake returned to slightly above control. Thereafter, both the nucleoside uptake and incorporation remained below control values for the next 3 hours. A similar time course pattern of uridine incorporation has been demonstrated by Borthwick and Bell (1975), assaying for RNA polymerase B activity in cortisol treated rat thymus nuclei.

Protein Inhibition

In order to further investigate the nature of the cortisol effect on ^{3}H-uridine metabolism in cell line I-407, additional experiments were carried out with cortisol and the protein synthesis inhibitor cycloheximide. The object of this series of experiments was to study the effects of inhibited protein synthesis on both normal and hormone-mediated uptake and incorporation of ^{3}H-uridine into this cell system.

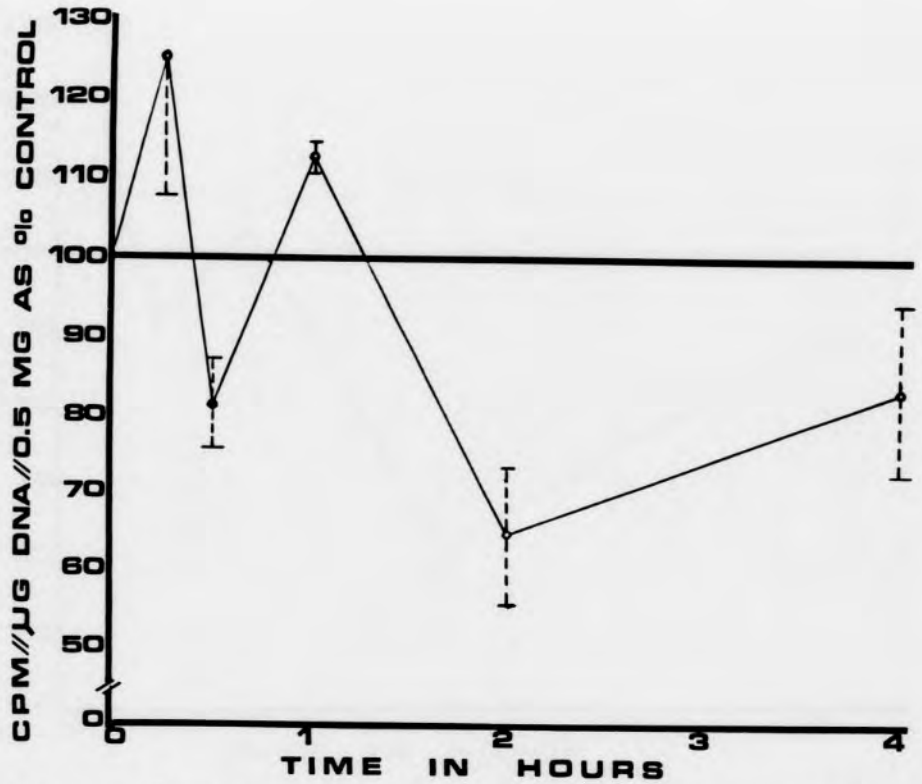

FIGURE 2. EFFECT OF CORTISOL ON ^{3}H-URIDINE UPTAKE AS A FUNCTION OF TIME. Cortisol (0.05 µg/ml) was incubated with cells for various periods of time. Cells were pulsed with 1.25 µCi ^{3}H-uridine/flask for 15 or 30 minutes. ^{3}H-uridine uptake was measured as described in Methods. Results are expressed as percent control (mean ± SEM). Data were calculated as CPM/µg DNA/0.5 mg cell pellet. Typical CPM for ^{3}H-uridine controls ranged from 810 to 1080 CPM. Differences between each time studied were significant to $p<0.05$.

