Lodish Model and Regulation of Ribosomal Protein Synthesis by Insulin-Deficient Chick Embryo Fibroblasts†

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ABSTRACT: The production of ribosomal proteins in chick embryo fibroblasts that have been deprived of insulin is depressed to a much greater degree than that of most or all other cell proteins. Previous observations ruled out explanations for the preferential decrease in ribosomal protein formation that depend upon a selective destruction of ribosomal protein messages or a regulatory role for nascent ribosomal ribonucleic acid. The proposition has now been examined that ribosomal protein messenger ribonucleic acids (mRNAs) in the hormone-deficient chick embryo cells have a lower affinity for a limiting, early acting component of the initiating machinery than do most other cell messages and, in consequence, suffer from a translational disadvantage. The approach that was used depends upon the findings of Lodish and others that all mRNAs are not initiated with equal ease, that inhibitors of elongation favor the initiation of low-affinity mRNAs, and that agents that dampen an early step in initiation discriminate against the low-affinity messages. The idea was tested by comparing the effects of various inhibitors on the rates of synthesis of total cell protein and individual nonribosomal proteins, on the one hand, with those of individual ribosomal proteins, on the other. The results fit the Lodish model and are consistent with the conclusions that ribosomal protein mRNAs are more poorly initiated in the resting fibroblasts than are most or all other cell messages and that this condition is largely or entirely responsible for the low rate of ribosomal protein formation.

Chick embryo fibroblasts that have been deprived of insulin make r proteins[1] at a severely diminished rate, whereas the formation of most or all other cell proteins is much less depressed (DePhilip et al., 1980). Two conceivable deficiencies that might have explained the preferential reduction in the synthesis of r proteins have been excluded (DePhilip et al., 1980). The resting cells have not destroyed a major part of their r protein messages nor does the availability of nascent rRNA govern the production of r proteins. A third possibility, that the hormone-deficient cells have modified a majority of their r protein messages so that they cannot be read, has been weakened (F. Sato, R. A. Ignotz, K. Tsukada, and I. Lieberman, unpublished experiments). These observations would seem to put the focus on a mechanism that selectively restricts the translation of capable r protein mRNAs in the insulin-deprived cells.

Some species of mRNA have greater affinities than others for components of the initiation system (Lodish, 1971; Lodish & Jacobsen, 1972; Lodish & Desalu, 1973; McKeehan, 1974; Palmiter, 1974; Temple & Lodish, 1975; Golini et al., 1976; Kabat & Chappell, 1977; Baglioni et al., 1978; Herson et al., 1979; Di Segni et al., 1979; Strome & Young, 1980). As a consequence, the translation of low-affinity mRNAs is severely restricted under conditions where they must compete with messages of higher affinity for a limiting initiation factor (McKeehan, 1974; Temple & Lodish, 1975; Golini et al., 1976; Kabat & Chappell, 1977; Baglioni et al., 1978; Herson et al., 1979; Di Segni et al., 1979; Strome & Young, 1980).

Drugs that inhibit the growth of polypeptide chains provide one means of distinguishing among mRNAs with different initiation frequencies. Thus, inhibitors of elongation preferentially reduce the translation of high-affinity messages (Lodish, 1971; Lodish & Desalu, 1973; Sonenshein & Brawerman, 1976; Jen et al., 1978; Strome & Young, 1980). Such an action might be anticipated since interference with the rapid movement of a newly initiated ribosome away from the site of initiation would be expected to have a more adverse effect on a mRNA with a high rather than a low initiation rate constant.

Inhibitors that act on initiation at or before the binding of the 40S subunit to the mRNA, on the other hand, show the opposite selectivity. Such agents preferentially depress the translation of messages with low initiation rate constants (Lodish, 1974; Nuss et al., 1975; England et al., 1975; Nuss & Koch, 1976a,b; Herson et al., 1979). Low-affinity messages, it would seem, can less well tolerate a dampening of their interaction with the initiation system than can mRNAs of high affinity.

The major aim of this study was to test the idea that r protein messages in the resting chick embryo fibroblasts are more poorly initiated than most or all other mRNAs. To this end, comparisons have been made of the effects of various inhibitors of translational elongation and initiation on the synthesis of individual basic r and non-r proteins. It was hoped that the results would shed light not only on the regulation of r protein synthesis in the hormone-deprived chick cells but also, indirectly, on the mechanism of insulin action.

Materials and Methods

Materials. Inhibitors of protein synthesis were obtained as follows: anisomycin, from the National Institutes of Health; MDMP, as gifts from Dr. Donald P. Weeks, The Institute for Cancer Research, Philadelphia, PA, and Dr. Robert Baxter, Sittingbourne Research Centre, Sittingbourne, Kent, England; 3-bromooxindole-3-acetic acid, the precursor of 3-methyleneoxindole, through the kindness of Dr. Sergio Abreu, Fordham University, Bronx, NY; cycloheximide, emetine, and puromycin, from Sigma. Crystalline bovine insulin was from Sigma, and radioactive compounds and counting solutions were from New England Nuclear. Radioactive and unlabeled leucine were in the L form. Specific activities of [^{3}H]leucine

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[1] Abbreviations used: r protein, ribosomal protein; rRNA, ribosomal ribonucleic acid; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; MDMP, D-[2-(4-methyl-2,6-dinitroanilino)]-*N*-methylpropionamide.

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ranged from 40 to 60 Ci/mmol.

Tissue Culture. Primary cultures of chick embryo fibroblasts were prepared and grown in basal medium (minimal essential, Eagle) containing 4% calf serum as previously described (DePhilip et al., 1979). Confluency was reached by 3 days of incubation, and the cultures were used 2 or 3 days later.

Radioactive Labeling and Extraction of Protein for Electrophoresis. At the time of implantation of the primary cells, 3 μCi of [^{14}C]leucine (270 mCi/mmol) was added to each culture. [^{3}H]Leucine (400 μCi/culture, unless otherwise indicated) was dried with a stream of air and dissolved in leucine-free basal medium immediately before use. Labeling of cells with [^{3}H]leucine was in a modified basal culture medium that contained 0.1 mM instead of 0.4 mM leucine. Protein was extracted from the doubly labeled cells by a modification of the procedure that was used previously (DePhilip et al., 1980). The attached cells were washed twice with ice-cold basal medium, and they were then detached by scraping and collected by centrifugation. The sedimented cells were broken by freezing and thawing 5 times, and the homogenate was stirred (0 °C) for 45 min in 2 mL of a solution of 67% acetic acid, 0.1 M $MgCl_2$, and 10 mM dithiothreitol. Insoluble material was discarded by centrifugation (8000*g*, 15 min), to each supernatant fraction was added 300 μg of unlabeled r protein, and the mixture was precipitated with acetone and dried in vacuo (Roberts & Ashby, 1978). Finally, the dry powder was dissolved in 200 μL of a solution of 8 M urea, 0.02 M Tris (pH 8.6), 1 mM EDTA, 0.026 M boric acid, and 5% 2-mercaptoethanol (v/v) (Lastick & McConkey, 1976). With the modified procedure, the extracts contained only about 60%, as opposed to more than 90%, of the radioactivity and the protein of the cells, but entry of the basic proteins into the first-dimension gel was improved more than 2-fold, whereas the ^{3}H/^{14}C ratios of the resolved basic proteins were not changed. Unlabeled r proteins were prepared from 12-day-old chick embryos.

