THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 239, No. 11, November 1964
*Printed in U.S.A.*

# Breakdown and Reassembly of Rat Liver Ergosomes after Administration of Ethionine or Puromycin*

SAUL VILLA-TREVINO,† EMMANUEL FARBER, THEOPHIL STAEHELIN, FELIX O. WETTSTEIN, AND HANS NOLL‡

*From the Department of Pathology and Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213*

(Received for publication, March 31, 1964)

It is now generally recognized that ribosomal aggregates (ergosomes or polysomes) rather than single ribosomes (73 S monomers) are the structures normally engaged in protein synthesis in the cytoplasm of rat liver (1–4) and other mammalian cells (5–13), as well as in leaves of higher plants and in microorganisms (14–16). The conclusion that each ergosome is held together by one continuous strand of messenger ribonucleic acid is supported by the observations that (*a*) breakdown of ergosomes into single ribosomes and loss of function follow the addition of traces of pancreatic ribonuclease (1, 8, 17), (*b*) breakdown *in vivo* follows inhibition of messenger ribonucleic acid synthesis with actinomycin (2, 4, 10), and, most directly, (*c*) proportionality exists between the molecular weight of messenger ribonucleic acid and the number of ribosomes in the aggregate from which it was isolated (18).

Kinetic analysis (3, 11, 12, 16, 19) of the functional state of ergosomes strongly supports the hypothesis (8, 15, 20) of the tape mechanism of protein synthesis. According to this concept, the read-out process by which the genetic message is translated into the colinear (21, 22) structure of a growing polypeptide chain requires the movement of mRNA[1] through a condensing site on each ribosome. The process begins with the successive attachment of single ribosomes to one end of the messenger strand and terminates with their release at the other end, each with its finished polypeptide chain. Thus, during the lifetime of a messenger RNA molecule *in vivo*, the aggregates engaged in protein synthesis are maintained in a steady state by the prompt reattachment of monomers. The spacing of the ribosomes on the mRNA strand, and hence the aggregate size corresponding to an mRNA of a given length, is determined by the rate at which ribosomes move along the messenger (read-out rate) relative to the rate of monomer attachment. Because of the breakdown and assembly of aggregates resulting from mRNA turnover, the steady state size distribution of an intracellular ergosome population would also be dependent upon the rate of mRNA turnover relative to the rate of the read-out process (16). Data published for rat liver and bacteria indicate that the average time for assembling a polypeptide chain is short compared with the average life of the messenger (23–26).[2]

According to these considerations, the steady state *in vivo* is characterized by a rapid cycling of ribosomes along the length of mRNA molecules and a much slower breakdown and replacement of the mRNA strands. It follows that suppression of mRNA synthesis should cause a *gradual* inhibition of protein synthesis reflecting the slow breakdown of ergosomes by degradation of pre-existing mRNA molecules. In contrast, interference with the cycling of ribosomes over the length of mRNA by events such as premature detachment of ribosomes from mRNA, defective reattachment, or an increased rate at which ribosomes move along the messenger relative to the rate of monomer attachment would be expected to cause a *rapid* reduction in ergosome size until a new steady state is established. Evidence in support of these concepts was obtained by studying the effects of two different inhibitors of protein synthesis, ethionine and puromycin, that are considered to act at different points in this complex series of molecular interactions (27, 28).

Previous studies with ethionine had shown that in female rats this ethyl analogue of methionine (29) causes a rapid drop of the hepatic adenosine triphosphate concentration (30), a depression of nuclear ribonucleic acid synthesis (31), and a delayed inhibition of protein synthesis (32). These findings suggested that ethionine inhibits mRNA synthesis, presumably by trapping adenine as *S*-adenosylethionine and thus limiting the supply of adenosine triphosphate (28, 33). To obtain further evidence for this hypothesis, we examined the size distribution of ergosomes as a function of time after administration of ethionine. When it was found that ethionine did indeed cause a breakdown of aggregates similar to that observed after actinomycin treatment (2, 4, 10), it became particularly important to study the effects of adenine and methionine upon the ergosome distribution in the ethionine-treated animal. Since these compounds are known to reverse the inhibition of protein synthesis induced by ethionine (32, 34), their use offered an opportunity to observe whether restoration of protein synthesis was accompanied by a reassembly of single ribosomes into aggregates. Such a phenomenon would be essential if our hypothesis of the mechanism of ethionine action and of the structure and function of ergosomes were correct.

For comparison, the effects of puromycin on the size distribution of ergosomes *in vivo* were studied in an effort to confirm and

* This work was supported in part by Research Grants AM 05590, CA 06074, and AI 04303 and Training Grants 2G 135 and 2E 80 from the United States Public Health Service, by Grants E 129 and P 318 from the American Cancer Society, by the Life Insurance Medical Research Fund, and by the Beaver County Cancer Society.

† Rockefeller Foundation Fellow, 1958 to 1962, and Sarah Mellon Scaife Fellow, 1962 to 1964.

‡ Supported by Research Career Development Award GM K3 15, 274 from the United States Public Health Service.

[1] The abbreviations used are: mRNA, messenger ribonucleic acid; sRNA, soluble ribonucleic acid.

[2] H. Noll, H. Oura, T. Staehelin, and F. O. Wettstein, unpublished work.



Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

to extend to a system *in vivo* previous observations that, despite the inhibition of protein synthesis, the characteristic guanine triphosphate-dependent breakdown of aggregates, which is normally coupled to amino acid incorporation *in vitro*, was accelerated in the presence of this drug (3).[3]

## EXPERIMENTAL PROCEDURE

White female Wistar rats (Carworth Farms) maintained on Purina diet and weighing 150 to 200 g, were deprived of food overnight and treated with injection as indicated in the figure legends. Aqueous solutions of 0.153 M DL-ethionine or DL-methionine and of 0.053 M adenine were used for injections. Puromycin HCl, generously supplied by Dr. Nestor Bohonos of Lederle Laboratories, was administered in aqueous solution neutralized with KOH. Controls received equal volumes of 0.9% NaCl solution. Intraportal injections were carried out after laparotomy during Nembutal anesthesia (6 mg/100 g of body weight).

