ancestral and familiar territory. Of the 15 long-distance migrants (*Z. l. gambelii* and *Z. atricapilla*) no more than eight would normally be expected to return without displacement. Although the six of these two races which did return are from a small sample, the fact that any returned seems indeed remarkable. It should be noted that none of the seven *Z. l. pugetensis* which had already returned to San Jose from Louisiana returned from displacement to Laurel.

Of the 638 birds displaced for the first time we would normally have expected 30 percent, or about 198, to return. The nine which returned (seven *Z. atricapilla* and two *Z. l. gambelii*) comprise only about 4 percent of those normally expected to return to traps at San Jose after a summer on the breeding grounds. Most of these 638 birds were from populations peripheral to the banding station (*3*), and the presence of returned birds in the surrounding suburbs would not have been detected. One of the birds which returned was captured about a kilometer from the banding station.

Because these birds returned the following winter (none in the same winter), it is presumed that they found their way first to their ancestral nesting ground (Fig. 1) and then returned to San Jose in normal migration. The return to the nesting ground was probably accomplished for the most part in spring during the normal period of migration. This hypothesis is supported by the chance recovery of one *Z. atricapilla* on 13 May 1963 at Penetanguishene, Ontario (Fig. 1). This recovery was nearly on the direct line from the release area at Laurel (21 March 1963) to the presumed nesting grounds in northwestern North America.

These data support the hypothesis that small passerines of the genus *Zonotrichia* have an innate ability to home, probably by means other than chance, from artificial displacements as far as across the continent of North America. The returns observed must be accounted for by a mechanism which includes an ability to home from a geographical area beyond their experience. The ability to home is better demonstrated in long-distance migrants than in short-distance migrants (*Z. l. gambelii* and *Z. atricapilla* in contrast to *Z. l. pugetensis*) and is better developed in adults than in immature birds. These findings further suggest that experience in homing, such as return to San Jose from displacement to Baton Rouge, improves homing performance from another remote location such as return to San Jose from displacement to Laurel.

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Collagenolytic Activity of Intact and Necrotic Connective Tissue

Abstract. *Isotonic saline extracts of both intact and necrotic skin of the rat were capable of releasing over 50 percent of the hydroxyproline content of soluble collagen as dialyzable, peptide-bound amino acid only after prior, limited proteolytic activation of trypsin. These "activated" extracts could also solubilize insoluble collagen to release dialyzable hydroxyproline containing peptides. This collagenolytic activity was maximal at* p*H 5.5 and was not inhibited by soybean trypsin inhibitor, ethylenediaminetetraacetic acid, or heavy metal salt. The "activated" extracts showed no general proteolytic activity toward denatured hemoglobin. The collagenolytic activity was destroyed both by heat and by extensive tryptic proteolysis.*

Results of studies (*1*) in which radioisotope has been incorporated into collagen indicate that most dermal collagen is relatively inert metabolically, with 50 percent of the labeled insoluble collagen remaining in the tissue for as long as 300 days. Despite this, the collagen content of rat skin is markedly reduced within 24 hours after onset of necrosis or stress (*2*). This catabolism of collagen, it has been suggested (*3*), proceeds by disaggregation of isotonic collagen into soluble precursors which are then denatured thermally into gelatins. These gelatins could then be attacked by cathepsins. However, this thermal denaturation of dermal collagen has recently been shown to be unlikely (*4*). An alternative mechanism to explain the rapid catabolism of collagen would be that a specific collagenase degrades insoluble collagens into diffusable breakdown products; this explanation has also been considered unlikely since various investigators (*5*) could not demonstrate any collagenolytic activity in necrotic tissue.

To learn more of the mechanism of collagen catabolism, we have studied the capacity of extracts of the connective tissue to release dialyzable, peptide-bound hydroxyproline from solutions of purified soluble collagen and from suspensions of purified insoluble collagen. Neutral soluble collagen was prepared from dilute acid extracts of calf skin (*6*) and purified by repeated successive precipitations with salt at acid and neutral *p*H (*7*). This presentation had an intrinsic viscosity of 14 dl/g and an optical rotation of −350 deg. The acid-insoluble residue after preparation of soluble collagen was washed exhaustively with dilute acid and water and it served as an insoluble collagen substrate.