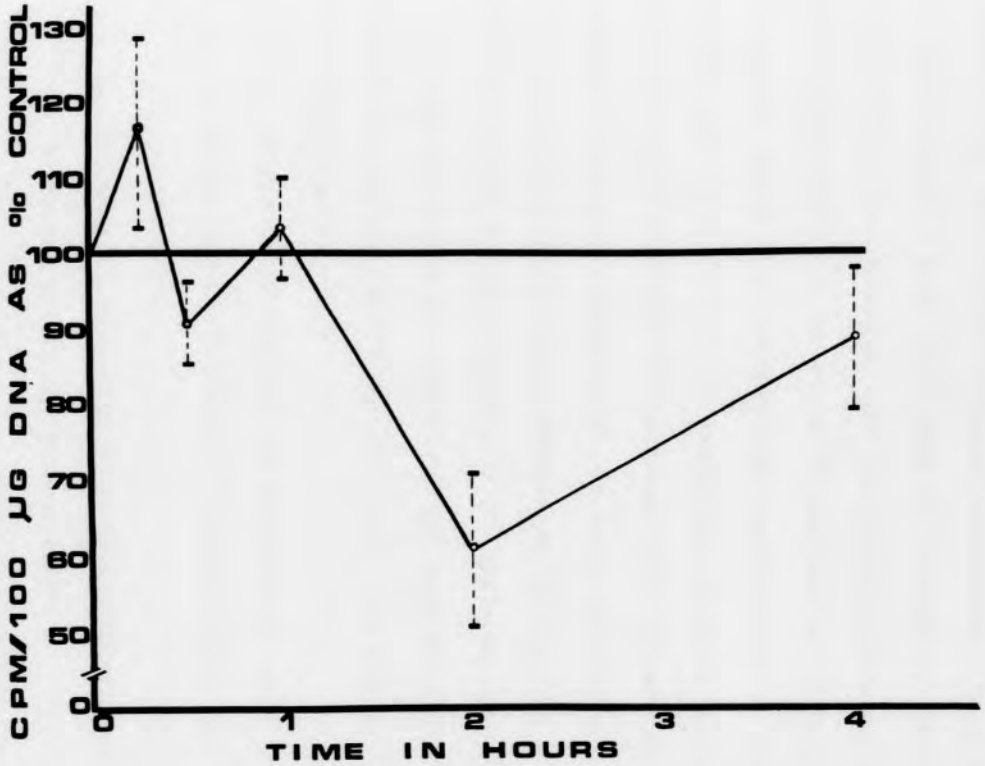

FIGURE 3. EFFECT OF CORTISOL ON ^{3}H-URIDINE INCORPORATION AS A FUNCTION OF TIME. Cortisol (0.05 µg/ml) was incubated with cells for various periods of time. Cells were pulsed with 1.25 µCi ^{3}H-uridine/flask for 15 or 30 minutes. ^{3}H-uridine incorporation was measured as described in Methods. Results are expressed as percent control (mean ± SEM). Data were calculated as CPM/100 µg DNA. Typical CPM/100 µg DNA for ^{3}H-uridine controls ranged from 24,900-34,000 CPM. Differences at each time were significant to $p<0.05$.

Prior to combined inhibitor and hormone experiments, dose response studies were performed to determine the cycloheximide concentration necessary to inhibit cellular protein synthesis. Table VI shows the results of the two separate duplicate experiments. Cells were incubated for 30 minutes with both ^{14}C-leucine and ^{3}H-uridine. Leucine and uridine uptake (TCA-soluble fraction) and incorporation (TCA-insoluble fraction) were measured using the procedures as outlined in the Methods section. The results showed that cycloheximide concentrations in the range 100-200 µg/ml have greater than 90% inhibitory effect on protein synthesis as measured by ^{14}C-leucine incorporation into the macromolecular acid insoluble fraction. Consequently, 100 µg/ml was used as the concentration for cycloheximide in subsequent experiments in order to avoid possible side effects higher concentrations of the inhibitor might produce.

Upon initiation of hormone and cycloheximide studies, the double-labelled precursor procedure was continued. The reasons for utilizing this technique were to:

1. determine if cortisol had an effect on early protein metabolism by measuring ^{14}C-leucine uptake and incorporation;
2. discover if inhibition of protein synthesis affected ^{3}H-uridine uptake and incorporation;
3. utilize ^{14}C-leucine as a check to ensure the cycloheximide was actually inhibiting protein synthesis.

Cells were treated simultaneously with the hormone and/or cycloheximide for 15 and 30 minute time periods. Each experiment consisted of four variables: cortisol, cortisol plus cycloheximide, cycloheximide and control.