The mass distribution of ergosomes was analyzed by zone centrifugation of the deoxycholate-treated postmitochondrial supernatant followed by automatic scanning of the gradient at 254 mμ (2). Centrifugation was carried out with approximately 0.3-ml samples for 2½ hours at 25,000 r.p.m. and close to 0°, with a Spinco SW 25.1 rotor and concave exponential sucrose gradients (10 to 34%).

Incorporation of radioactive leucine into protein was measured by planchet counting after precipitating the protein from the liver fractions or from the whole liver homogenate with an equal volume of cold 10% trichloroacetic acid and washing to remove soluble components, lipid, and nucleic acids (31).

## RESULTS

A typical series of sedimentation patterns, reproduced in Fig. 1, illustrates progressive stages in the breakdown of liver ergosomes produced by a single dose of ethionine in female rats. The process begins with a conspicuous increase of the monomer and dimer peaks, clearly visible as early as 2½ hours after the administration of ethionine (Fig. 1*B*). It is virtually completed after 4 hours (*C*) and remains unchanged for at least 24 hours (*D*). The observed patterns are clearly distinguishable from those produced by random cleavage with an endonuclease (3). The breakdown of ribosomal aggregates is accompanied by a corresponding inhibition of hepatic protein synthesis. It was found previously that incorporation of leucine into liver protein both *in vitro* and *in vivo* reaches a minimum value within 4 hours after the administration of ethionine and remains depressed for at least 24 hours (32).[4]

Inhibition of protein synthesis by ethionine in female rats can be prevented by ATP or adenine administered at the same time as, or within 2 hours after, ethionine (32, 34). In addition, ATP or methionine injected 5 hours after the ethionine produces a partial reversal of the inhibition already present. In view of these previous findings, it was of interest to examine whether the breakdown of ergosomes into single ribosomes induced by ethionine could be reversed by the administration of adenine alone or in combination with methionine. The sedimentation patterns reproduced in Figs. 2 and 3 clearly indicate the rapid reassembly

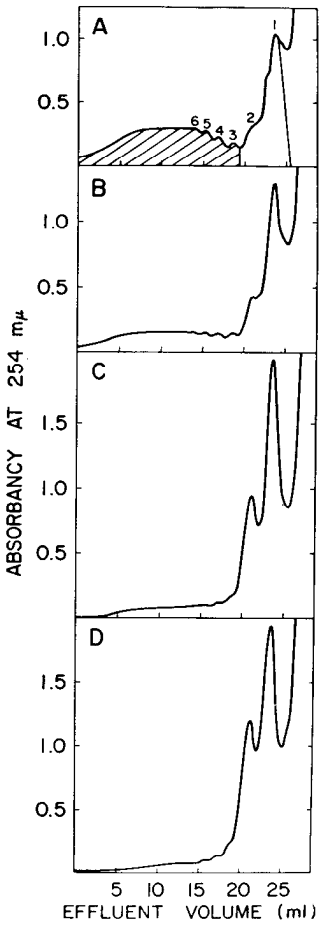


FIG. 1. Sucrose gradient patterns illustrating the breakdown of liver ergosomes as a function of time after injection of a single dose of ethionine (1 mg per g of body weight). *B* to *D*, progressive stages of ergosome breakdown 2½, 4, and 24 hours after ethionine injection. The *single hatched area* corresponds to the proportion of ergosomes ≧ (73 S)$_3$ in the untreated controls (*A*) and was used as a reference standard. In all measurements of particle size distribution, the dimers were included in the monomer fraction because of the strong tendency of monomers to form dimers under the conditions of these experiments (2). The peak positions of the 73 S monomer and its oligomers are marked by the numbers *1* to *6*.

of ribosomes into aggregates under these conditions. A characteristic feature of the reassembly process is the preferential formation of very large aggregates containing 20 and more ribosomes (Figs. 2, *B* and *C*, and 3, *C*, *D*, and *E*).

The quantitative aspects of this process are summarized in Fig. 4. The re-formation of aggregates was measured from the appropriate integrated areas in the sedimentation diagrams by computing the proportion of ribosomes present as monomers plus dimers or as aggregates ≧ (73 S)$_3$. The amino acid incorporation activity corresponding to a given distribution of aggregate size was determined by measuring the amount of $^{14}$C-leucine incorporated into the total liver proteins during the 15 minutes before the rats were killed. In Fig. 4, we have plotted the fraction of ergosomes and the amino acid incorporation activity (both expressed as percentage of the values of the untreated controls) as a function of time after restoration of mRNA synthesis with a single dose of adenine or adenine plus methionine. It is evident that after treatment with adenine plus methionine,

[3] H. Noll, F. O. Wettstein, and T. Staehelin, results reported at the meetings of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 19, 1963.

[4] S. Villa-Trevino and E. Farber, unpublished work.

