the overshoot voltage of the presynaptic spike (*3–5*).

The results of these studies, if also applicable to mammalian cortical neurons subjected to topical penicillin actions, lead us to restate the following hypothesis concerning the manner in which penicillin induces cortical epileptiform activity (*2*). Increased excitatory synaptic coupling in a critically large neuronal aggregate would greatly potentiate the effectiveness of preexisting recurrent excitatory pathways. This would create precisely those conditions requisite for augmented positive feedback that leads to generation of paroxysmal discharges within a large poplation of cortical neurons linked by axon collateral excitation. Indeed, a recent account of iontophoretic application of penicillin onto single cortical cells (*6*) lends further support to this proposed explanation by showing that a clear increase in test EPSP amplitude was produced by iontophoresis and, even more importantly, that full paroxysmal discharges were produced only upon surface topical application that affected much larger groups of cells.

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References and Notes

1. G. F. Ayala, S. Lin, C. Vasconetto, *Science* **167**, 1257 (1970).
2. M. Dichter and W. A. Spencer, *J. Neurophysiol.* **32**, 649 (1969); *ibid.*, p. 663; H. Matsumoto, G. F. Ayala, R. J. Gumnit, *ibid.*, p. 688; G. F. Ayala, H. Matsumoto, R. J. Gumnit, *ibid.* **33**, 73 (1970).
3. B. Katz and R. Miledi, *J. Physiol.* **192**, 407 (1967).
4. See A. Mauro, F. Conti, F. Dodge [*J. Gen. Physiol.* **55**, 497 (1970)] for the behavior of the squid axon transmembrane potential during applied discrete transmembrane currents.
5. T. H. Bullock, *J. Neurophysiol.* **11**, 343 (1968).
6. G. Walsh, *Neurology* **20**, 415 (1970).
7. This work was performed while we were guests of the Stazione Zoologica di Napoli with the support of the American Tables Committee of the American Institute of Biological Sciences. Financial assistance was received from St. Paul-Ramsey Hospital Medical Research Foundation and other local sources. We thank the Grass Instrument Company and Tektronix, Inc., for lending research equipment for use in Europe; and the Upjohn Corporation for donating the uniform batch of sodium penicillin. Work performed in the United States was partially supported by the National Institute of Neurological Diseases and Stroke (grants NB 5466, NB 05980, and NS 09361).

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Age-Associated Changes in the DNA of Mouse Tissue

Abstract. *The template activity of DNA with calf thymus DNA polymerase has been studied in sections of fixed brain, liver, and heart tissues from young and senescent mice. The enzyme catalyzed greater incorporation of deoxyribonucleotide monophosphates into nuclei of old mouse neurons, astrocytes, Kupffer cells, and heart muscle fibers. The results are interpreted as the accumulation of DNA strand breaks with aging.*

Questions of aging at the molecular level have been particularly difficult to pursue because of the problem of detecting subtle molecular changes. In this report we will present findings which may have some relevance to alterations in the DNA of certain cell populations of very old mice and may therefore aid those who have been addressing themselves to questions of differentiation and aging at the molecular level.

Calf thymus DNA polymerase requires partially denatured DNA with a 3′-hydroxyl group facing a single-stranded region of at least 20 nucleotides to catalyze the synthesis of complementary strands (*1*). This enzyme reaction has been carried out in fixed cell nuclei and the incorporation has been detected by an autoradiographic technique (*2–5*). Previously, nuclei of ethanol-fixed cells did not incorporate labeled deoxyribonucleotide triphosphates in the presence of the enzyme unless these cells were first treated with acid (*2–5*), alkali (*4*), or dimethyl sulfoxide (*4*) in order to denature DNA. Of particular interest are observations made on differentiating lens fibers (*3, 4*). With increasing degeneration, fiber cell nuclei, treated to denature DNA, incorporated increasing amounts of radioactivity in the presence of calf thymus DNA polymerase. This result was interpreted to indicate DNA strand scission in degenerating nuclei (*3, 4*). We now report the template activity of DNA in sections of fixed brain, heart, and liver tissues from young and senescent mice.

