Using Vogel's method (*13*), we prepared α-naphthylurethane derivatives for the compound extracted from quackgrass, for phenol, and for *m*-cresol. The melting points were, respectively, 132°–134°C, 134°C, and 127°C. The literature gives the melting point for phenol as 133°C and for *m*-cresol as 128°C. Infrared absorption spectra for the α-naphthylurethane of the unknown again corresponded with that for pure phenol, while the spectrum for *m*-cresol was decidedly different.

Preliminary tests seemed to indicate a similarity between phenol and the substance isolated from aqueous extracts of quackgrass rhizome tissue by ascending paper chromatography. The agreement between the infrared absorption spectra of prepared derivatives of the unknown material and those of phenol are more striking. Further evidence that the unknown substance is phenol was provided by the carbon, hydrogen, and nitrogen determinations of the phenylurethane derivatives prepared from the rhizome extract and those calculated for the phenol derivative of phenylurethane. For carbon: unknown, 74.37 percent; phenol, 73.22 percent. For hydrogen: unknown, 5.43 percent; phenol, 5.21 percent. For nitrogen: unknown, 6.44 percent; phenol, 6.57 percent. The percentage oxygen was found by the difference and was 13.76, while the calculated percentage was 15.10.

Dalapon has long been recognized as an effective herbicide fairly phytotoxic to quackgrass. Conditions of stress associated with its use are postulated as a causative factor which results in the increase of phenol in rhizome tissue (*14*).

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23 January 1963

Breakdown of Rat-Liver Ergosomes in vivo after Actinomycin Inhibition of Messenger RNA Synthesis

Abstract. *Ribosomes isolated from rat liver occur predominantly in the form of aggregates (ergosomes) corresponding to multiples of 73S particles held together by messenger RNA. After injecting rats with actinomycin, these aggregates gradually break down in vivo to 73S monomers and 113S dimers. We conclude that the observed breakdown results from the degradation of messenger RNA and the prevention by actinomycin of the synthesis of new messenger RNA.*

From evidence which was obtained with rat liver ribosomes, we have concluded that the functional unit of protein synthesis is a ribosomal aggregate which we have called ergosome (*1*); it consists of at least five 73*S* particles held together by messenger RNA (mRNA). Exposure of ergosomes to minute concentrations of ribonuclease causes a progressive fragmentation by random cleavage into smaller oligomers $(73S)_4$, $(73S)_3$, and so forth; this process thus results in the accumulation of free 73*S* monomers. This breakdown into smaller aggregates is accompanied by a rapid loss of potentiality for protein synthesis; some residual activity is associated with the 113*S* dimers, whereas the 73*S* monomers are completely inactive. Our conclusion that the ribonuclease-sensitive structure responsible for the integrity of the ergosome is in fact messenger RNA was further supported by experiments, with P^{32}-labeled phosphate, to detect rapid synthesis. An RNA fraction of high specific activity had become associated with purified ribosomes isolated 30 minutes after injecting the radioactive phosphorus. Upon treatment of the ribosomes with ribonuclease, or during incubation to effect the incorporation of amino acids, a large proportion of this rapidly labeled fraction was degraded into acid-soluble products, whereas the bulk of the ribosomal RNA remained stable (*2*). Moreover, dissociation of the labeled ribosomes into subunits at low Mg^{++} concentrations followed by sedimentation analysis in a sucrose gradient gave distribution patterns (*3*) analogous to those obtained with ribosomes from *Escherichia coli* containing labeled mRNA (*4*).

From the rapid synthesis of mRNA in stationary cells it must be inferred that breakdown occurs at the same rate so as to maintain steady-state conditions.

Our experiments were designed to obtain direct evidence for the breakdown in vivo of mRNA associated with ribosomes. Thus, if there is a rapid turnover in vivo of mRNA and if it is also responsible for the structural integrity of ergosomes, one would expect that inhibition of mRNA synthesis should result in the breakdown of ergosomes into 73*S* particles. This prediction was borne out by experiments with actinomycin C_3, a substance which specifically inhibits DNA-dependent RNA synthesis (*5*, *6*).