Gel Electrophoresis and Radioactivity Counting. Basic proteins were separated from the bulk of the cell proteins and from each other by two-dimensional gel electrophoresis as previously described (DePhilip et al., 1980). The second-dimension slab gels were stained and destained according to Cleveland et al. (1977).

Ribosomal proteins were named according to Ramjoué & Gordon (1977). Proteins designated as nonribosomal were not detectable in stained electrophoretograms of acetic acid extracts of washed polyribosomes. Non-r proteins were assigned letters.

Plugs from the slab gels were subjected to a third electrophoretic step in $NaDodSO_4$ cylinder gels (Laemmli, 1970), exactly as before (DePhilip et al., 1980). Non-r proteins a, b, h, i, j, k, and l, however, were counted directly from the slab gels since they were too faintly stained to be seen in the $NaDodSO_4$ gels. Spots a and b, undoubtedly, represented a mixture of different proteins.

For counting, the stained bands from the $NaDodSO_4$ gels were cut out and kept in 10% methanol for 2–4 h when they were blotted, quartered, and incubated at 38 °C for 3 or 4 days in 10 mL of 7.5% Protosol in Econofluor. Plugs (4.8 mm in diameter) to be counted directly from the slab gels were kept in water for 2 h and were blotted, quartered, and incubated at 38 °C for 2 or 3 days in 10 mL of 5% Protosol in Econofluor. The decolorized and swollen gels were discarded before counting. More than 80% of the radioactivity was consistently extracted from the gels.

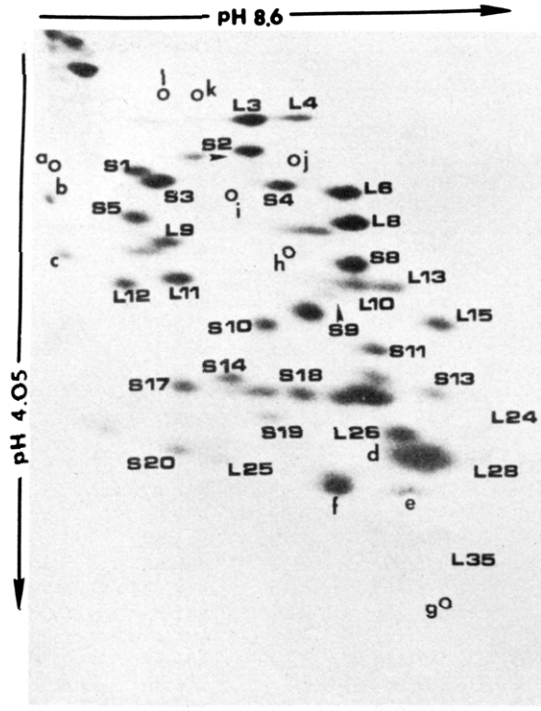

FIGURE 1: Map of basic proteins. The circles indicate the positions of non-r proteins that did not contain enough stain to be seen in the photograph of the slab gel.

There was no carry-over of ^{3}H into the ^{14}C channel, and only one-half of the ^{14}C spilled over into the ^{3}H channel. Carry-over of ^{14}C into the ^{3}H channel was estimated by adding 1 μL of [^{14}C]leucine (20 000 cpm) to vials that had already been counted. All samples of individual proteins were counted to a probable error of less than 4% (Cooper, 1977).

Rates of Synthesis of Total Cell Protein. Relative rates of formation of total protein were estimated from the ^{3}H/^{14}C ratios of the cell homogenates. To a 25-μL sample of a homogenate were added 500 μg of bovine serum albumin and 2 mL of 10% trichloroacetic acid–50 mM leucine. Insoluble material was collected on a filter and was washed with trichloroacetic acid, ethanol, and ether. The dried residue was then suspended in 2 mL of 0.05 M NaOH, and, after boiling for 30 min, 0.2 mL of the supernatant fluid was counted. Carry-over of ^{14}C into the ^{3}H channel was measured as for the resolved proteins.

Results

Electrophoretic Map of Basic Proteins. Figure 1 shows the distribution of the stained r and non-r proteins in the slab gel. Only the spots that were studied in quantitative experiments are numbered.

Effect of Cycloheximide on the Synthesis of r Proteins As Judged by Fluorography of Slab Gels. Two-dimensional gels were examined fluorographically as a preliminary step in studying the relative effects of cycloheximide on the synthesis of basic r and non-r proteins. Unlabeled confluent cultures were allowed to incorporate [^{3}H]leucine in the absence and presence of the drug, acetic acid extracts of the labeled cells were fortified with carrier r proteins, the basic proteins were resolved, and the slab gels were stained and fluorographed. Figure 2A,B represents the fluorograms of the proteins from the control and cycloheximide-treated cells, respectively. It can be seen that cycloheximide increased the labeling of the r proteins relative to that of the non-r proteins.

Quantitation of the Effects of Cycloheximide. As described under Materials and Methods, the synthesis of basic r and

Table I: Effects of Cycloheximide on Radioactivities and A_i Values of r Proteins and Non-r Proteins[a]

