gested that this failure might have been due to the presence of blood, containing enzyme inhibitors. This, however, is unlikely to be the case in the experiments reported here as macroscopic blood whenever present was carefully removed. Differences in methodology are more likely to contribute to the conflicting results from various laboratories but, whatever the cause, the existence of lipoprotein lipase in the adipose tissue of man in physiologically significant quantities still remains an open question.

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Lysosomal Enzymes in Bruised Tissue.* (30299)

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A great amount of literature concerning biochemical and physiological anomalies in disease and following injury of various types has appeared in the past few years. Investigations into the nature of the bruising phenomenon, however, have been the prime interest of Hamdy *et al,* who studied the biochemical changes resulting from blunt injury (1,2), the effect of ascorbate on healing(3), developed a method for estimating the age of a bruise(4) and measured changes in tissue conductivity and permeability as a result of bruising(4). In addition, Hamdy *et al* observed increased proteolytic activity, optimally active at pH 8.0, in bruised tissue and an accelerated healing upon injecting streptokinase or trypsin into bruises(5). Rate of healing was also affected by such factors as severity of contusion, environmental temperature, previous bruise history, and passive transfusion of blood from a previously bruised animal to one not previously bruised(2,3,6,7).

These changes, as well as investigations into other types of traumas(8-13), suggested the presence of high levels of hydrolytic activity as a result of trauma. The massive proteolysis, necrosis and phagocytic invasion observed by Schmittle and Rogers(14) during pathological and histochemical studies on experimentally inflicted poultry bruises further implicated the function of hydrolytic enzymes. Accordingly, the present study deals with a relatively recently recognized cellular organelle, the lysosome, which is known to contain an array of hydrolytic enzymes(15).

The main objectives of this study were to determine the effects of bruising on the following lysosomal(15) enzymes (β-galactosid-

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ase, β-glucuronidase, acid deoxyribonuclease and acid ribonuclease). Factors other than bruising which may affect the activities of these enzymes were examined and attempts were made to differentiate free and lysosomal bound forms of these enzymes in muscle tissues.

Materials and methods. Bruising and sampling. Bruising, sampling and preparation of 20% tissue homogenates were effected as previously described(16).

Enzyme assays. 1. *Total activities. DNase and RNase.* Total DNase and RNase activities were determined by the method of Schneider and Hogeboom(17), except that $MgSO_4$ was omitted from the reaction mixture since it did not stimulate activity in the tissue homogenate. The DNase enzyme reaction mixture contained 0.5 mg of highly polymerized DNA (Nutritional Biochemicals Corp.), 0.5 ml of 20% tissue homogenate and 0.05 M sodium acetate buffer (pH 5.2), in a total volume of 2.0 ml. The RNase assay mixture was similar except the substrate used was 1.5 mg thymus RNA (Nutritional Biochemicals Corp.) and the pH of the acetate buffer was 5.0. RNase reactions were stopped by addition of 1.0 ml of 10% perchloric acid (PCA) containing 0.25% uranyl acetate; DNase reactions were stopped by addition of 1.0 ml of 12% PCA. Following refrigeration for one hour and centrifugation, the resultant supernates were diluted 1/5 in distilled water and absorbancy measured at 260 mμ using a Beckman DU spectrophotometer. The blank in each case was the reaction mixture which was incubated without RNA or DNA. The appropriate nucleic acid was added immediately prior to addition of the PCA.

β-Glucuronidase. The β-glucuronidase assay was patterned after that of Fishman *et al* (18). Enzyme reaction mixtures contained 0.4 ml of 0.00125 M phenolphthalein glucuronide (Mann Research Laboratories), 0.5 ml of 20% tissue homogenate and 1.0 ml of 0.2 M sodium acetate buffer (pH 5.2). A separate instrument blank was prepared for each tissue. This blank, containing buffer and homogenate, was incubated simultaneously with the enzyme-substrate reaction tubes. Enzyme-substrate reaction times were either 10 minutes or 1 hour at 37°C. At the end of the reaction time, substrate was added to the blank and was followed immediately by the alkaline reagent(18) to stop the reaction. Following centrifugation, the absorbancy of the supernatant from all the reaction tubes was measured at 500, 550 and 600 mμ in a Bausch & Lomb "Spectronic 20." To minimize non-specific background haze, a base line was drawn from the absorbance at 500 mμ to that at 600 mμ. The difference between this base and the maximum at 550 mμ was considered to be absorbance due to phenolphthalein liberated from the substrate.