Actinomycin C_3 the structure of which has been elucidated by Brockmann *et al.* (*7*) is biologically equivalent to actinomycin C_1 (*8*), but differs from C_1 in the peptide chains: C_3 contains D-alloisoleucine in place of the D-valine residue of C_1. The compound named actinomycin D in the older nomenclature is identical with C_1. For injection, a concentrated solution of the crystalline substance (*9*) in ethanol (10 mg/ml) was diluted with saline to a final concentration in the range of 100 to 250 μg/ml.

Male albino rats weighing 350 g were injected intraperitoneally or in the penile vein and kept under observation until they were killed by decapitation 4 to 13 hours later. The livers were removed quickly, weighed, minced with scissors, and homogenized with Littlefield's Medium A (*10*) (2.0 ml/g tissue) in a Potter-Elvehjem device with 12 strokes of a motor driven Teflon pestle. The supernatant fluid (PM) obtained after removing unbroken cells, debris, nuclei, and mitochon-

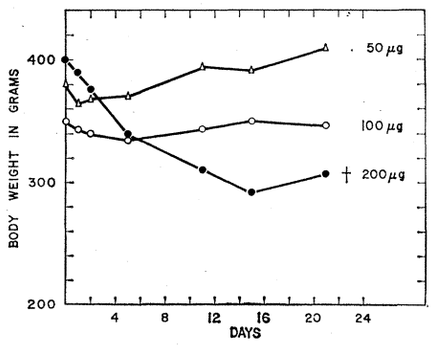

Fig. 1. Effect of different doses of actinomycin C_3 on weight of rats. Each of three rats received a single dose intraperitoneally as indicated. The animal injected with 200 μg died after 23 days, the others survived and appeared healthy.

dria by centrifugation at 20,000*g* for 10 minutes was clarified by the addition of 1.5 ml of 10 percent sodium deoxycholate (DOC) per 10 ml PM and immediately subjected to analysis by zone centrifugation.

The effect of different concentrations of DOC on the release and biological activity of ribosomes has been studied in detail. The disruption of the lipoprotein reticulum structure is not a simple function of the DOC concentration, but depends on the stoichiometry of the reactants. Hence, in order to determine the conditions optimal for completely dissolving the microsomes without loss of biological activity, a constant quantity of microsomes was exposed to various concentrations of DOC. The results in Table 1 show that, for the amount of PM supernatant used in these experiments, DOC concentrations of 1.1 to 1.3 percent gave optimal yields of biologically active ribosomes. Ribosomes were purified and the DOC, which interferes with amino acid incorporation, was removed by zone centrifugation through a sucrose solution the upper layer of which was 0.5*M* and the bottom 2.0*M* (*1*). With this method, the recovery of ribosomes and the distribution of aggregate sizes in the pellet are determined by the centrifugation time. Under our conditions most of the ergosomes, that is, particles of size $\geqslant (73S)_5$, are recovered in the pellet after 4 hours. Complete sedimentation of the smaller particles requires about 8 hours of centrifugation. Ribosomes thus purified have been characterized with respect to chemical composition, physical properties, and biological activity in protein synthesis (*1*). The data indicate that the ribosomes are highly pure and not attached to fragments of endoplasmic reticulum.

Table 1. Effect of DOC concentrations in PM supernatant on recovery and amino acid incorporation activity of ribosomes.

DOC in PM supernatant (%)	Ribosomal RNA recovered per 1.0 g liver (mg)*	C^{14}-leucine incorporated (count/min)	
		By ribosomes recovered per 1.0 g liver†	Per mg of RNA
0.7	1.18	7400	6250
1.1	1.03	7300	7100
1.3	1.03	7470	7250

* RNA estimated from the absorbancy at 260 mμ by the relation 20 absorbancy units = 1.0 mg of RNA. Recovery of ribosomes from pellet obtained after 4 hours of centrifugation through 2*M* sucrose at 105,000*g*. † The standard incorporation mixture contained the following additions: 0.2 ml ribosomes equivalent to 0.6 g liver, about 4 mg enzyme protein (*p*H 5) (*10*), 0.1 μmole each of 19 L-amino acids excluding leucine, and 0.04 μmole DL-leucine-1-C^{14} with a specific activity of 10.2 mc/mmole. The incubation time was 30 minutes.

