GDH, by immunologic and kinetic criteria, appears quite different in structure and does not have the same allosteric response.

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Actinomycin D: An Effect on Rat Liver Homogenates Unrelated to Its Action on RNA Synthesis

Abstract. *Liver homogenates prepared from rats injected with high doses of actinomycin D show a decrease in polyribosome content and in amino acid incorporation. These effects are not observed in rat liver not subjected to homogenization and are independent of the inhibition of RNA synthesis caused by the antibiotic.*

Actinomycin D inhibits DNA-dependent RNA synthesis in both mammalian (*1*) and bacterial (*2*) systems. However, certain effects of the antibiotic cannot be explained on this basis alone. For example, a rapid loss of pre-existing RNA has been described in mouse fibroblast cells (*3*) and in Ehrlich ascites tumor cells (*4*) when these cells are exposed to the antibiotic. Further, although cell-free preparations from the livers of rats given large doses of the drug show diminished amino acid incorporation into protein and a striking reduction in the number of polyribosomes (*5*), our studies indicate that these effects are not demonstrable in the same livers *not* subjected to cell fractionation. Our observations indicate that actinomycin D diminishes the protein-synthesizing capacity of rat liver homogenates by a mechanism independent of its action on RNA synthesis.

Table 1. Differences in effects of actinomycin (5 mg/kg, injected intraperitoneally) on rat liver slices and on microsomal fractions. The data in each line are from a single experiment. The results shown in Fig. 4 are from a separate experiment not shown in this table.

Time after injection (hr)	Effect (% of control)			
	Liver slices		Microsomes	
	Orotic acid-C^{14}	Leucine-C^{14}	Leucine-C^{14}	Polyribosomes*
11	29	103	37	
11	18	90	34	
11				21
11				37
2.5	88	96	62	
2.5	100			65

* Estimated from sucrose gradient analyses.

We have recently presented evidence (*6*) that the livers of rats injected with actinomycin D in an amount of 1.5 mg/kg of body weight incorporate amino acid into protein normally, despite a reduction of over 90 percent in labeling of the RNA of the microsomal fraction, which contains the bulk of cytoplasmic messenger activity (*7*). This work supports the proposition that the bulk of rat liver cytoplasmic messenger RNA is stable. On the other hand, Staehelin *et al.* (*5*) have reported, and we have confirmed, that ribosome preparations from livers of rats given 5 mg of actinomycin per kilogram show a profound impairment in amino acid incorporation. As described by the same authors (*5*), these preparations manifest a marked reduction in polyribosomes and an increase in monomeric ribosomes (Fig. 1). Similar effects can be demonstrated in microsomal fractions from rats treated with 5.0 mg of actinomycin D per kilogram.

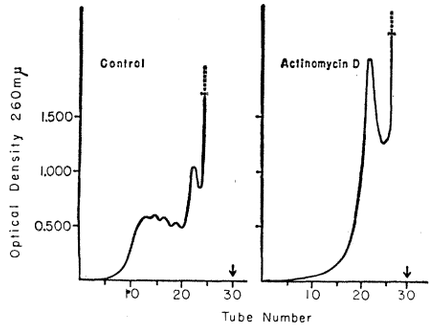

Fig. 1. Sucrose gradient centrifugation of liver particles from control and actinomycin D-treated rats. Livers from control, male, white rats (200 to 220 g) and from rats injected intraperitoneally 11 hours earlier with actinomycin (5 mg/kg) (dissolved in propylene glycol, 5 mg/ml, and diluted with an equal volume of 0.9 percent NaCl immediately before administration) were homogenized in 3 volumes of a medium containing tris *p*H 7.5 (0.01*M*), KCl (0.01*M*), and $MgCl_2$ (0.002*M*), at 0°C. The postmitochondrial supernatant fractions were made 1 percent with respect to sodium deoxycholate, and 1 ml was layered on a 25 ml, 10 to 30 percent sucrose gradient prepared in the same buffer. Centrifugation was at 25,000 rev/min for 2.5 hours at 4°C. After centrifugation the tube contents were removed through a needle inserted into the bottom of the tube and were analyzed for absorbance at 260 mμ by passage through a Gilford continuous flow cell. Polyribosomes were found in tubes 8 to 20; the peak at tube 22 represents "monoribosomes" (80*S* particles).