TABLE VI

Dose of Cycloheximide (µg/ml)	CPM/µg DNA/0.5 mg as % of Control		CPM/100 µg DNA as % of Control	
	TCA-SOLUBLE		TCA-INSOLUBLE	
	^{14}C-leucine	^{3}H-uridine	^{14}C-leucine	^{3}H-uridine
100	90.6	82.5	8.9	80.9
150	105.7	82.2	7.3	71.2
200	99.6	86.2	7.8	77.0

^{14}C-leucine and ^{3}H-uridine uptake and incorporation into cells was measured as a function of cycloheximide dosage. Cells were exposed to cycloheximide, 1.25 µCi/flask of ^{3}H-uridine and 0.95 µCi/flask ^{14}C-leucine for 30 minutes. Data are in percent control as the average of two experiments. Typical ^{14}C-leucine datum was 10 CPM/µg DNA/0.5 mg and 20,000 CPM/100 µg DNA for TCA-soluble and TCA-insoluble controls, respectively. Typical ^{3}H-uridine datum was 62.5 CPM/µg DNA/0.5 mg for TCA-soluble and 46,670 CPM/100 µg DNA for TCA-insoluble controls, respectively.

The data would indicate that cortisol has more than one immediate effect. As mentioned above, there appears to be increased uridine uptake (20%) and incorporation (15%) at 15 minutes (Table VIII). Although ^{14}C-leucine incorporation remains at or near control levels, cortisol rapidly inhibited amino acid transport into the cell by 30% at 15 minutes (Table VII). This was followed by a 20% decrease in leucine incorporation at 30 minutes. Cortisol has been found to inhibit amino acid uptake into cells of skeletal muscle, spleen and thymus tissue. Additional evidence has been reported to show cortisol inhibits amino acid uptake in HeLa, JTC-4 and L-929 cell lines (reviewed by Riggs, 1970), but this appears to be the first report showing leucine uptake inhibition to occur as rapidly as 15 minutes. Cycloheximide appears to mimic cortisol by inhibiting amino acid uptake to a similar extent both at 15 and 30 minutes after treatment (Table VII). The inhibitor also stopped ^{14}C-leucine incorporation by approximately 95% with or without the presence of cortisol.

Cycloheximide also blocked cortisol's early effect on uridine metabolism by inhibiting uridine uptake at 15 minutes while simultaneously decreasing nucleoside incorporation to below control levels (Table VIII). At 30 minutes, the inhibition of uridine uptake by cycloheximide continued. It appeared uridine incorporation remained below control levels after 30 minute cortisol treatment, although wide variations made analysis difficult. These inhibitor effects appeared to be similar in the presence or in the absence of hormone at 30 minutes.

Throughout the above series of inhibitor and hormone experiments, 100 µl aliquots were removed from each PBS wash. Aliquots were counted

TABLE VII

Effects of Cortisol and Cycloheximide
on ^{14}C-Leucine Uptake and Incorporation

Time	N	Treatment	CPM of ^{14}C-leucine/ µg DNA/0.5 mg as % of Control	CPM of ^{14}C-leucine/ 100 µg DNA as % of Control
			TCA-Soluble	TCA-Insoluble
15 min.	4	Hormone	69.3± 3.5	107.8±6.8*
	4	Hormone & Cycloheximide	73.9± 5.6	5.8±0.8
	4	Cycloheximide	69.0± 1.8	5.6±0.6
30 min.	3	Hormone	58.7± 8.9	81.5±5.1*
	3	Hormone & Cycloheximide	70.2± 6.6	2.4±0.4
	3	Cycloheximide	52.5±11.8	1.9±0.4

Either cortisol (0.05 µg/ml), cycloheximide (100 µg/ml) or a combination of both was added and incubated for indicated times. Cells were pulsed with 0.95 µCi ^{14}C-leucine/flask for 15 minutes. Results are expressed as percent control (mean ± SEM). N is number of duplicate samples. In a typical experiment, control TCA-soluble and TCA-insoluble data were 220 CPM/µg DNA/0.5 mg and 318 CPM/100 µg DNA, respectively.

*Difference between these two values was significant $p<0.05$.

TABLE VIII

Effects of Cortisol and Cycloheximide on ^{3}H-Uridine Uptake and Incorporation

Time	N	Treatment	CPM of ^{3}H-uridine/ µg DNA/0.5 mg as % of Control	CPM of ^{3}H-uridine/ 100 µg DNA as % of Control
			TCA-Soluble	TCA-Insoluble
15 min.	7	Hormone	121.1±13.3*+	115.8± 8.0**
	4	Hormone & Cycloheximide	78.7± 8.0*	90.7± 7.4
	4	Cycloheximide	84.7±11.6	101.0± 5.1
30 min.	9	Hormone	80.7± 4.0+	89.0± 4.3**
	3	Hormone & Cycloheximide	77.8±13.9	94.3±17.3
	3	Cycloheximide	61.2±14.5	70.3±20.4

Either cortisol (0.05 µg/ml), cycloheximide (100 µg/ml) or a combination of both was added and incubated for indicated times. Cells were pulsed with 1.25 µCi ^{3}H-uridine for 15 minutes. Results are expressed as percent control (mean ± SEM). N is number of duplicate samples. In a typical experiment, control TCA-soluble and TCA-insoluble data were 75.5 CPM/µg DNA/0.5 mg and 58,630 CPM/100 µg DNA, respectively.

