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STUDIES ON AMINO ACID INCORPORATION IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

SEASONAL CHANGES IN PROTEIN SYNTHESIS

HECTOR J. RODRIGUEZ * and JESSE T. YATES

Renal Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110 (U.S.A.)

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Summary

Amino acid incorporation in isolated toad bladder epithelial cells was investigated by examining the rate of radiolabel incorporation into trichloroacetic acid-insoluble material. The results demonstrated that during the summer months, a large fraction of the radiolabel incorporated was not inhibited by cycloheximide and did not seem to correspond to incorporation of the radioactive amino acid into elongating peptides. A similar effect was not observed in other organs of the toad (e.g. liver, spleen). During the winter months, however, virtually all the acid-insoluble radiolabel appears to correspond to incorporation of the amino acid into elongating peptides. Ouabain, an inhibitor of sodium transport, inhibited amino acid incorporation into acid-insoluble material by affecting the acid-soluble pool and this effect appeared to be independent of the effect of ouabain on sodium transport. The isolated epithelial cells appeared to derive the energy for protein synthesis almost entirely from glycolysis and in this regard they resemble certain tissue culture cells.

Introduction

The urinary bladder of the toad (*Bufo marinus*) has been used as a model system to investigate the electrophysiological properties of transepithelial

* To whom correspondence and reprint requests should be addressed.

transport of sodium chloride [1,2] and its hormonal regulation [3—7]. The regulation of transepithelial sodium transport by some steroid (aldosterone) and non-steroid (insulin) hormones appears to involve the activation of transcriptional and/or translational mechanisms [3—6]. Studies on the nature of the stimulatory effects of both aldosterone and insulin on transepithelial sodium transport in the toad bladder, and on the modulation of the hormonal response by inhibitors of transcription (actinomycin D, cordycepin, α-amanitin) and translation (cycloheximide, puromycin) clearly suggest that these hormones may regulate Na transport by stimulating the production of specific species of messenger RNA and/or proteins [5,8,9]. Consistent with this view is the observation that, in the toad bladder, aldosterone stimulates the incorporation of [^{3}H]uridine into a polyadenylated species of RNA with properties similar to those of messenger RNA [3] as well as the production of 'newly synthesized proteins' [4]. Likewise, induction of a specific species of 'messenger' RNA by insulin in the isolated toad bladder has been reported [10]. The nature of the macromolecular components (RNA, proteins), believed to modulate the hormonal effects on Na transport, has not been clearly defined in part because the biochemical properties of the isolated toad bladder are poorly understood. A large body of experimental evidence indicates that the transport properties of the isolated toad bladder are confined to the epithelial cell layer [1,11,12]; hence, understanding of the kinetics of amino-acid incorporation and protein synthesis in the isolated epithelial cells may provide insight into the molecular events that underlie the regulatory effects of certain hormones on sodium transport in this model system. In addition, understanding of the biochemical properties of the epithelial cells may throw light into the nature of specific pathways responsible for the translocation of ions and water [13], as well as the coupling between certain metabolic pathways and cellular processes that govern the overall transport rates [14,15]. We have recently developed an enzymatic method to isolate epithelial cells from the toad bladder that eliminates the need for scraping of the tissue. This method yields isolated cells, and few 'clumps', whose properties are comparable or better than those of 'scrapings' obtained by conventional collagenase treatment and scraping of the mucosal surface of the bladder. We have employed this method to investigate the kinetics of amino acid incorporation into proteins in isolated toad bladder epithelial cells and we report here the results of these experiments.

Materials and Methods

Preparation of isolated cells. Experiments were performed on urinary bladders of female toads of Dominican origin. Housing and handling of the toads were as previously described [17]. Each toad was perfused, via the ventricle, with at least 200 ml of Ringer solution of the following composition: NaCl, 90 mM; $NaHCO_3$, 24 mM; KCl, 3 mM; $CaCl_2$, 1 mM; KH_2PO, 0.5 mM; $MgSO_4$, 0.5 mM; and gentamicin 10 μg/ml. The pH was adjusted to 7.8 ± 0.1 by bubbling with a mixture of 97% O_2/3% CO_2. The osmolality was 230 ± 10 mosM/kg H_2O. The protocol for isolation of epithelial cells was as follows: the bloodless bladders were minced into fine pieces and incubated at 25°C in

Ringer solution containing 0.1 mM calcium and 0.15% collagenase for 10 min. The bladder segments were then treated with 2 mM EDTA for 10 min and resuspended in Ringer solution containing 0.1 mM calcium, 0.15% collagenase, and 133 units/ml of DNAase. Incubation was continued for 60 min. After 30 min of incubation, 'shearing forces' were applied by drawing the suspension of bladder segments into a syringe. The supernatant fluid of this incubation contained isolated epithelial cells. The isolated cells were washed by centrifugation at $1000 \times g$ for 10 min; the washing procedure was repeated twice, and the cells were then resuspended in an appropriate volume of Ringer solution, filtered twice through gauze (Topper Sponges, Johnson and Johnson, New Brunswick, NJ) and processed for biochemical analysis. Virtually all the isolated cells were intact and viable as assessed by light microscopy and exclusion of supravital dyes (trypan blue, erythrosine B). In some experiments cell isolation was carried out in specially prepared calcium-free modified Eagle medium (MEM L-liquid Cat. No. 320-1095, Gibco, NY) or amphibian culture medium (Cat. No. 350—1835, Gibco, NY). The isolation protocol was identical to the one used for cells prepared in regular Ringer solution.

