Effect of Trauma and Infection on Lyozyme in Poultry Tissue* (33890)

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Lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) is widely distributed in biological material. Research investigators reported the presence of this enzyme in significant quantities in tears and milk (1, 2), serum plasma, and saliva (3), kidney secretions (4), and bird egg whites (2). The enzyme splits the peptidoglycan component of bacterial cell walls by hydrolyzing the β (1→4) glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine (5). Several investigators (4, 6, 7) demonstrated that increased urinary lysozyme activity occurs in patients with a variety of types of kidney diseases and leukemias. Jollès (8) suggested that this natural antibacterial enzyme aids in combating bacterial invasions in animals. McCarthy *et al.* (9) showed that 61–74% of poultry bruises harbored relatively large numbers of both aerobic and anerobic bacteria and that 36% of the gram-positive cocci isolated from these damaged tissues belonged to the genus *Staphylococcus* and that 22% of the predominant isolates were gram-negative rods which included coliform. The authors also established that age of bruise, environmental conditions, severity of the trauma and the presence of hemoglobin and its degradation products were among the factors affecting the microbial content and their growth in the bruised areas. These injured areas served as an ideal host for the many organisms immediately following bruise infliction and during the early stages of healing. Increase in lysosomal DNase, RNase, β-glucuronidase and β-galactosidase activities was noted in experimentally inflicted poultry breast bruises (10). These changes suggested the presence of high levels of hydrolytic activity as a result of trauma. Therefore, the present study was initiated as an attempt to correlate trauma and/or bacterial infection with the level of both free and bound lysozymes in poultry tissues. In addition, the effects of severity and age of the trauma on lysozyme concentration in tissues were also examined.

Materials and Methods. Trauma and sampling. Apparently normal, white leghorn chickens, 8–12 weeks old, 3–4 lb, kept in batteries in a constant temperature room (22°) were used. They were offered standard rations free of medication and water *ad libitum*. Trauma was inflicted on the pectoralis major muscle using the standard technique described by Hamdy *et al.* (11). Superficial bruises were produced using 1 blow; medium, 3 blows; and severe, 5 blows. The degree of experimental trauma was less than that often sustained during catching, crating, and transporting poultry for processing. At specified time intervals following the contusion of trauma, the birds were sacrificed by exsanguination; and the center of the bruise, bruise periphery, or normal (control) tissues were immediately excised, sliced, minced, and thoroughly mixed.

Assay of free and bound lysozyme activities. From each tissue sample, two 20% homogenates were prepared by blending the tissue with phosphate buffer for 1–2 min in a Servall Omnimixer at 16,000 rpm. The first homogenate was made with cold 0.066 *M* phosphate buffer (pH 7.2), containing 0.31 *M* sucrose, and used for the assay of the free lysozyme activity. The second homogenate was prepared with the same phosphate buffer–sucrose mixture but contained 0.1% Triton X-100 and was used to determine the total lysozyme activity. Bound activities were calculated by subtracting free activities from total activities. The tissue homogenates were centrifuged for 30 min at 74,000*g* in a refrigerated model L Spinco preparative cen-

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TABLE I. Effect of Trauma and Age on the Specific Activities (μg of lysozyme/g of tissue) of Different Forms of Lysozyme in Poultry Tissue.[a]

Sample examined	No. of birds	Lysozyme (μg/g of tissue; $\pm$ SD)			Ratio of free:total
		Total	Free	Bound	
Control	30	0	0	0	
Bruised 15 min	10	0	0	0	
12 hr	17	8.8 $\pm$ 2.5	6.1 $\pm$ 1.8	2.7 $\pm$ 0.7	1:1.4
(days)					
1	21	7.8 $\pm$ 2.2	4.6 $\pm$ 2.9	3.2 $\pm$ 0.4	1:1.7
2	18	4.9 $\pm$ 1.1	3.6 $\pm$ 1.8	1.3 $\pm$ 0.7	1:1.4
3	24	4.1 $\pm$ 2.8	2.5 $\pm$ 2.2	1.6 $\pm$ 0.6	1:1.6
4	21	2.2 $\pm$ 1.6	1.4 $\pm$ 1.5	0.8 $\pm$ 0.1	1:1.6
5	16	0	0	0	
6	6	0	0	0	

[a] No activities were detected in bruise periphery or in symmetrically located control muscle tissues.