Tissues were collected from both young (3 to 4 months old) and aged (30 to 35 months old) (C57BL/6 Cum × C3H/Anf Cum)F_1 mice without pathological lesions; these mice have a mean life-span of approximately 30 months (Fig. 1).

In acid-denatured preparations, all types of cell nuclei were labeled (with more than three grains per nucleus) if treated with calf thymus DNA polymerase. With the exception of old brain tissue (Fig. 1A), label was localized exclusively over the nuclei. The labeling intensity, however, varied from one cell type to another (Fig. 1A). For a given cell type, labeling intensity varied between young and senescent tissue preparations. In the cerebral cortex, a gradation of labeling existed among the six different cell layers. Labeling was al-

Table 1. Mean grain counts per nucleus. Three grains per nucleus were substracted as background. At least 200 nuclei of astrocytes and neurons, 50 Kupffer cell nuclei, and 100 heart muscle cell nuclei were scored for number of grains. The similarity between the means was tested statistically by the so-called Z test (*16*) [limit of significance: probability, $P(X < -Z \text{ or } X > Z)$, $2P < .10$]. Compared to the findings for neurons and astrocytes of undenatured young brain, the labeling intensities in these cell types differed significantly in undenatured old brain ($P = .011$) and acid-denatured young brain ($P = .00013$). On the other hand, mean grain counts between undenatured old brain and acid-denatured young brain did not differ significantly ($P = .11$). A detailed analysis has been described (*6*). The intensity of labeling in nuclei of acid-denatured, old Kupffer cells was statistically greater than that of acid-denatured, young Kupffer cell nuclei ($P = .0286$); of undenatured, young Kupffer cell nuclei ($P = .0028$); and of undenatured, old Kupffer cell nuclei ($P = .0898$). The labeling intensity in undenatured, old Kupffer cell nuclei was not statistically different from that in undenatured, young Kuffer cells ($P = .1371$). Labeling in nuclei of acid-denatured cells from old heart muscle differed statistically from labeling in nuclei of acid-denatured, young ($P = .0474$) and undenatured, young ($P = .0010$) heart muscle cells. Labeling in undenatured, old heart muscle nuclei was not statistically different from that in nuclei of acid-denatured, old ($P = .1240$) and undenatured, young ($P = .3681$) heart muscle.

Cell type	Mean grain counts per nucleus			
	Young		Old	
	Undenatured	Acid-denatured	Undenatured	Acid-denatured
Neurons and astrocytes	8.92	30	22.13	*
Kupffer cells	2	4.5	6.28	13.9
Cardiac muscle	0.67	1.58	3.5	8.63

* Nuclei were too heavily labeled to obtain grain counts.

ways greater in neurons and astrocytes from old brain than in the same cell types in young brain (Fig. 1, A and B). In tissue from both senescent and young brains, microglia were labeled less than neurons and astrocytes. In heart sections, nuclei of acid-denatured, old striated muscle cells were labeled more heavily than the corresponding cell type in preparations of young muscle cells (Fig. 1, C and D). In both young and aged livers, Kupffer cells were always labeled more intensely than hepatocytes (Fig. 1, E and F). Kupffer cell nuclei in senescent livers incorporated more radioactivity than those in young livers. Negative controls showed no nonspecific binding of radioactive precursor to tissue sections when DNA polymerase was absent from the reaction mixture.

Labeled nuclei were present in undenatured, ethanol-fixed sections of young and old brain (Fig. 2, A and B), although the labeling intensity was greater in old brain. Nuclei of heart muscle cells from aged animals incorporate significant radioactivity (Fig. 2C), whereas the nuclei in young heart cells were labeled only faintly (Fig. 2D). Kupffer cell nuclei were labeled in both old (Fig. 2E) and young liver sections, but those in the old sections were labeled more intensely. Although hepatocytes in young liver preparations did contain label, it was not as evident as in old hepatocytes.

To test for statistical differences in labeling intensity, grain count analyses were performed. Although the background grain count was approximately 0.5 grain per equivalent nuclear area for all groups, three grains per nucleus were substracted as background. The grain count distributions are of the Poisson type (*6*). The mean grain counts are shown in Table 1; a statistical verification of our observations is included in the legend to the table.