Amino acid incorporation into proteins. For these experiments, the isolated epithelial cells were pre-incubated for 30 min at 25° C in Ringer solution containing 10 mM glucose and bubbled with 97% O_2/3% CO_2. The cells were then pulse-labeled with radioactive amino acids for different times. The reaction was stopped by diluting the cell suspension with Ringer solution containing 1000-fold excess of non-radioactive amino acid and cooling the cells at 0—2° C. In a separate set of experiments, a chase with 1000-fold excess of non-radioactive amino acid was performed at 25° C for periods varying from 30 to 180 min before cooling the cells down to 0—2° C. The results obtained were similar with either type of approach. After cooling for 10 to 15 min, the cells were washed 4 to 5 times in Ringer solution containing 1 mM non-radioactive amino acid by centrifugation at $10\,000 \times g$ for 2 min in a Beckman microfuge. The radioactivity in the supernatant of the final cell wash was essentially at background levels. The washed cell pellet was then resuspended in 5.7% sucrose containing 1.5 mM EDTA, 1 mM $NaHCO_3$, and 5 mM Tris-HCl, pH 8.1 and sonicated with a Branson sonicator fitted with the micro tip. This medium has been shown to facilitate complete rupture of toad bladder epithelial cells by sonication [16, 17]. The proteins of a sample of sonicate were precipitated with cold 10% trichloroacetic acid containing 1 mM non-radioactive amino acid, the pellet was washed 3 to 4 times in the same solution, resuspended in 1 N NaOH, and the radioactivity associated with the acid insoluble material was determined by scintillation counting. Appropriate quenching corrections were applied. The remainder of the sonicate was processed for determination of protein by the method of Lowry et al. [18]. For the experiments in which the acid soluble pool was quantitated, a sample of sonicate was treated with cold 10% trichloroacetic acid containing 1 mM non-radioactive amino acid, centrifuged at $10\,000 \times g$ for 2 min, and the radioactivity in the supernatant was determined by scintillation counting. The pellet was resuspended in 1 N NaOH and processed as previously described.

Enzymatic proteolysis of radioactivity incorporated into isolated epithelial cells. A suspension of isolated cells was pulse-labeled with radioactive amino

acids for periods of 30 to 60 min. The reaction was stopped by cooling the tubes of 0—2°C and 'chasing' the radioactivity with 1 mM non-labeled amino acid for 30—45 min. The cells were washed 3—4 times in Ringer solution, resuspended in a small volume (approx. 0.5 ml) of 5.7% sucrose containing 1 mM $NaHCO_3$, 1.5 mM EDTA and 5 mM Tris-HCl, pH 8.1 and sonicated. Solutions of proteolytic enzymes (5 μg/ml) were prepared in the following buffers: 100 mM phosphate buffer, pH 7.5, for pronase and trypsin; 100 mM phosphate buffer, pH 7.5, with 5 mM cysteine and 1 mM EDTA for papain. For proteolytic digestion, a small aliquot of the cell sonicate (0.05—0.1 ml) was diluted in 1 ml of the corresponding solution of proteolytic enzyme. A small volume (0.0025—0.003 ml) of toluene was added to each tube to prevent bacterial growth [17,19] and the tubes were incubated at 37°C for 48—56 h. Control tubes containing samples of cell sonicate in buffer were incubated in parallel. At the end of the incubation period, a sample of the enzymatic digest was loaded in a column packed with Sephadex G-50 and equilibrated with a buffer of the following composition: 10 mM Tes (*N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid), pH 7.5, 10 mM EDTA, and 0.5% SDS. Addition of SDS was necessary to insure complete recovery of the radioactivity loaded on the column. Elution was carried out at room temperature and the radioactivity associated with the fractions collected from the column was quantitated by scintillation counting.

Materials

The toads used in these experiments (Dominican subspecies) were supplied by W.A. Lemberger Assoc. (Germantown, WI). Collagenase (Type I from *Chlostridium histolyticum*), deoxyribonuclease from bovine pancreas (2000 U/vial), cycloheximide, protease from stretomyces griseus (Pronase) Type VI, trypsin Type I, leupeptin hemisulfate, lima bean trypsin inhibitor Type II-L, and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., (St. Louis, MO). Actinomycin D was supplied by Merck, Sharp and Dohme (West Point, PA) and Sephadex G-50 by Pharmacia Fine Chemicals (Piscataway, NJ). Specially prepared Ca^{2+}-free MEM Earle's liquid medium (Cat. No. 320-1095) and Ca^{2+}-free amphibian culture medium (Cat. No. 350-1835) were purchased from Grand Island Biological Co. (Grand Island, NY). The amphibian medium was supplemented with Gentamicin (10 μg/ml). Modified Eagle medium contained 0.4 mM L-isoleucine. The amphibian culture medium contained 0.22 mM L-leucine and 0.22 mM L-isoleucine, however, the total content of these amino acids was larger but undetermined because this medium was supplemented with fetal bovine serum and whole egg ultrafiltrate. L-[4,5-^{3}H]Leucine (spec. act. 120—135 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). L-[U-^{14}C]glutamine (spec. act. 0.25 Ci/mmol), L-[^{35}S]Methionine (spec. act. 460—822 Ci/mmol), and L-[*methyl*-^{3}H]methionine (spec. act. 2 Ci/mmol) were purchased from New England Nuclear Co. (Boston, MA). All other reagents were obtained from different suppliers without apparent effect on the results.

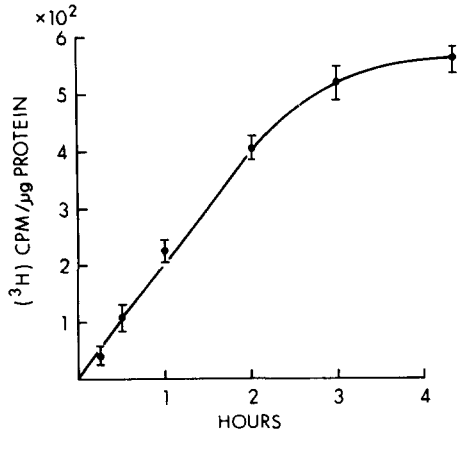
Results

1. Effect of time, protein concentration and composition of the incubation medium on amino acid incorporation

When the isolated epithelial cells were pulse-labeled with [^{3}H]-leucine and the radioactivity incorporated into acid-insoluble material was determined at different times, incorporation of the isotope was linear for periods of up to three hours (Fig. 1). Longer incubations (4—18 h) did not result in substantial increases of radiolabel incorporation into acid-insoluble material. As shown in Fig. 2, incorporation of [^{3}H]leucine into epithelial cells was linear for cell protein concentrations in the range of 10—100 μg. Thereafter, the rate of incorporation became constant or decreased slightly. Changes in the composition of the incubation medium did not influence the linearity of incorporation of [^{3}H]leucine (Fig. 2).