These preparations incorporate 35 percent as much C^{14}-labeled leucine into protein as material from untreated rats and show an apparent detachment of single ribosomes from endoplasmic reticulum (*8*) (Fig. 2). However, inhibition of isotope incorporation into microsomal RNA is only slightly greater in the livers of rats given 5 mg of the drug than that observed in rats given 1.5 mg/kg (*6*). Indeed, 3.0 mg of actinomycin results in 99 percent inhibition of isotope incorporation into microsomal RNA, but in no reduction in incorporation of C^{14}-labeled leucine by the microsomal fraction in vitro. Therefore, the effects of the higher dose of actinomycin D on amino acid incorporation and on polyribosomes in homogenates require an

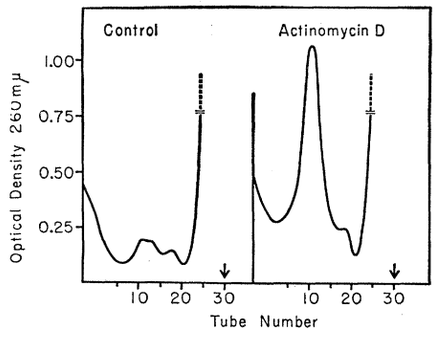

Fig. 2. Sucrose gradient centrifugation of microsomal fractions from control and actinomycin D-treated rats. Conditions were the same as those described for Fig. 1, except for the omission of deoxycholate. In both control and treated samples the bulk of the optically dense material was recovered from the pellet. This material had ratios of protein to RNA that are characteristic of microsomes. There was a large quantity of membrane-free, 80*S* particles in the fraction from treated animals (tubes 10 to 12).

explanation other than that of inhibition of messenger RNA synthesis (*5*, *8*).

Two series of experiments demonstrate that livers of rats given high doses of actinomycin undergo profound changes during cell fractionation procedures. First, electron micrographs of unfractionated liver do not show the changes observed after homogenization. Thus, while few polyribosomes are demonstrable in *homogenates* prepared from the livers of rats treated with 5 mg/kg and examined after centrifugation through a sucrose gradient (Fig. 1), abundant polyribosomes are visible in electron micrographs of the same livers *not subjected to homogenization* (Fig. 3). Further although sucrose gradient centrifugation of microsomal preparations from treated animals shows that large numbers of ribosomes have been freed from the endoplasmic reticulum (Fig. 2), very few ribosomes are seen free of membranes in the electron micrographs (Fig. 3).

Second, amino acid incorporation has been compared in slices and cell-free systems prepared from the same livers. As shown in Fig. 4 and Table 1, incorporation of C^{14}-labeled leucine is unimpaired in slices but is markedly depressed in microsomal fractions of livers from treated animals. These experiments provide further evidence that the fractionation procedure has pronounced effects on liver from treated animals. Unequivocal proof that these effects are not the consequence of an inhibition of RNA synthesis is available from experiments in which rats were killed 2½ hours after injection of actinomycin D (5 mg/kg). At this time interval, in some animals, little or no inhibition of incorporation of C^{14}-labeled orotic acid into RNA is yet demonstrable in vivo or in slices. As shown in Table 1, however, the microsomal fractions from the livers of such animals manifest both impaired amino acid incorporation and a diminution in polyribosomes. Neither parameter is as markedly affected as it is in material from rats killed 11 hours after injection of the antibiotic.

The nature of the actinomycin effect is not yet defined. Addition of actinomycin D, in concentrations up to 100 μg/ml, to microsomal preparations does not reduce amino acid incorporation into protein and does not cause breakdown of polyribosomes, as also shown by Staehelin *et al.* (*5*). Mixing experiments with polyribosomes from control preparations fail to demonstrate factors promoting their breakdown in homogenates of livers from rats treated with high doses of actinomycin.