Soluble collagen (1.25 mg/ml) was dissolved in cold 0.1*M* acetate buffer (*p*H 5.5) containing 0.5*M* $CaCl_2$ (*8*). Four milliliters of this solution were incubated for 16 hours at 12°C with 1.0-ml samples of various tissue extracts, and the hydroxyproline concentration of these solutions before and after 48 hours of dialysis at 4°C against 500 volumes of buffer was determined (*9*). The low temperature prevented thermal denaturation of the substrate, since no change in either viscosity or optical rotation was noted under these conditions when buffer was substituted for the tissue extracts. The *p*H used is optimal for a mammalian collagenolytic activity (*8*).

Isotonic saline extracts of necrotic wounds 48 hours after they had been induced by croton oil (*2*) and of intact uninjured skin from 24 injured male Sprague-Dawley rats (250 to 270 g) were prepared (*10*) from three pools representing eight animals each. After dialysis and lyophilization, solutions of these extracts were diluted in acetate

Table 1. Percentage of insoluble collagen hydroxyproline rendered soluble by variously treated tissue extracts, and the percentage of the solubilized hydroxyproline that was dialyzable.

Extracts	Solubilized	Dialyzable
Trypsin control *	0	0
Wound extract control	0	0
Wound extract treated with trypsin at *p*H:		
3.5	0	0
5.5	10 ± 1	58 ± 3
7.5	0	0
Intact skin extract control †	0	0
Intact skin extract treated with trypsin at *p*H:		
3.5	0	0
5.5	9 ± 1	54 ± 3
7.5	0	0

* Substrate plus buffer plus trypsin. † Extract to which trypsin was added after addition of soybean trypsin inhibitor.

buffer to contain 10 mg of extract per milliliter. Portions of these solutions were incubated with 1 mg of crystalline bovine trypsin (*11*) at *p*H 3.5, *p*H 5.5, or *p*H 7.5 for 10 minutes at 20°C. After exposure to trypsin, 2 mg of crystalline soybean trypsin inhibitor (*11*) was added to these solutions, and the *p*H was brought to 5.5.

These trypsin-treated solutions were incubated with purified soluble collagen, as already described. Other portions of these tissue extracts were mixed with soybean trypsin inhibitor, and then trypsin was added to the mixture. Solutions prepared in this "unactivated" fashion served as tissue extract controls and were also incubated with soluble collagen. Finally, controls were prepared in buffer containing trypsin alone (1 mg/ml). The percentage of the total hydroxyproline lost during dialysis was determined and corrected for volume changes.

These results indicated that extracts of both intact skin and necrotic wounds could render more than 50 percent of the total hydroxyproline dialyzable only after trypsin treatment at *p*H 5.5. The trypsin alone rendered only about 4 percent of the hydroxyproline dialyzable, and therefore the substrate had not been substantially denatured to gelatin. Obviously there would be no appreciable tryptic activity at *p*H 3.5 to "activate" the collagenolytic activity of these extracts, while the amount of tryptic activity at *p*H 7.5 would be very large and apparently it destroyed the collagenolytic activity. At *p*H 5.5 and 20°C, however, only enough tryptic activity would be available for limited proteolysis but not enough to destroy the collagenase activity.

The general proteolytic activity of the trypsin "activated" extracts from both wounded and intact skin after the addition of soybean trypsin inhibitor was assayed with denatured hemoglobin according to the method of Anson (*12*). Neither the extracts from wounds nor from the intact skin were active proteolytically under these conditions. Similarly, this concentration of soybean inhibitor (2 mg/ml) could inhibit completely all the proteolytic activity of the added trypsin.

The apparent collagenolytic activities of these "activated" fractions were markedly reduced by exposure to 65°C for 30 minutes before incubation with the soluble collagen substrate. The fact of this thermal inactivation of the collagenolytic activity, when combined with the observation that this activity was also lost when the fractions were treated with trypsin for too long a time (60 minutes) at *p*H 5.5, or with too much trypsin (5 mg/ml), or at a *p*H closer to the optimum of trypsin (*p*H 7.5), suggests strongly that the collagenolytic activity of these fractions was associated with a protein moiety, and hence was due to an enzyme.

