are lured to 2-methylheptadecane from 10 a.m. to 10 p.m. (sunset 8 p.m.), while a laboratory population from the same material mates exclusively at night.

Sex pheromones can also play an important role in effecting reproductive isolation in the *aurantiaca* complex even though 2-methylheptadecane is common to them all. Specificity could be effected by the use of secondary chemicals (*9*) or by differences in the release of the primary attractant, or both. Field responses (Table 1) (*10*) of males in the *aurantiaca* complex to 2-methylheptadecane suggest that other chemicals may be involved. Males of *aurantiaca, immaculata,* and *rubicundaria* are easily attracted into the traps, whereas *lamae* and males of the undescribed Black Hills species approach the traps upwind but rarely enter. Upon reaching the vicinity of the trap, males walk or hover a few centimeters over the herbage and get to about 0.1 to 1 m from the trap before abruptly flying 10 m or more away. Lower concentrations of attractant did not change this behavior. *Nigricans* never oriented toward the traps, and exceedingly few of the feral population were actually caught—although males in laboratory bioassays are attracted to filter paper disks containing 2-methylheptadecane and often attempt copulatory movements. *Ferruginosa* males were never trapped in the field. No orientation flight was noted in daytime observations, but such behavior could have occurred at night during the normal period of mating. Varying the rate of release of 2-methylheptadecane and modifying possible visual clues with the use of black traps or of dead females as calling models did not seem to change the field responses of males of these various species. It was interesting to find that 2-methylpentadecane, 2-methylhexadecane, 2-methyloctadecane, 2-methylnonadecane, 4-methylheptadecane (*4*), and *n*-octadecane were totally unattractive to tiger moth males. 2,15-Dimethylhexadecane did attract some *immaculata* males, but it was about one-tenth as attractive as 2-methylheptadecane.

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8. The gland which produces the pheromone is situated between the eighth and ninth abdominal segments. In calling, the female raises its wings slightly from the rooflike resting position and protracts and retracts the ovipositor and gland at short (< 2-second) intervals. Night observations were made with a low intensity light filtered with Kodak Wratten filters 29 and 92 (> 6100 Å).
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10. 3M Co., white Sectar traps were deployed a few centimeters above the herbage and baited with 20 μl of synthetic compound inside a polyethylene cap [see (*1*)] or with rubber septa containing 10, 30, 100, or 1000 μg of attractant, or with a saturated rubber septum.
11. We thank Dr. D. Habeck for conducting the Florida field studies, J. Muller and Dr. A. Shapiro for help in locating *H. nigricans* colonies, D. Angell for invaluable assistance with the mass spectrometry, R. W. Mackey, Jr., for chemical synthesis, and Dr. J. G. Franclemont for helpful discussions.

30 September 1970

Sea Urchin Embryos Are Permeable to Actinomycin

Abstract. *When eggs and cleaving embryos of the sea urchin are exposed to [^{3}H]actinomycin D, they become radioactive, and autoradiograms show that the radioactivity is inside the cells. At midcleavage, nuclei are more radioactive than cytoplasm. Extraction and chromatography of the intracellular labeled compounds identify them as actinomycin D and a water-soluable derivative. Conversion does not take place outside the cells. Treatment of embryos for 90 minutes with actinomycin D inhibits synthesis of RNA by more than 90 percent, leaving unaffected turnover of the pCpCpA terminals in transfer RNA. These data justify earlier interpretations of actinomycin experiments with embryos and justify use of the drug as a tool in the analysis of gene expression.*

Actinomycin has been used as an inhibitor of RNA synthesis in studies on gene expression during sea urchin development (*1*). Recent claims have been made to the effect that actinomycin fails to enter the embryos before hatching (*2*), and on this ground doubts have been expressed as to the validity of conclusions drawn from such experiments. Concepts evolved from actinomycin experiments are of some importance in developmental biochemistry (*3*); it is therefore important to be certain of the permeability of early stages to the drug. Results of a critical examination prove that the drug does penetrate eggs and cleavage stages.