β-Galactosidase. The procedure of Sellinger *et al*(19) was employed for β-galactosidase. The reaction mixture, composed of 1.0 ml of 20β tissue homogenate and 1.0 ml of pH 5.0 pyridine-HCl buffer containing 2.5 μmoles o-nitrophenyl β-D-galactoside, was incubated at 37°C for 30 minutes. Enzyme denaturation was accomplished by adding 3.0 ml of 3.75% trichloroacetic acid. After the denatured substances were removed by centrifugation, the yellow color of nitrophenol liberated from the galactoside substrate was developed with 1.25 M glycine—Na_2CO_3 buffer (pH 10) and the absorbancy determined at 410 mμ using a Bausch & Lomb "Spectronic 20."

Protein estimations on tissue homogenates were used to express specific activities of the enzymes studied. Protein measurements were affected with the alkaline copper reagent of Gornall *et al*(20).

2. *Free and bound activities.* Tissue homogenates and substrates were prepared in cold 0.25 M sucrose. Free activity was measured directly on 20% tissue homogenates in an overall reaction mixture of 0.25 M with respect to sucrose, whereas, total activities, with the exception of β-glucuronidase, were measured similarly in the presence of 0.2% Triton X-100‡(21). The bound activity was obtained by subtracting the free activity from the total activity. Due to a turbidity formed when Triton X-100 was placed in an alkaline medium, the total activity of β-glucuronidase was measured after rapidly freezing and

‡ Triton X-100 was donated by the Rohm & Haas Co., Philadelphia, Pa.

thawing the tissue homogenate 6 times.

Results and discussion. Lysosomal activities and age of bruise. β-Glucuronidase, β-galactosidase, DNase and RNase activities were determined in tissues from 36 bruised and 4 control chickens at different intervals post contusion. Samples excised from bruised fowl included the bruise (discolored portion of injured tissue) and the periphery (not obviously discolored area immediately surrounding the bruise). Changes in activities of β-galactosidase, RNase, DNase and β-glucuronidase, as well as acid phosphatase(16), as a response to bruise infliction were strikingly similar in trend though differing in magnitude (Fig. 1, 2, 3, 4). Maximum activities of all these enzymes were attained 3 to 4 days post bruising. By the sixth and seventh days, activities were declining but were still significantly higher than corresponding controls. For β-galactosidase, the maximum activity was 2.7 times higher than corresponding control activities. With RNase, DNase and β-glucuronidase, these maxima represented 10-, 13- and 10-fold increases over control activities, respectively. The earlier detection of increased enzyme (1 day post bruising) in assays of the latter 3 enzymes as compared