(*, +, **) Differences between these paired values were significant $p<0.05$.

in the same manner as mentioned in Methods with the time course experiments. The samples were counted to determine if the cycloheximide had an effect on facilitated uridine transport out of the cell. This was necessary to monitor any changes that occurred in uridine transport which might be brought about by a cycloheximide effect on the cell membrane.

The data (Table IX) indicate that the cycloheximide had no effect on uridine facilitated transport out of the cell. In a typical experiment, the 100 µl TCA-soluble aliquot from each flask had counts 3-5 times higher than the corresponding final wash. Both the TCA-soluble and wash aliquots were measured in CPM/µg DNA/0.5 mg. As shown in Table IX, there appears to be no significant differences in the wash data that result from differences in treatment.

A preliminary experiment was performed testing how selective inhibition of rRNA synthesis would affect cortisol action on I-407 cells. Low doses of approximately 0.042% actinomycin D have been found to preferentially inhibit rRNA transcription in HeLa (Derman and Darnell, 1974). A dose close to 0.042% (0.1 µg/ml) was incubated with the I-407 cells for 15 minutes. Incubations were performed with and without cortisol. The results are shown in Table X. Although decisive conclusions cannot be made on the basis of one experiment, it is interesting to note that the inhibitor failed to halt the hormone stimulated rise in ^{3}H-uridine uptake and incorporation. In the absence of cortisol, low doses of actinomycin D inhibited precursor uptake and incorporation 35 and 55% respectively. This would suggest the early 15 minute hormone stimulated increase in uridine incorporation does not involve synthesis of new rRNA.

TABLE IX

Effect of Cortisol and Cycloheximide
on Radioactivity in PBS Washes

	CPM/ g DNA/0.5 mg			
Wash	Hormone	Hormone & Cycloheximide	Cycloheximide	Control
PBS	423±102	375±108	407±111	325±95
1	54±15	50±15	52±17	52±16
2	20± 5	20± 6	20± 5	21± 6
3	14± 3	15± 4	13± 4	13± 4
4	11± 3	12± 4	11± 4	11± 3

Cells were exposed to ^{3}H-uridine and either hormone, cycloheximide, hormone and cycloheximide or control for 15 minutes. One hundred µl aliquots were solublized in 0.5 ml NCS, combined with liquid scintillation fluor and their radioactivity measured in a liquid scintillation counter. Results are expressed as CPM/µg DNA/0.5 mg (mean ± SEM) of 10 flasks.

TABLE X

Effect of Low Dose Actinomycin D and Cortisol on ^{3}H-Uridine Uptake and Incorporation

Treatment	CPM of ^{3}H-uridine/ µg DNA/0.5 mg as % of Control	CPM of ^{3}H-uridine/ 100 µg DNA as % of Control
	TCA-Soluble	TCA-Insoluble
Hormone	121.1	115.8
Hormone & Actinomycin D	125.8	109.5
Actinomycin D	66.7	45.5

Low doses of actinomycin D (0.1 µg/ml) were incubated simultaneously with hormone (0.05 µg/ml) or hormone vehicle for 15 minutes. Results are expressed as percent control. Control TCA-soluble and TCA-insoluble data averaged 12.75 CPM/µg DNA/0.5 mg and 8840 CPM/100 µg DNA, respectively.

Electrophoresis

A preliminary experiment was performed to separate labelled RNA from both hormone-treated and control cells on polyacrylamide-agarose gels (see Methods). RNA was extracted using a hot (55^{o}) phenol procedure that selectively retains high molecular weight nuclear A-U rich RNA (see Methods). Figure 4 shows cortisol's apparent shift to higher molecular weight RNA species after 15 minutes. This would indicate cortisol induced changes in hnRNA synthesis. This RNA species has been suggested as being the source of mRNA precursors (see Introduction).