2. Effect of amino acid concentration in the incubation medium on the rate of radiolabel incorporation

To investigate the rate of radiolabel incorporation as a function of the concentration of amino acid in the incubation medium, isolated epithelial cells were pulse-labeled with [^{3}H]leucine and [^{14}C]glutamine at different specific activities obtained by the addition of non-radioactive amino acid. As shown in Table I, the rates of incorporation, expressed as dpm/μg protein per 30 min, decreased as the concentration of amino acid in the medium was increased



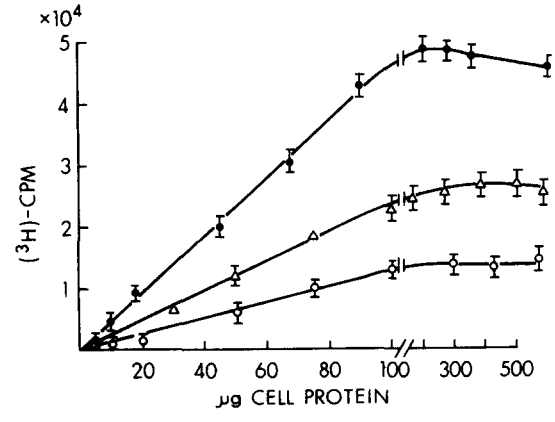


Fig. 1. Effect of time on incorporation of radioactivity into acid-insoluble material. Isolated epithelial cells were incubated in amphibian Ringer and pulse-labeled with [^{3}H]leucine for different times, as described under Methods. The values plotted are the means ± S.E. of 6 experiments. In 3 experiments a 120-min 'chase' with non-labeled leucine was done at the end of the pulse-labeling period. Since the results were similar to incubations terminated without 'chase', the values have been pooled.

Fig. 2. Effect of cell protein concentration on incorporation of radioactivity into acid-insoluble material. Epithelial cells were incubated in amphibian Ringer solution (●), amphibian culture medium (○) and modified Eagle medium (△). The cells were pulse-labeled with [^{3}H]leucine for 2 h and the radioactivity incorporated into acid-insoluble material was determined as described under Methods. The differences in radioactive incorporation reflect differences in the specific activity of the label (cf. Methods section). The values plotted are the means ± S.E. of 4 to 5 experiments in each medium.

TABLE I

EFFECT OF AMINO ACID CONCENTRATION ON INCORPORATION OF [^{3}H]LEUCINE AND [^{14}C]GLUTAMINE IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

Isolated epithelial cells were incubated in amphibian Ringer solution and pulse-labeled with [^{3}H]leucine and [^{14}C]glutamine for 30 min. The concentration of amino acids in the incubation medium was varied by adding appropriate volumes of a stock solution of non-radioactive amino acid. The values are the mean ± S.E. of 4 experiments.

Concentration of amino acid (M)	dpm/μg protein/30 min	
	^{3}H	^{14}C
10^{-6}	256 ± 10	22 ± 0.8
10^{-5}	125 ± 11	12 ± 0.5
10^{-4}	59 ± 7	5 ± 0.3
10^{-3}	28 ± 2	—

from 10^{-6} M to 10^{-3} M. The decrease, however, could not be accounted for by the differences in specific activity since a 10-fold difference in the amino acid concentration resulted in a 2-fold decrease in the rate of incorporation. The results were similar with both isotopes and also when the cells were pulse-labeled with [^{35}S]methionine (results not shown). These results suggested that, within the range of amino acid concentrations employed in these experiments, the acid-insoluble radioactivity might not entirely correspond to incorporation of the labeled amino acid into proteins, since under these conditions one would expect saturation kinetics with respect to different concentrations of amino acids in the incubation medium. This suggestion was strengthened by the observation that the inhibitor of protein synthesis cycloheximide resulted in only partial inhibition of the rate of radiolabel incorporation, from [^{3}H]leucine and [^{14}C]glutamine, into acid insoluble material (Table II). Radiolabel incorporation into acid insoluble material was essentially abolished when the cellular proteins were denatured by boiling or exposing the cell suspension to trichloro-

TABLE II

EFFECTS OF BOILING, TRICHLOROACETIC ACID AND CYCLOHEXIMIDE ON INCORPORATION OF [^{3}H]LEUCINE AND [^{14}C]GLUTAMINE IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

Isolated cells were divided in 4 groups; the first group served as control; the second group was exposed to cycloheximide (F.C.: 1 μg/ml, $3.5 \cdot 10^{-6}$ M); the third group was treated with 10% trichloroacetic acid at 4°C for 5 min and the fourth group was heated to 90°C for 5 min. The cells were then pulse-labeled (in Ringer solution) for 30 min with [^{3}H]leucine and [^{14}C]glutamine (both at 10^{-5} M) and the radioactivity associated with acid-insoluble material was determined as described under Materials and Methods. Cycloheximide was added 30 min before labeling. The values are the means ± S.E. of 5 experiments.

	dpm/μg protein/30 min	
	^{3}H	^{14}C
Control	91 ± 3	9.1 ± 0.5
Cycloheximide	79 ± 61	7.9 ± 1
Trichloroacetic acid	18 ± 1	0.79 ± 0.02
Boiling	15 ± 1	0.9 ± 0.01

acetic acid prior to pulse-labeling with radioactive amino acids, thus suggesting that the radiolabel associated with acid-insoluble material could not be accounted for by non-specific adsorption or 'trapping' of the isotope into the epithelial cells.