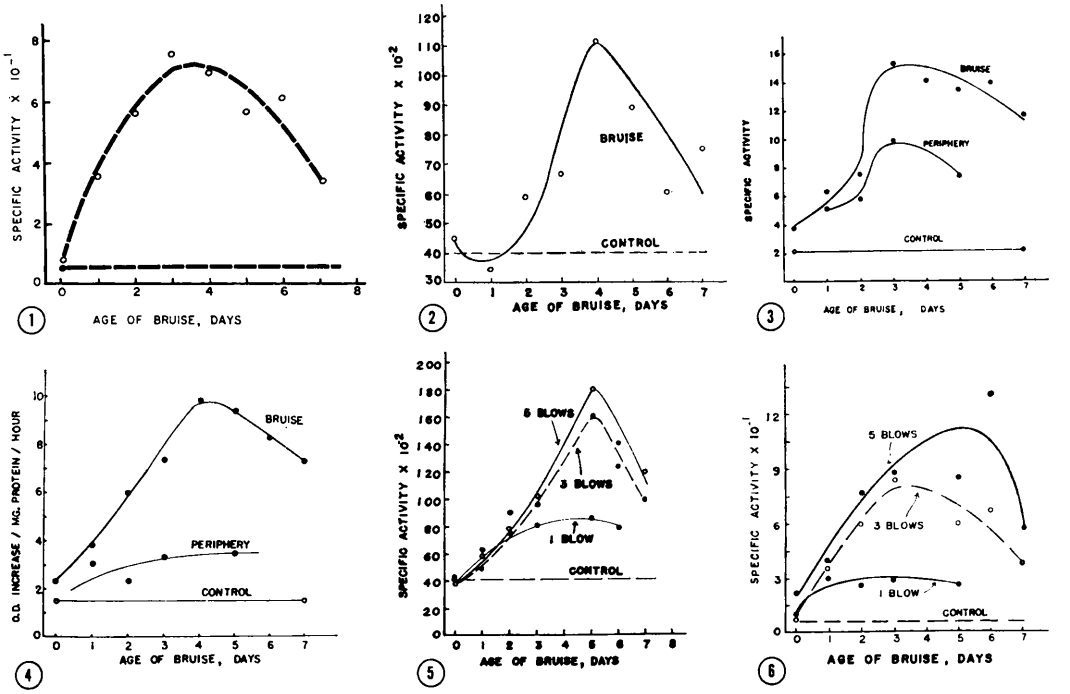

FIG. 1. Effect of age of bruise on β-glucuronidase activity present in experimentally inflicted poultry bruises. Specific activity = μg phenolphthalein liberated per mg protein per 1 hr incubation at 37°C.

FIG. 2. Effect of age of bruise on β-galactosidase activity present in experimentally inflicted poultry bruises. Specific activity = millimicromoles of nitrophenol liberated per mg protein per 30 min incubation at 37°C.

FIG. 3. DNase II activity in control muscle, bruise and bruise periphery as a function of the age of bruise. Specific activity = increase in Beckman DU units absorbance at 260 mμ per 1 hr incubation at 37°C.

FIG. 4. Effect of age of bruise on ribonuclease activity in bruise and periphery.

FIG. 5. Effect of severity and age of bruise on β-galactosidase levels in poultry bruises. Three groups of chickens were bruised. The first received 1 blow, the second 3 blows and the third 5 blows. Specific activity = millimicromoles nitrophenol liberated per mg protein per 30 min incubation at 37°C.

FIG. 6. Effect of severity and age of bruise on β-glucuronidase levels in poultry bruises. Three groups of chickens were bruised. The first group received 1 blow, the second 3 and the third 5 blows. Specific activity = μg phenolphthalein liberated per mg protein per 1 hr incubation at 37°C.

to acid phosphatase(16) and β-galactosidase (2 days post bruise) is probably a reflection of the greater *in vitro* manifested response and, in a sense, may be more apparent than real. Whether the magnitudes of these responses *in vivo* are of the same order as their *in vitro* behaviour indicates is not known. Factors which might influence activities measured *in vitro* include homogenization procedures, cleanliness of glassware, purity of chemicals and relative efficiency of each enzyme system, *i.e.*, optimum pH, temperature, ionic strength of reaction mixture, time of incubation, proper substrate, proper ratios of substrate and enzyme concentrations and presence or absence of inhibitors and activators.

While activities of these enzymes were elevated in both the bruise and the bruise periphery, the bruise itself was the site of highest activities. Schmittle and Rogers(14) found this portion of bruises to be highly necrotic and to contain many infiltrating leucocytes. This agrees with reports on certain lysosomal enzymes in experimental wounds (22,23) and malignant growth(24-27) where the enzymes were concentrated in leucocytes, malignant cells and proliferating epithelium. According to deDuve(28), the probable function of lysosomes in necrosis and cellular repair is to digest components of dead and dying cells. This would clear the area for regeneration of cells and the products of the digestion would help to nourish the new growth.