In addition to brain, heart, and liver, we attempted to apply this technique to lymphatic tissue such as spleen, bone marrow, and lymph node. In all cases template activity of DNA was so high that it obscured any apparent differential existing between tissues from the two different age groups. This difficulty

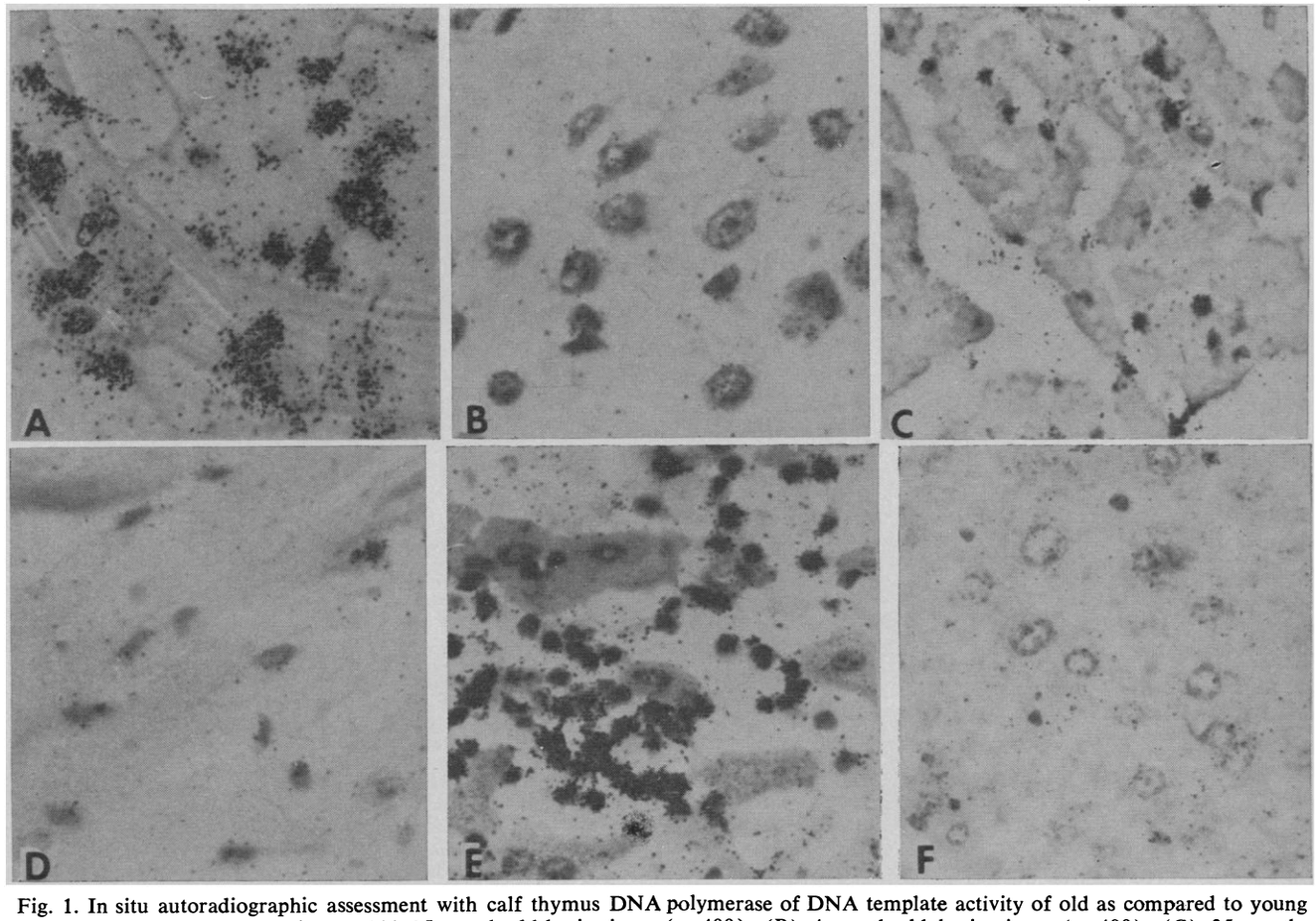

Fig. 1. In situ autoradiographic assessment with calf thymus DNA polymerase of DNA template activity of old as compared to young adult, acid-denatured mouse tissues; (A) 35-month-old brain tissue (× 400), (B) 4-month-old brain tissue (× 400), (C) 35-month-old heart tissue (× 400), (D) 4-month-old heart tissue (× 400), (E) 35-month-old liver tissue (× 400), (F) 4-month-old liver tissue (× 400). Brain tissue was stained with gallocyanin. Heart and liver tissues were stained with toluidine blue. Tissues from brain, liver, and heart of young mice (3 to 4 months old) and senescent mice (30 to 35 months old) were fixed in 80 percent ethanol immediately after they were killed; the tissues were then embedded in paraffin ($t_m = 52°$ to 53°C). Sections (3 to 5 μm) were divided into two groups. One group was treated with 0.01*N* HCl (30 minutes at room temperature) to denature DNA present in situ and was designated as "acid-denatured." The other group was not treated with acid and was designated "undenatured." Preparations were incubated with calf thymus DNA polymerase reaction mixture for 60 minutes (*2*, *3*). The reaction mixture contained a large excess of the enzyme, [^{3}H]deoxyadenosine triphosphate (5.7 c/mmole; Schwarz BioResearch), and other unlabeled deoxyribonucleoside triphosphates—deoxythymidine, deoxyguanosine, and deoxycytidine triphosphates. The nonspecific binding of labeled precursor was checked in tissue sections from both acid-denatured and undenatured groups by incubating them with the reaction mixture from which DNA polymerase was omitted. After incubation, sections were rinsed with 0.15*M* NaCl (ice cold) and washed in 5 percent trichloroacetic acid containing 1 percent inorganic pyrophosphate to remove unincorporated labeled precursor. Slides were prepared for autoradiography as previously described (*3*).