trifuge; the supernatants were collected and the sediments were discarded. Lyophilized cells of *Micrococcus lysodeikticus* (ML 36), prepared in 0.066 *M* phosphate buffer (pH 7.2) according to the procedure of Shugar (12), were used as the substrate. Four and one-half ml of cell suspension (substrate) and 0.5 ml of the tissue supernatant were mixed in a cuvette. Optical density (OD), measured in a Bausch and Lomb Spectronic 20 at 450 mμ, was made initially and after 3-min incubation at 25°. Standard solutions of the reference enzyme, *i.e.*, egg white lysozyme, were also assayed in the same manner and the decrease in OD of the substrate was plotted against various concentrations of lysozyme. The lysozyme concentration (μg lysozyme equivalent/g tissue) of the tissue samples was computed by comparing the change in OD to the standard curve of lysozyme.

Bacterial cultures. Two cultures were used for tissue infection: (a) a virulent marker strain (MS) of *Staphylococcus aureus* coagulase and deoxyribonuclease positive, mannitol negative and phage type 52/52A/80 (obtained from R. A. Vogel, V. A. Hospital, Atlanta, Ga.). This culture was similar to other coagulase-positive *S. aureus* which were among the predominant organisms isolated from bruised tissue; (b) a nonpathogenic culture of *Escherichia coli* K-12 (obtained from W. J. Payne, Bacteriology Dept., Univ. of Ga.). Flasks of nutrient broth were inoculated with an active culture of the test organism and incubated for 18–24 hr at 37°. The cells were harvested by centrifugation, washed 3 times, and resuspended in sterile saline at the desired cell concentration.

Infection technique. A saline suspension (0.25 ml) containing the desired concentration of viable cells was injected intramuscularly into the pectoralis major of bruised and control birds as previously reported (13). Injection of the bruised tissues was performed always at the center of the traumatized area 0.5 hr after contusion. The number of living cells present in the saline suspension at the time of injection was determined by plating appropriate dilutions on violet red bile agar (Difco) for *E. coli* or on mannitol salt agar for *S. aureus* (MS).

Results. Effect of trauma and age on lysozyme activity in tissue. Free and total lysozyme activities were assayed in tissues obtained from 30 normal (control) and from 127 traumatized (medium, 3 blows) chickens. Three samples were excised from each traumatized bird immediately following trauma and at specified time intervals thereafter. These samples included the discolored area (bruise), the bruise periphery (not obviously discolored area immediately surrounding the bruise) and the symmetrically located control muscle on the same bird. The results (Table I) indicate that no lysozyme activit-

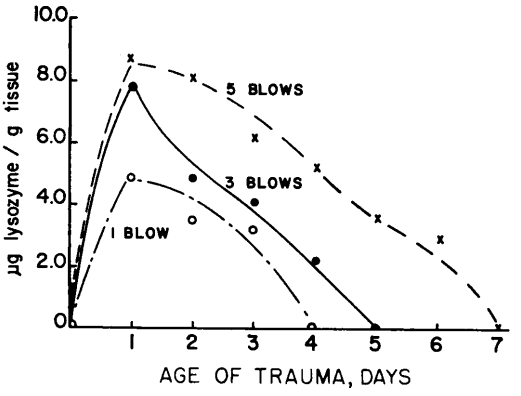

FIG. 1. Effect of severity and age of bruise on total lysozyme levels in poultry bruises.

ies were detected in any normal tissue and for convenience the lysozyme concentration in these tissues will not be reported in the following results as it was always zero. Neither free nor bound lysozyme activities were found in bruised tissue 15 min following trauma as well as in both the bruise periphery and in the symmetrically located control tissue at any time during healing. On the other hand, trauma elicited the release of considerable lysozyme activities both in the free and bound form in the traumatized tissue after 12 hr of injury. The free specific activity (μg lyxozyme equivalent/g tissue) reached a maximum level at 12 hr and the bound after 1 day. The specific activities of both forms of enzyme declined thereafter to reach the normal (zero) level on the fifth day. It is of interest to point out that the ratio for the mean value of the free specific activity of lysozyme to the total specific activity ranged from 1:1.4 to 1:1.7 and was somewhat consistent as healing progressed. As the standard deviation indicated, there was considerable range of enzyme activity about the mean during healing of the tissue. Evidence of complete healing, as detected by morphological observation was noted 5 days after trauma and coincided with the absence of lysozyme activities in the tissue.