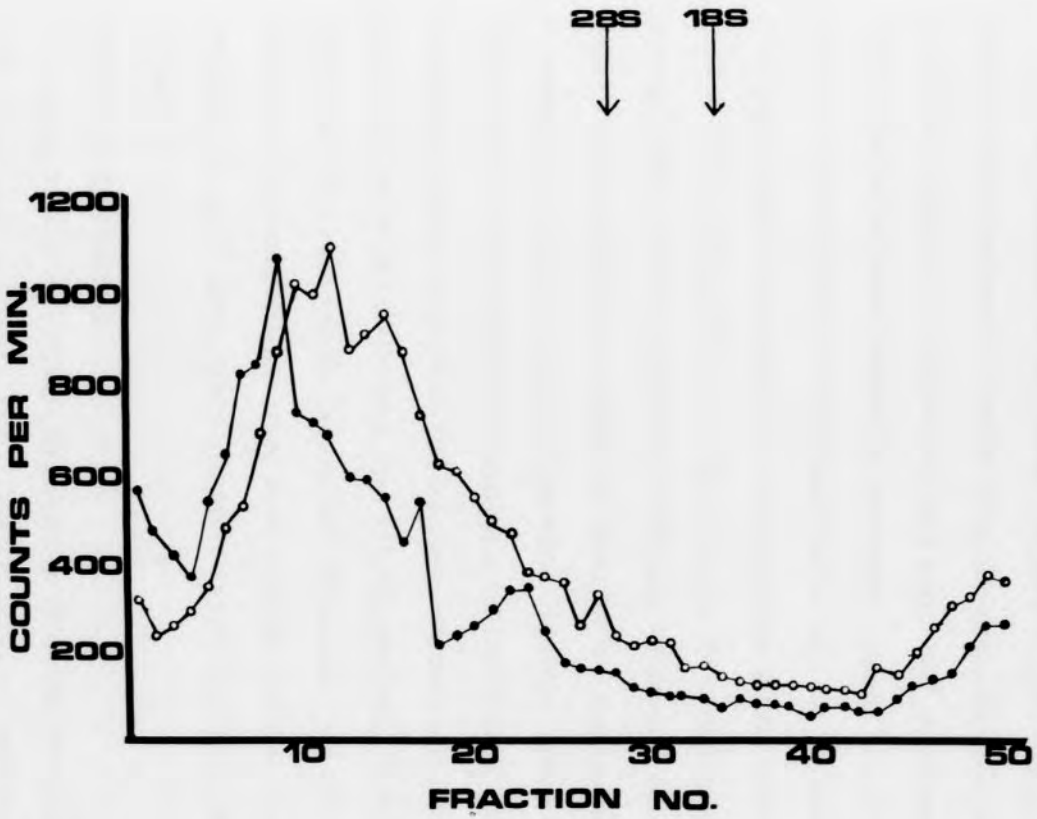

FIGURE 4. ELECTROPHORESIS OF Hn RNA. Cells were exposed to hormone or hormone vehicle and (5, 6-^{3}H)-uridine for 15 minutes. HnRNA was extracted using a hot (55°) phenol procedure as described in Methods. Equivalent optical density (O.D.) units were applied as 50 µl aliquots to polyacrylamide-agarose gels. RNA samples were separated electrophoretically for 3 hours. • - hormone-treated hnRNA; o - control hnRNA.

DISCUSSION

The discussion of this study requires a short review of the system and techniques employed. The use of a cultured cell line allowed the effects of cortisol to be measured in a controlled environment without interaction with other tissues or compounds. Intact cells made it possible to measure total cellular reaction to cortisol incubation. This included hormonal alterations in both precursor uptake and incorporation. The TCA separation technique, while allowing the measurement of nucleoside and amino acid transport (see Methods), could only measure non-specific macromolecular changes. No specific RNA species was measured as a separate entity using this technique. However, specific metabolic inhibitors and electrophoretic separation of RNA allowed the study of hormone effects on particular RNA species. Consequently, it was possible to analyze the results obtained from the TCA studies. These results were similar to data reported in the steroid literature. In the following discussion, references were often made to other steroids, especially estrogen, as their early actions seem to follow a pathway similar to cortisol.

