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Mechanism of Hormonal Induction of Tyrosine Aminotransferase Studied by Measurement of the Concentration of Growing Enzyme Molecules

(pactamycin/hepatoma cells/dexamethasone)

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ABSTRACT **Proteins labeled in cultured hepatoma cells grown in the presence of radioactive amino acids and pactamycin consist primarily of growing polypeptide chains initiated before addition of the antibiotic. Nascent tyrosine aminotransferase (EC 2.6.1.5) molecules were labeled in this way, and their radioactivity after completion was measured immunologically. Cells exposed to the synthetic adrenal steroid, dexamethasone, contain 10- to 18-times as much nascent enzyme as uninduced cells. These results indicate that induction control is not at the level of polypeptide-chain elongation or release, but probably operates on either the amount of messenger RNA or the rate of specific polypeptide-chain initiation.**

Addition of certain adrenal steroids to a line of cultured rat hepatoma (HTC) cells causes an increase in the *de novo* rate of synthesis of the enzyme, tyrosine aminotransferase (EC 2.6.1.5) (TAT) (1). This increase might be due to (*i*) an increase in the amount of TAT-specific mRNA, (*ii*) an increase in the number of ribosomes translating an unchanged amount of TAT mRNA, (*iii*) an increase in the rate at which each ribosome traverses the TAT mRNA, or (*iv*) removal of a block in translation behind which ribosomes have accumulated. Possibilities (*i*) and (*ii*) imply that more nascent chains of TAT are present in hormonally induced than in uninduced cells; while (*iii*) and (*iv*) would mean that nascent TAT chains would remain at the same concentration, or even decrease, when TAT synthesis was induced. To investigate these possibilities and to quantitate the growing chains of TAT, we have taken advantage of the fact that pactamycin prevents initiation but not completion of polypeptide chains (2). Therefore, by addition of radioactive amino acids to a growing HTC-cell culture simultaneously with pactamycin, it is possible to label only chains that had been initiated before the inhibitor was added. The radioactivity incorporated into completed TAT under these conditions gives an estimate of the concentration of nascent enzyme molecules.

The experiments reported below show that steroid-induced cells contain an increased concentration of nascent TAT molecules roughly in proportion to the increased rate of enzyme synthesis. From these results, it seems likely that induction is due either to an increased amount of specific message or to an increase in the specific initiation of the messenger for TAT.

MATERIALS AND METHODS

Materials. Mixtures of 15 radioactive amino acids were obtained from New England Nuclear. The specific radioactivity of the [^{3}H]aminoacids ranged from 1–44 Ci/mmol and the [^{14}C]aminoacids from 0.08–0.38 Ci/mmol. Pactamycin was a gift from the National Cancer Institute-Cancer Chemotherapy National Service Center. Cycloheximide was obtained from Sigma. Dexamethasone, a gift from Merck, Sharpe and Dohme, was prepared as a 1 mM solution in absolute ethanol.

Cell Growth, Induction of TAT, and Incorporation of Amino Acids. Suspension cultures of HTC cells were grown in modified S-77 medium containing no fetal-calf serum and 10% calf serum (3). Synthesis of TAT was induced with dexamethasone (4). Cells were collected for labeling after 15–20 hr of growth, when they had reached a density of 4–6 $\times$ 10^5/ml. 1–2 hr before the cells were collected, one-tenth volume of fresh growth medium was added, and incubation at 37° was continued. Cells were then harvested, washed in labeling medium (see below), and suspended at a density of 5 $\times$ 10^7/ml. Induced cells were pooled and incubated in labeling medium containing 1 μM dexamethasone, and uninduced cells, in labeling medium without dexamethasone but containing 0.1% ethanol. Labeling medium (which contained reduced concentrations of amino acids and vitamins) was prepared by addition of $^1/_{20}$ volume of modified S-77 growth medium, $NaHCO_3$ (1 mg/ml), and dialyzed calf serum (final concentration, 10%) to the inorganic constituents of modified S-77 medium. In the experiments reported in Fig. 3 and Table 1, adenine (0.16 mM) and uridine (32 μM) were added to suppress labeling of purines and pyrimidines (5).