Amino acid incorporation was measured as previously described (*1*). The standard incorporation mixture, adjusted to 1.0 ml, contained the following reagents in μmoles: $MgCl_2$, 6.2; HCl-tris buffer *p*H 7.6, 30; 2-mercaptoethanol, 2.0; adenine triphosphate (ATP), 1.0; guanine triphosphate (GTP), 0.4; K phosphoenolpyruvate, 10; crystalline pyruvate kinase (0.01 mg), C^{14}-leucine, soluble enzymes, and ribosomes as specified. After incubation at 37°C, the reaction was stopped by adding 1 ml 1*M* NaOH. The reaction mixtures were kept at room temperature for 1 hour to remove the radioactive leucine bound to transfer-RNA. In the zero-time controls, the samples were kept at 0°C and immediately subjected to this treatment. The protein was precipitated with $HClO_4$ containing Hyflo Super-Cel, filtered, washed, and assayed for radioactivity in a liquid scintillation counter (*1*). Counting efficiency was about 50 percent.

Zone centrifugation analysis was carried out with a Spinco SW 25 rotor. Test material was layered in the form of an inverted gradient (*11*) over 27.5 ml of an exponential sucrose gradient (10 to 34 percent) and centrifuged for the specified periods. The bottom of the plastic tube was punctured with a hypodermic needle and the fluid was collected by passage through a continuous flow ultraviolet-absorption cell attached to a strip chart recorder. A constant-flow rate was maintained with a precision pump. The sedimentation constants were estimated by comparison of the peak positions with an experimental reference curve obtained by calibration of the sucrose gradient with standard ribosome preparations. The numerical *S* values were computed on

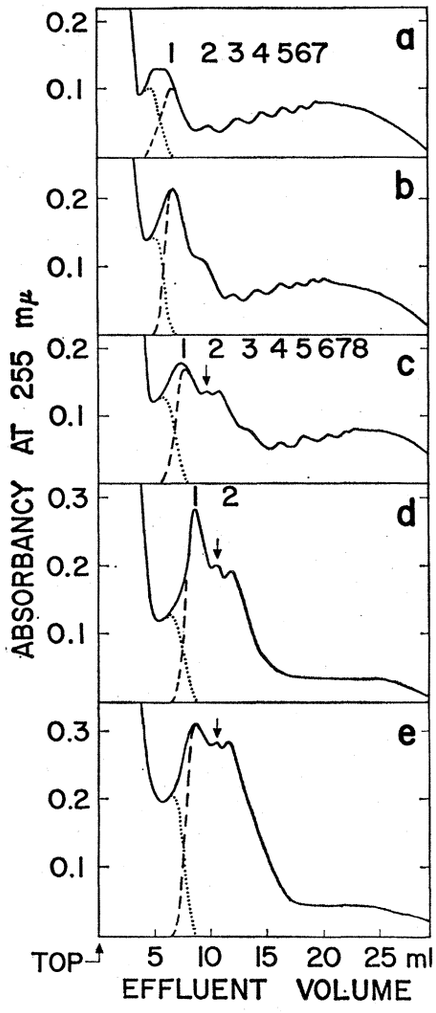

Fig. 2. Sucrose gradient patterns illustrating the breakdown of liver ergosomes after injection of actinomycin C_3 into rats. (*a*) No actinomycin control; input: 0.3 ml PM supernatant; centrifugation time: 2.5 hours at 25,000 rev/min. (*b*) 0.20 mg actinomycin intravenously, sacrificed 4 hours after injection. Input and centrifugation as in (*a*). (*c*) 1.0 mg actinomycin intravenously, sacrificed 4 hours after injection; input: 0.4 ml; centrifugation time: 3 hours at 25,000 rev/min. (*d*) 1.0 mg actinomycin intravenously + 1.0 intraperitoneally, 3 hours later; sacrificed 8 hours after first injection; other conditions as in (*c*). (*e*) 1.0 mg actinomycin intraperitoneally, sacrificed 13 hours after injection. Other conditions as in (*c*). One rat each was used for experiments *b* to *d*, 2 rats for (*e*). The dotted lines indicate the leading edge of the metalloprotein peak and the arrows the position of the 98*S* peak. The peak position of the 73*S* monomer and its oligomers are marked by the numbers 1 to 8.