may be attributed to the significantly higher lysosomal activity present in these rapidly dividing cell populations, as compared to the tissues of very low or zero mitotic rates.

In contrast to earlier observations (*2–5*) we observed, in this experiment, DNA polymerase–catalyzed incorporation in certain types of cell nuclei of undenatured brain, heart, and liver from both young and senescent mice. There could be one or more reasons for this discrepancy. First, an unexplained differential denaturation of DNA in astrocytes, neurons, heart muscle cells, and Kupffer cells during fixation could have affected our present findings. If this is true, it would appear that fixation affects cells in senescent tissues more than in young tissues. If our results are due to an autolysis of cells because the fixation of tissue was incomplete or too slow, we would expect to see a nonrandom pattern of labeling, in which cells in the interior of tissues showed proportionally greater labeling than that in the more exposed exterior regions; since we saw no such patterns in any of the tissues examined, fixation is probably not the reason for labeling in undenatured tissues. Second, the precursor of higher specific activity used in our experiment may have increased the sensitivity of the method over that in earlier experiments (*2–5*). However, at least in the lens, this does not seem to have played any significant role (*3, 4*). Third, the types of tissue used in our experiment were different from those used in the earlier studies, which could account for the discrepancy in results. We favor the last of these reasons and further conclude that the observed incorporation into trichloroacetic acid–insoluble material of undenatured cell nuclei is due to DNA polymerase–catalyzed synthesis along the template DNA in these nuclei, which was probably present prior to the fixation. However, direct, highly sensitive tests not currently available are needed to clearly resolve questions on the mechanisms by which DNA acquired template activity when the mouse reached the age of 3 to 4 months.