The absence of demonstrable in vitro effects of actinomycin suggests an in

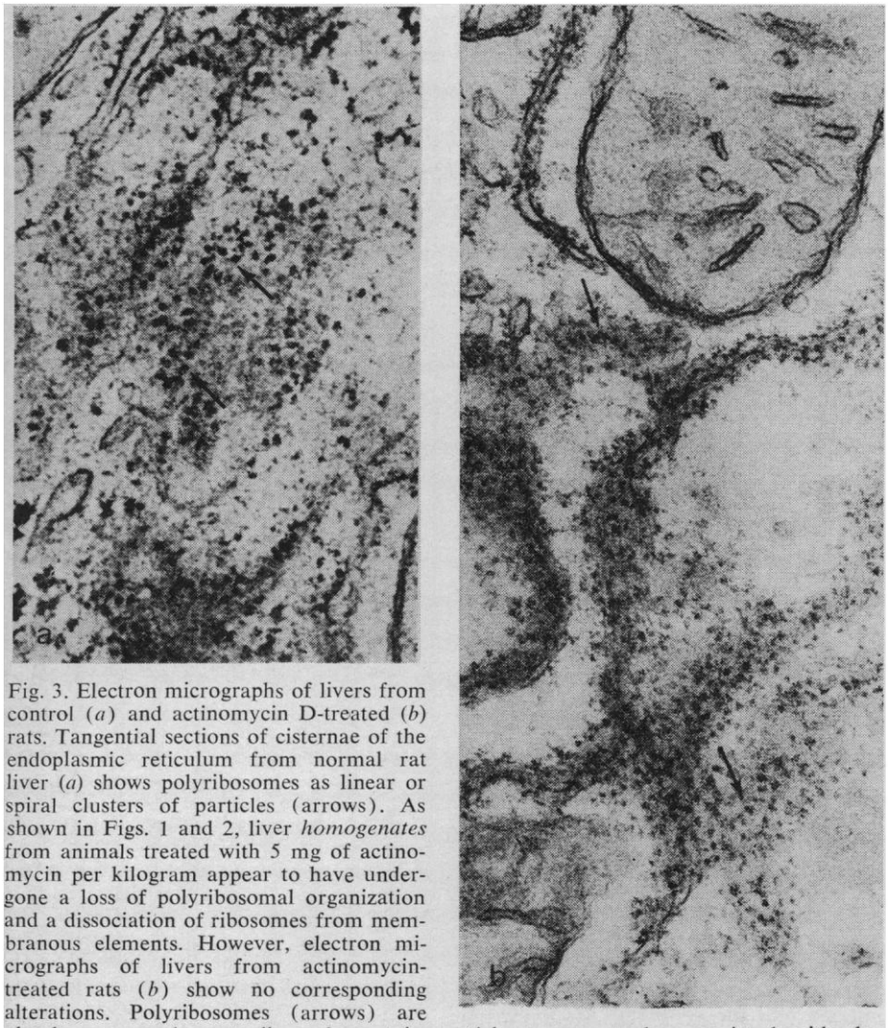

Fig. 3. Electron micrographs of livers from control (*a*) and actinomycin D-treated (*b*) rats. Tangential sections of cisternae of the endoplasmic reticulum from normal rat liver (*a*) shows polyribosomes as linear or spiral clusters of particles (arrows). As shown in Figs. 1 and 2, liver *homogenates* from animals treated with 5 mg of actinomycin per kilogram appear to have undergone a loss of polyribosomal organization and a dissociation of ribosomes from membranous elements. However, electron micrographs of livers from actinomycin-treated rats (*b*) show no corresponding alterations. Polyribosomes (arrows) are clearly seen, and most ribonucleoprotein particles appear to be associated with the membranes of the endoplasmic reticulum. The livers were fixed for electron microscopy in osmium tetroxide, dehydrated in ethanol, and embedded in araldite. Sections showing silver to gold interference colors were stained in uranyl acetate and lead hydroxide and examined in a Siemens Elmiskop I operating at 80 kv.

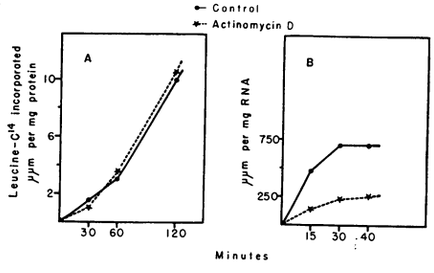

Fig. 4. Incorporation of C^{14}-labeled leucine into liver slices (*A*) and microsomal fractions (*B*). Livers were removed from control rats and from animals injected 11 hours earlier with actinomycin (5 mg/kg of body weight). A portion of each was used for preparation of liver slices (*12*), and the remainder was homogenized and fractionated to yield microsomal and *p*H 5 fractions. Incorporation is expressed in micromicromoles of leucine-C^{14} per milligram of protein in the slices, and per milligram of RNA in the microsomal fractions. Incorporation cannot be compared quantitatively, for the undoubtedly large amino acid pool in the liver slices was not measured. Liver slices were incubated with leucine-C^{14} or orotic acid-C^{14} in Krebs-Ringer bicarbonate medium at 37°C, with 95 percent oxygen and 5 percent carbon dioxide in the gas phase. Specific activities of total liver RNA and of protein samples were determined by procedures previously summarized (*7*). The microsomal fractions from control and treated animals were both incubated with control *p*H 5 fractions, as described elsewhere (*6*).

vivo action, which is not, however manifest until the tissue is subjected to fractionation procedures. The fact that more breakdown of polyribosomes is observed 11 hours after injection of the drug than at 2½ hours (Table 1) points to a slow and progressive process. This action of actinomycin is reminiscent of the effects of certain hepatotoxins, including carbon tetrachloride (*9, 10*) and ethionine (*11*). It is of interest that carbon tetrachloride leads to breakdown of polyribosomes at dose levels which have no detectable effect on liver RNA synthesis (*10*).