Preliminary Experiments

Experiments were first performed to find the lowest dose at which I-407 cells would consistently respond to cortisol. Cortisol doses below 0.05 µg/ml (1.7×10^{-7}M) failed to elicit a statistically consistent negative response in terms of ^{3}H-uridine uptake and incorporation (Table V) as evidenced by the relatively large standard error of the

mean (SEM). The reasons for this were not readily discernable, but possibly the lower hormone concentrations were not sufficient to activate the number of receptors needed to stimulate a cortisol response in all the cells. The reasons for the lack of response must remain speculative since little work has been performed in the area of suboptimal steroid hormone response.

Studies were also conducted to determine a fetal bovine serum (FBS) concentration which would allow normal cell growth. There were no statistically significant differences in dose response between cells grown at 5 and 10% with the three hormone concentrations studied (Table V). The experiments yielded similar results in both the TCA-soluble and TCA-insoluble fractions. The higher serum concentration was selected since it enabled the cells to grow in a more rapid and consistent manner, possibly by increasing the cells' ability to attach to the surface of the culture flask (Temin et al., 1972). All cells were grown in 10% FBS for later experiments.

Time Course of Cortisol Action

Figures 2 and 3 show the effects of cortisol over time in both TCA-soluble and TCA-insoluble fractions of I-407 cells. The former measured ^{3}H-uridine uptake, the latter ^{3}H-uridine incorporation (see Methods). At 15 minutes, both uridine uptake and incorporation increased 20 and 15% respectively. This was followed by a rapid decrease of 40% (uptake) and 25% (incorporation) at 30 minutes. A similar decrease for both parameters was also found at 2 and 4 hours after cortisol treatment.

An interesting hormone effect observed was the increase in uridine uptake (30%) and incorporation (15%) between 30 and 60 minutes. This may

reflect either the synthesis of mRNA that produced the later inhibition found at 2 and 4 hours or an alteration in uridine transport. The available data fail to clarify the cause of this second increase to control levels. A review of the literature failed to show a similar temporary increase during a general inhibitory effect in any other system.

This inhibitory pattern of cortisol action was similar to that reported by Borthwick and Bell (1975). Using cortisol-treated isolated thymus nuclei, the authors found cortisol elicited a small, early (15 minute) increase in RNA polymerase B activity followed by a drop to control lvels. Treatments longer than 60 minutes caused decreases in both RNA polymerase A and B activity to below control levels. Thus, the pattern of cortisol stimulated RNA polymerase activity in isolated thymus nuclei appears to be similar but not identical to the pattern of RNA synthesis found in intact I-407 cells.

Early temporary increases in RNA polymerase B activity have been found also in uterine and liver nuclei after estrogen and cortisol treatment, respectively (Glasser et al., 1972; Schmid and Sekeris, 1972; Borthwick and Smellie, 1975). In both systems, the temporary increase was followed by a sustained increase in both RNA polymerase A and B activity. The above investigators are in agreement that one of the early steroid actions in target cells is the stimulation of a temporary rise in RNA polymerase B activity which leads to sustained changes in both RNA polymerase A and B activity. Although the above polymerase assays were performed in cell-free systems, the general early pattern is similar to data obtained in this study using intact tissue.

An alternate mode of cortisol action in I-407 cells is suggested by the similarity between Figure 2 and 3. It is possible that early cortisol action alters the rate of nucleoside transport through the membrane, thus controlling the amount of labelled nucleoside available for incorporation. This argument has been advanced for estrogen by Billings et al. (1969a, b) and for cortisol by Waddell et al. (1976).

Waddell and his colleagues have presented evidence that large doses (500 μg/ml) of the cortisol analog 6α-methylprednisolone caused an immediate (3 minute) 90% inhibition of ^{3}H-uridine uptake into human leukemic lymphoblastoid cells. This is in contrast to the 20% increase above control levels reported here (Figure 2). However, the dosage used by Waddell's group is 10^{4} times larger than used in this investigation and at this high concentration the steroid hormone could have a detergent effect on membrane lipids (Munck, 1965).

While studying the <u>in vitro</u> effect of estrogen on uterine RNA metabolism, Billings and his colleagues (1969a, b) found the nucleotide pool specific activity seemed to increase 1-2 hours before any increases in RNA specific activity. This increase was evident after actinomycin D inhibited uridine incorporation. The authors surmised that the increase in RNA radioactivity was caused by estrogen-stimulated nucleoside uptake into the uterus. However, Oliver (1971) found no difference in uptake and incorporation times after separating nucleoside transport from its incorporation. She has attributed the increases in nucleoside activity to increased utilization of nucleoside pools by RNA synthesizing processes. A similar conclusion was also reached by Hagenbuckle et al. (1976) using thyroxine, a hormone thought to be similar in action to steroids (Gorski and Ganon, 1976).