	cycloheximide (μg/mL)									
	0		0.03 (29%)[b]		0.06 (45%)		0.1 (55%)		0.25 (71%)	
protein	$^3H/^{14}C$ (cpm)	A_i	$^3H/^{14}C$ (cpm)	A_i	$^3H/^{14}C$ (cpm)	A_i	$^3H/^{14}C$ (cpm)	A_i	$^3H/^{14}C$ (cpm)	A_i
S1	1010/293	3.45	1223/198	8.70	1672/225	13.5	2257/332	15.1	1309/264	17.1
S2	940/283	2.46	1126/285	5.56	1441/306	8.56	1282/324	8.79	708/258	9.46
S3	1002/286	3.50	778/159	6.89	1461/235	11.3	1839/332	12.3	1467/312	16.2
S4	1197/425	2.82	1935/408	6.68	2181/348	11.4	2191/449	10.8	1522/397	13.2
S5	375/99	3.79	388/68	8.03	473/75	11.5	774/138	12.5	352/116	10.5
S8	949/244	3.89	1214/206	8.30	1393/189	13.4	1889/285	14.7	1367/263	17.9
S9	453/122	3.71	898/141	8.97	766/98	14.2	945/153	13.7	499/113	15.2
S10	802/222	3.61	975/191	7.19	1252/193	11.8	1379/252	12.2	811/208	13.4
S11	716/212	3.38	1070/210	7.18	1102/177	11.3	1302/252	11.5	705/204	11.9
S13	472/154	3.06	952/226	5.93	815/155	9.56	884/198	9.92	594/193	10.6
S14	649/137	4.74	908/152	8.41	959/136	12.8	717/127	12.5	488/117	14.4
S17	636/209	3.04	893/211	5.96	1111/205	9.85	1161/243	10.6	690/213	11.2
S18	666/210	3.17	1101/197	7.87	1357/199	12.4	1586/242	14.6	914/203	15.5
S19	288/100	2.88	438/82	7.52	489/72	12.3	569/95	13.3	473/102	16.0
S20	397/136	2.92	433/95	6.42	526/94	10.2	806/161	11.1	504/137	12.7
L3	674/302	2.23	944/227	5.85	1069/210	9.25	805/197	9.10	797/243	11.3
L4	308/99	3.11	406/88	6.50	294/50	10.7	323/65	11.0	253/78	11.2
L6	1528/439	3.48	2018/384	7.40	2009/317	11.5	2583/462	12.4	1549/399	13.4
L8	736/259	2.84	981/212	6.52	1447/237	11.1	1683/303	12.3	1267/285	15.3
L9	617/151	4.09	698/136	7.23	354/56	11.5	761/141	12.0	425/116	12.6
L10	387/114	3.39	484/94	7.25	500/77	11.8	344/63	12.1	346/97	12.3
L11	259/58	4.46	274/46	8.39	653/96	12.4	315/60	11.7	296/101	10.1
L12	512/194	2.64	567/136	5.87	1061/187	10.3	1041/239	9.67	868/173	10.4
L13	201/83	2.42	264/70	5.31	245/49	9.09	194/46	9.37	204/73	9.64
L15	352/112	3.14	421/85	6.97	524/87	11.0	511/97	11.7	371/103	12.4
L24	257/52	4.94	251/43	8.22	276/42	11.9	288/49	13.1	242/58	14.4
L25	284/67	4.24	581/107	7.65	420/69	11.1	390/76	11.4	337/94	12.4
L26	248/80	3.10	359/76	6.65	439/72	11.1	459/91	11.2	277/87	11.0
L28	236/58	4.07	608/88	9.73	575/68	15.4	658/87	16.8	362/79	15.8
L35	123/31	3.97	255/45	7.98	311/45	12.6	222/37	13.3	204/51	13.8
a	2025/137	14.8	727/61	16.8	900/105	15.6	1191/156	17.0	486/108	15.5
b	4765/386	12.3	1861/238	11.0	1915/303	11.5	1613/301	11.9	666/219	10.5
c	1220/180	6.77	888/161	7.77	845/189	8.13	686/193	7.90	420/176	8.23
d	3929/589	6.67	2915/539	7.62	2483/562	8.03	2347/704	7.41	904/608	5.12
e	4005/681	5.88	1845/370	7.02	2345/639	6.67	2079/702	6.58	1215/631	6.64
f	1893/381	4.97	1344/333	5.68	929/345	4.90	1108/501	4.91	449/403	3.84
g	451/177	2.55	355/167	2.99	235/149	2.87	271/209	2.88	144/187	2.65
h	2358/174	13.5	1149/156	10.4	591/90	11.9	990/207	10.6	714/198	12.4
i	730/161	4.53	417/139	4.22	403/150	4.88	543/219	5.51	251/194	4.46
j	647/110	5.88	479/125	5.40	457/140	5.94	397/150	5.88	377/167	7.78
k	1025/91	11.3	675/85	11.2	530/89	10.8	426/81	11.7	311/95	11.3
l	868/52	16.7	591/52	16.0	535/56	17.4	575/76	16.8	293/60	16.8

[a] Pairs of confluent cultures of chick embryo cells that had been grown up with [^{14}C]leucine were washed and then preincubated for 30 min in 2 mL of modified basal medium (0.1 mM leucine). At zero time, the preincubation medium was replaced with 2 mL of fresh modified basal medium that contained cycloheximide, as indicated, and [^3H]leucine was added after 30 min. Labeling was for 30 min. The cultures were then washed with ice-cold basal medium, the cells of the paired cultures were harvested, pooled, and extracted with 67% acetic acid, and 300 μg of unlabeled r proteins was added to each of the extracts. Estimation of f and resolution of individual basic proteins with two-dimensional gels followed by electrophoresis of plugs in $NaDodSO_4$ gels were as described under Materials and Methods. [b] The values in parentheses represent the percent inhibitions of [^3H]leucine incorporation into total cell protein.

non-r proteins was measured with cells that had been grown up with [^{14}C]leucine and were then briefly labeled with [^3H]leucine. The ^{14}C content of an individual protein provided a measure of its recovery. A minor modification of the equation of Dennis (1974) was used to relate the synthesis of protein i to that of total cell protein. In the modified equation

$$A_i = \frac{^3H/^{14}C \text{ in i protein}}{f}$$

f adjusts for the relative rates of total protein synthesis in the control and experimental cells as measured by the $^3H/^{14}C$ ratios of cell homogenates. The $^3H/^{14}C$ ratio of the control cells was set at 1.0, and the values of f for the experimental cells were the quotients of the ratios of the homogenates of experimental cells/control cells.

Four concentrations of cycloheximide that reduced the incorporation of [^3H]leucine into total cell protein by 29–71% were studied. The effects of the drug on the radioactivities and A_i values of 30 r proteins and 12 non-r proteins are shown in Table I. It can be seen from the table, first, that the ^{14}C contents of the individual proteins were not changed by cycloheximide, indicating that the drug had no effect on the stability or extractability of the preformed r proteins. Second, cycloheximide raised the $^3H/^{14}C$ ratios of the r proteins by as much as 2-fold, whereas it depressed those of the non-r proteins to the same degrees as those of the total cell protein. Finally, the A_i values of the r proteins, unlike those of the non-r proteins, were gradually elevated by the increasing levels of cycloheximide to a maximum of 4–5-fold.

In six additional experiments with a level of cycloheximide (0.07 μg/mL) that inhibited total protein synthesis by about 50%, the $^3H/^{14}C$ ratios of the r proteins were invariably raised. The increases with the different batches of cells varied, however, from 1.3- to slightly more than 2-fold.

Lack of Effect of Cycloheximide on the Extractability of Nascent r Proteins with Acetic Acid. Two trivial explanations