Standard culture techniques were

Table 1. Radioactivity in nucleic acids from control and actinomycin-treated embryos labeled with ^{32}P. Percentages of distributions of total radioactivity (count/min) were obtained by summation in appropriate parts of the gradients shown in Fig. 4. Nucleic acid preparations were treated with deoxyribonuclease prior to gradient sedimentation and the radioactivity (count/min) in the DNA was all in oligodeoxynucleotides sedimenting at less than 4*S*. The number of counts per minute in the DNA was determined by resistance to alkaline hydrolysis. Total incorporation of ^{32}P in the presence of actinomycin was 0.3 of that in controls, hence 0.3 times the values in column 2 gives the radioactivity in each RNA and DNA fraction, labeled in actinomycin, expressed as percentage of total incorporation in controls. Values in column 3 can be expressed as percentages of corresponding fractions in column 1, and subtracting such percentages from 100 gives the percentage *inhibition* of incorporation into each fraction, attributable to presence of the antibiotic. Since 4*S* RNA labeling is unaffected, actinomycin treatment did not alter the uptake of precursors. The percentage of inhibitions (column 4) is an explicit measure of decrease in synthesis. Actinomycin D, Act. D.

Fraction	Radioactivity (% total count/min)		Act. D incorporation (% control total count/min)	Inhibition (% by fractions)
	Control	Act. D		
RNA > 4*S*	65.4*	15.5*	4.7	**92.8**
"RNA ≤ 4*S*"	34.6*	84.5*		
DNA	22.2	41.0	12.3	**44.6**
4*S* RNA	12.4†	43.5†	13.0	**—5.0**

* These are distributions of all labeled nucleic acids and sum to 100 percent. † The radioactivity in the 4*S* RNA was determined by difference.

used for embryos of *Lytechinus pictus* and *Arbacia punctulata* (*4*). For autoradiography of fixable actinomycin, unfertilized eggs or embryos were incubated in seawater containing [^{3}H]actinomycin D (*5*) at 20 or 40 μc/ml (2.5 or 5 μg/ml) for 1 or 2 hours, washed twice in seawater, fixed in Smith's or Carnoy's solution, embedded in paraffin, and sectioned at 7 μm. Slides coated with Kodak NTB-2 emulsion were exposed at 4°C. Developed autoradiograms were stained with safranin and light green.

Intracellular radioactivity was detected in the autoradiograms (Fig. 1). Silver grains were distributed abundantly over the cytoplasm as well as over nuclei of early stages; at midcleavage and later, the grains tended to be more concentrated over nuclei and mitotic figures. This is consistent with the large cytoplasmic fraction of total DNA in eggs and during early cleavage (*6*). Backgrounds in the autoradiograms were low, and there was no evidence for accumulation of radioactivity on the external cell surfaces.

Uptake and binding of actinomycin was time-dependent. Autoradiograms of cells treated for 1 or 2 hours show much higher grain density than those exposed to actinomycin for 5 minutes only. As was expected from their behavior toward other solutes, unfertilized eggs were less permeable than zygotes and embryos; grain densities over egg sections were always lower than those over cleavage stages (Fig. 1).

Autoradiographic detection of radioactivity is, for several reasons, inefficient; hence it was important next to prove that radioactivity in the cells was due to the presence there of labeled actinomycin, rather than to a contaminant. For this purpose, 16- to 32-cell stages were incubated with [^{3}H]actinomycin D (50 μc/ml) for 2 hours, washed very thoroughly, collected, and homogenized in distilled water. Carrier actinomycin was added, and the homogenate was extracted with butanol. The aqueous and organic phases were chromatographed in parallel with authentic actinomycin D and with marker amino acids. The two systems used were (i) butanol, acetic acid, water (60 : 15 : 25) for 12 to 16 hours and (ii) *n*-butyl ether, ethyl acetate, 2 percent naphthalene β-sulfonic acid (3 : 1 : 4) (*7*). Chromatograms were dried and stained with ninhydrin, and sequential segments of the paper were cut out and counted.

All radioactivity in the butanol extract was coincident with authentic actinomycin D (Fig. 2) in both separation systems. About half of the intracellular radioactivity remained, however, in the aqueous phase. This ran as a single peak with R_F less than that of actinomycin. Control experiments established that (i) the [^{3}H]actinomycin D was chromatographically identical with authentic actinomycin D (that is, there was no labeled contaminant), and (ii) [^{3}H]actinomycin D was completely stable in the incubation medium (that is, *no* conversion of the extracellular drug took place during the time in which half of the intracellular drug had been rendered nonextractable with butanol).