3. Studies on the nature of the acid-insoluble material

(a) Effects of ammonium sulfate and boiling. To test the prediction that the acid-insoluble material was associated with cellular proteins, sonicates of labeled cells were boiled for 5—10 min or subjected to treatment with trichloroacetic acid and ammonium sulfate. The sonicates were then centrifuged at 48 000 × *g* for 20 min and the radioactivity associated with the supernatant and pellet fractions was quantitated by scintillation counting. As shown in Table III, treatment with trichloroacetic acid resulted in precipitation of approximately 80% of the radioactivity incorporated into the epithelial cells. Likewise, ammonium sulfate treatment of cell sonicates, at concentrations of 60% and 100%, resulted in precipitation of a fraction of radioactivity comparable to that obtained with trichloroacetic acid. In contrast, a relatively small fraction (10%) of the cellular radioactivity was precipitated by boiling the cell sonicate (Table III). These results were consistent with the interpretation that the bulk of the acid-insoluble radioactivity was indeed associated with proteins because it could be quantitatively precipitated by high concentrations of ammonium sulfate. However, the experiments did not indicate whether the protein associated radioactivity corresponded to amino acid incorporation into elongating peptides or to incorporation of the radiolabel into prosthetic groups (e.g. carbohydrates, lipids). The lack of precipitation of the cellular radioactivity by boiling may suggest that the labeled proteins were relatively resistant to thermal denaturation; alternatively, it may be consistent with incorporation of the radiolabel into molecular species other than proteins.

(b) Effects of cycloheximide and actinomycin D on amino acid incorporation. Table II shows that amino acid incorporation from [^{3}H]leucine or [^{14}C]-

TABLE III

EFFECTS OF BOILING, TRICHLOROACETIC ACID AND AMMONIUM SULFATE ON [^{3}H]LEUCINE RADIOACTIVITY INCORPORATED IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

Isolated epithelial cells were incubated in Ringer solution and pulse-labeled for 60 min with [^{3}H]leucine. The cells were then washed (4—5 times) in Ringer solution and sonicated. Samples of sonicate were heated at 90°C for 5—10 min or treated with 10% trichloroacetic acid and different concentrations (30%, 60%, 100%) of ammonium sulfate at 0—2°C for 30 min. The sonicates were centrifuged at 48 000 × *g* for 20 min and the radioactivity associated with pellet and supernatant fractions was determined by scintillation counting. $n = 3$.

	% cpm	
	Supernatant	Pellet
Boiling	90 ± 5	11 ± 1
10% Trichloroacetic acid	20 ± 1	80 ± 7
30% $(NH_4)_2SO_4$	47 ± 3	53 ± 3
60% $(NH_4)_2SO_4$	19 ± 1	81 ± 6
100% $(NH_4)_2SO_4$	14 ± 1	87 ± 5

glutamine into acid-insoluble material, was only partially inhibited by cycloheximide. To further explore the role of inhibitors of protein and RNA synthesis on amino acid incorporation, we examined the effects of these inhibitors on cells exposed to different doses of the inhibitors. As shown in Fig. 3, prolonged exposure of the cells (up to 18 h) to 10 μg/ml ($3.5 \cdot 10^{-5}$ M) cycloheximide resulted in 36—40% inhibition of the rate of radiolabel incorporation. By contrast, prolonged treatment with actinomycin D inhibited amino acid incorporation by approximately 90%. It was also noted (Fig. 3) that the time dependence of the inhibitory effect was different with cycloheximide and actinomycin D. With actinomycin D there was a progressive increase in the inhibitory effect with time; by contrast, the maximal inhibitory effect of cycloheximide was already apparent after 2 h of incubation and did not change appreciably thereafter. The results were similar with higher or lower doses of either inhibitor. The results of these experiments were consistent with the previous observations and suggested that a substantial fraction of the radiolabel incorporated into acid-insoluble material, although associated with proteins, could not be entirely accounted for by incorporation of the label amino acid into elongating peptides. The time dependence of the inhibitory effect observed after prolonged incubation (18 h) in the presence of actinomycin D is difficult to explain, in view of the fact that this inhibitor has been shown to enhance the translation of available messages [20] as well as to decrease the rate of degradation of certain proteins [21]. The results with cycloheximide, however, can be readily interpreted, because this inhibitor appears to selec-

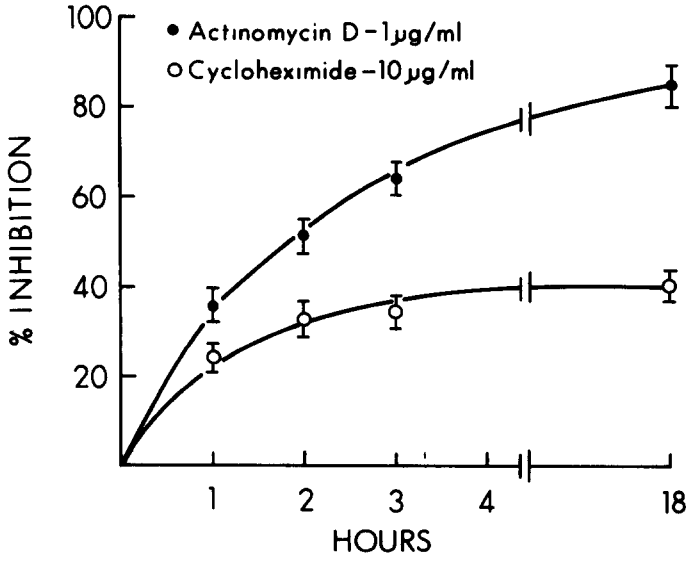


Fig. 3. Effect of cycloheximide and actinomycin D on radioactive incorporation into acid-insoluble material. Isolated epithelial cells were incubated in Ringer solution containing cycloheximide or actinomycin D for 1—18 h. The cells were then pulse-labeled with [^{3}H]leucine for 1 h and the radioactivity incorporated into acid-insoluble material was determined as described under Methods. Control cells were incubated under identical conditions. The values shown represent the percent inhibition of radiolabel incorporation, as compared to control, after different times of exposure to the inhibitors.

tively block initiation and elongation of peptides at the ribosomal level [22].