Table 2. Amino acid incorporation activity of ribosomes isolated from the liver of normal and actinomycin treated rats.

Ribosome input (mg RNA)*	Ergosome content (%)†	C^{14} leucine incorporated (count/min)‡		
		Per assay ‡	Per 0.1 mg	
			Total RNA	Ergosomal RNA
	Standard ribosomes from normal rat liver (4 hr pellet)			
0.60	88	3210	535	607
	Standard ribosomes, actinomycin added in vitro (10 μg/ml)			
0.60	88	3230	538	612
	Ribosomes from actinomycin-treated rats (5 hr pellet); Fig. 3a			
0.33	63	1290	390	620
	Ribosomes from actinomycin-treated rats (8 hr pellet); Fig. 3b			
1.30	34§	2600	200	588

* Estimated from the absorbancy at 260 mμ where 20 Absorbancy Units = 1.0 mg RNA. † The ergosome content of the ribosome preparation was defined as the fraction of particles with aggregate size $\geq$ $(73S)_5$ and was computed from the appropriate integrated areas in the automatically recorded sedimentation patterns. ‡ The standard incorporation mixture contained the following additions: ribosomes equivalent to the specified amounts of RNA; 0.3 ml "postmicrosomal" supernatant (*1*); and 0.02 μmole DL-leucine-1-C^{14} with a specific activity of 21.5 mc/mmole. Actinomycin was mixed with the supernatant before addition to the incubation mixture; incubation time 10 min. § This value was computed from the sucrose gradient patterns obtained after 2 hours centrifugation at 25,000 rev/min. A 4-hour sedimentation diagram of this preparation is shown in Fig. 3*b*. The ergosome content of this preparation (34 percent) is higher than the value (20 percent) estimated for the parent PM supernatant (Fig. 2*e*), because of some loss of lighter particles during rinsing of the pellet.

the basis of 73*S* for the monomeric ribosomal particle unit which represents an average of published values.

The toxicity of actinomycin in mice and rats has been studied in detail by Philips *et al.* (*12*). In order to establish the most suitable dosage range for our experiments, a preliminary titration of toxicity was carried out with rats weighing 350 to 400 g. In agreement with the results of Philips *et al.*, we found that intravenous or intraperitoneal injection of 0.5 to 1.0 mg of actinomycin C_3 caused acute toxic effects and death of the animals after 24 to 48 hours. Lower doses produce a chronic toxic disorder characterized by a rapid loss of weight during the first week followed by a gradual emaciation and death after several weeks. Rats receiving 100 μg or less recovered after an initial loss of weight (Fig. 1).

The breakdown of the ergosome structure resulting from the action of actinomycin is clearly revealed in the sedimentation patterns reproduced in Fig. 2. The sedimentation diagram of the PM supernatant from control rats shows the characteristic sequence of seven peaks ranging in position from 73*S* to 259*S*. The peaks at 113, 147, 178, 206, 234, and 259*S* represent oligomers of the 73*S* particles corresponding to the series $(73S)_2$, $(73S)_3$, and so forth (*1*). The 73*S* peak is partially covered by a slower ultraviolet-absorbing component visible in the gradient as a sharp band of intense yellow color that is probably associated with a metalloprotein (*1*). The size distribution of the population of aggregates shows a peak near the heptamer with a large proportion of unresolved heavy aggregates sedimenting between 250*S* to 500*S*. The patterns obtained with different animals are reproducible if the isolation procedure is carried out rapidly at temperatures close to 0°C. Under these conditions, 60 to 70 percent of the ribosomes are present as ergosomes, that is, aggregates of particle size $\geqslant (73S)_5$. Although the steady-state fraction of ergosomes in intact cells is not known, it is possible that it is much higher and that the 30 to 40 percent of smaller particles in the cell-free extracts are breakdown products formed during the isolation procedure.