Effect of severity of bruise. Superficial bruises were inflicted on 46 birds, medium bruises on 110 birds, and severe bruises on 97 birds. Bruised tissue samples from each group of birds were examined at intervals. The results showed that increasing the severity of contusion elicited an increase in lysozyme activities (Fig. 1). Maximum total activities occurred on the first day postbruise regardless of the force applied to inflict the trauma. Severe bruises had approximately 2-fold higher activity of total lysozyme compared to the superficial bruise for the first 3 days postbruise, and little difference was noted between the lysozyme concentration in severe and medium bruises on the first day. On the second through the fourth day the total lysozyme activities in severe bruises were 2-fold higher than those of the medium bruises. Free lysozyme activities (Fig. 2) reached a maximum within 1 day in superficial and medium bruises and within 2 days in severe bruises followed by a gradual decrease thereafter. The free activities in superficial, medium, and severe bruises averaged 55.7%, 64.2%, and 70.4% of the total lysozyme activities, respectively.

Lysozyme activity 1 day postinfection. Three groups of birds were used. The first consisted of 8 bruised and 6 normal (unbruised) birds and each (bruised and normal tissues) was injected with 3.2×10^6 cells of *S. aureus* (MS); the second group consisted of 10 bruised and 10 unbruised (normal) birds and each was injected with 4.7×10^6 cells of *E. coli* K-12; the third group of 21 bruised and 10 normal birds served as controls. One day after the bacterial injection, tissue samples from all the birds in the three groups were obtained for lysozyme analysis. Figure 3 indicates that both S. *aureus* and *E.*

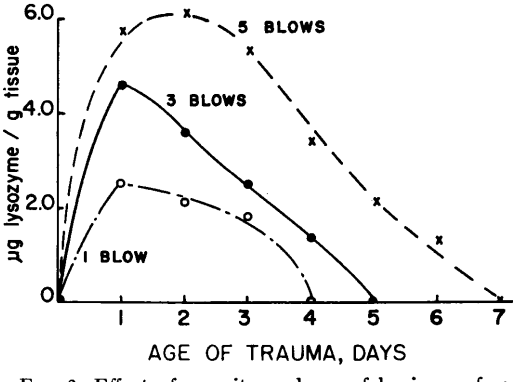

FIG. 2. Effect of severity and age of bruise on free lysozyme levels.