Most available evidence would indicate the early action of steroids is to alter RNA synthesis. The use of the acid separation technique to study hormone-mediated nucleoside transport (Oliver, 1971; Hagenbuckle et al., 1976) support this concept. The same technique, used in this study (see Methods), yields a pattern of uridine uptake and incorporation similar to that seen for cortisol-activated thymus RNA polymerase B activity (Borthwick and Bell, 1975). The significance of this work was the demonstration, for the first time, of the early (15 minute) and continuous parallel hormonal response of uridine uptake and incorporation in an in vitro cell system.

Inhibitor Studies

Treatment of I-407 tissue with cycloheximide and ^{14}C-leucine produced interesting results. Table VII showed the effect of cortisol on ^{14}C-leucine metabolism. One definite result was a 30% decrease in amino acid uptake observed when cells were treated for 15 minutes with either hormone, cycloheximide or a combination of the two. As previously mentioned in the Results, inhibition of amino acid uptake has been reported to occur in many tissues treated with cortisol including HeLa cells (Mohri, 1967). However, a search of the literature has failed to show other reports to this effect as early as 15 minutes after exposure to hormone. The lack of significant differences at both 15 and 30 minutes among the three experimental variables would suggest the possibility that cortisol and cycloheximide work independently to inhibit amino acid uptake. Cycloheximide inhibition of protein synthesis may block the formation of a labile leucine amino acid transport protein, while cortisol may induce the synthesis of a protein that interferes with amino acid

transport. After 30 minutes incubation, the hormone-cycloheximide treatment appeared to result in higher amino acid uptake than the separate hormone or inhibitor treatments. However, this increase was not found to be statistically significant.

Although the cortisol-induced decrease in amino acid uptake did not appear to affect protein synthesis after 15 minutes, there was a significant 25% decrease in leucine incorporation by 30 minutes. This decrease may have been caused by hormonal inhibition of amino acid uptake first seen at the 15 minute time period, although the inhibition of leucine uptake and subsequent incorporation have been reported to be independent events (Munro, 1970).

Cycloheximide inhibition of protein synthesis appears to block the cortisol-induced increase of ^{3}H-uridine uptake at 15 minutes (Table VIII). The protein inhibitor reduced nucleoside uptake to 78% of control which continued through the 30 minute hormone treatment. It would appear from the above ^{14}C-leucine incorporation data (Table VII) that cycloheximide acts to block translation of a protein or group of proteins necessary for uridine uptake. The fact that this decrease occurs so quickly after exposure to inhibitor would indicate this transport protein is labile and its continuous translation is necessary to maintain uridine uptake at control levels.

Cycloheximide alone also acted to eliminate the cortisol-induced increase in uridine incorporation at 15 minutes. One possible explanation for this is the decrease in uridine uptake discussed above may lower the supply of substrate necessary for RNA synthesis. An alternate possibility is that cycloheximide may prevent cortisol-induced translation

of proteins that increase RNA synthesis. A similar response to cycloheximide is found in cortisol-treated thymus cells. Cycloheximide has been found to block the hormone-induced inhibition of glucose uptake in thymus cells which would indicate new protein synthesis was necessary for thymus response to cortisol (Mosher et al., 1971; Munck et al., 1972). Although there is direct evidence for an early steroid-induced protein in the uterus (Gorski and Barnea, 1970; Gorski and DeAngelo, 1970; Cohen and Hamilton, 1975; Katzenellenbogen and Gorski, 1975), this is the first report that indicates a relation between early protein synthesis and uridine incorporation in a catabolic system.

A preliminary experiment was performed to investigate how selective inhibition of rRNA synthesis affected cortisol action in I-407 cells. Low doses (0.1 μg/ml) of actinomycin D used in this experiment have been reported to block rRNA, but not hnRNA synthesis (Derman and Darnell, 1974). Table X shows that cortisol's effect on labelled uridine uptake and incorporation was not impaired by the inhibitor. Cells treated only with actinomycin D showed a 35% decrease in nucleoside uptake and 55% inhibition in uridine incorporation. Although valid conclusions cannot be based on a preliminary experiment, the results would suggest cortisol stimulated uridine incorporation despite a general inhibition of than 50%. This would indicate that at 15 minutes, the increased uridine incorporation in cortisol-treated cells was not entering the rRNA fraction.