A comparison between nuclei of young and senescent cells shows that the senescent nuclei are more intensely labeled, a finding which reflects an age-associated increase in either DNA denaturation or strand scission. If denaturation of DNA were the sole change associated with aging, then the labeling intensities in both young and senescent cell nuclei after acid treatment could be expected to reach the same level. Since incorporation is higher in senescent nuclei of the neuron, astrocyte, and Kupffer cell after acid denaturations, we conclude that, with aging, nuclear DNA in these cells undergoes strand scission similar to that seen in terminally differentiating lens fibers (*3, 4*) and to that produced after x-irradiation (*6*) (see legend to Table 1). Since calf thymus DNA polymerase only accepts single-stranded template DNA (*1*), it can be inferred that age-associated increase in incorporation is perhaps due to single strand breaks (*7*). This conclusion is contingent on the assumptions that, with aging, the self-absorption coefficient (*8*) does not change and that the DNA-protein complex does not dissociate (*9*), which otherwise might increase the susceptibility of DNA to damage during and after fixation (*3, 4*). Our conclusion is in agreement with earlier predictions (*10*). We could show no statistical differences among labeling intensities in nuclei of undenatured heart muscle cells from young and old mice (see legend to Table 1).

Nondividing cells such as astrocytes, neurons, and heart muscle cells (*11*) are labeled more readily than microglia. In