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

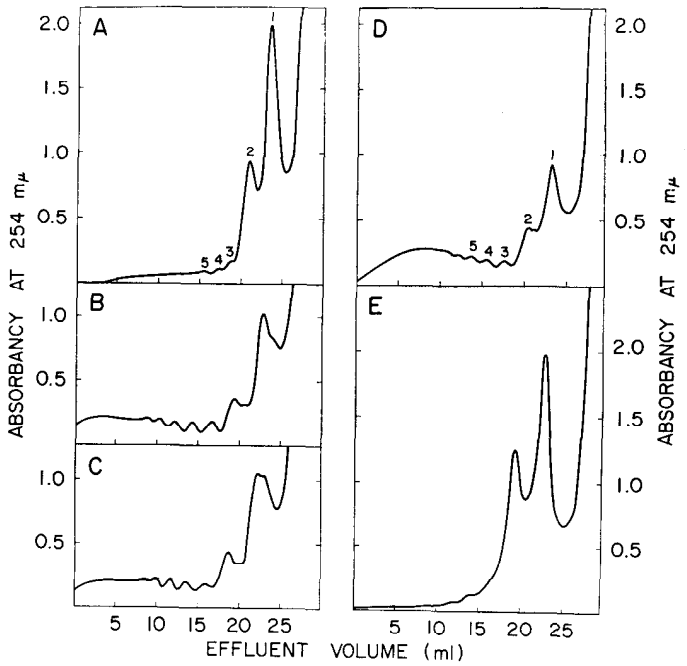


FIG. 2. Sucrose gradient patterns illustrating reassembly of ergosomes upon reversal of ethionine action by adenine. With the exception of the control (*D*), all rats received 1 mg of ethionine per g at zero time. Extensive ergosome breakdown at 5 hours (*A*) and 10 hours (*E*) is contrasted by recovery observed 2½ hours (*B*) and 5 hours (*C*) after injection of 0.162 mmole of adenine per 200 g of body weight at 5 hours. All animals received 5 μc of uniformly labeled L-leucine-$^{14}$C (131 mc per mmole) 15 minutes before they were killed. All injections were intraperitoneal.

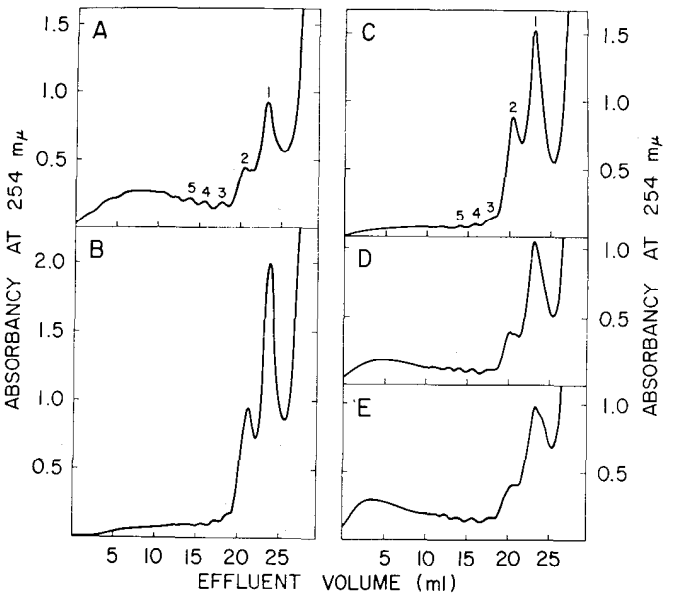


FIG. 3. Sucrose gradient patterns illustrating reassembly of ergosomes upon reversal of ethionine action by adenine plus methionine. With the exception of untreated control (*A*), all animals were treated at zero time with ethionine as indicated in Fig. 2. *B*, ergosome breakdown at 5 hours. Intraperitoneal injection of adenine (0.162 mmole/200 g) plus methionine (equimolar to the ethionine dose) at 5 hours produces progressive reassembly shown ½ (*C*), 1 (*D*), and 3 (*E*) hours later. All rats received 5 μc of L-leucine-$^{14}$C into the portal vein 15 minutes before they were killed.

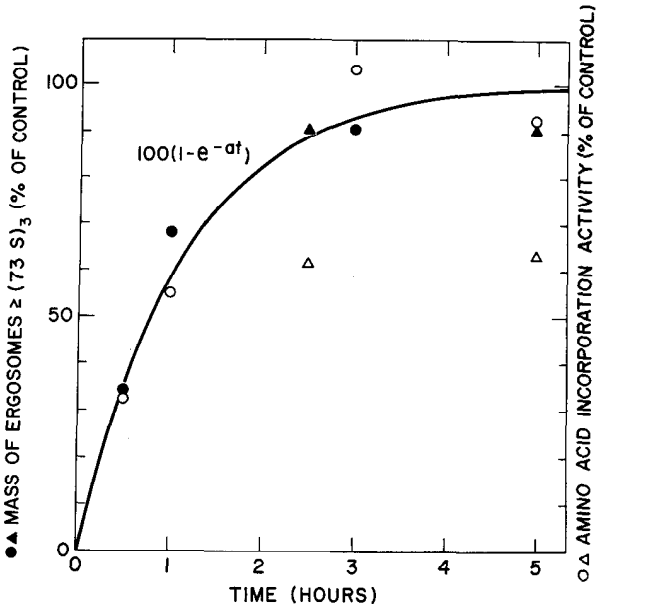


FIG. 4. Kinetics of reassembly of ergosomes and restoration of protein synthesis. The data from Fig. 2 (▲) and Fig. 3 (●), together with the corresponding amino acid incorporation activities (△ and ○), are plotted as a function of time as explained in the text. *The solid line* is the function $100(1 - e^{-at})$. The patterns in Figs. 2 and 3 and the corresponding amino acid incorporation values (Table I) are representative of a larger series of similar data and are reproducible within about ±10%.

the recovery of protein synthesis is proportional to the re-formation of ergosomes and essentially complete within 3 hours. After treatment with adenine alone, the rate of protein synthesis appears to be restored only to the extent of about 60%, even though the ergosome fraction has reached the control level. This might indicate that, whereas the rapid return of the ATP concentration to normal by adenine is able to reverse the inhibition of mRNA synthesis, methionine is required to repair another ethionine-induced defect in the protein-synthesizing system, such as in the methylation of sRNA (*cf.* Farber (33)).