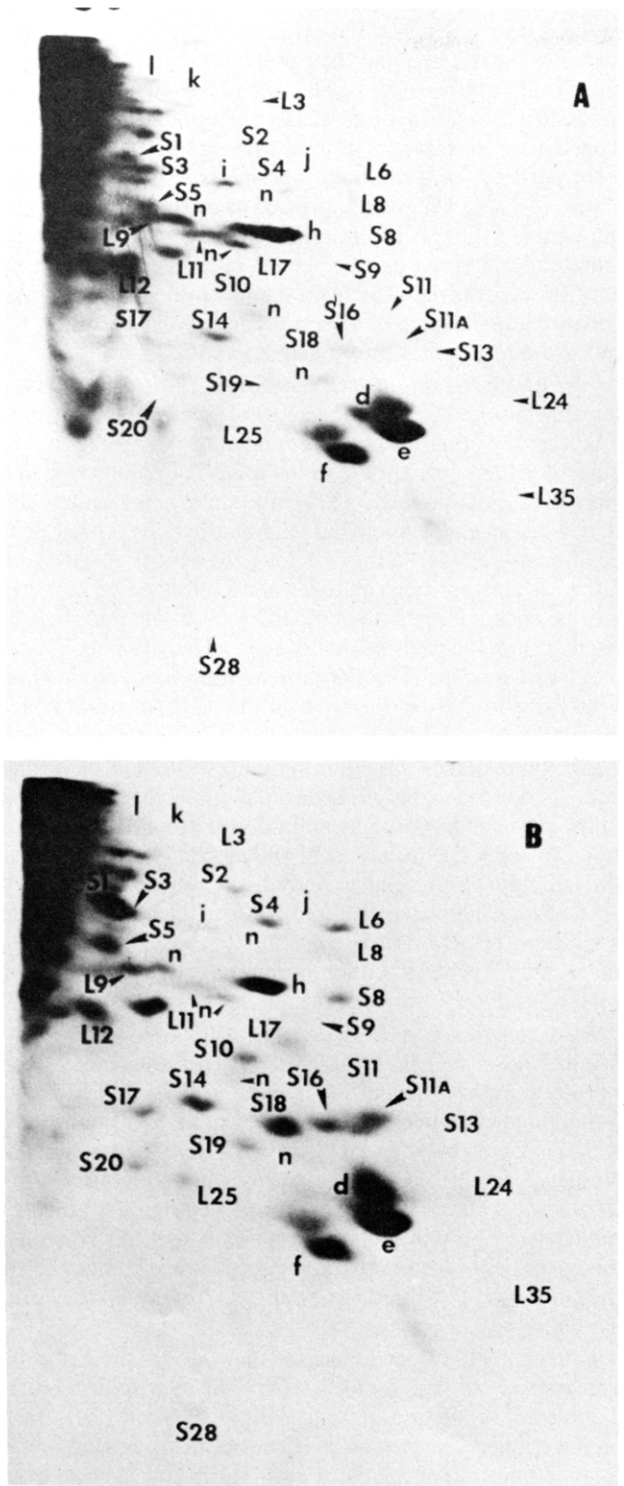

FIGURE 2: Preferential effect of cycloheximide on the synthesis of r proteins as visualized by fluorography. Two pairs of unlabeled cultures of chick embryo fibroblasts were washed and preincubated for 30 min in modified basal medium (0.1 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified basal medium, and each of one of the pairs of cultures received, in addition, 0.14 μg of cycloheximide. After 30 min, the cultures were labeled with [^{3}H]leucine for 0.5 h, the pooled cells from each pair of cultures were extracted with 67% acetic acid, and carrier r proteins (control, 600 μg; cycloheximide, 300 μg) were added to the extracts. Approximately 5×10^6 cpm from each extract were used for two-dimensional electrophoresis. The slab gels were then stained, impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974), dried, and exposed to X-ray film for 20 days at −80 °C. The non-r proteins that had not been assigned specific names are designated by n. Panels A and B: control and cycloheximide, respectively.

Table II: Lack of Effect of Cycloheximide on the Extractability of Nascent r Proteins with Acetic Acid[a]

	^{3}H/^{14}C			
	control		cycloheximide	
protein	before chase	after chase	before chase	after chase
S1	2.67	2.49	5.32	5.16
S2	2.12	2.37	3.40	4.12
S3	3.16	2.63	4.63	4.95
S4	2.14	2.32	4.23	4.76
S8	2.96	2.93	5.12	5.09
S10	2.90	2.74	4.35	4.68
S11	2.69	2.79	3.72	4.70
S20	2.38	2.08	3.81	3.99
L4	2.80	2.18	4.37	3.99
L6	2.82	2.28	4.84	3.76
L8	2.22	2.22	4.23	4.63
L13	1.71	1.95	3.42	3.74
L15	2.65	2.26	4.21	3.82
L26	2.66	2.15	4.33	3.81
L28	3.43	2.88	7.03	5.75
a	13.1		8.90 (16.5)[b]	
b	12.3		7.85 (14.5)	
c	5.68		3.56 (6.59)	
d	3.84		2.19 (4.05)	
e	3.72		2.33 (4.31)	
f	3.13		1.76 (3.26)	
g	1.86		1.12 (2.08)	
h	9.56		4.89 (9.05)	
i	5.49		3.22 (5.96)	
j	4.17		3.03 (5.61)	
k	10.9		5.13 (9.50)	
l	15.2		8.15 (15.1)	

[a] Confluent cultures of chick embryo cells that had been grown up with [^{14}C]leucine were washed and then preincubated for 30 min in modified basal medium (0.1 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified medium, and one-half of the cultures received, in addition, 0.14 μg of cycloheximide. Labeling with [^{3}H]leucine was from 30 to 60 min. At the end of this time, pairs of control and drug-treated cultures were extracted with 67% acetic acid, and the radioactive medium from the rest of the cultures (five/group) was replaced with 2 mL of unlabeled medium containing 4.4 mM leucine. After 2.5 h of chase, the cultures were harvested, and the cells were mixed with those from 24 unlabeled cultures. Polyribosomes, prepared from the postmitochondrial fraction with sodium deoxycholate, were extracted with 67% acetic acid. Finally, carrier r proteins were added to the acetic acid extracts of the whole cells, and basic proteins were resolved from all the preparations by two or three electrophoretic steps. [b] The numbers in parentheses represent the values of A_i ($f = 0.54$).

were considered for the ability of cycloheximide to elevate the ^{3}H/^{14}C ratios of the r proteins. The first, a destruction of ^{14}C-prelabeled molecules in the drug-treated cells, could not account for the results of Figure 2 and, in addition, was eliminated by the data of Table I. According to the second, cycloheximide would have induced cellular changes that increased the extractability with acetic acid of newly made r proteins, considered to be in the nucleus (Warner, 1979), but not of the preformed, ^{14}C-labeled cytoplasmic molecules.

The second possibility was tested with [^{14}C]leucine-prelabeled cells that were given [^{3}H]leucine for 30 min in the absence or presence of cycloheximide and were then chased for 150 min in unlabeled, inhibitor-free culture medium. Under the conditions that were used, the chase was immediately effective (DePhilip et al., 1980). Extraction of protein with 67% acetic acid was from the whole cells at the end of the 30-min labeling period and from polyribosomes after the chase. Table II shows the results that were obtained with 15 r and 12 non-r proteins. It can be seen from the table that exposure of the cells to the drug did not affect the extractability

Table III: Comparison with Cycloheximide of the Effects of Other Inhibitors of Elongation and of MDMP on the A_i Values of r and Non-r Proteins[a]