Isolation of Soluble Proteins and Immunological Assay for TAT. After incubation with labeled amino acids, the cells were harvested and lysed by hypotonic shock and brief homogenization (6). The postribosomal supernatant (S_{105}) was prepared by centrifugation for 90 min at 105,000 $\times$ *g*. For immunological assays, S_{105} was heated (7), and the supernatant solution from the heat precipitation was further purified on small DEAE-cellulose columns (Beck, Beck, and Tomkins, in preparation). Recovery of TAT activity through these two purification steps was 30–65%. A portion of the DEAE-cellulose eluate was immunoprecipitated with sheep antibodies to TAT as described (7), except that the KCl concentration was adjusted to 0.15 M and bovine serum albumin (fraction V from bovine plasma, Armour Pharmaceutical Co.) was added to 5 mg/ml. The washed immunoprecipitates were suspended in 10% trichloroacetic acid, collected on Whatman 3MM filter discs, and dried. The discs were placed in 10 ml of toluene containing Omnifluor (New England Nuclear) and 0.016 N NCS tissue solubilizer (Amersham/Searle), and incubated 18–24 hr at room temperature before they were counted.

Abbreviations: TAT, tyrosine aminotransferase; PTH, phenylthiohydantoin; HTC, hepatoma cells.

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TABLE 1. *Effect of pactamycin on amino-acid incorporation into the NH_2-terminal position of soluble proteins**

Treatment of ^{3}H-labeled HTC cells	Fraction	^{3}H (dpm)	^{14}C (dpm)	Ratio ^{3}H/^{14}C	R†
Pactamycin	NH_2-terminal	469	817	0.57	0.029
	Internal	400,000	20,330	19.7	
Control	NH_2-terminal	334	105	3.2	0.26
	Internal	305,700	25,000	12.2	

* Uninduced cells were incubated with [^{3}H]aminoacids for 10 min in the presence or absence of 2 μM pactamycin. S_{105} fractions obtained from each of these incubation mixtures were combined with soluble proteins derived from cells incubated overnight with [^{14}C]aminoacids but no pactamycin. Edman degradation was performed, and the PTH amino-acid derivatives were purified by chromatography. No PTH amino acids were detected when the trifluoracetic acid cleavage step was omitted, indicating that no free radioactive amino acids were carried through the procedure. NH_2-terminal labeling was calculated from the radioactivity running with the solvent front on silica-gel chromatography. Radioactivity in internal positions was determined by counting the solid residue remaining after extraction of the PTH amino acids.

† R is defined in the *text*. $R_{pactamycin}/R_{control} = 0.11$. Correction for the effect of pactamycin on the number average molecular weight of labeled soluble proteins (see *Appendix*) yields $(\alpha/\beta) = 0.13$.

NH_2-Terminal Analysis. Suitable portions of ^{3}H- and ^{14}C-labeled S_{105} fractions were combined, hydrolyzed with 0.5 N NaOH for 30 min at 37°, exhaustively dialyzed against water, and then lyophilized. Edman degradation was performed on 3 mg of this material as described by Gray (8), except that the products of the coupling reaction were extracted three times successively with benzene, ethanol, and acetone; and the products of the subsequent cleavage reaction were extracted with three aliquots of butyl acetate and then back-extracted with water. The resulting butyl acetate phase was dried, and the phenylthiohydantoin (PTH) amino acids were chromatographed on silica-gel sheets (Eastman) in chloroform–methanol–formic acid 70:30:2. In this system, nearly all PTH amino acids travel with the solvent front (9). The chromatogram was cut into strips, and the radioactivity was eluted by overnight incubation at room temperature in scintillation fluid containing 0.032 N NCS tissue solubilizer. The plastic backing and the silica-gel sheet were then removed from the scintillation vial, and the eluted material was counted. Counting efficiencies and ^{14}C spill were determined in each sample with internal standards. Background was determined by parallel experiments on unlabeled material.