The more polar metabolite was soluble in cold trichloroacetic acid, was ninhydrin negative, and continued, after hydrolysis in 6*N* HCl, to migrate as a single peak with decreased R_F (change from 0.5 to 0.4). Apparently it is not any of the amino acids or cyclic peptides of the parent actinomycin and it is not a macromolecule. It is presumably a breakdown product of the heterocyclic ring. This acid-soluble product would probably not be detected autoradiographically.

Completion of the argument required assay of the effectiveness of actinomycin as an RNA synthesis inhibitor in the same experimental situation as was employed for the penetration tests. This was done in two ways. Kinetic experiments (Fig. 3) showed that the rate of incorporation of [^{3}H]uridine into RNA falls to less than 5 percent of the control rate within 90 minutes after exposure of the embryos to actinomycin D (20 μg/ml). There is a fast component of the inhibition, so that a significant effect is always seen even in the first few minutes after addition of the drug. During the short labeling periods employed, no significant radioactivity from [^{3}H]uridine found its way into DNA (*8*).

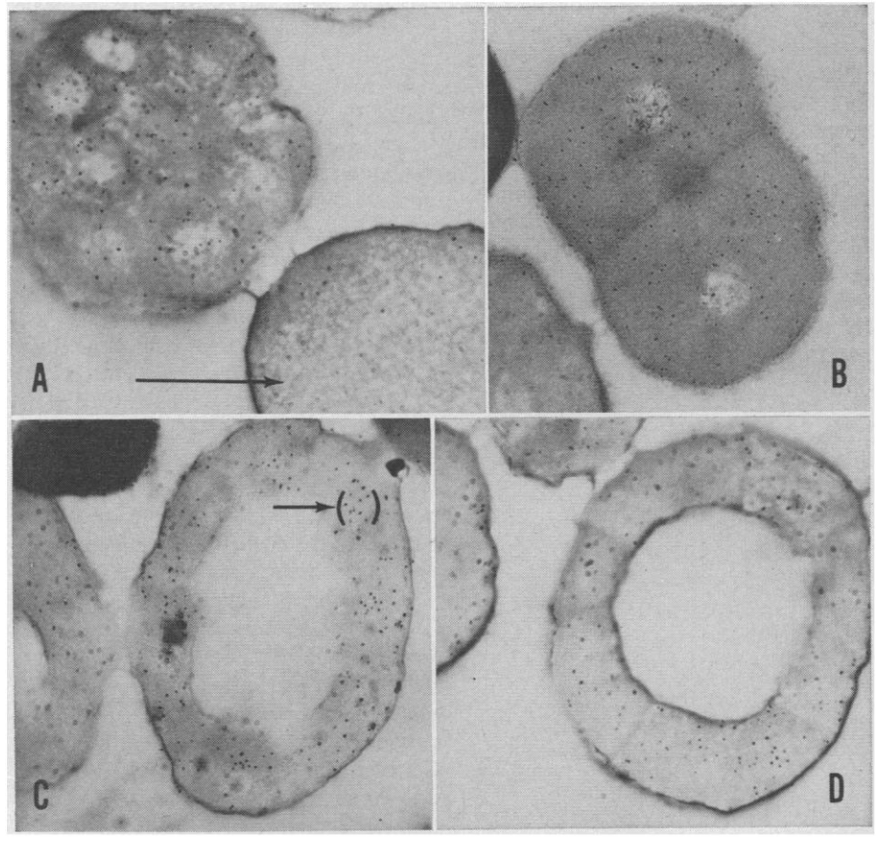

Fig. 1. Autoradiograms of sectioned eggs and embryos exposed to [^{3}H]actinomycin D; in A, C, and D embryos were incubated for 1 hour with 20 μc/ml (2.5 μg/ml) and in B, for 2 hours at the same dose. The photographic exposure for A, C, and D was 2 weeks, and for B it was 4 weeks. (A) A section of a midcleavage embryo and a section of a unfertilized egg (arrow) in the same culture. Silver grains over both sections, but more over the embryo, with tendency for clustering near and over nuclei (lighter stain, circular profiles). (B) Two-cell stage, first telophase just completed. Abundant grains over cytoplasm and nuclei. (C and D) Embryos at midcleavage ($\sim$ 100 cells). Nuclei fill a large fraction of total cell volume and are not in focus (the emulsion plane is); location and limits of one nucleus indicated by parentheses and arrow in C. Grains tend to be concentrated over the nuclei. [Approximately $\times$ 600]