Injection of a lethal dose of actinomycin causes a distinct shift toward smaller aggregates which, after 4 hours, is reflected in a conspicuous rise of the 73*S* and 113*S* peaks (Fig. 2, *b* and *c*). The effect is somewhat more pronounced with a 1 mg dose, which causes death after about 24 hours, than with a five times smaller dose which kills the animal only after prolonged chronic toxicity. At the end of 8 hours a dramatic change has taken place: most of the ergosomes have broken down into 73*S* monomers and 113*S* dimers, and the peaks resulting from the larger aggregates have disappeared. The distribution pattern observed after 13 hours was similar, except that the relative proportion of dimers was larger (Fig. 2, *d* and *e*).

The sedimentation diagrams in Fig. 2 show that a relatively constant fraction of the ultraviolet-absorbing material remained in a highly aggregated state even at the end of 13 hours after the injection of large actinomycin doses and sedimented as a diffuse band between 200 to 500*S*. The nature of this material was examined in further experiments. The PM supernatant from two rats, which had received 1.0 mg actinomycin 13 hours before they were killed, was divided into two portions and centrifuged through a double layer consisting of 0.5-*M* and 2.0*M* sucrose solution for 5 and 8 hours respectively. By this procedure the dense nucleoprotein particles are separated from the soluble proteins in the PM supernatant (*1*). The purified ribosomal pellets were then resuspended and analyzed in a sucrose gradient (Fig. 3, *a* and *b*). The sedimentation pattern of the pellet from the 8-hour centrifugation (Fig. 3*b*) shows a particle size distribution corresponding to that of the PM supernatant (Fig. 2, *d* and *e*), indicating that most of the ribosomes had been recovered in the preceding purification. In contrast, the pellet from the 5-hour centrifugation contained most of the ergosomes but only 15 to 20 percent of the 73*S* and 113*S* particles originally present in the PM supernatant. The sedimentation pattern of this purified heavy-aggregate fraction shows the clearly resolved group of peaks characteristic of ergosome preparations (Fig. 3*a*). Hence, the material which sedimented as a diffuse band in the gradient of the original PM supernatant was not an artifact, but consisted of well-defined ribosomal aggregates. Moreover, Table 2 shows that the amount of protein synthesized by these purified ribosome preparations was proportional to their ergosome content. Comparison with ribosome preparations from normal rat liver reveals that the extent of orgosome breakdown resulting from the action of actinomycin in vivo is accompanied by a corresponding loss of biological activity in vitro. On the other hand, addition of actinomycin to the incorporation mixtures in vitro had no effect on protein synthesis in the concentration range tested (0.1 to 10 μg/ml). These results also demonstrate that a residual fraction corresponding to about 20 percent of the original nucleoprotein resists breakdown and remains biologically active for prolonged periods even after administration of high doses of actinomycin.

An interesting feature of the ergosomal breakdown process observed

after treatment with actinomycin is the accumulation of a relatively large proportion of dimers in addition to the 73*S* monomeric particles and the appearance of a 98*S* peak. More extensive data on this point, to be published in a forthcoming paper, indicate that the 73*S* particles tend to form dimers by unspecific aggregation. Similarly, the 98*S* particles might arise from attachment of a 30*S* subunit to a 73*S* ribosome. This would also explain the appearance of a 53*S* peak in these preparations (Fig. 3*b*).

The occurrence of a rapidly labeled RNA of high specific activity in the nuclei or whole cells of mammalian origin has been reported from several laboratories (*13*), and its function as a genetic messenger for protein synthesis has been implied. A characteristic feature of nuclear RNA synthesis is its sensitivity to actinomycin which causes 90 to 100 percent inhibition in concentrations of 1 to 10 μg/ml (*5*, *6*). Equivalent doses are lethal for mammals (*12*). Actinomycin is taken up very rapidly by mammalian cells and accumulates in the nuclei (*5*). It