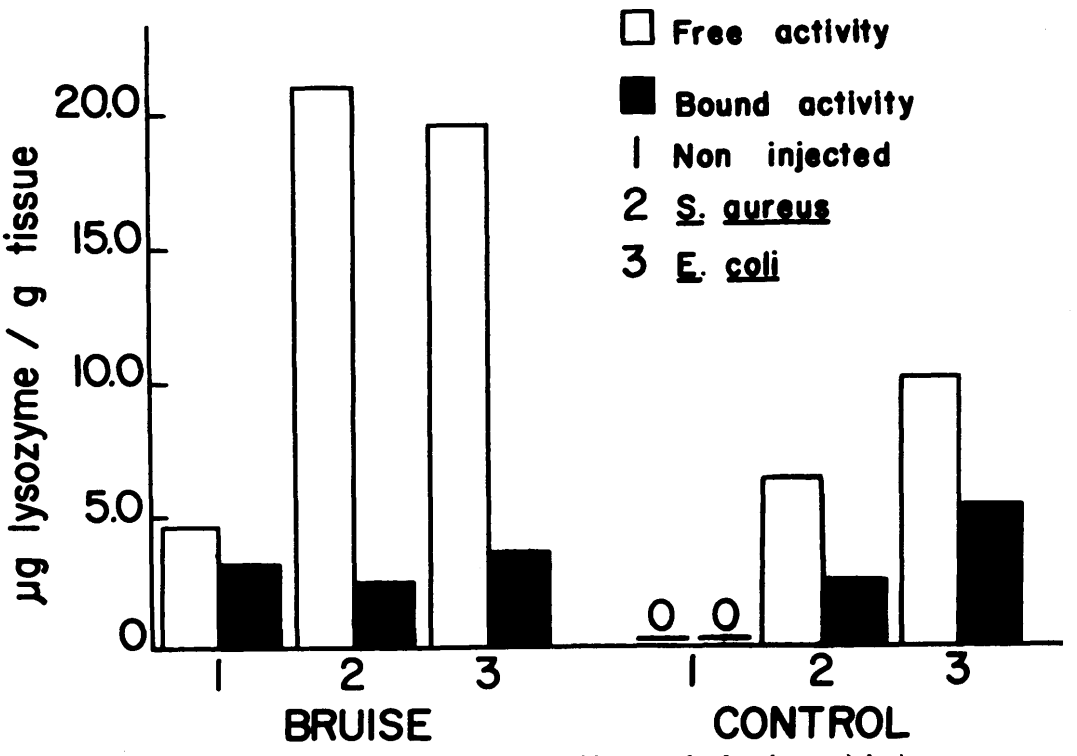

FIG. 3. Effect of infection and bruising on free and bound lysozyme levels 1 day postinfection.

coli infections caused a marked increase in lysozyme activities. However, bruised infected tissues had 3-fold higher concentration of total lysozyme (free and bound) than those of the bruised noninfected tissues, whereas the concentration of free lysozyme in the *S. aureus*-infected and in the *E. coli*-infected bruised tissues was 4.6- and 4.3-fold higher, respectively, than those noted in bruised noninfected tissues. Bound activities in all bruised infected and noninfected tissues were approximately the same. On the other hand, the tissues of *E. coli*-infected unbruised birds had twice as much free lysozyme activity as the bruised noninfected tissues, whereas the tissues of *S. aureus*-infected unbruised birds had only slightly more free lysozyme activities than the bruised, noninfected tissues.

Effect of infection and bruising on lysozyme during healing. Previous experiments revealed that infection and bruising enhanced the liberation of lysozyme in tissues. Therefore, this experiment was performed to follow the time-course and the distribution of lysozyme activities in bruised and infected tissues during healing. Two groups of birds were used. The first consisted of 40 bruised and 42 unbruised (normal) birds and each bird was infected with 3.2×10^6 cells of *S. aureus*. The second group consisted of 65 bruised and 34 unbruised (normal) birds and each was infected with 4.7×10^6 cells of *E. coli*. Tissue samples were collected at intervals, from birds of both groups, for lysozyme determination. Figure 4 depicts the distribution of the different forms of lysozyme in tissue as a result of trauma and infection with *S. aureus*. All forms of lysozyme, reported as specific activity, increased immediately following trauma and infection to a value of 10 µg of lysozyme/g of tissue. This was followed by a rapid increase to reach maximum level within 1 day for both total and free forms of lysozyme and within 2 days for the bound form. This increase was followed by a rapid decline thereafter. It is of interest that the specific activity of free lysozyme was noted in these tissues for as long as 13 days (2.5 µg of lysozyme/g of tissue). Figure 5 represents the results obtained when trauma-

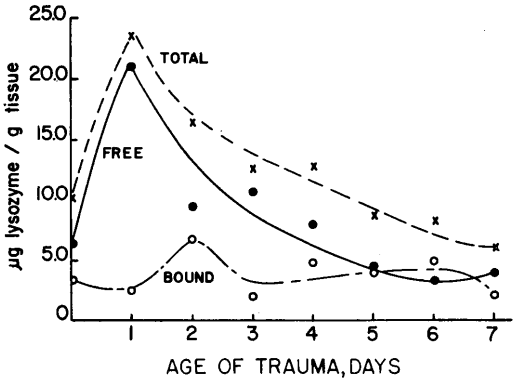

FIG. 4. Distribution of total, bound, and free lysozyme in S. aureus-infected bruised tissue as a function of age of bruise.