Effect of severity of bruising. Three groups of 24 chickens each were contused. One group was afflicted with a superficial bruise (1 blow), another with a medium bruise (3 blows), and the third group with a severe bruise (5 blows). Increasing the severity of contusion caused an increase in maximum activity of the lysosomal enzymes studied (Fig. 5, 6, 7, 8) and an observable delay in the time post contusion required to reach this activity. These results were similar to those established by Hamdy *et al*(5) for proteolytic activity measured at pH 8.0 in that increasing severity of injury prolonged the duration of enzyme activity, *i.e.*, 2 to 4 days for the proteinase and 3 to 7 days for the lysosomal hydrolases. However, increasing severity of bruising did not elicit an increased maximum proteolytic activity (pH 8.0). The delay in attaining maximum activity may suggest that the increased activities were not stimulated by direct action of hormones. This may be indicative of invading phagocytes as the source of increased enzymes.

Free and bound lysosomal enzymes. Although normal skeletal muscle is relatively deficient in lysosomal enzymes, Zalkin *et al* (29) found bound enzymes in rabbit leg muscle and much increased activity in vit E-deficient and dystrophic rats. Tappel *et al* (30) reported bound and free lysosomal enzymes in the leg muscle of dystrophic mice and in the pectoral muscle of dystrophic chickens. In the present study, free and bound β-glucuronidase, RNase and DNase activities increased during healing of poultry bruises (Fig. 9, 10, 11).

β-Glucuronidase appeared to be 100% free in normal muscle and about 75% free in bruises immediately after infliction. RNase and DNase activities were 100% bound in control muscle and in zero hour bruises. (It should be noted, however, that activities of these 3 enzymes at these times were extremely low and, therefore, were subject to gross error.) Average bound activities representing 78, 85, and 51% of total activities of RNase, DNase and β-glucuronidase, respectively, were found in 2- to 5-day-old bruises.

Two possible sources of lysosomes in bruised tissue are invading leucocytes(31,15, 28) and the muscle cells themselves(15,30). No attempt was made in this study to evaluate the source of the enzymatic activities.

Summary. Lysosomal DNase, RNase, β-glucuronidase and β-galactosidase activities in experimentally inflicted poultry breast bruises increased by the second day post contusion, reached a maximum between the third and fifth day and then declined toward normal as the bruises showed gross morphological healing. The magnitude of enzymatic response as a result of bruising was not the same for each enzyme, but all enzymes showed the same trends in activity. Activities were elevated to a greater extent in the center of the bruise than at the edge. Normal muscle

from bruised chickens had activities similar to tissues excised from unbruised chickens. The effects of increasing the severity of muscular injury were to increase the maximum activity response and, also, the time required to reach it. Free, bound and total DNase, RNase and β-glucuronidase activities were determined as a function of age of bruise. All forms of the enzymes were found to increase during the healing process.