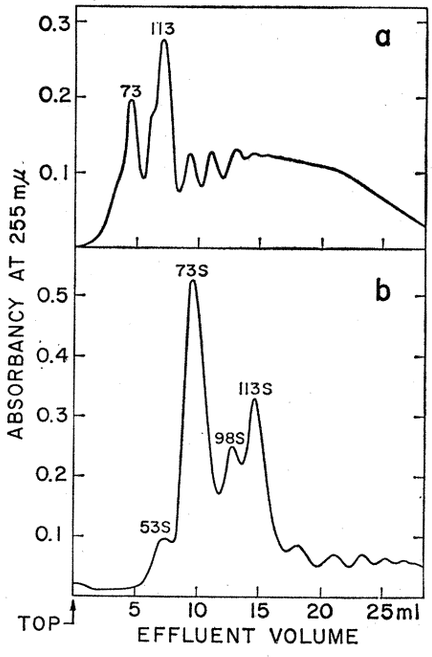

Fig. 3. Sucrose gradient patterns of purified ribosomes isolated from PM supernatant of actinomycin-treated rats. Ribosomes recovered from pellet obtained after centrifugation of PM supernatant through a double layer of sucrose for (*a*) 5 hours; (*b*) 8 hours. Input: ribosomes containing approximately (*a*) 1 mg; (*b*) 1.6 mg RNA. The PM supernatant was from the same actinomycin-treated rats as in Fig. 2*e*. Centrifugation time: (*a*) 2 hours; (*b*) 4 hours at 25,000 rev/min.

combines specifically with DNA both in vivo and in vitro and hence blocks the DNA-dependent RNA polymerase (*5*, *14*).

In a previous paper we have presented evidence for the association of mRNA with ribosomes to form characteristic aggregates which represent the functional units of protein synthesis. The structural model of the ergosome, which we predicted on the basis of extensive physicochemical and biochemical studies in vitro (*1*), has been confirmed by the striking electron micrographs of Warner, Rich, and Hall (*15*). The present results add further support to these interpretations. Viewed in the context of the highly specific mechanism of actinomycin action, both the breakdown of ergosomes in vivo and the characteristic toxic effects observed after administration of actinomycin to animals are consistent with the concept of a metabolically unstable messenger that is synthesized in the nucleus and subsequently used as template in ribosomal protein synthesis. This interpretation assumes that actinomycin has no direct effect on the structural and functional integrity of ergosomes but acts indirectly by inhibition of the synthesis of mRNA which ultimately results in the depletion of ribosome-associated mRNA. Control experiments have indeed shown that the incorporation activity and sedimentation properties of ergosomes are unaffected by high concentrations (10 μg/ml) of actinomycin in vitro. Furthermore, the ribosomal particles which are released as a result of mRNA breakdown are structurally intact, and the rats remain physically active for many hours after they have lost most of their liver ergosomes.

The mechanism of mRNA breakdown in vivo and its average rate of turnover in normal liver cells are not known. The difficulties in obtaining precise kinetic data with whole animals are obvious. Our results indicate that 50 to 80 percent of the ribosome-associated mRNA breaks down within 4 to 8 hours after injection of actinomycin doses that are expected to inhibit mRNA synthesis almost completely. A somewhat similar approach has been used by Levinthal *et al.* for measuring the turnover rate of mRNA in bacterial cells (*6*). It is uncertain, however, whether the rate of mRNA breakdown observed after inhibition of its synthesis by actinomycin is the same as under steady state conditions in normal cells. Nevertheless, our method for measuring the breakdown of mRNA by its effect on the ergosome structure in vivo offers a convenient tool for the study of cellular regulatory mechanisms. Its application to the elucidation of the mechanism of such dramatic metabolic shifts as in virus infection, enzyme induction, antibody synthesis, and so forth, appears particularly promising. Moreover, the production with actinomycin of "messenger-free" ribosomes under physiological conditions opens a new approach for reconstruction experiments with either synthetic or natural messengers (*16*).

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16. Supported by grant No. IN58B from the American Cancer Society Institutional Research Grant Committee and grants No. E-1500 and E-4303 from the U.S. Public Health Service. One of us (F.O.W.) was supported by training grant No. 2E-80, and another (H.N.) was supported by senior research fellowship No. SF-274 from the U.S. Public Health Service.

10 January 1963