According to our concept, the kinetics of the reassembly of ergosomes and of the restoration of protein synthesis (Fig. 4) should reflect the kinetics by which newly synthesized mRNA accumulates in the cytoplasm if the attachment of free ribosomes to mRNA is not rate-limiting. The accumulation of mRNA in the cytoplasm as a function of time ($t$) is expected to approach the 100% saturation level as the rate of mRNA breakdown becomes equal to the rate ($a$) of its synthesis and release into the cytoplasm. The *solid line* in Fig. 4 has been calculated from the theoretical saturation function $100(1 - e^{-at})$ and gives a satisfactory fit to the experimental data for a rate of mRNA synthesis corresponding to $a = 0.0144$ min$^{-1}$ and a messenger half-life, $t_{\frac{1}{2}}$, of $0.693/a = 48$ minutes. A similar value (approximately 60 minutes) for the rate of renewal of cytoplasmic mRNA has been obtained from studies of the kinetics by which $^{32}$P is incorporated into mRNA.[1]

Puromycin, in contrast to actinomycin and ethionine, causes an immediate release of 73 S ribosomes, accompanied by a shift toward smaller aggregates in the size distribution of ergosomes (Fig. 5). The mass distribution has shifted from a peak position

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

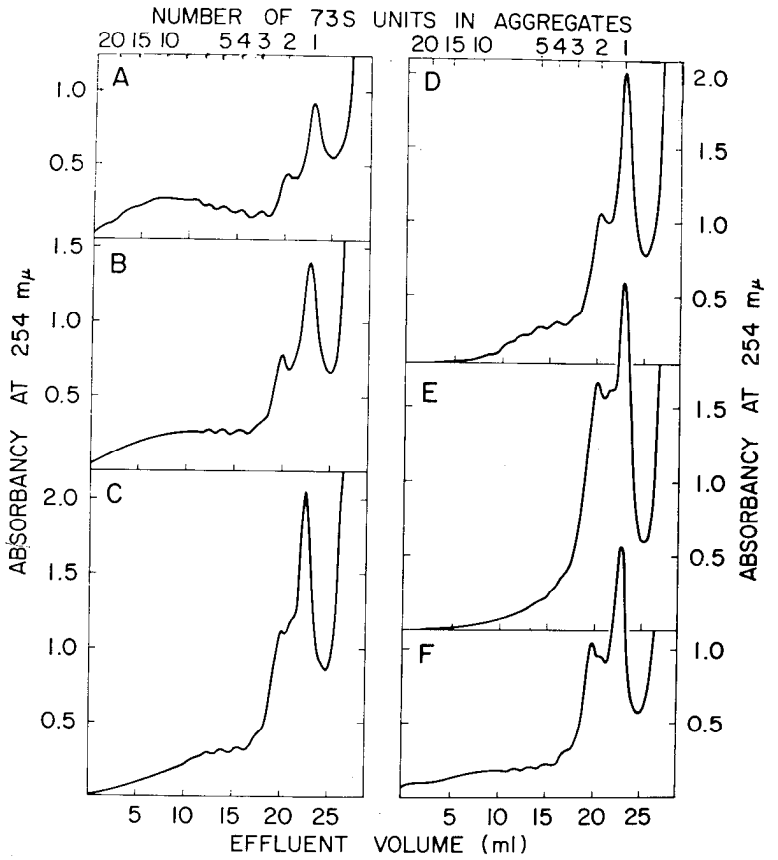


FIG. 5. Sucrose gradient patterns illustrating the breakdown of ergosomes as a function of time after injection of puromycin; details of the experiment as shown in Table I and discussed in the text.

corresponding to an aggregate size of (73 S)$_{6\text{-}10}$ (Fig. 5*A*) to a new position in the trimer to tetramer region (Fig. 5, *C* and *D*). The kinetics of this process, illustrated in Fig. 6*A*, shows that within 20 minutes the ergosome population drops to one-third of its original mass and then declines only very slowly during the following 3 hours (Fig. 5*E*). Incorporation of radioactive amino acids into protein was more than 99% inhibited when labeled amino acids and puromycin were injected simultaneously into the portal vein (Table I). This inhibition was complete within 5 minutes, the shortest time interval measured, indicating that inhibitory concentrations of the drug reach the ribosomal sites as rapidly as the radioactive amino acids in the form of their sRNA derivatives. The smaller ergosomes, characteristic of the population observed after the shift in distribution produced by puromycin, were found to be functional when tested for amino acid incorporation activity in a cell-free system without puromycin. There is no indication that this population of smaller ergosomes represents a selected class of aggregates, *e.g.* aggregates endowed with a more stable messenger. On the contrary, the specific activity of pulse-labeled mRNA isolated from ergosomes after puromycin treatment was comparable to that of the controls.

The puromycin-induced changes are reversible with time. A substantial fraction of the larger ergosomes has reappeared 3½ hours after the second 15-mg injection of puromycin (Fig. 5*F*), and the size distribution of the pattern has shifted toward that of the control (Fig. 5*A*). Protein synthesis has recovered to the extent of about 50% (Table I). Taken together, these results