Suspensions of insoluble collagen were made up in 0.1*M* acetate buffer (*p*H 5.5) to contain 10 mg of collagen per milliliter. These suspensions were similarly treated at 22°C with the various tissue extracts described and the amount of peptide-bound hydroxyproline solubilized was determined after extensive centrifugation in the cold (24,000*g* for 1 hour). The percentage of the solubilized hydroxyproline which was dialyzable was also determined in triplicate. The results of this experiment (Table 1) indicate that only after treatment with trypsin at *p*H 5.5 did either the wound or the intact skin extract solubilize peptide-bound hydroxyproline. Also, slightly less than half of the solubilized collagen could not be dialyzed. The activity of these fractions toward insoluble collagen was also thermolabile and was destroyed by large amounts of trypsin activity.

It is possible that the losses of hydroxyproline from within the dialysis bags could have resulted from some unique properties of activated fractions to effect the adsorption of hydroxyproline-containing polypeptides to the surface of the bag itself. Therefore duplicate 5-ml portions from the supernatant obtained after incubating both the unactivated and activated extracts of intact skin with 50 mg of insoluble collagen (50 μmole of hydroxyproline) were dialyzed in the cold against 5 ml of buffer for 120 hours. No peptide-bound hydroxyproline was present in the dialyzate obtained from the incubation of unactivated tissue extract with insoluble collagen, but hydroxyproline (2.1 μmole) was found in the dialyzate from the supernatant from the incubation of activated extract with insoluble collagen. This represents about 37 percent of the solubilized hydroxyproline content of this supernatant. Therefore the losses of peptide-bound hydroxyproline from within the dialysis bags were associated with the appearance of hydroxyproline in the dialyzing fluid.

Table 2. Effects of various enzyme and substrate concentrations upon the percentage of hydroxyproline solubilized from insoluble collagen at *p*H 5.5 after 16-hour incubation at 32°C.

Concentration (mg/ml)	Solubilized (%)
Enzyme *	
1	1.9
2	3.9
5	9.8
10	18.8
Substrate †	
5	8.4
10	9.9
15	12.0
20	5.1

* Using 10 mg of insoluble collagen per milliliter. † Using 5 mg of intact skin extract per milliliter.

The effect of varying the concentration of the activated extract from 0 to 10 mg/ml on the percentage of insoluble hydroxyproline that becomes soluble after 16-hour incubation at 32°C and *p*H 5.5 was studied. The concentration of activating trypsin was maintained at 1 mg per 10 mg of tissue

Table 3. Effects of incubation temperature, *p*H, and time on the percentage of hydroxyproline solubilized (% sol.) from 10 mg of insoluble collagen per milliliter by 5 mg of proteolytic activated extracts of intact connective tissue per milliliter.

Temperature *		H-ion concentration †		Time ‡	
°C	% sol.	*p*H	% sol.	Hr	% sol.
4	1.8	4.0	1.2	2	2.5
12	3.0	4.5	2.4	3	8.4
22	5.8	5.0	6.0	4	10.0
32	10.4	5.5	10.4	8	10.4
		6.0	4.8	16	9.8
		6.5	2.4		
		7.0	0.6		
		7.5	0.0		

* At *p*H 5.5 for 16 hours. † At 32°C for 16 hours. ‡ At *p*H 5.5 and 32°C.

extract. The effects of increasing the concentration of the insoluble collagen substrate from 0 to 20 mg/ml were also studied at *p*H 5.5 and 32°C, with 5 mg per milliliter of activated extract. In similar fashion, the effect of varying the time, *p*H, and temperature of incubation on the percentage of insoluble hydroxyproline from insoluble collagen (10 mg/ml) by activated extract (5 mg/ml) was studied. More than half of the hydroxyproline solubilized in these experiments was dialyzable.

These results (Tables 2 and 3) demonstrate (i) an essential linearity of response with enzyme concentration; (ii) "excess substrate inhibition" at concentrations exceeding 15 mg of insoluble collagen per milliliter; (iii) a linear increase in collagenolysis with temperature, and approximate doubling of enzyme activity with each increment of 10°C; (iv) a *p*H optimum of about 5.5; (v) and that the bulk of the reaction was completed at *p*H 5.5 within 4 hours. With respect to properties (ii) and (iv), the collagenolytic activity of the connective tissue appears similar to that described for pancreatic collagenase (*8*).