Treatment of cell sonicates with organic lipid solvents (e.g. ethanol-ether, acetone-water) resulted in extraction of 18%—30% of the acid-insoluble radioactivity. Appropriate experiments with three different amino acids (leucine, lysine, methionine) labeled with ^{3}H, ^{14}C or ^{35}S showed that the partial effect of cycloheximide on radiolabel incorporation was not unique to leucine.

(c) Column chromatography of the acid-insoluble material and effects of proteolytic enzymes. The experiments described have been interpreted as evidence that in the isolated epithelial cells of the toad bladder, pulse labeled with radioactive amino acids, the bulk of the radioactivity which is precipitated by trichloroacetic acid appears to be associated with protein species, but only a fraction of such radioactivity (30%—40%) seems to correspond to incorporation of the radiolabel into elongating peptides and hence to reflect protein synthesis. To further explore this issue, we performed chromatographic analysis of cell sonicates, as well as the fractions precipitated with trichloroacetic acid and ammonium sulfate, and in addition, we subjected cell sonicates to proteolytic

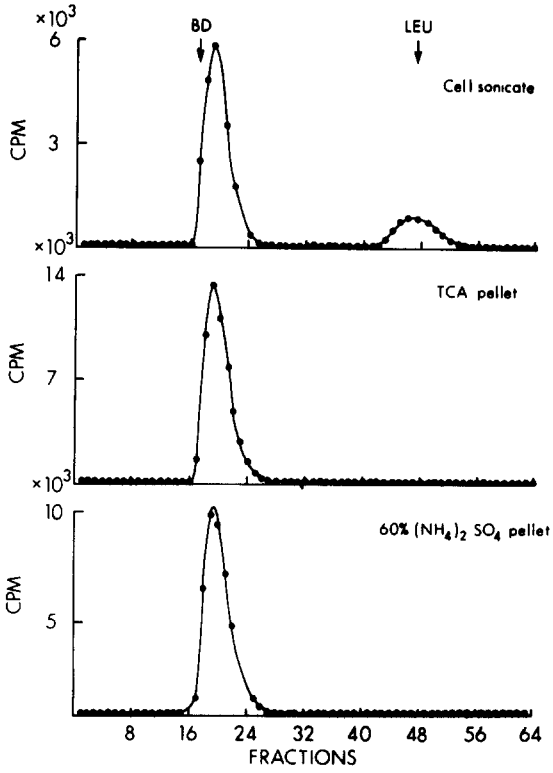


Fig. 4. Column chromatography (Sephadex G-50) of sonicates of toad bladder epithelial cells treated with trichloroacetic acid and ammonium sulfate. Samples of sonicates of isolated cells (pulse-labeled with [^{3}H]leucine) were heated with 10% trichloroacetic acid and 60% $(NH_4)_2SO_4$ at 0—2°C for 20 min. The samples were centrifuged at 48 000 × *g* for 30 min. The pellets were resuspended in 10 mM Tes, 10 mM EDTA, 0.5% SDS (pH 7.5) by sonication, loaded on a 50 × 1.5 cm column packed with Sephadex G-50 and eluted with the same buffer. SDS was required to prevent 'trapping' of radioactive material. Cell sonicates served as controls (upper panel). BD and Leu: position of elution of blue dextran and free leucine.

digestion and examined the products by column chromatography. As shown in Fig. 4, the untreated cell sonicate yielded a large radioactive peak that eluted near the void volume of the column and corresponded to about 80—85% of the radioactivity loaded on the column and a smaller peak that eluted near the position of free amino acid and accounted for about 15—20% of the total counts. Fig. 4 also shows that treatment of cell sonicates with 10% trichloroacetic acid or 60% ammonium sulfate resulted in quantitative recovery of the peak eluting near the void volume of the column, whereas the second peak was eliminated. These results provided unequivoval evidence that the majority of the cell-associated radioactivity was present in a macromolecular complex and they were consistent with the interpreation that the material was indeed associated with cellular proteins. If the radioactivity present in this macromolecular complex does correspond to amino acid incorporation into elongation peptides, it then follows that treatment with proteolytic enzymes should degrade this material into species of lower molecular weight. To test this prediction, sonicates of labeled cells were subjected to proteolytic digestions with pronase,

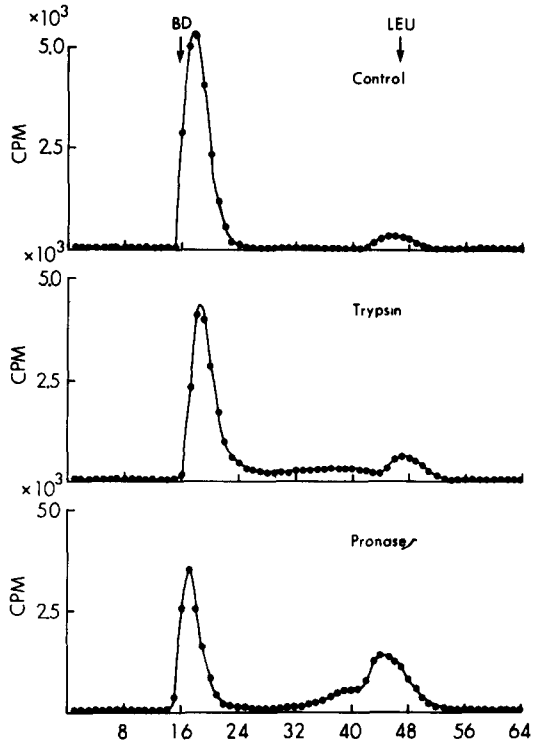


Fig. 5. Column chromatography (Sephadex G-50) of proteolytic digest of epithelial cells prepared from summer toads. Isolated cells were pulse-labeled with [^{3}H]leucine, sonicated and subjected to proteolytic digestion as outlined under Methods. The digest was chromatographed in a 50 × 1.5 cm column eluted with 10 mM Tes, 10 mM EDTA, 0.5% SDS (pH 7.5). The percents of radioactivity recovered in fractions 15—23 were 85% in controls, 65% in trypsin and 60% in pronase incubations. Fractions 24—40 contained 3% of counts in controls, 19% in trypsin and 6% in pronase incubations. Fractions 41—54 contained 10% of counts in controls, 16% in trypsin and 28% in pronase incubations. BD and Leu: position of elution of blue dextran and free leucine.