tized tissues were infected by *E. coli*. No lysozyme activity was detected in these tissues immediately following trauma and infection, whereas, on the first day, both total and free lysozyme increased rapidly to reach a peak followed by a gradual decline thereafter to reach a very low level on the seventh day posttrauma and infection. No lysozyme was evident in these tissues after 13 days. Most of the lysozyme activity was in free form in both staphylococcus and *E. coli*-traumatized tissues averaging 64.5% of total in the former and 75.4% in the latter tissues for the entire experimental period of 7 days. Unbruised, infected birds in both groups had no lysozyme activity immediately following infection. On the first day the total, free, and bound lysozyme activities of the *S. aureus*-infected tissue increased to reach a maximum level of 8.8, 6.2, and 2.4 μg of lysozyme/g of tissue, respectively, followed by a rapid decline on the fourth day to a value of 1.5, 1.2, and 0 μg of lysozyme/g of tissue. Thereafter, both total and free activities remained slightly elevated but constant during the entire experiment. Bound lysozyme activity was not detected in these tissues on days 4–7. In *E. coli*-infected tissue, total, free, and bound activity, on the first day, followed the same pattern previously observed for *S. aureus*-infected tissue. However, the magnitude of the concentrations of all three forms was almost twofold that noted in *S. aureus*-infected tissues. The lysozyme activities in all forms gradually decreased on the fourth day to 2.1, 1.9, and 0.3 μg of lysozyme/g of tissue for total, free, and bound, respectively. Slight and gradual decrease, in both total and free lysozyme concentrations, was evident in these tissues up to the seventh day. Most of the lysozyme, in *S. aureus*- and in *E. coli*-infected tissues, was in the free form and represented an average of 75.6% and 76.5% of the total, respectively, for the entire experiment.

Discussion. Previous studies from our laboratory revealed that bruising enhances the activities of various lysosomal enzymes and that the magnitude of the enzymatic response as a result of bruising was not the same for each enzyme, but all enzymes studied showed the same trend in activity. Characterization of free and bound lysozyme activities in bruised tissues revealed that bruising elicited the release of free lysozyme in the damaged areas. Within 1 days postbruise, the concentration of lysozyme (μg lysozyme equivalent/g of wet tissue) increased from 4.9 in a superficial bruise to 7.8 in a medium bruise and to 8.8 in a severe bruise. The percentage free lysozyme also increased in these tissues from 51% in superficial, 59% in medium, to 65% in severe bruises. The increase of free lysozyme activity in the bruised area may be due to the activity of polymorphonuclear leukocytes invading the tissues (3) as well as to the injury and autolysis of the tissue cells. Schmittle and Rogers (14) found that bruised tissue contained many infiiltrating leukocytes.

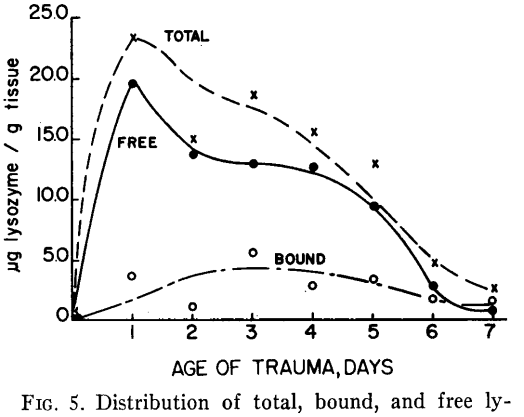

FIG. 5. Distribution of total, bound, and free lysozyme in *E. coli*-infected bruised tissue as a function of age of bruise.

The enhancement of lysozyme concentration in bruised tissue was also affected by infection of the bruised tissues. Immediately following trauma and infection, the traumatized tissue exhibited a considerable activity of both bound and free lysozyme in the *S. aureus*-infected but not in *E. coli*-infected tissue. This may be due to the response of the animal to the virulent staphylococcus culture compared to the avirulent *E. coli*. It is also possible that this rapid increase in lysozyme concentration may be due to the activity of the pathogenic staphylococcus bacteria in the traumatized area. Within 1 day *S. aureus*-infected bruises exhibited an activity of 23.5 (μg lysozyme equivalent/g of tissue) as compared to 8.7 in control-infected tissues and *E. coli*-infected bruised tissues had approximately the same value as that of staphylococcus. In the *E. coli*-infected control tissues, the total lysozyme was twice that noted for *S. aureus*-infected control tissues. Again, it is of interest to note that 89% of total lysozyme activity was free in *S. aureus*-infected bruises and 84% in *E. coli*-infected tissues on the first day posttrauma. Ribble (15) showed that intravenous injection of typhoid vaccine, purified endotoxin, and Newcastle disease virus into rabbits resulted in elevation of plasma lysozyme concentration. Greatly elevated serum and urine lysozyme levels were observed in rats with transplanted chloroleukemia (16). Osserman and Lawlor (4) indicated that serum and urine lysozyme content increased in monocytic and in monomyelocytic leukemia and that this enzyme is a product of the proliferating monocytes. Other authors found increases in lysozyme content of the kidneys of rats with Jensen sarcoma (17), and increases in the kidney and spleen of mice challenged with BCG zymosan and bacterial endotoxins (18). Jensen *et al.* (19) reported that induced inflammation resulted in the accumulation of high concentration on lysozyme at tissue sites where the inducer (glycogen or aluminum silicate) was injected. These authors also presented evidence implicating leukocytes as the source of free lysozyme at inflammatory sites. The presence of high levels of free lysozyme in bruised and in bruised-infected tissues make these tissues an excellent source for further studies to investigate the role of this enzyme in healing and/or in combating bacterial infections.