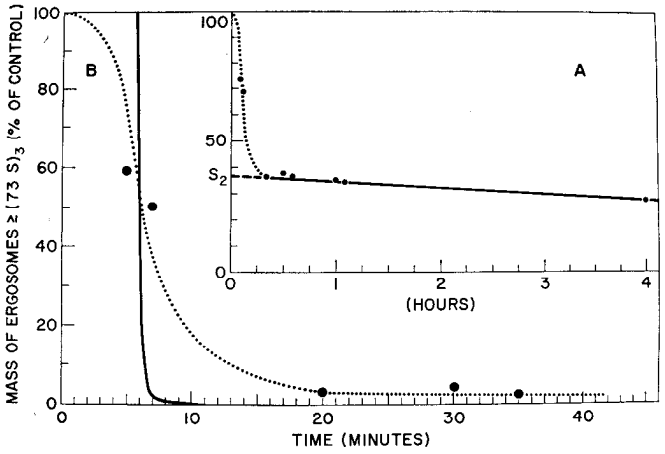


FIG. 6. Kinetics of ergosome breakdown produced by puromycin. Rapid drop of ergosome mass (····) to new steady state level $S_2$ (——) is shown in *A*. In *B* the time scale has been expanded, and the ordinate recalibrated, by setting $S_2$ equal to zero and the ergosome mass of untreated controls equal to 100%. The theoretical curve (——) has been computed from the known mass distribution of the control (Fig. 5*A*), and from the data in Fig. 7, *Model B*, by assuming a synchronized doubling of the rate, $V_2$, at which ribosomes move relative to mRNA. The *dotted line* connecting the experimental points is expected to approximate the kinetics under actual experimental conditions.

suggest that the release of monomers and the change in the distribution of aggregate sizes produced by puromycin reflect a shift to a new steady state followed by a gradual return to the original situation as the puromycin is eliminated from the cells.

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

TABLE I

*Effect of puromycin on incorporation of L-leucine-$^{14}C$ into total liver proteins in vivo*

| Experiment | Sedimentation pattern* | Dose of puromycin per injection | Timing of injections† | | Time of killing | $^{14}$C-Leucine incorporation into protein | |
|---|---|---|---|---|---|---|---|
| | | | Puromycin | $^{14}$C-Leucine | | | |
| | | *mg* | *hrs* | | *hrs* | *μμmoles/mg protein* | % |
| I | A | —‡ | — | 0 | $\frac{1}{12}$ | 4.00 | 100 |
| | B | 15 | 0 | 0 | $\frac{1}{12}$ | 0.01 | <1 |
| | C | 15 | 0 | $\frac{1}{6}$ | $\frac{1}{4}$ | 0.11 | 3 |
| II | — | —‡ | — | 0 | $\frac{1}{2}$ | 4.85 | 100 |
| | D | 15 | | $\frac{1}{2}$ | 1 | 0.05 | 1 |
| | E | 15 | 0, 1, 2, 3 | $3\frac{1}{2}$ | 4 | 0.01 | <1 |
| | F | 15 | 0, $\frac{1}{2}$ | $3\frac{1}{2}$ | 4 | 2.34 | 48 |

* Fig. 5.

† The rats were treated with injection intraportally in Experiment I and intraperitoneally in Experiment II.

‡ Control animals (indicated by dashes) were injected with NaCl instead of puromycin.

## DISCUSSION

Cytoplasmic protein synthesis involves a complex chain of molecular intractions beginning with the synthesis of the message in the nucleus and ending with its read-out in the cytoplasm and eventual destruction. Since the individual interactions in this chain are probably coupled to each other by self-regulatory feedback mechanisms, the use of inhibitors that act at different points should make it possible to dissect the system and gain insight into the mechanism of these interactions and the nature of the controls to which they are subject. In the present study, we have examined the effects of ethionine and puromycin on hepatic protein synthesis in rats at the molecular level.

Previous conclusions (28, 32) that ethionine inhibits protein synthesis primarily by trapping the ATP necessary for mRNA synthesis have been supported by the demonstration of the expected parallelism between ergosome breakdown and reduction of protein synthesis, and the reversibility of both effects by methionine and adenine.

Previous results have shown that the loss of incorporation activity *in vitro* of ribosomes from ethionine-treated rats follows the rapid decline of the intracellular ATP concentration with a delay of about $1\frac{1}{2}$ hours and is nearly complete after 3 hours (32). In the case of bacteria, attempts have been made to estimate the normal turnover rate of mRNA from the first order rate at which pre-existing mRNA decays after blocking new synthesis (24). It seems difficult to apply this approach to the interpretation of the ethionine effects because of the following complicating factors. RNA synthesis ceases only gradually and the decline of the incorporation activity *in vitro* of ribosomes is delayed and fails to exhibit first order kinetics. In addition, it is by no means certain that the decay rate of mRNA observable after inhibition of its synthesis is normal.

Similar criticism applies to estimates of mammalian mRNA turnover derived from experiments with actinomycin (2, 4, 10). Although rapid inhibition of RNA synthesis in rat liver by high actinomycin concentrations has been shown (35),[2] an even longer lag appears to precede ergosome breakdown and inhibition of protein synthesis *in vitro* than in the case of ethionine (2). The reason for this lag, which seems to reflect a temporary inhibition of mRNA breakdown, is obscure. Moreover, no precise study has been made of the kinetics of these processes after inhibition with high doses of actinomycin.

A novel approach to determining the renewal rate of cytoplasmic mRNA, not complicated by the gradual cessation of synthesis and delayed breakdown of mRNA, was made possible by measuring the kinetics of reassembly of ergosomes and restoration of protein synthesis after the inhibition of mRNA synthesis was reversed with a combination of adenine and methionine. The results indicate that the reversal proceeds with a half-time of about 50 minutes. Whether this value can be taken as a measure of the average rate of mRNA turnover under normal steady state conditions depends upon the validity of the assumption that the synthesis of the normal spectrum of mRNA molecules is resumed immediately at the normal rate. It is obvious from our data that, compared with the usual steady state size distribution, a rather pronounced shift toward heavy aggregates occurs during the reversal process, resulting in a peak of mass distribution in the region of eicosamers $(73\ S)_{20}$. In view of the known homeostatic regulation of hepatic secretion, this might reflect an increased output of the large messenger for albumin synthesis to compensate for previous losses. Whatever the reason, in view of the complex events triggered by ethionine (28, 33), a simple return to the original steady state conditions upon reversal of ethionine action is hardly to be expected. Nevertheless, the estimates of the average mRNA turnover rate in rat liver cells derived from the kinetics of ergosome breakdown and reassembly are in fairly close agreement with each other and with the half-life of about 60 minutes determined by radioisotope labeling under steady state conditions.[2]