	(ΔA_i with test drug)/(ΔA_i with cycloheximide)			
protein	anisomycin	emetine	puromycin	MDMP
S1	0.96	0.87	−0.03	−0.07
S2	0.86	1.33	0.11	−0.03
S3	0.95	1.19	0.09	−0.08
S4	0.95	0.95	0.01	−0.06
S5	0.91	1.33	0.23	−0.12
S7	1.53	0.93		0.04
S8	1.26	1.24	0.11	0.07
S10	0.72	1.07	−0.13	−0.07
S11	1.10	1.26	−0.01	−0.15
S17	0.97	1.47	0.21	0.06
S20	1.46	1.29	0.14	−0.09
L3	0.90	0.78		0.12
L4	1.07	0.99		−0.13
L6	0.90	1.00	0.08	0.18
L8	1.19	1.31	−0.01	0.13
L11	1.33	1.20	0.08	−0.03
L13	1.35	1.01		−0.11
L17	1.06	1.36	0.06	−0.12
L26	1.07	1.26	−0.01	−0.19
L28	0.94	0.88		−0.01
a	0.94	1.21	1.38	0.97
b	0.94	0.95	1.17	0.96
c	1.00	0.88	1.24	1.14
d	1.47	1.47	0.98	0.73
e	1.11	1.52	0.94	0.77
f	1.49	1.36	0.79	0.81
g	0.96	1.46	0.93	0.94
h	1.02	0.99	0.99	0.80
i	1.18	1.11	1.12	0.95
j	0.87	0.97	0.95	0.86
k	1.22	0.89	0.91	0.93
l	1.15	1.02	1.17	0.92

[a] All the inhibitors except MDMP were dissolved in water. The stock solution of MDMP (20 mg/mL) was in ethanol, and dilutions were made in water. A culture treated with MDMP received less than 5×10^{-4} mL of ethanol. The results of five separate experiments are shown. Each was comprised of pairs of cultures with no inhibitor, cycloheximide (0.07 μg/mL), and the test drug. Treatment of cultures, labeling conditions, and resolution of basic proteins were exactly as those described for Table I. The concentrations of the test drugs used and the values of f, respectively, were as follows: anisomycin, 0.06 μg/mL, 0.46; emetine, 0.6 μg/mL, 0.51; puromycin, 1.5 μg/mL, 0.64; MDMP, 3.5 μg/mL, 0.47. The values of f for cycloheximide in the separate experiments ranged from 0.48 to 0.56. The results are presented as the quotients of the differences from the control values of A_i (no drug) produced by the test drug/cycloheximide.

of the newly made r proteins. Thus, the $^3H/^{14}C$ ratios of the r proteins from the briefly labeled cells and the polyribosomes were the same whether or not the cultures had been treated with cycloheximide.

Other Inhibitors of Protein Synthesis. Three additional inhibitors of elongation, anisomycin (Grollman, 1967), emetine (Grollman, 1968), and puromycin (Yarmolinsky & de la Haba, 1959), and an agent that is considered to suppress the joining of the 60S subribosome to the initiation complex, MDMP (Weeks & Baxter, 1972), were compared with cycloheximide for their abilities to increase preferentially the A_i values of the r proteins. It can be seen from Table III that two of the inhibitors of elongation, anisomycin and emetine, were as effective as cycloheximide. Puromycin and MDMP, on the other hand, had no effect.

Translational Inhibitors and α_r. The chick embryo cells use all or virtually all newly made r proteins for the production of cytoplasmic ribosomes (DePhilip et al., 1980; Table II). This made it possible to study the effects of translational inhibitors on r protein synthesis by an independent procedure that involved neither the use of a culture medium with a reduced level of leucine, the extraction of proteins with acetic acid, nor the resolution of the r proteins by gel electrophoresis. Confluent cultures were labeled with [^{3}H]leucine for 2.5 h in unmodified basal medium that was supplemented with cycloheximide, anisomycin, or puromycin, and 60S subribosomes were purified from the polyribosome fraction. The results, shown in Table IV, were expressed as α_r (Schlief, 1967), the quotients of the specific activities of the ribosomal core protein/total cell protein. It can be seen from the table that, just as with the resolved proteins, cycloheximide and anisomycin enhanced the labeling of the r proteins relative to that of total cell protein, whereas puromycin had little or no effect.

Agents That Inhibit an Early Step in Initiation. Hypertonicity, most commonly an excess of NaCl, has been shown to inhibit the initiation of translation at or before the step that binds the 40S subribosome to the mRNA (Wengler & Wengler, 1972; Saborio et al., 1974), and there is reason to believe that 3-methyleneoxindole has a similar action (Abreu & Lucas-Lenard, 1977). Table V shows the results that were obtained when confluent cultures of ^{14}C-prelabeled cells were labeled with [^{3}H]leucine in modified basal medium that had been supplemented with three levels of NaCl or 3-methyleneoxindole. The 23 r proteins that were examined have been listed roughly in the order of the increasing effect of the highest level of NaCl on the values of A_i.

The table makes the following points. First, hypertonicity and 3-methyleneoxindole behaved similarly, adding support to the status of the drug as an inhibitor of an early initiation step. Second, the influence of the agents on the labeling of the individual r proteins varied all the way from little or no preferential inhibition (S3 and S10) to an almost complete inhibition (L3, L4, and L15). Finally, the A_i values of 11 of the 12 non-r proteins either were unaffected or were raised by the inhibitors, but one, that of spot c, was lowered.

Incorporation of [^{3}H]Leucine into Total Cell Protein in Methionine-Free Culture Medium. The resistance of some r proteins to the action of the hypertonic culture medium and 3-methyleneoxindole could have been due to contamination with non-r proteins whose $^3H/^{14}C$ ratios were higher than those of the r proteins. One way to test this possibility was to fluorograph stained gels in order to see if the stained and radioactive r protein spots were superimposable. To increase the intensities of the fluorographic spots, it was planned to label the cells with [^{35}S]methionine in a methionine-free culture medium.

Pain et al. (1980) have reported that the initiation of protein synthesis is reduced by 60% when Ehrlich ascites cells are deprived of lysine or glutamine. Figure 3 shows that, for the period studied, the rate of [^{3}H]leucine incorporation into the total cell protein of the chick embryo fibroblasts was hardly affected by the omission of methionine from the culture medium.

Effects of Hypertonic Culture Medium on the Labeling of r Proteins As Judged by Fluorography of Stained Slab Gels. Confluent chick embryo cultures were labeled with [^{35}S]-methionine for 30 min in methionine-free basal medium and in the same medium supplemented with 120 mM NaCl. Acetic acid extracts of the labeled cells were fortified with carrier r proteins, the mixtures were subjected to two-dimensional gel electrophoresis, and the slab gels were stained and fluorographed. Exposure times were adjusted to compensate for the lower counts in the inhibited extract.