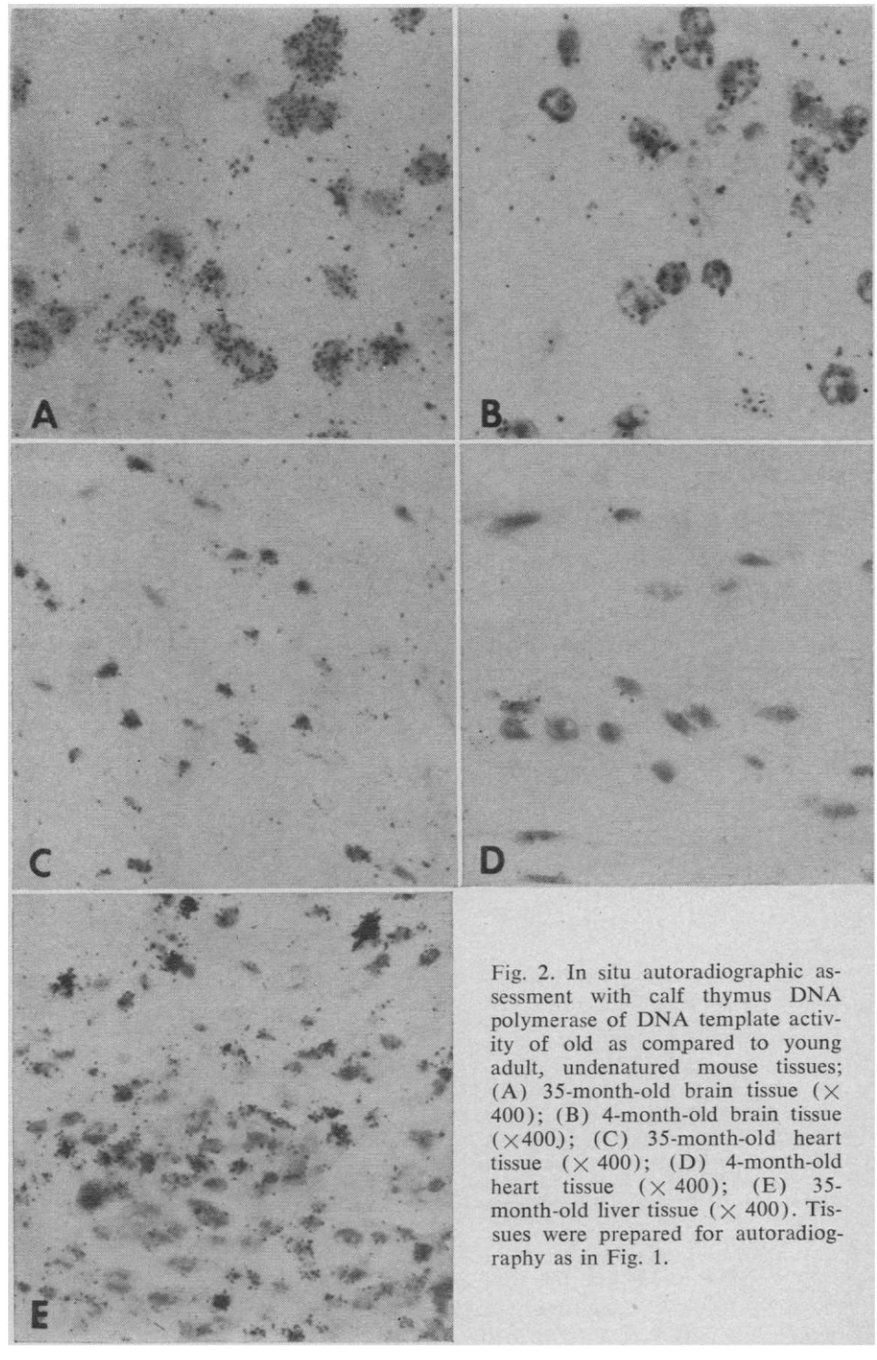

Fig. 2. In situ autoradiographic assessment with calf thymus DNA polymerase of DNA template activity of old as compared to young adult, undenatured mouse tissues; (A) 35-month-old brain tissue (× 400); (B) 4-month-old brain tissue (×400); (C) 35-month-old heart tissue (× 400); (D) 4-month-old heart tissue (× 400); (E) 35-month-old liver tissue (× 400). Tissues were prepared for autoradiography as in Fig. 1.

the liver, however, Kupffer cells, which have long generation times, are heavily labeled, whereas stationary phase hepatocytes are only slightly labeled. Thus, DNA in microglia and hepatocyte nuclei inherently seems to be either more resistant to damage by strand scission or is more effectively repaired when damaged. At least, with age, a progressive stabilization of DNA-protein complex occurs in the liver (*9*).

Our results imply that, with aging, the nuclear DNA in certain cells may accumulate damage such as single strand breaks. Using 10-kr whole-body x-irradiation (*6*), which results in induction of a lethal central nervous system syndrome (*12*), to induce primarily single strand breaks, we have been able apparently to duplicate the age-associated changes that occur naturally in DNA of senescent brain cells. Such changes could arise if DNA-repair enzymes (*13*) are progressively lost or if age-associated increases in the release of hydrolytic enzymes occur. Evidence does exist demonstrating the age-associated increase in lysosomes (*14*) and lysosomal enzyme activities (*15*). A more direct proof of accumulation of strand breaks with aging and the resolution of mechanisms involved must await development of adequate physical and chemical techniques. Finally, our histochemical data should not be considered as support for any single generalized theory of aging. However, these data are consistent with any theory dealing with alterations in the information content of *certain specific* cell populations at the genome, such as molecular aging theories involving error-inducing mechanisms.