Finally, in separate experiments, the activity of both necrotic and intact rat skin on insoluble collagen was not inhibited by 0.01*M* EDTA, 0.001*M* *p*-chloromer-curibenzoate, or soybean trypsin inhibitor (2 mg/ml). Almost all of this collagenolytic activity was inhibited by 0.05*M* phosphate buffer, however.

These results suggest that both necrotic and intact connective tissue contain a protein component which, after limited proteolytic activation, breaks the primary peptide backbone structure of collagen. This collagenolytic activity of the connective tissue was not inhibited by a mercuric salt; nor was it associated with any general proteolytic activity. It therefore appears that this collagenolytic activity could not result from general tissue cathepsin activity, but rather was relatively specific for collagen. Finally, since this collagenolytic activity was not inhibited by EDTA, it could not have resulted from bacterial collagenase contamination of these tissue extracts (*13*).

It is difficult to explain why the necrotic wound should not contain any "activated" collagenolytic activity. Perhaps the activated enzyme was digested by the various peptidases which presumably exist in necrotic wounds, and was thus destroyed, or perhaps it was rapidly drained away from the area of the necrotic wound into the circulation along with the breakdown products of collagen. Both of these explanations seem possible, but, without supporting data, they remain speculative.

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Immunity and Susceptibility toward Cheek Pouch Transplants of a Mouse Leukemia

Abstract. *Intravenous sensitization with spleen or leukemic cells of mice and certain rats immunized Syrian hamsters toward cheek pouch heterografts of a mouse leukemia. By contrast, sensitized hamsters given cortisone became more susceptible to the challenging leukemic graft. Neither response was elicited by sensitization with cells of the guinea pig, Syrian hamster, rabbit, or human being.*

Heightened immunity toward cheek-pouch heterografts of a mouse leukemia, AK-4, can be induced in the Syrian hamster by prior exposure of the hamster to the leukemic cells or to normal AKR spleen cells. By contrast, administration of cortisone with the leukemic cells or spleen cells results in increased susceptibility. Cortisone alone does not have this effect.

Increased susceptibility evoked in this way resembles immunologic enhancement in some respects, but appears to be dependent upon some special property of the cheek pouch, which is not yet fully understood. Immunity to AK-4 heterografts, on the other hand, is much less site-specific and can be induced by exposing the hamster to cells from strains of mice other than AKR (*1*).

The present experiments (*2*) were done to determine whether susceptibility can be similarly induced by exposing the cortisone-treated hamster to out-of-strain mouse cells, and whether sensitization with sufficiently "foreign" cells fails to result in either susceptibility or immunity.

The results of these experiments indicate that both susceptibility and immunity are not only specifically induced states, but are probably related immunologic states, since their specificity is approximately the same. Neither state is evoked by sufficiently "foreign" sensitizing cells; and which immunologic behavior is evoked by less "foreign" cells appears to depend upon whether or not the hamster is exposed to cortisone at the time of exposure to antigen.

The sensitizing out-of-strain or "foreign" (Table 1) cells were taken from the normal spleens or experimental neoplasms of four strains of mice, Wistar or Sprague-Dawley rats, guinea pigs, and rabbits. Cells were also obtained from a human leukemic spleen (*3*), and from the normal spleens of Syrian hamsters of the same closed but not pure-bred colony which provided the experimental subjects (*4*). For the sensitization, 1.0×10^7 of these unwashed, dissociated spleen or leukemic cells were injected intravenously into different groups of hamsters by retro-orbital puncture. Four weeks after sensitization all of the hamsters were challenged with 1.0×10^7 AK-4 leukemic cells implanted into the right cheek pouch. Part of each group was treated with cortisone (*5*) at the time of sensitization, and cortisone treatment was continued to the end of the experiment, usually 3 to 4 weeks after challenge.

Criteria for evaluating the subsequent behavior of the challenging AK-4 cells inoculated into the cheek pouch, were