Electrophoresis

A preliminary experiment was performed to separate labelled RNA from both hormone-treated and control cells on polyacrylamide-agarose gels (see Methods). RNA was extracted using a hot (55^{o}C) phenol procedure

that selectively retains A-U rich nuclear RNA (see Methods). High molecular weight nuclear RNA (hnRNA) has been shown to be rich in adenine and uridine. A 15 minute cortisol treatment produced an apparent shift to higher molecular weight RNA (Figure 4). This, in conjunction with the actinomycin D data (Table X), would suggest cortisol induced changes in hnRNA synthesis. This RNA species has been reported as being the source of mRNA precursors (see Introduction).

General Conclusions

From the data obtained it was concluded that:

1. The longer term (2-4 hours) effect of cortisol on I-407 cells was inhibitory in regards to uridine uptake and incorporation, but shorter term studies (15-60 minutes) produced variations in response to the hormone.
2. The general pattern of ^{3}H-uridine uptake and incorporation showed a rise above control levels after 15 minutes followed by a decrease at 30 minutes. Since both fractions followed a similar pattern, it is difficult to conclude from this evidence alone whether short-term changes were due to cortisol alterations in uridine uptake or incorporation processes.
3. Cortisol inhibited ^{14}C-leucine uptake by 30% at 15 minutes and 40% at 30 minutes. Leucine incorporation remained near control values at 15 minutes but showed an 18% decrease at 30 minutes. This suggests cortisol inhibited protein synthesis possibly by blocking amino acid uptake.

4. Inhibition of translation by cycloheximide mimiced the effect of cortisol on amino acid uptake causing a 30% decrease in leucine transport into the cells. This suggests protein synthesis is necessary to maintain amino acid transport in I-407 cells.

5. In a preliminary experiment, hormone-treated and control cells were exposed to low doses of actinomycin D which selectively inhibit rRNA synthesis. At 15 minutes, hormone treated cultures showed a 25% increase in uridine uptake while incorporation was slightly above control levels. Untreated cells showed substantial decreases in both parameters indicating cortisol affects synthesis of one or more species of non-rRNA. Possibly affected is hnRNA, thought by some investigators to contain precursors of mRNA (see Introduction).

6. hnRNA was preferentially extracted from hormone-treated and control cells and separated by polyacrylamide-agarose electrophoresis. This preliminary experiment showed a shift in synthesis to higher molecular weight RNA in cells treated with cortisol for 15 minutes. This evidence in conjunction with the actinomycin D data, would indicate a possible early cortisol effect on hnRNA of I-407 cells.

Future Experiments

The following is a list of some of the most pertinent problems left unanswered by the present study:

1. One problem is determining whether cortisol has a direct effect on uridine transport. Experiments similar to those of Oliver (1971) might be performed using a non-metabolized but transported nucleoside analog such as cytosine arabinoside. Also specific inhibitors of nucleoside transport such as thioguanosine, thioinosine or 4-Nitrobenzyl-6-mercaptoinosine (NBMI) could be utilized to block uridine uptake (Eilam and Cabantchick, 1977). This would allow transport to be studied as a separate entity.
2. Labelled uridine might be administered to the cells three to four hours before cortisol administration. This would establish a steady-state of uridine both inside and outside the cell and would eliminate possible changes in specific activity caused by a sudden influx of labelled nucleoside into the medium (Billings et al., 1969; Puckett and Darnell, 1975).
3. Polysome isolation procedures (Wettstein et al., 1963) (see Methods) may be used to isolate cytoplasmic messengers. It may be possible to detect hormone-stimulated changes in polysome preparations by separation of RNA using oligo d(T)-cellulose chromatography (Aviv and Leder, 1972; Bantle et al., (1976) and polyacrylamide-agarose electrophoresis (see Methods).

4. Inhibitors cycloheximide (protein), α-amantim (RNA polymerase B) and low doses of actinomycin D (RNA polymerase A) might be used with and without the hormone to determine the importance of each inhibited substances to steroid action. Inhibitors might be given before or after cortisol as well as simultaneously in these experiments.
5. Electrophoretic separation of cellular soluble protein could be done to determine if the hormone does stimulate or inhibit the early synthesis of an "induced protein".

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