trypsin, chymotrypsin and papain as described under Methods and the digestion products were analyzed by column chromatography. As depicted in Fig. 5, prolonged digestion with pronase and trypsin yielded limited degradation of the material eluting near the void volume of the column. The percent of radioactivity eluting in this position was 85% in the control incubation, 65% in the trypsin incubation, and 60% in the pronase incubation which indicates that between 23—28% of the large molecular weight material was degraded by proteolytic digestion. In addition, pronase digestion yielded degradation products of lower molecular weight. These results are quantitatively similar to those obtained in the experiments of cycloheximide inhibition and clearly suggest that only about 1/3 of the radioactivity incorporated into cells can be accounted for by incorporation of the amino acid into elongating peptides. Similar results were obtained with chymotrypsin, and papain (results not show shown). Mild acid or alkaline hydrolysis did not change the chromatographic behavior of the radioactivity associated with the labeled epithelial cells, thus indicating that under the experimental conditions used in our studies there was no substantial labeling of the glycocalyx [16,17].

(d) Effect of cycloheximide on amino acid incorporation in other organs of the toad. To explore the question of whether the limited effect of cycloheximide on amino acid incorporation in isolated epithelial cells was a phenomenon unique to this tissue, we investigated the effects of the inhibitor on the incorporation of radioactivity from [^{3}H]leucine into acid-insoluble material in other organs of the toad. The results of these experiments are shown in Table IV. It can be seen that after 1 h of incubation in the presence of [^{3}H]leucine, cycloheximide produced only a 26% inhibition of radiolabel incorporation in isolated epithelial cells. By contrast, a comparable dose of the inhibitor produced between 70—80% inhibition of radiolabel incorporation in slices of spleen, liver, and the submucosal and serosal tissue of the urinary bladder. Of interest is the fact that a small effect of the inhibitor (31%) was also observed in the heart. These observations indicate that the partial effect of cycloheximide on radiolabel incorporation into acid-insoluble material, although not unique to the epithelial cells of the toad bladder (it also occurs in

TABLE IV

EFFECT OF CYCLOHEXIMIDE ON INCORPORATION OF [^{3}H]LEUCINE IN DIFFERENT ORGANS OF THE TOAD

Slices (approx. 1 mm thick) of liver, spleen and heart were obtained with a Stadie-Riggs microtome. The non-epithelial bladder tissue was obtained by scraping the cells off a hemibladder and mincing the tissue in small segments. The slices and isolated epithelial cells were pulse-labeled with [^{3}H]leucine for 1 h in the presence and absence of cycloheximide (10 μg/ml, approx. $3.5 \cdot 10^{-5}$ M). After washing, the cells and slices were sonicated and the acid-insoluble material was determined by scintillation counting. The values represent the percent inhibition over control in 3 experiments.

	% inhibition
Bladder epithelial cells	26 ± 0.8
Bladder non-epithelial tissues	72 ± 2
Liver	75 ± 1
Spleen	80 ± 0.5
Heart	31 ± 2

the heart), is not shared by the non-epithelial structures of the bladder or by other organs of the animal.

(e) Effects of inhibitors of proteolysis on radioactive incorporation. As shown in Table V, leupeptin had no effect on the rate of radiolabel incorporation. By contrast, phenylmethylsulfonyl fluoride and the trypsin inhibitor from lima bean resulted in a 15% increase in the rate of incorporation into acid-insoluble material, thus indicating that the contribution of proteolysis by the isolated epithelial cells was relatively small. Table V also shows that the inhibitory effect of cycloheximide (30—32%) was the same in the presence or absence of inhibitors of proteolysis. NaF, an inhibitor of anaerobic glycolysis, is known to produce almost complete inhibition of the rate of degradation of tyrosine aminotransferase and cellular proteins in HTC cells [28]. This effect has been attributed to depletion of the cellular levels of ATP [23]. Prolonged exposure of isolated epithelial cells to 10 mM NaF resulted in a decrease in the rate of incorporation of radiolabel from [^{3}H]leucine of a magnitude comparable to that observed with cycloheximide (38%). Of interest is the observation that the addition of cycloheximide to cells treated with NaF did not produce a further inhibition of the rate of radiolabel incorporation (Table V).

4. Effects of ouabain and Na-free Ringer on the rate of amino acid incorporation

The interference that protein synthesis is involved in the mechanism of action of certain hormones that affect sodium transport in the isolated toad bladder (aldosterone, insulin) has been deduced from the effects of inhibitors of RNA and protein synthesis on the response of this tissue to hormonal stimulation [4,6,9]. To investigate any potential role of Na on the rate of amino acid incorporation in the isolated epithelial cells, we examined radiolabel incorporation into acid-insoluble material in cells pulse-labeled with [^{35}S]-methionine and exposed to ouabain or incubated in the absence of Na in the Ringer. As shown in Table VI, ouabain resulted in about 50% inhibition of the rate of radiolabel incorporation whereas in Na-free Ringer medium only about 10% inhibition of incorporation was observed. By comparison, cycloheximide

TABLE V

EFFECTS OF NaF AND INHIBITORS OF PROTEOLYSIS ON INCORPORATION OF [^{3}H]LEUCINE IN ISOLATED TOAD BLADDER EPITHELIAL CELLS

The isolated cells were pulse-labeled for 2 h. The inhibitors and NaF were added 30 min before pulse-labeling. Cycloheximide was used at a final concentration of 10 μg/ml. The values are the means ± S.E. of 4 experiments.

	cpm/μg protein	
	— Cycloheximide	+ Cycloheximide
Control	875 ± 51	592 ± 41
Leupeptin ($1.4 \cdot 10^{-4}$ M)	870 ± 62	587 ± 57
PMSF ($1.1 \cdot 10^{-4}$ M)	1008 ± 95 *	672 ± 62
NaF (10^{-2} M)	539 ± 42 *	518 ± 51
Lima bean trypsin inhibitor (0.1 μg/ml)	987 ± 51	683 ± 49

* Statistically significant difference from control, $P < 0.02$.