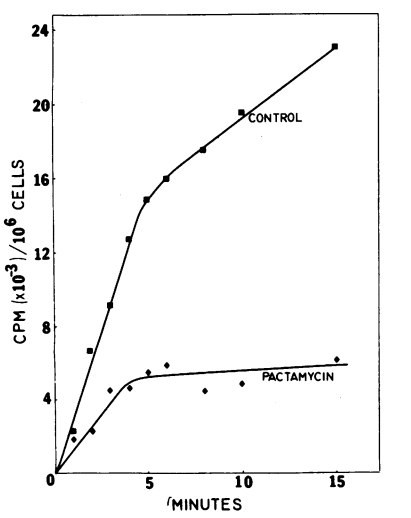

FIG. 1. Rate of inhibition of amino-acid incorporation by pactamycin. [^{3}H]aminoacids (16 μCi/ml) were added to two 1.5-ml suspensions of uninduced HTC cells and to one, pactamycin was added to a final concentration of 2 μM. Aliquots (0.1 ml) were sampled at the indicated times after incubation at 37° into phosphate-buffered saline (25 mM potassium phosphate buffer pH 7.6–0.1 M NaCl) at 0° to stop further incorporation. These cells were washed once with phosphate buffered saline and then incubated 50 min at 37° in 1 N KOH to hydrolyze aminoacyl tRNA. Proteins were precipitated by addition of two volumes of 20% trichloroacetic acid. After incubation overnight at 4°, precipitates were collected and counted.

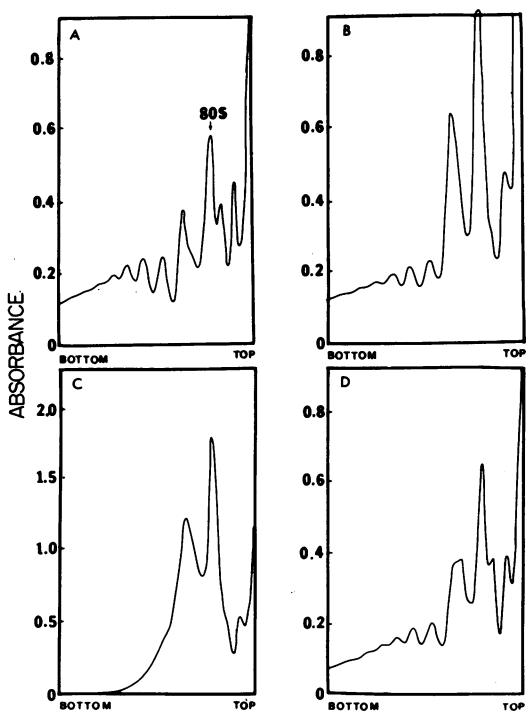

FIG. 2. Sucrose density-gradient sedimentation of HTC cell lysates after incubation with or without pactamycin and cycloheximide. Uninduced cells were concentrated and four aliquots were prepared. One aliquot (*A*) was immediately diluted in 40 ml of phosphate-buffered saline at 0°. A second (*B*) was incubated 10 min at 37° and then diluted into cold phosphate-buffered saline. Pactamycin (2 μM) was added to the remaining two aliquots (*C* and *D*), and cycloheximide (0.2 mM) was also added to *D*. Both were incubated 10 min at 37° and then diluted in cold phosphate-buffered saline. All four samples were centrifuged, resuspended in lysis buffer [10 mM Tris.HCl (pH 7.4)–1.5 mM $MgCl_2$–1.0 mM KCl], and 0.5% Nonidet P-40 detergent (Shell) was added. Lysis was completed by drawing the cell suspension into a Pasteur pipet several times. Aliquots from each lysate were layered onto 5-ml gradients (15–30% sucrose in lysis buffer) and centrifuged 35 min at 45,000 rpm (SW 50.1 rotor; Beckman Spinco ultracentrifuge). Absorbance at 254 nm was scanned with an ISCO UV flow-through cell. Position of the 80S particle was determined by plotting log of sedimentation distance against log of polysome number (14).