Figure 4A,B, the fluorograms of the control and hypertonically treated cells, respectively, shows that the intensities

Table IV: Effects of Inhibitors of Translation on α_r [a]

expt	inhibitor	inhibitor concn (μg/mL)	total protein synthesis		ribosome synthesis	
			cpm/μg	% inhibition	cpm/μg of core protein	α_r
1	none		336		47	0.14
	cycloheximide	0.017	284	16	54	0.19
		0.035	238	29	57	0.24
		0.07	181	46	62	0.34
		0.21	109	68	36	0.33
2	none		253		35	0.14
	anisomycin	0.07	150	41	49	0.33
		0.35	53	79	17	0.32
	cycloheximide	0.07	128	49	50	0.39
3	none		251		31	0.12
	puromycin	1.5	133	47	21	0.16
		3.0	85	66	14	0.17
	cycloheximide	0.07	110	56	43	0.39

[a] Confluent cultures of chick embryo cells (24 cultures/group) were preincubated for 30 min in 2 mL of basal medium (0.4 mM leucine). At the end of this time, the medium was replaced with 2 mL of fresh basal medium containing 10 μCi of [^{3}H]leucine and the indicated level of inhibitor. After 2.5 h of labeling, the cells were harvested and homogenized, and polyribosomes were prepared from the postmitochondrial supernatant fraction as described for Table II. Purification of 60S derived subribosomes from the polyribosomes and the estimation of the specific activities of the proteins of the cell homogenates and the 60S derived cores were exactly as previously detailed (DePhilip et al., 1979).

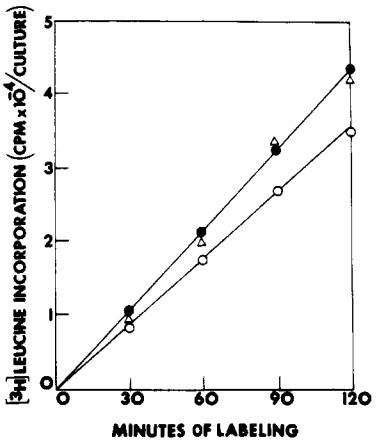

FIGURE 3: Incorporation of [^{3}H]leucine into total cell protein in methionine-free culture medium. Confluent cultures of chick embryo fibroblasts were washed twice and then preincubated for 45 min in 2 mL of basal medium that lacked methionine. Control cultures were treated in the same way with complete basal medium. At zero time, the media were replaced with 2 mL of fresh methionine-free or complete basal medium, as appropriate, one-half of the deprived cultures each received, in addition, 0.2 μmol of methionine, and all the cultures were given 2 μCi of [^{3}H]leucine. Protein synthesis was stopped at the times indicated, and acid-insoluble radioactivity was measured as described under Materials and Methods. (○) Preincubated and labeled in methionine-free medium; (●) Preincubated in methionine-free medium and labeled in the same medium supplemented with methionine; (△) Preincubated and labeled in complete basal medium.

of the majority of the non-r spots were similar in the two X-ray films. The reduced intensities of at least some of the non-r spots, for example, d, e, f, and h, were unexpected (d, e, and f are histones) and may have resulted from the combined impact of the methionine deficiency and the excess NaCl. The effects of hypertonicity on the labeling of the r proteins ranged from almost a complete suppression (for example, L3, L4, and L15), to a marked but less dramatic reduction (for example, S1, S8, and L17), to little or no preferential inhibition (S3, S10, and S14).

The refractory behavior of S3, S10, and S14 did not appear to be an artifact due to radioactive contamination. Although the X-ray spots were slightly larger than the stained ones, the outlines of the radioactive and stained spots were identical. Radioactive contamination may, however, have contributed to the high A_i values of several of the other r proteins in the experiment with [^{3}H]leucine. Thus, the fluorograms show that excess NaCl preferentially depressed the labeling of S1, L11, and L17, for instance, to a greater extent than was indicated by the values of Table V.

Lack of Effect of Hypertonic Culture Medium on the Stability of Nascent r Proteins. The reduction in the A_i values of the r proteins by hypertonicity could have resulted from a preferential inhibition of synthesis or from an enhanced rate of degradation of nascent molecules. To distinguish between these possibilities, ^{14}C-prelabeled cells were given [^{3}H]leucine for 30 min in isotonic or hypertonic medium, and they were then chased for 1 h. The extraction of protein was from whole cells after both the labeling and the chase periods. For the sake of comparison, the experiment included cultures that had been labeled and chased in isotonic medium containing 10 μg/mL actinomycin D. In the absence of rRNA formation, newly made r proteins in the chick embryo cells decay with half-lives that range from about 15 min to more than 1 h (DePhilip et al., 1980).

Table VI shows the values of A_i of ten r and six non-r proteins at the end of the labeling period and the fates of the proteins during the chase period. It can be seen that hypertonicity destabilized none of the r proteins, not even those that decayed rapidly in the absence of rRNA synthesis, nor did the excess NaCl cause any of the newly made non-r proteins to break down.

Discussion

Chick embryo fibroblasts were grown to confluency in a medium that contained [^{14}C]leucine. The resting cells, in which r protein synthesis had fallen to a low level, were used to compare the effects of various inhibitors of translation on the incorporation of [^{3}H]leucine into total cell protein and individual non-r proteins, on the one hand, and individual r proteins, on the other. The ^{14}C content of a resolved protein served as a measure of its recovery, the ^{3}H/^{14}C ratio as a measure of its synthesis or accumulation.

Levels of cycloheximide that reduce the ^{3}H/^{14}C ratios of total cell protein and of individual non-r proteins by as much as 70% have the opposite effect on the 30 r proteins that were examined. The ^{3}H/^{14}C ratios of the r proteins are raised by up to 2 times.

Table V: Effects of Hypertonic Culture Medium (NaCl) and 3-Methyleneoxindole (MOI) on the Values of A_i of r and Non-r Proteins[a]

	A_i (NaCl)/A_i (no NaCl) NaCl added (mM)				A_i (MOI)/A_i (no MOI) MOI (μg/mL)		
protein	0	85	110	125	5	10	20
S3	6.12	0.82	0.87	0.84	0.93	0.82	0.74
S10	5.95	0.96	1.04	1.18	0.99	1.04	0.79
S1	5.53	0.74	0.79	0.74	0.94	0.76	0.71
L11	7.24	0.77	0.63	0.74	1.07	0.65	0.44
L17	6.86	0.81	0.80	0.77	0.99	0.67	0.56
S11	5.69	0.73	0.71	0.56	0.91	0.71	0.66
S13	6.18	0.78	0.71	0.65	0.85	0.66	0.52
S17	7.36	0.69	0.67	0.52	1.11	0.72	0.53
S18	5.03	0.70	0.68	0.53	0.89	0.55	0.45
L12	5.88	1.61	0.76	0.58	0.82	0.50	0.50
L24	7.72	0.85	0.84	0.52	0.90	0.77	0.58
S2	4.17	0.53	0.48	0.37	0.77	0.49	0.42
S4	4.32	0.63	0.60	0.41	0.81	0.73	0.49
S5	6.11	0.62	0.57	0.38	0.83	0.53	0.43
S8	5.60	0.79	0.73	0.41	0.84	0.68	0.47
L8	5.00	0.55	0.47	0.33	0.73	0.32	0.22
L13	4.32	0.63	0.60	0.41	0.70	0.51	0.59
L15	5.17	0.56	0.48	0.26	0.86	0.43	0.44
S20	4.22	0.41	0.34	0.22	0.76	0.44	0.34
L3	3.42	0.44	0.29	0.19	0.69	0.21	0.15
L4	5.63	0.52	0.37	0.10	0.74	0.38	0.17
L10	4.91	0.67	0.53	0.11	0.73	0.39	0.11
L28	6.79	0.40	0.31	0.10	0.76	0.47	0.29
a	20.1	0.96	0.98	0.72	1.00	1.05	1.01
b	23.8	0.96	0.87	1.09	1.02	1.06	1.06
c	11.2	0.74	0.61	0.49	1.00	0.81	0.65
d	11.3	1.25	1.34	1.17	0.97	1.03	0.79
e	7.37	1.01	1.43	1.89	0.99	1.05	1.15
f	6.20	0.96	1.27	1.82	0.89	1.12	1.25
g	2.79	0.74	0.83	1.03	1.05	0.82	1.14
h	25.2	0.91	1.23	1.03	1.42	1.08	0.85
i	8.83	1.21	1.44	1.43	0.88	0.98	1.12
j	15.4	0.88	1.00	1.10	1.19	0.87	1.02
k	18.3	1.21	1.52	1.38	1.01	1.22	1.11
l	27.8	1.04	1.24	1.25	1.06	1.21	1.13