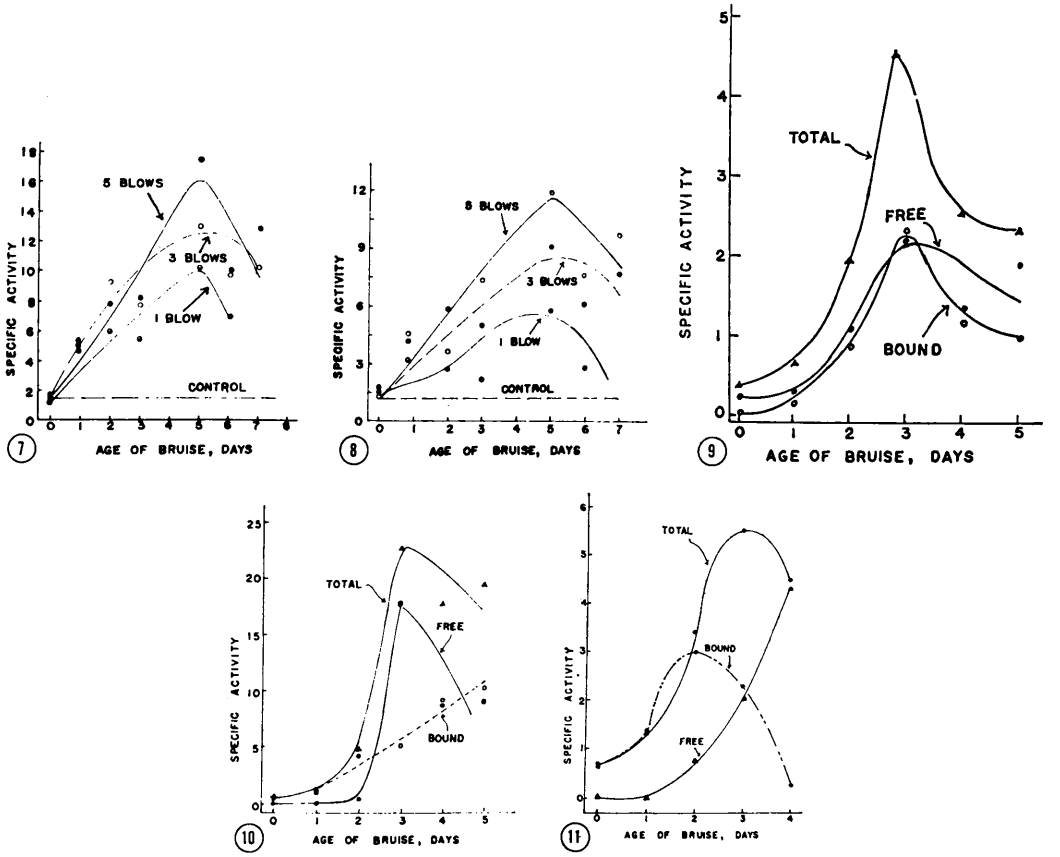

FIG. 7. Effect of severity of bruise and age of bruise on DNase II levels in poultry bruised. Three groups of chickens were bruised. The first received 1 blow, the second 3 and the third 5 blows. Specific activity = the number of DU absorbance units (260 mμ) increased per mg protein during 1 hr incubation at 37°C.

FIG. 8. Effect of severity and age of bruise on RNase specific activity in poultry bruises. Three groups of chickens were bruised. The first group received one blow, the second 3 and the third 5 blows. Specific activity = number of DU absorbance units increased during 1 hr incubation at 37°C per mg protein.

FIG. 9. Effect of age of bruise on total, free and bound β-glucuronidase activities in tissue homogenates. Total activities were determined directly on sucrose homogenates in presence of 0.2% Triton X-100. Free activities were determined directly on sucrose homogenates. Bound activities were calculated by subtracting free from total activities. All activities were expressed as μg of phenolphthalein liberated per mg protein per 10 min incubation at 37°C.

FIG. 10. Effect of age of bruise on total, free and bound forms of RNase measured in tissue homogenates. Total activities were determined in presence of 0.2% Triton X-100. Free activities were determined directly on sucrose homogenates. Bound activities were calculated by subtracting free from total activities. All activities were expressed as number of DU absorbance units increased per mg protein during 10 min incubation at 37°C.

FIG. 11. Distribution of total, bound and free acid DNase in bruised tissue homogenates as a function of age of bruise. Free activities were determined on sucrose homogenates in presence of 0.2% Triton X-100. Bound activities were obtained by subtraction of free from total activities. All activities were expressed as number of DU absorbance units (260 mμ) increased per mg protein per 10 min incubation at 37°C.

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Some Biological Changes in Traumatized Poultry Muscle.* (30300)

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Decreases in the adenosinetriphosphate (ATP) content of various tissues after different types of injury have been reported, usually accompanied by an increase in inorganic phosphate(1-4). Uyeki(3) reported that the predominant changes produced by X-irradiation were on the adenosinetriphosphate levels, and that the magnitude and the temporal sequence of this decrease varied from one species to another. Less significant changes were noted in the total adenosine nucleotide, adenylic acid, and adenosinediphosphate levels of spleen in rats, whereas in the mouse the adenylic acid levels increased 65 to 75% above the normal level 3 to 7 days following exposure to irradiation. Engel(1) and Jordan and Gray(4) found

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