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References and Notes

1. F. J. Bollum, *J. Biol. Chem.* **234**, 2733 (1959); *ibid.* **235**, 2399 (1960); *ibid.* **237**, 1945 (1962); in *Progress in Nucleic Acid Research*, J. N. Davidson and W. E. Cohn, Eds. (Academic Press, New York, 1963), p. 1; in *Genetics, Elements, Properties and Function*, D. Shugar, Ed. (Academic Press, New York, 1967), p. 3; M. Yoneda and F. J. Bollum, *J. Biol. Chem.* **240**, 3385 (1965).
2. F. J. Bollum, *J. Cell. Comp. Physiol.* **62** (Suppl. 1), 61 (1963); R. C. von Borstel, D. M. Prescott, F. J. Bollum, *J. Cell Biol.* **29**, 21 (1966).
3. S. P. Modak, R. C. von Borstel, F. J. Bollum, *J. Cell Biol.* **39**, 93a (1968); *Exp. Cell Res.* **56**, 105 (1969).
4. S. P. Modak and F. J. Bollum, *Exp. Cell Res.* **62**, 421 (1970).
5. S. Liebecq-Hutter and R. Goutier, *Int. J. Radiat. Biol.* **13**, 449 (1967).
6. S. P. Modak and G. B. Price, *Exp. Cell Res.*, in press.
7. It is also possible that the increased incorporation in acid-denatured old nuclei may be due in part to an opening up of DNA strands at the regions of possible double strand breaks.
8. W. Maurer and E. Primback, *Exp. Cell Res.* **33**, 8 (1964).
9. H. P. von Hahn and E. Fritz, *Gerontologia* **12**, 237 (1966); M. J. Pyhtila and F. G. Sherman, *Biochem. Biophys. Res. Commun.* **31**, 340 (1968); R. L. Herrman, A. R. O'Meara, A. P. Russel, L. E. Dowling, *Int. Congr. Gerontol. Proc. 8th* **1**, 141 (1969).
10. K. Atwood, in *Proceedings of a Conference on Aging and Levels of Biological Functions* (Univ. of Chicago Press, Chicago, 1965), p. 1; F. Verzar, *Exp. Gerontol.* **3**, 69 (1968).
11. Z. A. Medvedev, *Advan. Gerontol. Res.* **1**, 181 (1964); A. Deyl, *Exp. Gerontol.* **3**, 91 (1968).
12. S. G. Wilson, *J. Neuropathol. Exp. Neurol.* **19**, 195 (1960); T. Samorajski, C. Rolsten, H. J. Curtis, *Anat. Rec.* **168**, 203 (1970).
13. S. R. Pelc, *Exp. Gerontol.* **1**, 215 (1965).
14. K. D. Bachman, *Virchows Arch. Abt. A Pathol. Anat.* **323**, 133 (1953).
15. Youhotsky-Gore and K. Pathmanathan, *Exp. Gerontol.* **3**, 281 (1968); A. Elens and R. Wattiaux, *ibid.* **4**, 131 (1969).
16. K. A. Brownlee, *Statistical Theory and Methodology in Science and Engineering* (Wiley, New York, 1960).
17. We thank Dr. F. J. Bollum for advice and for the calf thymus DNA polymerase. We thank Dr. F. Chino for advice and W. D. Gude for technical assistance. Research jointly sponsored by NIH grant CA-08487, and the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation. S.P.M. received financial support from National Cancer Institute grant CA-10375 (accorded to Dr. J. Sisken) during completion of a part of this work at the University of Kentucky, Lexington. G.B.P. is a PHS predoctoral fellow (5-F01-GM-40, 756-03).

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Nest Predation Affecting the Breeding Season of the Clay-Colored Robin, a Tropical Song Bird

Abstract. *Predation seems more important than food availability in determining the reproductive period of the clay-colored robin* Turdus grayi. *It breeds in the dry season when food resources are low. The rainy season brings more food but breeding stops, possibly because of greatly increased nest predation.*

Fluctuations in food resources have been named as the ultimate factor responsible for the short and distinct breeding seasons found in many tropical birds (*1*). The predation rate on nests is often higher in tropical than in temperate areas (*2*). I now report a case where predation seems more important than food abundance in setting the breeding season of a tropical bird.

Fifty-six nests of the clay-colored robin *Turdus grayi* were found in a 57-hectare area of Summit Gardens, Panama Canal Zone (*3*), before or during the time eggs were being laid in them. The range of dates of clutch initiations was 26 February to 17 May 1970 (median date 7 April).

Most breeding attempts (70 percent) were in the dry season, which in this area begins in December and ends in late April or early May. In 1970, after 20 April the rains were frequent enough that the ground remained continuously damp. Soon thereafter the robins ceased breeding.

Weighing nestlings gave an index to food abundance throughout the breeding season. Fledgling weights were lowest during the main part of the breeding season and some nestlings died of starvation (*4*). As the rains became more frequent in mid-April, fledgling weights increased and there was no starvation.

The increase in fledgling weight was largely due to the addition of earthworms to the diet. They became available after the ground became damp or flooded following rains. Fruit is abundant during the dry season (*5*) and may furnish adult robins with easily obtainable food, but the fast growth of the young probably requires protein-rich invertebrate food (*6*).

Why don't the birds breed later and thus fledge more and heavier young? The reason may be the increasing amount of nest predation that occurred after the rains began (*7*) (Table 1). Another indication of higher predation pressure may be that 64 percent of nests were lost in earlier stages (containing eggs) after 20 April. Before this,

Table 1. A comparison of nests lost to predators with successful nests with clutches started before and after 20 April. That date marks the beginning of the rainy season. A nest was considered to be successful if it fledged at least one young. Chi-square was 4.20 with 1 degree of freedom; $P < .05$.

Time	Lost (No.)	Successful (No.)
Before	21	15
After	17	3