Available data seem to indicate that mRNA of liver cells is heterogeneous with respect to turnover rate. Thus, even after a high dose of actinomycin which inhibits RNA synthesis completely, an appreciable fraction (20 to 30%) of the ergosomes resist breakdown for extended time periods (2, 4). Experiments suggesting heterogeneity of mRNA with regard to lifetime in regenerating rat liver have been reported by Giudice and Novelli (36). However, in experiments with high doses of actinomycin and ethionine, the bulk of mRNA seems to be unstable and to break down within a few hours after the onset of the degradation process. Estimates of the half-life of this metabolically active mRNA fraction, based on the rate of ergosome assembly after reversal of ethionine inhibition as well as on $^{32}P_i$ incorporation rates, give values on the order of about 1 hour and are in reasona-

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

ble agreement with the rates of induction observed for several liver enzymes (37).

Puromycin is known to stop protein synthesis immediately by attachment to the growing end of the nascent polypeptide chains (38, 39). In addition, we have shown in this study that it causes a rapid breakdown of ergosomes to a new steady state level, characterized by a shift to smaller aggregates corresponding to an approximately 2-fold reduction of all aggregate sizes. Among conceivable explanations, two principal mechanisms must be considered in the light of our present concept; puromycin causes a shift in the steady state (*a*) by increasing the breakdown of mRNA relative to the rate of its synthesis, or (*b*) by increasing the rate at which ribosomes move along the messenger tape relative to their rate of reattachment to the messenger.[5] As evident from Fig. 7, the two mechanisms are distinguishable by several features. Thus, an increased rate of mRNA breakdown would cause a reduction in the *number* of ergosomes by a constant factor without affecting their size distribution. On the other hand, an increased read-out rate, premature release, or both would result in wider spacing of the ribosomes along the messenger and hence in the reduction of the *size* of ergosomes without changing their their number. In view of the drastic shift in size distribution toward smaller aggregates after puromycin treatment, it is evident that our results rule out an effect of puromycin on mRNA breakdown, but are compatible with the postulated effect on the read-out mechanism.[6] Moreover, a 3-fold reduction of the ergosome mass by accelerated mRNA degradation according to *Model A* in Fig. 7 would require more than 1 hour, *i.e.* at least 3 times longer than indicated by our kinetic experiments (Fig. 6). This interpretation is further strengthened by observations of Levinthal *et al.* (40), who reported that stopping protein synthesis with puromycin did not change the normal rate of messenger decay in cultures of *Bacillus subtilis*.

In contrast, the alternative mechanism (*b*) predicts an extremely rapid transition to the new steady state. It may be calculated from published data (18, 26) that, in liver cells, ribosomes move with respect to mRNA at a velocity of more than 800 nucleotides per minute. Thus, the expected shift should occur within less than 2 minutes. The data in Fig. 6*B* suggest a somewhat slower transition time; however, broadening effects would be expected from the normal delay in equilibration.[6] It

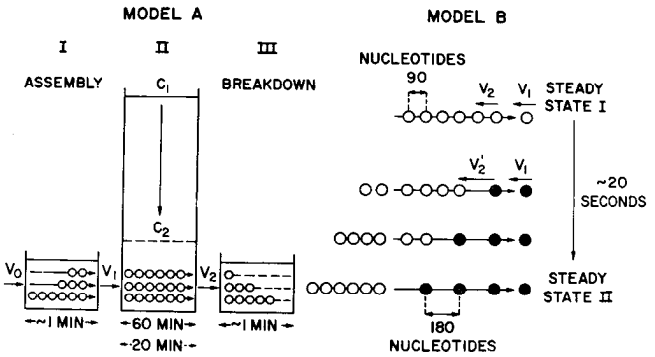


FIG. 7. Models to explain shift in steady state levels of ergosome population. *Model A*, individual ergosomes (schematically represented by hexamer) are assembled within 1 to 3 minutes during Stage *I* and enter fully functional Stage *II* at rate $V_1$ (= rate, $V_0$, of mRNA synthesis). The rate, $V_2$, of ergosome breakdown, defined as flow into Stage *III*, is proportional to the decay constant, $k$, of mRNA and the concentration, $C$, of ergosomes in Stage *II*: $V_2 = kC$. The steady state level, $C_1$, is established when $V_2 = kC_1 = V_1$. If the decay constant of mRNA is tripled ($k' = 3k$) so as to shorten its half-life from about 60 to 20 minutes, the original steady state level, $C_1$, in Stage *II* drops exponentially to a new steady state level, $C_2$, corresponding to one-third of the original value, $C_1$. Assuming that assembly and breakdown of individual ergosomes are coupled to protein synthesis (3, 16) and hence occur at the rapid rate of the read-out process, a hexamer is estimated to spend less than 2 minutes or about 3% of its lifetime in Stages *I* and *III*, since it takes about 2 minutes to assemble the 574 amino acids of a rat albumin chain (26) and 18 ribosomes to fill its messenger at the normal spacing of 90 nucleotides per ribosome (18). *Model B*, single ribosomes (73 S monomers) attach to the head of mRNA at a given maximal rate, $V_1$ (about 0.16 particle per second) and move along the mRNA tape at their maximal speed, $V_2$ (about 14 nucleotides per second). This results in the normally observed spacing of 90 nucleotides between ribosomes (18). If puromycin causes a doubling of $V_2$, a shift to a new steady state takes place (within 30 seconds for hexamers) in which all of the original mRNA molecules contain a fraction of the original number of ribosomes in wider spacing.

is obvious that effects of such short duration are difficult to measure with precision in intact animals and with the methods employed in these experiments. More accurate are the measurements of the mass distribution before and after the shift to the new steady state. From the distribution of ribosomal mass with respect to aggregate size in the control, we calculate that the observed loss of 64% of ergosomal ($\geq$ trimers) mass would correspond, on the average, to an almost 2-fold reduction of all aggregate sizes. In agreement with the postulated mechanism of puromycin action, we find a shift toward smaller aggregates corresponding to a nearly 2-fold reduction of the original aggregate sizes. This effect manifests itself most conspicuously in the complete disapparance of the largest ergosomes corresponding to the approximate aggregate sizes $(73\ S)_{15}$ to $(73\ S)_{30}$ (Fig. 5).