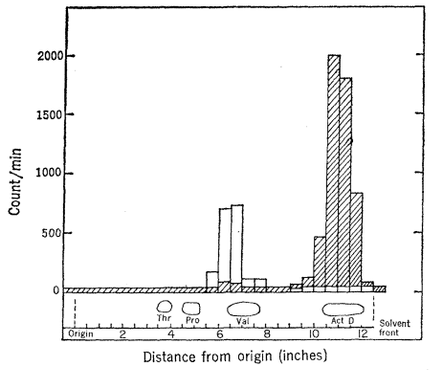

Fig. 2. Chromatography of butanol and aqueous fractions in a butanol, acetic acid, and water system. Open bar histogram, aqueous phase. Hatched bars, butanol phase. The R_F for the modal radioactive fraction was 0.5 for the aqueous phase and 0.9 for the butanol phase.

Size distribution of the RNA was examined after labeling with ^{32}P in the presence and absence of actinomycin D (Fig. 4) (*9*). Controls synthesized normal amounts of 10*S*, 20*S*, and larger molecular weight material. Drug-treated embryos produced almost no labeled RNA heavier than 4*S*. Much of the slowly sedimenting radioactivity in preparations such as these is in acid-insoluble DNA fragments arising from digestion with deoxyribonuclease. Sensitivity to alkaline hydrolysis was therefore used to determine radioactivity in RNA itself. Data from such tests, together with those from the experiment of Fig. 4, were used in the calculations summarized in Table 1. After a 2-hour incubation with actinomycin D (20 μg/ml), synthesis of RNA larger than 4*S* was reduced by more than 90 percent. Radioactivity in 4*S* RNA is mainly in the pCpCpA terminal of transfer RNA (tRNA) (*10*), and this was unchanged, while labeling of DNA was reduced in proportion to the slowing of cleavage (*1, 11*). Normal end-labeling of the tRNA shows that the entry of labeled RNA precursors into the pools was not affected by actinomycin. Therefore, decreased incorporation into high-molecular-weight RNA is synonymous with inhibition of synthesis.

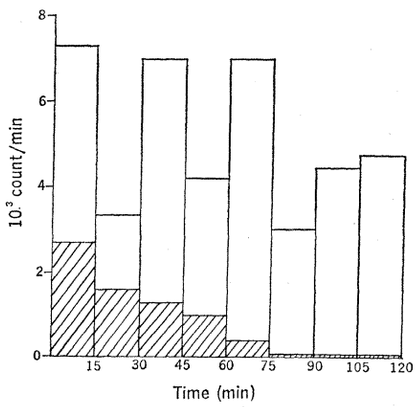

Fig. 3. The rate of incorporation of uridine into RNA. Morulae were treated with actinomycin D or kept in plain seawater as controls. Samples were labeled for 15 minutes with [5-^{3}H]uridine (5 μc/ml). Material from sodium dodecyl sulfate lysates and insoluble in trichloroacetic acid was collected on Millipore filters for liquid scintillation counting. Open bars, controls. Hatched bars, 20 μg of actinomycin D per milliliter was added at time zero.

These results contradict the earlier conclusion of Thaler, Cox, and Villee (*2*). In part, the disagreement can be attributed to their use of ^{14}C-labeled actinomycin with specific activity a thousand times less than that of the tritiated drug. Even their hatched embryos, which were claimed on the basis of bulk measurements to have incorporated actinomycin, showed too few silver grains per section (about ten) for useful interpretation, especially in view of difficulties inherent in the use of ^{14}C β-particles for high-resolution autoradiography. The argument that actinomycin does not enter cleavage stages was accompanied by data showing an amount of the drug associated with each embryo equal to that in the same volume of medium. Such data would argue for a very considerable penetration of actinomycin, unless the association were at the external cell surface. The autoradiograms presented by Thaler *et al.* could not be used to decide such a question, but those obtained with the tritiated drug can be used and reveal clearly an intracellular site.