Summary. Characterization of lysozyme free, bound, and total in traumatized tissues revealed that trauma elicited the release of free lysozyme in damaged areas. Within 1 day, lysozyme (μg lysozyme equivalent/g of wet tissue) increased from 4.9 in superficial to 7.8 in medium and to 8.8 in severe trauma. The percentage free lysozyme in these tissues was 51% of total in superficial, 59% in medium, and 65% in severe trauma. No lysozyme was detected in control tissue, in periphery of damaged area, or in symmetrically located control muscle on the same traumatized birds. Ratio of total to free lysozyme concentration was 1:0.6 to 1:0.7 and was constant during healing. Enhancement of lysozyme concentration in traumatized tissue was also affected by bacterial infection. Within 1 day *S. aureus*-infected traumatized tissue had 23.5 μg lysozyme equivalent/g of tissue compared to 8.7 in control-infected tissue; *E. coli*-infected traumatized tissues had the same value as that of *S. aureus*. In control *E. coli*-infected tissues, total lysozyme was twice that for *S. aureus*-infected control tissues. Most of the lysozymes in infected tissues were in free form. The increase of lysozyme in damaged areas is apparently due to activity of polymorphonuclear leukocytes invading the tissue and to injury and autolysis of tissue cells.

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Studies on the Inactivation of Thyrotropin-Releasing Hormone (TRH)* (33891)

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Thyrotropin-releasing hormone (TRH)[1] has been found in the hypothalamus of several species including man (1–4). Porcine, bovine, and ovine TRH have been obtained in a high state of purity (2, 5–7). Although TRH stimulates release of thyrotropin (TSH) from the anterior pituitary *in vivo* and *in vitro*, it was never clearly shown to exist in peripheral or hypophyseal portal blood. The main difficulty in demonstrating its presence in blood is due to the fact that the bioassay methods available for its detection are not sensitive enough to detect minute quantities of TRH activity. Another difficulty is that TRH is rapidly destroyed by an enzyme present in plasma and some tissues (8, 9). This report describes some of the characteristics of the blood enzyme responsible for inactivation of TRH. The term "enzyme," as used in this report, does not imply that we are dealing with a purified enzyme system.

Materials and Methods. TRH bioassay. The TRH bioassay is an indirect procedure based on the ability of TRH, injected *in vivo*, to elicit a release of TSH from the anterior pituitary gland. The elevation of endogenous TSH increases the rate of release of labeled hormone from the thyroid gland. This is measured by an increase in blood radioactivity over that of control values. Female white Swiss mice weighing 15–20 g on arrival in the laboratory, were fed a low iodine diet for 3–4 weeks. The day before the assay the mice were injected intraperitoneally with 5 μCi of sodium iodide ^{125}I along with 0.085 μg of triiodothyronine (T3) dissolved in 0.01 *N* NaOH:1% albumin solution. Twenty-four hr later, blood samples were withdrawn from the retro-orbital sinus of the eye (55 μl of heparinized blood). Plasma or other test materials were then injected into the tail vein. Two hr later a second blood sample was taken and corresponding samples counted for radioactivity to a 2% probable error in a scintillation spectrometer. Five to six mice were used per group. To ascertain whether TSH was present, some samples were assayed by the method of McKenzie (10). The difference in counts of the initial and second hour samples from experimental groups were analyzed by the non-parametric method of Wilcoxon or by the Student's *t* test as we have previously reported (11).

Plasma fractions were obtained from the Nutritional Biochemicals Co., Cleveland,

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[1] The terminology proposed by Schally *et al.* (2) will be used in this paper.