TABLE VI

EFFECT OF OUABAIN AND DIFFERENCES IN Na^+ CONCENTRATION ON RADIOACTIVE INCORPORATION FROM [^{35}S]METHIONINE

Cells were pulse-labeled for 2 h. Ouabain and cycloheximide were added 30 min prior to labeling. In choline Ringer, NaCl and $NaHCO_3$ were replaced by equimolar quantities of choline chloride and choline bicarbonate. The values are the means ± S.E. of 4 experiments.

	dpm/μg protein	
	Acid soluble	Acid insoluble
Control	269 ± 7	1080 ± 29
Cycloheximide (10 μg/ml)	271 ± 12	745 ± 42 *
Ouabain ($5 \cdot 10^{-4}$ M)	156 ± 2	511 ± 12 *
Choline Ringer	268 ± 5	962 ± 19

* Statistically significant difference from control. $P < 0.001$.

resulted in about 31% inhibition. Similar results were obtained with [^{3}H]-methionine and [^{3}H]leucine. The effect of ouabain, however, appears to be mediated by an effect of the inhibitor on the soluble pool of radioactive amino acids since estimation of the size of this pool (as acid-soluble radioactivity) showed a comparable decrease. The small inhibitory effect observed in the absence of Na in the medium did not attain statistical significance.

5. Evidence for a seasonal dependence of the inhibitory effect of cycloheximide

It has long been recognized that certain functions of amphibian epithelial and non-epithelial tissues are clearly affected by changes in the seasons [24, 25]. The reasons for these differences are not clearly understood but they may represent adaptive mechanisms to changes in environmental factors (food,

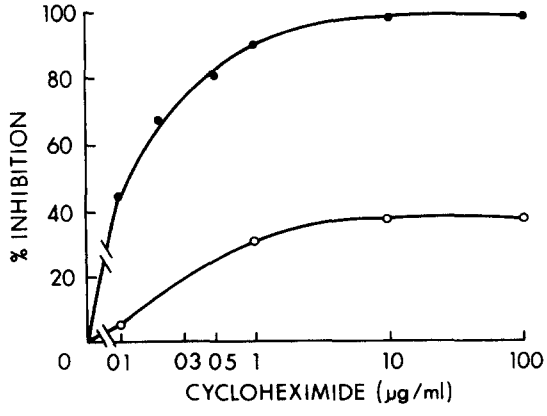


Fig. 6. Dose-response of the inhibitory effect of cycloheximide on incorporation of [^{3}H]leucine into acid-insoluble material. Cycloheximide was added 30 min prior to pulse-labeling of the epithelial cells. Labeling was carried out for 1—2 h. ●———●, cells obtained from winter toads; ○———○, cells obtained from summer toads. Each point represents the mean percent inhibition observed in 3 to 6 experiments for each condition.

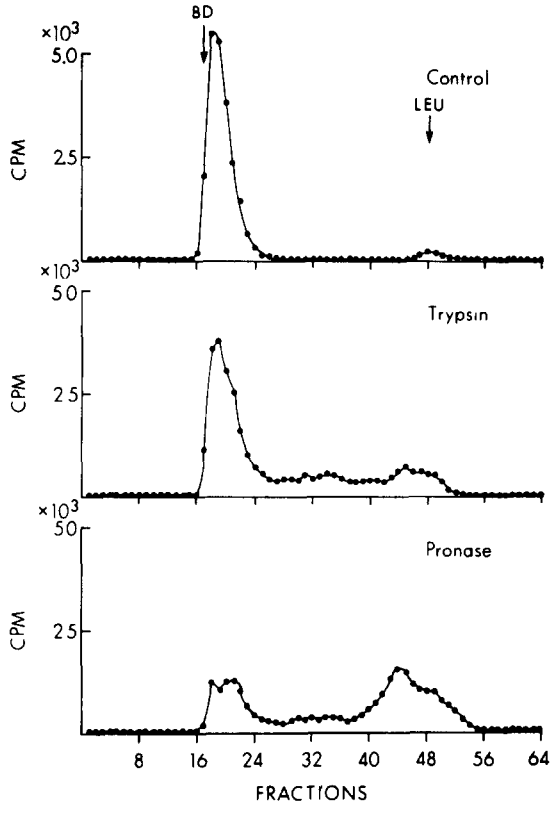


Fig. 7. Chromatographic analysis of proteolytic digest of epithelial cells prepared from winter toads. Experimental conditions were as described in the legend to Fig. 5. The percents of radioactivity recovered in fractions 16—22 were: 82% in controls, 58% in trypsin and 27% in pronase incubations. Fractions 23—40 contained 5% of counts in controls, 23% in trypsin and 20% in pronase incubations. Fractions 41—54 contained 5% of counts in controls, 18% in trypsin and 45% in pronase incubations. BD and Leu: position of elution of blue dextran and free leucine.