Assays and Determination of Radioactivity. TAT activity was determined by the method of Diamondstone (10) and protein, by the method of Lowry *et al.* (11). Acid-precipitable radioactivity was determined by the method of Mans and Novelli (12), except that the filter discs containing precipitated protein were incubated in scintillation fluid containing NCS as described for counting immunoprecipitates. This procedure effectively solubilizes radioactivity from the paper and improves counting efficiency, making this determination directly comparable with the immunoprecipitation procedure.

RESULTS

Effect of pactamycin on protein synthesis and polysome size distribution in HTC cells

Inhibition of protein synthesis in HTC cells by pactamycin was strongly concentration dependent between 0.1 and 1 μM, as has been reported in other systems (2, 13). A concentration of 2 μM was chosen for the experiments reported below. Incorporation of radioactive amino acids is reduced to 2% of the control rate after a 5-min incubation with this concentration of the antibiotic. Rates of labeling in the presence and absence of pactamycin are shown in Fig. 1. With the inhibitor present, incorporation of radioactive amino acids into soluble protein continues for a short period, reaching a plateau in 4–5 min. This result suggests that polypeptide-chain elongation is less inhibited than initiation, and that previously initiated chains are completed in the presence of the antibiotic.

These conclusions are further supported by the effects of pactamycin on the polysome size distribution. Although some polysome disaggregation is observed when cells are incubated in the absence of pactamycin (Fig. 2*B*), disaggregation is dramatically enhanced in the presence of the antibiotic (Fig. 2*C*); and as expected, it is prevented by cycloheximide (Fig. 2*D*). The large disome peak remaining after incubation in pactamycin is characteristic of these cells and does not indicate continued protein synthesis.

Polyacrylamide gel analysis of proteins completed in the presence of pactamycin

The soluble proteins prepared after 10-min incubation with pactamycin and [^{3}H]aminoacids were mixed with soluble proteins labeled for an equal period with [^{14}C]aminoacids in the absence of the inhibitor and subjected to electrophoresis on polyacrylamide gels (Fig. 3*A*). Shown for comparison is a gel run on a mixture of ^{3}H- and ^{14}C-labeled soluble proteins where both labels were incorporated in the absence of pactamycin (Fig. 3*B*). Proteins released in the presence of pactamycin are somewhat larger than those released in the absence of this antibiotic (weight average molecular weight is increased by 19%; number average molecular weight is increased by 14%). This presumably results from the fact that, within a given time of incubation, more copies of small proteins would be completed in the absence of pactamycin than in its presence because, with initiation unimpaired, shorter messages could be translated more frequently than longer ones. In any case, pactamycin does not cause release of abnormally short polypeptide fragments, indicating that polysome breakdown shown in Fig. 2 is not due to premature detachment of ribosomes from the message and consequent release of incomplete proteins.