[a] 3-Bromooxindole-3-acetic acid was dissolved in water (2 mg/mL) in which it was rapidly debrominated and decarboxylated to yield 3-methyleneoxindole (Hinman & Bauman, 1964). Solutions were made within 10 min of use. Pairs of confluent cultures of chick embryo cells that had been grown up with [^{14}C]leucine were washed and preincubated for 30 min in 2 mL of modified basal medium (0.1 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified basal medium that contained excess NaCl or 3-methyleneoxindole, as indicated. After 30 min, each culture received 800 μCi of [^{3}H]leucine, and harvesting was 0.5 h later. Extraction of protein from the cells was with 67% acetic acid, each extract received 300 μg of unlabeled r proteins, and resolution and counting of the individual basic proteins were as described under Materials and Methods. The values of f for the hypertonic cultures were 0.62, 0.47, and 0.22, and for those treated with MOI, 0.83, 0.56, and 0.28.

Cycloheximide does not elevate the ^{3}H/^{14}C ratios of the r proteins by triggering the destruction of a fraction of the ^{14}C-prelabeled r proteins. Nor does the drug act by stabilizing or enhancing the extractability with acetic acid of the nascent ^{3}H-labeled molecules. Rather, the inhibitor causes an absolute increase in the rate of production of the r proteins.

The stimulation of r protein synthesis by cycloheximide would seem to depend upon an inhibition of translational elongation and not on some secondary action of the drug. Thus, anisomycin and emetine, two other inhibitors of elongation, are just as effective as cycloheximide in selectively raising the rate of r protein formation in the resting chick embryo cells.

Thach and his co-workers (Jen at al., 1978; Ramabhadran & Thach, 1980) have studied the effects of cycloheximide, anisomycin, and emetine on the synthesis of viral and host proteins in ascites tumor and L cells. They found that all three agents interfere preferentially with the formation of viral protein but, contrary to the results with the chick embryo cells,

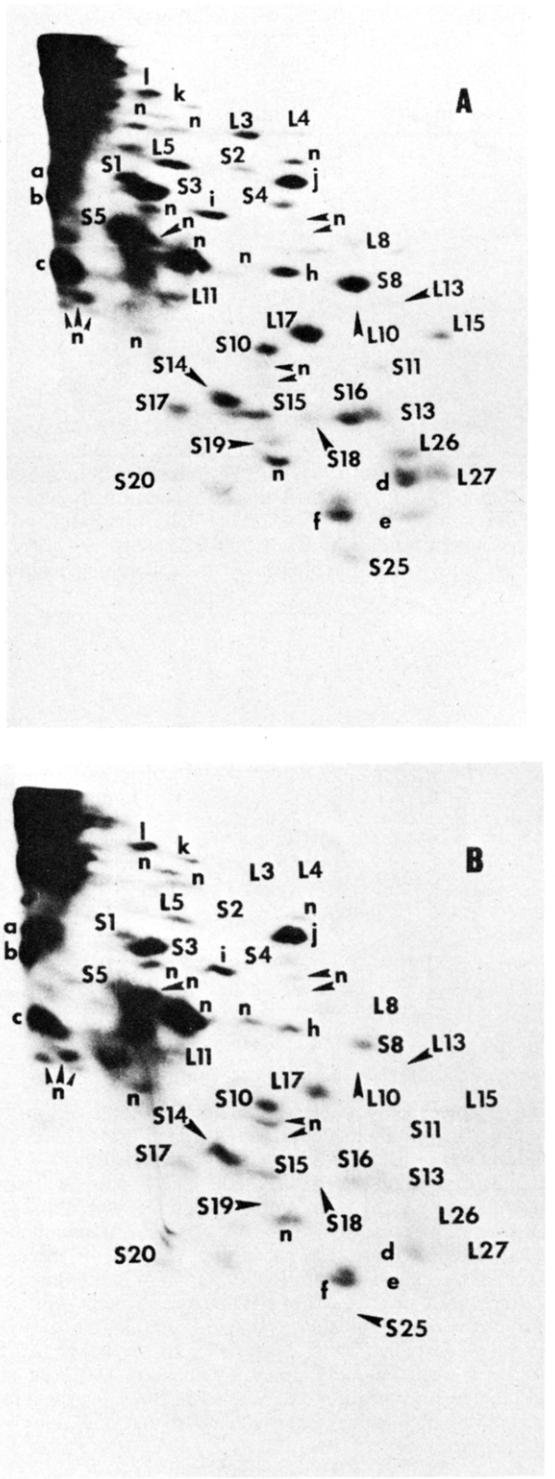

FIGURE 4: Effects of hypertonic culture medium on the labeling of r and non-r proteins as visualized by fluorography. The cultures were treated as described for Figure 2 except that preincubation and labeling were in methionine-free medium and the experimental cultures each received 250 μmol of NaCl instead of cycloheximide. Labeling was with 25 μCi/culture of [^{35}S]methionine (1050 Ci/mmol). The total acetic acid extracts (control, 13 × 10^{6} cpm; hypertonic, 4 × 10^{6} cpm) were applied to the gels, the gels were fluorographed as described for Figure 2, and the control gel was exposed for 48 h, the experimental gel for 128 h. The non-r proteins that had not been assigned specific names are designated by n. Panels A and B: control and excess NaCl, respectively.