light, etc.) that ultimately affect the overall cellular functions. All of the experiments previously described in this paper were conducted between the months of May and September (summer months). To investigate whether the limited inhibitory effect of cycloheximide was also reproduced in toads supplied to us during the winter months, similar experiments were conducted in the months of November through February. As shown in Fig. 6, the inhibitory effect of cycloheximide was markedly different in cells obtained from toads supplied during the summer months as compared to cells obtained from toads supplied during the winter months. During the winter months, cycloheximide produced almost 100% inhibition of the rate of radiolabel incorporation from [^{3}H]leucine into acid-insoluble material. Half-maximal inhibition was obtained at a concentration of 0.1 μg/ml ($3.5 \cdot 10^{-7}$ M) and essentially complete inhibition occurred at a concentration of 1 μg/ml ($3.5 \cdot 10^{-6}$ M). By contrast, during the summer months, the maximal inhibitory effect of cycloheximide was around 40% with concentrations as high as 100 μg/ml. Half-maximal inhibition occurred at about 0.4 μg/ml ($1.4 \cdot 10^{-6}$ M). Likewise,

during the winter months, proteolytic digestion of the labeled cells resulted in degradation of large fractions of the radioactivity with trypsin, chymotrypsin, or papain and of essentially all of the radioactivity with pronase (Fig. 7). These results are in marked contrast to those obtained during the summer months (Fig. 5), in which only limited proteolysis of the labeled cells was observed, and they indicate that during the winter months the majority of the radioactivity incorporated into acid insoluble pellets of epithelial cells does indeed correspond to incorporation of the labeled amino acid into elongating peptides as evidence by its sensitivity to cycloheximide and to proteolytic digestion.

Discussion

The experiments described in this paper indicate that when the isolated epithelial cells of the toad bladder are pulse-labeled with radioactive amino acids and treated with trichloroacetic acid, a large fraction of the radioactivity which is acid-insoluble, although associated with a macromolecular protein complex may not correspond to incorporation of the label into elongation peptides. This conclusion is based upon the dependence of radiolabel incorporation as a function of amino acid concentration in the medium, the effects of ammonium sulfate, cycloheximide, actinomycin D and lipid solvents on radiolabel incorporation and the effects of proteolytic enzymes on the chromatographic behavior of the labeled material. We have shown that the labeled material cannot be accounted for by incorporation of the radioactive amino acid into the glycocalyx or into lipid components of the isolated epithelial cells; however, the nature of the labeled macromolecular species in this system remains to be established.

The experiments on the effects of cycloheximide on radiolabel incorporation and enzymatic hydrolysis of the labeled material did suggest that the variable fate of radioactive amino acids in isolated epithelial cells appears to display seasonal variation. During the winter months, cycloheximide produced essentially complete inhibition of incorporation of radioactive amino acids into acid-insoluble material. The dose of inhibitor that produced 50% inhibition of incorporation (0.1 μg/ml) is of the same order of magnitude as the dose of cyclohexmide that has been shown to inhibit certain physiologic effects on sodium transport that appear to depend on induction of protein synthesis [9]. The inference that radiolabel incorporation during this time of the year corresponds to incorporation of the labeled amino acid into elongating peptides was strengthened by the demonstration that essentially all the labeled material could be degraded by proteolytic enzymes. By contrast, during the summer months, not only was the maximal inhibitory effect reduced substantially (35%—40%) but the dose required to attain 50% inhibition was increased approximately 4-fold. The partial inhibitory effect was associated with a resistance to degradation by proteolytic enzymes of the labeled material thus confirming our initial impression that during this time of the year a large fraction of the acid-insoluble material does not correspond to elongating peptides. Although seasonal differences in physiologic responses of amphibia and specifically of the toad bladder is a well documented phenomenon [24,25], it is possible that these results may reflect changes in handling and housing of the

toads by the supplier. Due to special difficulties with capturing and shipping the toads from Central America, a large supply of animals is stocked in late spring (May and June) and maintained in 'their natural environment'; hence, toads supplied during the summer months have been stored by the supplier for variable times, whereas between the months of September and May storing by the supplier is very short. Thus, it is quite possible that the observed seasonal differences may reflect changes in biological functions determined by the storing conditions of the supplier rather than true seasonal variations in the biology of the animal. In any case, these results emphasize the importance of the time of the year when performing experiments aimed at investigating the effect of hormones or other stimuli on protein synthesis in the isolated toad bladder. In addition, these results may in part explain the difficulty of several investigators in demonstrating an effect of certain hormones (e.g. aldosterone) on protein synthesis in the isolated toad bladder.

The effects of ouabain and sodium-free Ringer on the rate of amino acid incorporation in the isolated cells are interesting. The inhibitory effect of ouabain on radiolabel incorporation is best explained by a decrease in the soluble pool of radioactive amino acid. Ouabain treatment of the isolated cells produces an increase in the intracellular sodium concentration and a decrease in the intracellular K concentration. The increase in cellular Na would in turn decrease the rate of sodium influx and the rate of amino acid penetration since it is generally agreed that amino acid transport is energized by Na^+ gradients [26]. It is unlikely, however, that the effect of ouabain on the amino acid pool is due to changes in transmembrane sodium gradients because no appreciable effect on the amino acid soluble pool was observed in cells incubated in sodium-free Ringer solution. A similar effect of ouabain on amino acid uptake has been recently reported in squid giant axons [27]. Thus, it is possible that ouabain has a direct effect on the rate of amino acid transport into the cells; alternatively, the effect on amino acid transport may be due to the changes in transmembrane potassium gradients. The present experiments do not provide any information as to the pertinent mechanism.

Sodium fluoride is known to produce almost complete inhibition of the rate of degradation of tyrosine aminotransferase and cellular proteins in HTC cells (hepatoma tissue culture cells) [23]. In addition, many cultured cells (both malignant and healthy embryonic or adult cells) show a marked tendency to accumulate lactic acid and ketoacids in the medium [28] which suggests that glycolysis is the major source of energy for the metabolic activities of these systems. Of interest is the observation that exposure of isolated epithelial cells to a high concentration of sodium fluoride produced inhibition of radiolabel incorporation of a magnitude similar to that obtained with cycloheximide. However, the effects of cycloheximide and sodium fluoride were not additive. This suggests that in the isolated epithelium of the toad bladder the energy for protein synthesis is preferentially obtained from glycolysis although a contribution of oxidative metabolism cannot be ruled out.

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