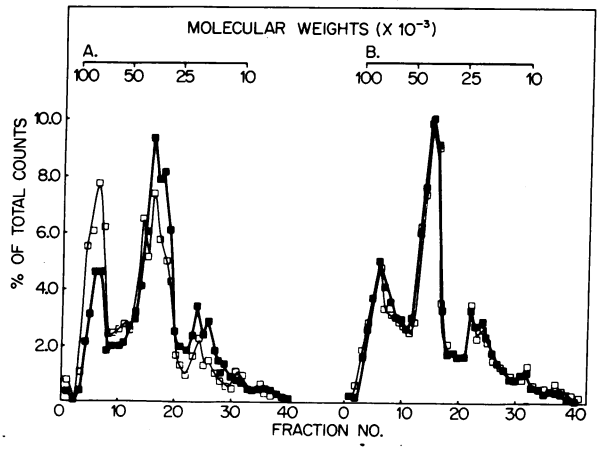

FIG. 3. Polyacrylamide gel analysis of soluble HTC cell proteins labeled in the presence or absence of pactamycin. Aliquots of the S_{105} fraction derived from ^{3}H- and ^{14}C-labeled uninduced cells (incubated for amino-acid incorporation 10 min) were mixed and subjected to electrophoresis on 15% polyacrylamide gels (15). The *ordinate* is expressed as percent of total cpm recovered in the gel. Molecular weights (*upper scale*) were determined by parallel gels containing protein standards. In gel *A*, pactamycin (2 μM) was added simultaneously with [^{3}H]amino-acids. ^{3}H incorporation in gel *B*, and ^{14}C incorporation in both gels *A* and *B* were done in the absence of this antibiotic. ^{3}H (□); ^{14}C (■). Total cpm recovered in gel *A*: ^{3}H = 18,930; ^{14}C = 4025; in gel *B*: ^{3}H = 22,220; ^{14}C = 4479. The number average molecular weight was 29,500 for proteins labeled in the presence of pactamycin and 25,900 for proteins labeled in its absence. The weight average molecular weight was 52,200 in the presence of pactamycin and 44,000 in its absence. Only ^{3}H distributions were used for these calculations because of the small number of counts in ^{14}C. We are indebted to Dr. Richard Bernstein for the computer analysis of these data.

Effect of pactamycin on incorporation of amino acids into the NH_2-terminal position of polypeptide chains

For confirmation that pactamycin preferentially inhibits initiation of protein synthesis, incorporation of radioactivity into the NH_2-terminal position of soluble proteins synthesized in the presence of the inhibitor was determined by Edman degradation and compared with internal labeling. To correct for differences in recovery of the PTH NH_2-terminal amino-acid derivatives throughout the procedure, the degradation was performed on a mixture of ^{3}H-labeled proteins synthesized in the presence of pactamycin together with a preparation of uniformly ^{14}C-labeled soluble proteins. Incorporation of radioactivity into the NH_2-terminal position of proteins (Table 1) is indeed preferentially inhibited by pactamycin.

Comparison of the effect of the inhibitor on NH_2-terminal labeling and on labeling of internal positions is given by the ratio, R, where

$$R = \frac{^3H/^{14}C \text{ in } NH_2\text{-terminal positions}}{^3H/^{14}C \text{ in internal positions}}.$$

R determined for the control incubation reflects the difference in labeling conditions with [^{14}C] and [^{3}H] aminoacid mixtures and also the fact that NH_2-terminal amino acids in HTC cells are not representative of the total amino-acid composition of these cells. Because of these considerations, the ratio, $R_{pactamycin}/R_{control}$, is the best indicator of the quantitative

TABLE 2. *Effect of pactamycin on incorporation of amino acids into TAT*

Treatment[a]	cpm in S_{105} proteins ($\times 10^{-6}$)	Immunoprecipitable cpm			cpm in TAT corrected[b] to activity in S_{105}	cpm in TAT expressed as % of S_{105} protein
		1st	2nd	Difference		
Pactamycin added simultaneously with [^{3}H]aminoacids[c]						
Induced	2.07	1418	34	1384	6918	0.33
		1473	32	1441	7198	0.35
Uninduced	5.88	332	9	323	1547	0.03[d]
		283	15	268	1283	0.02
No pactamycin[e]						
Induced	1.78	1861	47	1814	7825	0.44
		1797	62	1735	7485	0.42
Uninduced	7.01	824	31	793	2961	0.04
		425	28	397	1481	0.02
Pactamycin added 5 min before [^{3}H]aminoacids[f]						
Induced	0.61	137	7	130	907	0.15
		177	8	169	1183	0.19
Uninduced	0.75	59	12	46	292	0.04
		57	26	31	196	0.03

[a] Incubation with [^{3}H]aminoacid mixture was for a total of 10 min. Pactamycin, where added, was 2 μM. The specific activity of TAT, measured in the S_{105} fraction was 288 milliunits/mg of protein in induced HTC cells and 31 milliunits/mg of protein in uninduced cells.