Evidence from studies *in vitro* indicates that the amino group of puromycin forms a peptide bond with the carboxyl end of the growing polypeptide chain in competition with sRNA-bound amino acids (38, 39, 42). Apparently the read-out mechanism continues to function in the presence of puromycin (3, 12)[3] and whenever unfinished polypeptide chains are prematurely terminated by puromycin, new chains are started (43), presuma-

[5] A situation similar to *b* would arise if ribosomes traveling at normal speed were released prematurely before reaching the end of the message, as a consequence of derailment produced by tracking errors. Furthermore, faster read-out and premature detachment are not mutually exclusive, but might both occur as a result of loose tracking. Premature detachment would also produce an increased spacing of ribosomes along the messenger with corresponding breakdown of aggregates and shift to a new steady state size distribution. In contrast to the situation produced by faster read-out, however, the spacing of the ribosomes should increase with increasing distance from the starting point, as the chances of escaping tracking errors decrease. Although the spacing manifests itself in the distribution of aggregate sizes, the present data would not allow one to distinguish between the two situations. However, more recent experiments indicate that the mass ratio of messenger to ribosomal RNA as well as the length of mRNA per ribosome increases with aggregate size (results to be published). Other evidence suggesting that puromycin causes premature release was reported by Nathans (meetings of the Federation of American Societies for Experimental Biology, Chicago, April 1964) after this paper was submitted for publication.

[6] After this paper had been submitted, Williamson and Schweet published similar observations and interpretations (41). They found that in reticulocytes exposed to puromycin the shift toward smaller aggregates was complete within 10 minutes or less and was partially reversible upon removal of the drug.

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

bly with the amino acid specified by the codon next to the point of interruption. Since puromycin might attach itself to this amino acid in the following step, the initiation of new chains would be expected to end abortively in the production of puromycin-linked single amino acids or oligopeptides. The accumulation of acid- and alcohol-soluble peptide fragments and the beginning of new chains in the presence of puromycin have been reported (42, 43). Furthermore, the specific breakdown of ergosomes by release of monomers, characteristic of the read-out process *in vitro*, is accelerated in the presence of puromycin and requires GTP (3).[3] This indicates that whenever puromycin attaches itself to the activated carboxy group of an amino acid by displacing the terminal sRNA (44), it triggers mRNA transport. Thus, as the coding mechanism continues, in the presence of high puromycin concentrations, theoretically every second step could result in the attachment of a puromycin molecule to the amino acid fixed to the ribosomal site in the preceding step. If the selection of the correct aminoacyl-sRNA is rate-limiting, the skipping of every other sRNA selection step could well increase the read-out rate. Other plausible mechanisms might contribute to the postulated increase in the rate of ribosomal movement, release, or both. Thus, since sRNA is known to interact with both the ribosomal surface and the coding triplet of the messenger (45, 46), it would be expected to play an essential role in keeping the messenger codons in proper register with respect to the ribosomal condensing site. Temporary loss of this control during interaction with puromycin might cause slipping of the messenger tape, perhaps frequently resulting in premature detachment of ribosomes from mRNA.[7] On the other hand, it seems reasonable to assume that attachment of vacant monomers to the beginning of the message proceeds by a special mechanism that is independent of the read-out or release rate and, hence, not directly affected by puromycin. Thus, the experimental findings with puromycin are best explained by the hypothesis that this compound causes acceleration of the ribosomal movement, release process, or both, without a corresponding increase of the rate at which vacant monomers are attached to the beginning of the message. An important aspect of this hypothesis is the postulate that attachment requires a special mechanism which is separable from the read-out process, thus emphasizing the decisive role of the attachment mechanism in metabolic control. A similar conclusion has been reached by Goldstein, Goldstein, and Lowney (48).[8]

## SUMMARY

The injection of ethionine into female rats produces a breakdown of liver ergosomes and a parallel inhibition of protein synthesis, both reversible upon administration of adenine or methionine or adenine plus methionine. This result offers further support for the hypothesis that ethionine inhibits protein synthesis through an inhibition of messenger ribonucleic acid synthesis. From the kinetics of the reassembly process, an average half-life of messenger ribonucleic acid corresponding to about 50 minutes has been estimated. In contrast, puromycin causes a rapid but incomplete breakdown. This shift to a new steady state level is characterized by a nearly 2-fold reduction in the size of all ribosomal aggregates. These results are explained by a general hypothesis of the mechanism of puromycin action.

[7] A rather spectacular example of inaccurate tracking resulting in a misreading of the genetic code and produced by a family of antibiotics that bind to ribosomes has just come to light (47).

[8] We thank Dr. A. Goldstein for kindly sending us a preprint of his manuscript.