It is clear that the action of high doses of actinomycin D (5 mg/kg) on the protein-synthesizing apparatus is detectable only in liver subjected to fractionation. The effects observed cannot be attributed to the inhibition of RNA synthesis produced by the antibiotic. Thus, inhibition of protein synthesis and a decrease in polyribosomes in liver homogenates of rats given actinomycin in these doses cannot be used as indexes of turnover of messenger RNA.

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Evolution of a Catabolic Pathway in Bacteria

Abstract. *Genetic derepression of ribitol dehydrogenase in a mutant of* Aerobacter aerogenes, *strain 1033, enabled it to grow on xylitol, a substrate of the enzyme, but not its inducer. A derivative strain with an improved rate of growth on xylitol was obtained from the first mutant. The faster growth rate was made possible by the production of an altered ribitol dehydrogenase, as demonstrated by an increase in its activity on xylitol relative to ribitol and by its decreased heat stability.*

Since most compounds in the natural environments of microorganisms can be expected to undergo both acute fluctuations and gradual but profound shifts, it would seem that successful competition by a species should depend not only on its range of phenotypic adaptability but also on the flexibility of its genetic apparatus for the acquisition or deletion of metabolic functions. Such genetic alterations might arise either from mutational events or from recombination with foreign genomes. Although genetic transfers surely must play essential roles in biochemical evolution, this type of process serves mainly to facilitate the eclectic convergence of various genetic traits into more viable systems. Answers to how new functions arise, therefore, should in general be sought in terms of primary mutational events.

The aim of our studies is to discover examples of mutations which would enable an organism to utilize new resources. Assuming that new pathways must be built stepwise and grafted onto the preexisting metabolic network in the manner suggested by Horowitz (*1*), we can then study the problem of developing a new physiologic function for an enzyme (*2*).

Such an innovation might arise as follows: (i) If the structural gene of an inducible enzyme codes a protein which can also act on noninducing substrates, a mutation leading to the constitutive expression of this gene might allow the organism to utilize an additional substrate if its product happens to be an intermediate in the cell's metabolic network. (ii) The same purpose can be achieved if a mutation in a control gene of the above system allows the previously noninducing substrate to induce formation of the enzyme. (iii) If an enzyme is present at significant concentrations most of the time, mutations which alter its specificity, such that a new compound can be attacked, may likewise open up a new pathway.

Several examples are now available in which cells have been enabled to utilize a new substrate by genetic derepression of an enzyme: altrose-galactoside via β-galactosidase (*3*); β-glycerophosphate via alkaline phosphatase (*4*); putrescine via diamine-α-ketoglutarate transaminase (*5*); and D-mannitol via D-arabitol dehydrogenase (*6*).

We have chosen to study short-chain polyols as potential carbon sources not only because they provide interesting stereochemical problems but also because they probably enter bacterial cells freely by diffusion (*7*), thus obviating the necessity for the organism to develop new permeation systems. *Aerobacter aerogenes*, strain 1033, has been used for this study because this organism already has enzyme systems which enable it to grow on a number of polyols. In a preliminary investigation to see if mutations could endow this organism with the ability to use additional polyols, substrains were readily obtained which grew slowly on xylitol. This new growth property was conferred by the genetic derepression of ribitol dehydrogenase, for which xylitol is a substrate but not an inducer

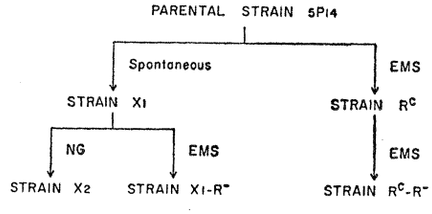

Fig. 1. Genealogy of mutants descended from parental strain 5P14. Strain X1, able to grow on xylitol, was selected by plating about 10^7 cells of strain 5P14 on xylitol agar, from which about one cell per 4×10^5 grew out. To obtain other mutants, ethyl methanesulfonate (EMS) (*19*) or 1-methyl-3-nitro-1-nitrosoguanidine (NG) (*20*) was used as mutagen. The treated cells were allowed a 100-fold growth in simple medium supplemented with arginine and guanine to complete genetic segregation and to eliminate unwanted auxotrophs. The cells were then plated on appropriate media for screening for the desired type of mutant. Strain R^c, selected for its constitutive utilization of ribitol, and strains X1-R^- and R^c-R^-, selected for their inability to ferment ribitol, were isolated from EMS-treated cells (*19, 21*) with appropriate modifications. Strain X2, which grows faster on xylitol, was isolated by recycling NG-treated cells of strain X1 on xylitol as the carbon source.