[b] cpm in TAT was divided by the fraction of TAT activity from the S_{105} fraction that was added to the immunoprecipitation reaction. This correction, which is derived from the incomplete recovery of TAT activity through the two purification steps and from the size of the aliquot of the DEAE-cellulose eluate that was added to the immunoprecipitation assay, was determined for each incubation mixture and ranged from 0.14–0.27.

[c] Induced: 7.5 ml incubation mixture, 60 μCi/ml; uninduced: 10 ml incubation mixture, 120 μCi/ml.

[d] In three experiments, uninduced cells gave values similar to those shown here; but in one experiment the label recovered in the immunoprecipitate from uninduced cells was 60–85% of the values obtained with induced cells. We have no explanation for this deviation.

[e] Induced: 3.0 ml incubation mixture, 20 μCi/ml; uninduced: 3.0 ml, 100 μCi/ml.

[f] Induced: 7.5 ml incubation mixture, 120 μCi/ml; uninduced; 10 ml, 120 μCi/ml.

effect of pactamycin on NH_2-terminal labeling of proteins (see *Appendix*).

Effect of pactamycin on synthesis of TAT

Proteins labeled and released during incubation with or without pactamycin were analyzed for total radioactivity and for radioactivity immunoprecipitable as TAT. Proteins labeled and released after 5-min preincubation with pactamycin were similarly analyzed (Table 2). When the quantity of specifically precipitated radioactivity is expressed as the percent of total radioactive protein in the original S_{105}, it can be seen that TAT labeled in the presence of pactamycin roughly parallels that labeled in the absence of the antibiotic. These results show that cells synthesizing TAT at the induced rate contain more growing TAT chains than do uninduced cells in proportion to the increased rate of enzyme synthesis. The slight reduction in the relative incorporation observed when pactamycin is present is accounted for by the increased weight average molecular weight of the proteins completed in the presence of this inhibitor (Fig. 3*A*). When cells were preincubated with pactamycin, the fraction of the total radioactivity incorporated into TAT did not increase, indicating that TAT synthesis is at least as sensitive to the inhibitor as is total protein synthesis.

DISCUSSION

From the experiments presented here we conclude that there are more growing chains of TAT in cells exposed to steroid inducer than in uninduced cells and, therefore, more ribosomes are involved in the synthesis of TAT. This conclusion depends on the demonstration that in HTC cells, as in other cells (2, 13), pactamycin preferentially inhibits polypeptide-chain initiation over elongation. This is evident for total protein synthesis from direct NH_2-terminal analysis (Table 1) as well as from the rate of amino-acid incorporation in the presence of the antibiotic (Fig. 1) and the protein-synthesis-dependent disaggregation of polysomes induced by the antibiotic (Fig. 2). TAT synthesis is not uniquely resistant to pactamycin inhibition because a 5-min prior incubation with the antibiotic inhibited incorporation into TAT at least as much as into other soluble proteins (Table 2).

When radioactive amino acids and pactamycin were added simultaneously, most of the radioactivity incorporated was in proteins whose synthesis had been initiated before addition of the inhibitor. In a 10-min incubation, about 13% of the total radioactivity incorporated into soluble proteins was due to escape from initiation inhibitions (Table 1).

It was important to show that nascent TAT molecules were released to the same extent in induced and uninduced cells. If control were exerted at the level of polypeptide-chain elongation or release, in uninduced cells only a small fraction of the growing enzyme molecules might be released after 10 min of incubation. This possibility was examined by preincubation of cells with pactamycin before adition of labeled amino acids (Table 2). If polypeptide-chain elongation were slower in uninduced cells, it should continue at the same rate

between 5 and 15 min after addition of pactamycin as in the period between 0 and 10 min. Since the incorporation into total soluble protein is decreased about 90% by preincubation, the fraction of radioactivity incorporated into TAT would have had to increase 10-fold. This obviously did not occur (Table 2). There may have been somewhat more unreleased TAT chains in uninduced cells than in induced cells after preincubation; however, since the level of radioactivity was small, this observation may not be significant.