Table VI: Stability of Nascent r Proteins in Cells in Hypertonic Culture Medium[a]

protein	A_i before chase			($^3H/^{14}C$ after chase)/($^3H/^{14}C$ before chase)		
	control	hyper-tonic	actino-mycin	control	hyper-tonic	actino-mycin
S2	5.36	1.45	5.39	0.94	1.37	1.10
S4	6.02	3.53	4.28	0.87	0.92	0.14
S5	7.83	3.96	7.76	0.75	0.72	0.50
S8	6.85	3.36	6.32	0.96	1.03	1.26
S20	5.62	1.37	6.43	0.99	0.88	1.18
L3	4.75	0.89	4.87	1.02	1.11	0.37
L8	5.77	1.85	5.13	1.01	0.93	0.42
L13	6.70	2.90	4.77	1.03	0.94	0.47
L15	6.12	1.81	4.75	0.94	1.09	0.23
L28	6.64	1.92	6.72	0.84	0.89	0.82
a	26.3	24.4	21.8	0.93	0.92	1.05
b	25.6	26.6	24.5	0.94	0.95	0.98
d	8.69	10.1	5.69	0.94	0.71	0.71
g	6.20	5.79	2.94	1.16	0.90	0.94
j	10.0	12.9	10.0	1.14	1.32	0.89
k	20.8	29.6	18.3	0.89	1.17	0.97

[a] Confluent cultures of chick embryo cells that had been grown up with [^{14}C]leucine were washed and preincubated for 45 min in 2 mL of modified basal medium (0.1 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified basal medium that had been supplemented with 250 μmol of NaCl or 20 μg of actinomycin D, as shown. After 15 min, each culture received 800 μCi of [^3H]leucine, and labeling was for 0.5 h. At the end of this time, pairs of cultures were extracted with 67% acetic acid, and the radioactive medium from the remainder (four/group) was replaced with 2 mL of unlabeled medium containing 4.4 mM leucine and excess NaCl and actinomycin D, as appropriate. After 60 min, the chased cultures were harvested, and the cells were mixed with those from 24 unlabeled cultures. The preparation and extraction of polyribosomes and the resolution and counting of the basic proteins of the whole cell and polyribosomal extracts were the same as described for Table II. The values of f for the hypertonic and actinomycin D treated cultures were 0.26 and 0.92, respectively.

not with equal effectiveness. Cycloheximide gives the largest discrepancy between viral and host protein production, emetine, the smallest. The graded effects of the agents were ascribed to an inhibition by anisomycin and emetine, but not by cycloheximide, of a process other than elongtion, perhaps a step in initiation. It would seem that the avian cells are resistant to the side effects of the two drugs.

According to the model of Lodish (1974), the selective enhancement of r protein synthesis by inhibitors of elongation means that the r protein mRNAs in the resting fibroblasts are initiated with a lower frequency than are most cell messages. Infrequent initiation, in turn, is taken as a sign that the r protein mRNAs have a poor affinity for a limiting component of the initiation system that acts at or before the binding of the 40S subunit to the mRNA. In the absence of elongation inhibitors, the limiting factor is used primarily for the initiation of high-affinity messages. A partial block of elongation impedes the initiation of the efficient mRNAs, thereby making the factor more available for use by the r protein messages.

Interpretation of the results with the inhibitors of elongation in terms of the Lodish model leads to the following predictions: (1) Puromycin will have no preferential effect on r protein synthesis. Premature termination of growing polypeptides would not be expected to raise the cellular level of the limiting initiation component. (2) Drugs, such as MDMP, that inhibit initiation after the 40S subunit has joined the mRNA will likewise be without selective action. (3) Agents that slow down an early step in initiation will preferentially depress r protein production.

The predictions were satisfied. Puromycin and MDMP do not change the rate of r protein synthesis relative to that of total cell protein. Excess NaCl and 3-methyleneoxindole, agents that are considered to inhibit an early step in initiation, preferentially depress the synthesis of at least most of the r proteins.

Hypertonicity was found to raise the values of A_i of some of the non-r proteins (the largest increases are with the histones, spots e and f) and to lower the value of one of the non-r proteins, spot c (Table V). The elevated values have been assumed to indicate that the mRNAs have higher initiation rate constants than the average of the functioning cell messages. The depressed A_i value of spot c, however, is not as easy to account for since inhibitors of elongation have little or no effect on the relative rate of synthesis of the protein (Tables I–III). Lodish & Froshauer (1977) encountered a somewhat similar situation with a viral protein. In analogy with the explanation they offered for the viral protein, it can be suggested that the initiation of spot c mRNA is no more limited in the untreated resting chick embryo cells than is, for example, that of spots g and h. Despite its normal behavior, however, spot c does require more of some component of the initiation machinery than do the messages for spots g and h. The critical factor is sensitive to excess NaCl, and, hence, the synthesis of spot c, but not of spots g and h, is preferentially affected by hypertonicity.

Cycloheximide stimulates the synthesis of all the r proteins that were examined to the same or about the same extent. The picture with excess NaCl and 3-methyleneoxindole, however, is more complex. The production of some species of r protein is inhibited to a much greater extent than that of total cell protein, at least three r proteins seem to be affected at best only a little more than is the bulk of the cell protein, and the rest of the proteins fall between the extremes. A similar discrepancy in the effects of cycloheximide and hypertonic culture medium has been described for late adenovirus proteins made in HeLa cells (Wolgemuth et al., 1980).

The discrepant results with cycloheximide and hypertonicity need not mean that the Lodish model is incorrect. It may serve some purpose to suggest an entirely hypothetical, but specific and plausible, set of conditions that would account for the discrepancy in terms of the model. It is proposed that the frequencies of initiation of the NaCl highly sensitive and highly resistant mRNAs are governed by two different early acting components of the initiation system. One of the components has a low affinity for the sensitive messages, and its activity is seriously affected by hypertonicity. The other factor has a poor affinity for the resistant messages, and it functions normally under hypertonic conditions. The frequency of initiation of the mRNAs with intermediate sensitivities to excess NaCl would be governed by both factors, and the contribution of each factor would vary for the individual r protein.

The NaCl highly sensitive r proteins need no further comment since the observations that were made accord perfectly well with the Lodish model and the present hypothesis. One facet of the results with the highly resistant r proteins does, however, need additional consideration. Thus, cycloheximide and a hypertonic culture medium both depress the frequency of initiation of the bulk of the cellular mRNAs, and both treatments might, therefore, be expected to increase the level of the NaCl-resistant factor in the cell sap. Yet, cycloheximide, but not hypertonicity, stimulates the rate of synthesis of the resistant r proteins.

This problem can be resolved within the bounds of the hypothesis by attributing three additional properties to the

NaCl-resistant initiation factor: the factor binds to a mRNA before, rather than along with, a 40S subribosome; the factor is unable to bind if a 40S or 80S particle is present at or near the 5′ end of the message; the factor is released from the message upon the attachment of a new 40S subunit.

In light of these additional properties, cycloheximide and hypertonicity should have very different effects on the frequency of the initiation of the NaCl-resistant mRNAs. Cycloheximide, in slowing the movement of ribosomal particles away from the 5′ portion of the message, would block the binding of the NaCl-resistant initiation factor to the high-affinity mRNAs. As a result, the supply of the factor for low-affinity messages would be increased. Hypertonicity, on the contrary, would not hamper the attachment of the factor to the high-affinity messages, but it would impede its release from them. The competitive relationship between the efficient mRNAs and the NaCl-resistant, low-affinity messages might, therefore, remain as it was under isotonic conditions.

It seems likely that insulin stimulates the production of r proteins by increasing the supply of the components of the initiation system that are limiting in the hormone-deficient chick embryo cells.

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