Extraction of actinomycin and of a derivative from homogenates of washed cells is, of course, entirely independent evidence in favor of the same point, particularly because the derivative is never found externally. Calculations based on our own data do show an intracellular concentration of actinomycin roughly equal to that in the medium, after 2 hours of incubation. The time needed to achieve this condition may, however, be much shorter than 2 hours, as is suggested by results such as those in Fig. 3.

A final point concerns the ability of embryos treated with actinomycin for a few hours and then washed to recover, and, with certain schedules of

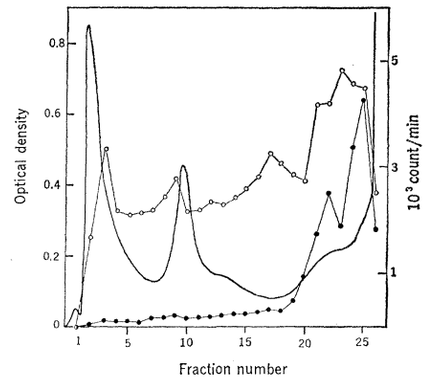

Fig. 4. Sucrose gradient sedimentation of ^{32}P-labeled RNA synthesized in the presence and absence of actinomycin. At the 8- to 16-cell stage ^{32}P (60 μc/ml) was added and incubation terminated after 1 hour. Nucleic acids were extracted by standard methods (*9*). Sedimentation was on sucrose gradients 15 to 30 percent in a Spinco SW41 rotor, 32,000 rev/min for 15 hours at 22°C. Continuous line, optical density (260 nm). Open circle data points, control radioactivity (acid insoluble). Solid circles, 20 μg of actinomycin D per milliliter was added 2 hours prior to addition of ^{32}P. Gradients were centrifuged together and were loaded with equal amounts of bulk RNA. Sedimentation from right to left.

treatment, to develop fairly normally. This too has been used to argue for impermeability of early stages to the drug (*2*). Such is not the case, for reasons already presented and because the recovery has this simple basis: DNA synthesis is virtually continuous during cleavage, the S phase occupying most of the cell cycle and the divisions becoming asynchronous after the third cycle. DNA synthesis continues in the presence of actinomycin (Table 1) and continues after the drug is washed out. Hence new DNA without bound actinomycin becames available for transcription as soon as the drug is removed, provided that this is done at an appropriate stage (*12*). The possibilty has been exploited in several recent studies (*13*). Our results prove that actinomycin D enters the cells of cleavage stage embryos and there blocks transcription. Experiments based upon these properties of the drug are therefore valid.

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15 October 1970; revised 21 January 1971 ■

Lesch-Nyhan Syndrome: Altered Kinetic Properties of Mutant Enzyme

Abstract. *Hypoxanthine-guanine phosphoribosyltransferase is virtually inactive in erythrocytes from patients with the classical Lesch-Nyhan syndrome. In one such patient, activity of this enzyme ranged from 8 to 34 percent of normal in erythrocytes when assayed with a very high concentration of magnesium 5-phosphoribosyl-1-pyrophosphate. In addition, the mutant enzyme exhibited sigmoidal kinetics with this substrate as well as an increased Michaelis constant for both guanine and hypoxanthine. These findings provide the first evidence for genetic heterogeneity within the group of patients with the Lesch-Nyhan syndrome.*

Hypoxanthine-guanine phosphoribosyltransferase (PRT) (E.C. 2.4.2.8) catalyzes the transfer of the 5-phosphoribosyl moiety of magnesium 5-phosphoribosyl-1-pyrophosphate (MgPRPP) to guanine and hypoxanthine to form their corresponding ribonucleotide derivatives, guanosine 5′-monophosphate (GMP) and inosine 5′-monophosphate (IMP). A genetic deficiency of this X-linked enzyme is associated with two clinical syndromes in man. A partial deficiency (with values up to 17 percent of normal in erythrocytes) leads to excessive uric acid production and the consequent development of uric acid calculi, hyperuricemia, and gouty arthritis; of these patients 20 percent exhibit mild neurologic symptoms (*1*). In contrast, the virtually complete deficiency of this enzyme in man (< 0.5 percent of normal activity) is associated with the Lesch-Nyhan syndrome, a bizarre behavioral disorder characterized by self-mutilation, choreoathetosis, spasticity, and mental retardation as well as increased uric acid production (*2*).