From these considerations, we conclude that the TAT that is labeled and released in the presence of pactamycin is a valid measure of the nascent enzyme molecules present at the time the inhibitor and the radioactive amino acids were added. The major conclusion, that induced cells contain more growing chains of TAT than uninduced cells, implies that the difference in the rate of synthesis of TAT between induced and uninduced cells is not due to differences in the rates of specific elongation or release of enzyme molecules, as has been suggested for control of TAT synthesis by cyclic AMP in neonatal rat liver (16). In our case, the difference must reside either in the amount of TAT messenger RNA available for translation, or in the rate of specific initiation of synthesis of this enzyme.

APPENDIX

Parameters obtained in NH_2-terminal analysis experiments

If a mixture of proteins is separated into size classes (e.g., polyacrylamide gel slices), the ith size class is made up of n_i protein molecules of molecular weight M_i. Then the number of NH_2-terminal residues in this size class is n_i, and the number of internal residues is given by $(M_i n_i/k) - n_i \approx (M_i n_i/k)$, where k is the average molecular weight of an internal residue.

For N size classes of proteins labeled in the absence of pactamycin, total ^3H incorporation into NH_2-terminal positions is given by $k_1 \sum_1^N n_i$; and total ^3H incorporation into internal positions by $(k_2/k)\sum_1^N M_i n_i$; where k_1 and k_2 are constants determined by amino-acid specific activities and the distribution of amino acids between NH_2-terminal and internal positions. The same expression describe uniformly ^{14}C-labeled soluble proteins with new constants (k_1 and k_2) defined by the [^{14}C]aminoacid mixture. Thus, for the control experiment (Table 1), the $^3H/^{14}C$ ratio for NH_2-terminal residues is k_1/k_1' and for internal residues is k_2/k_2' and $R_{control} = k_1k_2'/k_1'k_2$.

When pactamycin is present in the incubation, the size distribution of labeled soluble proteins is changed (Fig. 3*A*). To indicate this, the jth size class of proteins out of N' size classes is considered to be made up of n_j' protein molecules of molecular weight M_j'. If pactamycin alters the amount of radioactivity incorporated into NH_2-terminal positions by a factor α and the amount of radioactivity incorporated into internal positions by a factor β, the $^3H/^{14}C$ ratio for NH_2-terminal residues is given by

$$\alpha k_1 \sum_1^{N'} n_j' \Big/ k_1' \sum_1^N n_i.$$

Similarly, the $^3H/^{14}C$ for internal residues is

$$\beta k_2 \sum_1^{N'} M_j' n_j' \Big/ k_2' \sum_1^N M_i n_i$$

and the expression for $R_{pactamycin}$ reduces to,

$$\frac{\alpha}{\beta}\frac{k_1k_2'}{k_1'k_2}\left(\frac{\sum_1^N M_i n_i}{\sum_1^N n_i} \Big/ \frac{\sum_1^{N'} M_j' n_j'}{\sum_1^{N'} n_j'}\right) = \frac{\alpha}{\beta}\frac{k_1k_2'}{k_1'k_2}\frac{\bar{M}_c}{\bar{M}_p}$$

where $\bar{M}_c$ and $\bar{M}_p$ are the number average weights of soluble proteins labeled in the control and in the pactamycin experiments, respectively. From this

$$R_{pactamycin}/R_{control} = (\alpha/\beta)(\bar{M}_c/\bar{M}_p)$$

The value, α/β, is a measure of the relative contribution of labeling of NH_2-terminal residues and internal residues, and is dependent on the length of the labeling period. If it is assumed that this escape does not generate proteins of special composition, it can be shown that α/β is about equal to the fraction of radioactivity incorporated in the presence of pactamycin that is due to escape from initiation inhibition.

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