## REFERENCES

1. Wettstein, F. O., Staehelin, T., and Noll, H., *Nature*, **197**, 430 (1963).
2. Staehelin, T., Wettstein, F. O., and Noll, H., *Science*, **140**, 180 (1963).
3. Noll, H., Staehelin, T., and Wettstein, F. O., *Nature*, **198**, 632 (1963).
4. Korner, A., and Munro, A. J., *Biochem. and Biophys. Research Communs.*, **11**, 235 (1963).
5. Marks, P. A., Burka, E. R., and Schlessinger, D., *Proc. Natl. Acad. Sci. U. S.*, **48**, 2163 (1962).
6. Warner, J. R., Rich, A., and Hall, C. E., *Science*, **138**, 1399 (1962).
7. Warner, J. R., Knopf, P. M., and Rich, A., *Proc. Natl. Acad. Sci. U. S.*, **49**, 2163 (1963).
8. Gierer, A., *J. Molecular Biol.*, **6**, 148 (1963).
9. Marks, P. A., Rifkind, R. A., and Danon, D., *Proc. Natl. Acad. Sci. U. S.*, **50**, 336 (1963).
10. Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E., *Proc. Natl. Acad. Sci. U. S.*, **49**, 654 (1963).
11. Goodman, H. M., and Rich, A., *Nature*, **199**, 318 (1963).
12. Hardesty, B., Miller, R., and Schweet, R., *Proc. Natl. Acad. Sci. U. S.*, **50**, 924 (1963).
13. Zimmerman, E. F., *Biochem. and Biophys. Research Communs.*, **11**, 301 (1963).
14. Clark, M. F., Matthews, R. E. F., and Ralph, R. K., *Biochem. and Biophys. Research Communs.*, **13**, 505 (1963).
15. Gilbert, W., *J. Molecular Biol.*, **6**, 374 (1963).
16. Staehelin, T., Brinton, C., Wettstein, F. O., and Noll, H., *Nature*, **199**, 865 (1963).
17. Arnstein, H. R. V., *Biochem. J.*, **81**, 24p (1961).
18. Staehelin, T., Wettstein, F. O., Oura, H., and Noll, H., *Nature*, **201**, 264 (1964).
19. Hardesty, B., Hutton, J. J., Arlinghaus, R., and Schweet, R., *Proc. Natl. Acad. Sci. U. S.*, **50**, 1078 (1963).
20. Watson, J. D., *Science*, **140**, 17 (1963).
21. Sarabhai, A. S., Stretton, O. W., Brenner, S., and Boole, A., *Nature*, **201**, 13 (1964).
22. Yanofsky, C., Carlton, B. C., Guest, J. R., Helsinki, D. R., and Henning, U., *Proc. Natl. Acad. Sci. U. S.*, **51**, 266 (1964).
23. McQuillen, K., Roberts, R. B., and Britten, R. J., *Proc. Natl. Acad. Sci. U. S.*, **45**, 1347 (1959).
24. Levinthal, C., Keynan, A., and Higa, A., *Proc. Natl. Acad. Sci. U. S.*, **49**, 1631 (1962).
25. Zipser, D., *J. Molecular Biol.*, **7**, 739 (1963).
26. Jungblut, P. W., and Turba, F., *Biochem. Z.*, **337**, 88 (1963).
27. Yarmolinsky, M. B., and de la Haba, G. L., *Proc. Natl. Acad. Sci. U. S.*, **45**, 1721 (1959).
28. Farber, E., Shull, K. H., Villa-Trevino, S., Lombardi, B., and Thomas, N., *Nature*, **203**, 34 (1964).
29. Dyer, H. M., *J. Biol. Chem.*, **124**, 519 (1938).
30. Shull, K. H., *J. Biol. Chem.*, **237**, PC1734 (1962).
31. Villa-Trevino, S., Shull, K. H., and Farber, E., *Federation Proc.*, **22**, 237 (1963).
32. Villa-Trevino, S., Shull, K. H., and Farber, E., *J. Biol. Chem.*, **238**, 1757 (1963).
33. Farber, E., *Advances in cancer research, Vol. 7*, Academic Press, Inc., New York, 1963, p. 383.
34. Villa-Trevino, S., and Farber, E., *Biochim. et Biophys. Acta*, **61**, 649 (1962).
35. Merits, I., *Biochem. and Biophys. Research Communs.*, **10**, 254 (1963).
36. Giudice, G., and Novelli, G. D., *Biochem. and Biophys. Research Communs.*, **12**, 383 (1963).
37. Schimke, R. T., Sweeney, E. W., and Berlin, C. M., *Biochem. and Biophys. Research Communs.*, **15**, 214 (1964).
38. Allen, D. W., and Zamecnik, P. C., *Biochim. et Biophys. Acta*, **55**, 865 (1962).
39. Gilbert, W., *J. Molecular Biol.*, **6**, 389 (1963).
40. Levinthal, C., Fan, D. P., Higa, A., and Zimmermann, R. A.,

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012

*Cold Spring Harbor symposia on quantitative biology, Vol. 28*, Long Island Biological Association, Cold Spring Harbor, Long Island, New York, 1963, p. 183.
41. WILLIAMSON, A. R., AND SCHWEET, R., *Nature*, **202**, 435 (1964).
42. NATHANS, D., VON EHRENSTEIN, G., MONRO, R., AND LIPMANN, F., *Federation Proc.*, **21**, 127 (1962).
43. NORRIS, A., ARLINGHAUS, R., FAVELUKES, S., AND SCHWEET, R., *Biochemistry*, **2**, 1084 (1963).
44. WETTSTEIN, F. O., AND NOLL, H., *J. Molecular Biol.*, in press.
45. CANNON, M., KRUG, R., AND GILBERT, W., *J. Molecular Biol.*, **7**, 360 (1963).
46. KAJI, A., AND KAJI, H., *Biochem. and Biophys. Research Communs.*, **13**, 186 (1963).
47. DAVIES, J., GILBERT, W., AND GORINI, L., *Proc. Natl. Acad. Sci. U. S.*, **51**, 883 (1964).
48. GOLDSTEIN, A., GOLDSTEIN, D. B., AND LOWNEY, L. I., *J. Molecular Biol.*, **9**, 213 (1964).

Downloaded from www.jbc.org at Cinvestav IPN, on November 6, 2012