Activity of PRT in lysates of erythrocytes from a 10-year-old male with all of the classical features of the Lesch-Nyhan syndrome was approximately 0.2 percent of normal when assayed at a concentration of MgPRPP ($1 \times 10^{-3}M$) which is saturating for the normal enzyme. However, at a higher concentration of MgPRPP ($1 \times 10^{-2}M$), activity of this mutant enzyme ranged from 6 to 8 percent of normal with guanine as substrate to 34 percent of normal with hypoxanthine as substrate (Table 1). Extrapolating to saturating concentrations of these purine substrates, we found that the activity of the mutant enzyme was 12 percent of normal with guanine and 94 percent of normal with hypoxanthine. These values are within or above the range of PRT activity observed in gouty subjects with no neurological dysfunction. Hemolyzates from five other patients with the Lesch-Nyhan syndrome had no detectable activity (< 0.01 percent of normal) at MgPRPP concentrations as high as $1 \times 10^{-2}M$.

This mutant enzyme exhibited qualitatively as well as quantitatively altered kinetics with MgPRPP as the variable substrate. The effect of increasing MgPRPP concentration on rate of GMP production by normal and mutant PRT enzymes is shown in Fig. 1. The MgPRPP substrate curve observed with the mutant enzyme appeared to exhibit sigmoidal characteristics in contrast to the hyperbolic kinetics observed with the normal enzyme (*3*, *4*). The normal PRT enzyme has been

Table 1. Comparison of the kinetic properties of the normal and mutant hypoxanthine-guanine phosphoribosyltransferase (PRT) enzymes. Erythrocyte PRT enzyme activity was measured (*8*) with $1 \times 10^{-3}M$ MgPRPP (for the normal enzyme) or $1 \times 10^{-2}M$ MgPRPP (for the mutant enzyme) and either $1 \times 10^{-4}M$ [8-^{14}C]guanine (1.76×10^4 dpm/nmole, 1.15×10^5 dpm/nmole) or $1 \times 10^{-4}M$ [8-^{14}C]hypoxanthine (9.28×10^3 dpm/nmole) as purine substrates. The PRT activity in extracts of cultured skin fibroblasts (*9*) was measured similarly, except that 3.3 m*M* thymidine triphosphate was included to inhibit 5′-nucleotidase activity (*5*). The K_m of the normal enzyme for both MgPRPP and the purine substrates was estimated from a curve of $1/v$ plotted against $1/S$. The K_m of the mutant enzyme for guanine and hypoxanthine was also estimated from double reciprocal plots, while the $S_{0.5}$ for MgPRPP (that is, the MgPRPP concentration giving one-half of the maximum observed rate) was estimated from curves of v plotted against MgPRPP concentration. Product formation was proportional to enzyme concentration and time under the conditions of the experiments shown for both the normal and mutant enzymes.

Enzyme source		Phosphoribosyltransferase activity (nmole mg^{-1} hr^{-1})		Apparent $K_{m(MgPRPP)}$ (M)		Apparent $K_{m(guanine)}$ (M)	Apparent $K_{m(hypoxanthine)}$ (M)
Cell	Type	Guanine	Hypoxanthine	Guanine	Hypoxanthine		
Erythrocyte	Normal	98 ± 14*	97 ± 19*	2.5×10^{-4}		5.0×10^{-6}	1.7×10^{-5}
	Mutant	8.2 (12.1)†	33.7 (94.4)†	3.2×10^{-3}‡	2.8×10^{-3}‡	4.8×10^{-5}	1.8×10^{-4}
Fibroblast	Normal	141 ± 17§		1.0×10^{-4}			
	Mutant	10.4		$> 2.0 \times 10^{-3}$‡			

* Mean ± S.D. in 119 subjects. † Numbers in parentheses represent V_{max} of the mutant enzyme for each purine substrate calculated from the Michaelis-Menten formula ($V_{max} = v(K_m + S)/S$). § Mean ± S.D. in 13 normal cell strains. ‡ Values depict $S_{0.5}$ rather than K_m.