ting that almost all the LH was released from the pituitary in a surge lasting not more than 3 hr, and that the normal cycling ewe pituitary synthesizes approximately 0.5 mg of NIH-LH-S1 equivalent per day.

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Histological Effects of Procedural and Environmental Factors on Isolated Rat Heart Preparations* (33343)

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Studies of isolated tissue preparations constitute the basis of much of our present knowledge concerning biochemistry and physiology. A controlled environment and a thorough understanding of the variables involved in isolated tissue experimentation is of primary importance.

There were three objectives of this study: (i) to determine the effects of certain environmental conditions on the histological appearance of isolated rat heart preparations; (ii) to determine if significant differences exist between incubated and perfused preparations under identical conditions of treatment-duration and temperature; and (iii) to determine the best type of preparation for further isolated tissue studies.

The effects of duration of treatment, temperature of the environment, and the presence of various drugs on the morphology of the isolated rat right ventricular strip, isolated atria, and perfused whole heart were evaluated. Attention was focused on a possible explanation for the significant differences. All histological observations were made with a light microscope.

Isolated, incubated, and perfused hearts have been extensively employed in experimental procedures. A survey of the literature disclosed that few studies have been done on the histology of isolated preparations. Tanz (1) reported that 0.5 μg/ml of 9-alpha-fluorohydrocortisone greatly reduced the histological signs of cardiac degeneration which occurred in the isolated cat papillary muscle incubated at 38° for 6 hr. Tanz (2) subsequently reported that chlortetracycline at 20 μg/ml prevented loss of cross striations and increased the height of contraction in isolated cat papillary muscle over a 6-hr period of

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continuous stimulation. McCarty and Shideman (3) reported that the cat capillary muscle under tensions of 3.0 g at 37.5°, exhibited areas of early degeneration with areas of normal morphologic appearance also present. An increase in tension to 4.0 g resulted in severe degenerative changes, such as loss of cross striations, foamy cytoplasm and irregular and pyknotic nuclei. These changes were present regardless of whether the muscle was stimulated to contract or placed under static tension.

Materials and Methods. Right ventricular strip and atria preparation. The experiments were conducted on albino male, Cox rats weighing 200–280 g. Three rat hearts were used for each variable. A minimum of three slides were made from each heart and the results of at least nine slides were averaged. The slides were coded in order that the evaluation of the histology could be performed without knowledge of the treatment.

A modified Krebs–Henseleit bicarbonate solution (KHBS) (4) containing 1.22 m*M* calcium and 5.5 m*M* glucose was employed for the incubation media, rinsing solution and dissecting solution. Before each experiment, fresh solutions were prepared using distilled–double deionized water. The temperature of the rinsing solution was 23° and the dissecting bath was maintained at 9° by surrounding it with crushed ice. The temperature of the incubation media was maintained at the requisite temperature with a constant temperature bath. All solutions were continuously oxygenated with 95% O_2 and 5% CO_2.

The rats were decapitated by guillotining and their hearts were excised within 20–40 sec. The hearts were briefly rinsed to remove excess blood from the surface and chambers. Careful dissection of a strip of the right ventricular wall and complete excision of both auricles with the intervening isthmus was performed while the hearts were immersed in a dissecting bath at 9°. Both the right ventricular strip and the atria were mounted on plastic mounting plates by cotton thread. The thread was passed through the centers of the atrial preparation and through the end of the strip in the ventricular preparation. The time between decapitation and the completion of the mounting procedure was 4–6 min.

The mounted tissues were transferred to chambers filled with KHBS. The chambers were then placed in the constant temperature bath. After incubating the requisite period, the tissues were removed from the bath and immediately placed into Zenker's fixative. Zenker's fixative was chosen due to its rapid rate of fixation. The tissues were embedded in paraffin and sections were cut at 6 μ. Slides were stained with hematoxylin and eosin. Some special histological studies were performed using oil red O for fat and periodic acid Schiff (PAS) for glycogen.

The handling of the isolated tissues with tissue forceps during the dissection and mounting procedures resulted in discretely localized tissue damage. These damaged areas were not included in the total histological evaluation of the tissues.

The following grading system was used in the evaluation of the degenerative changes: Vacuoles: (+) 0–5 per 425 × field = slight; (++) 5-20 per 425 × field = some; (+++) 20–40 per 425 × field = moderate; and (++++) 40–100 per 425 × field = abundant. Loss of cross striations: (+) loss of distinctness and granular appearance of A and I bands; (++) complete loss of cross striations. Central interstitial edema: (+) = slight; (++) = some; (+++) = moderate; and (++++) = abundant.

Perfused whole heart preparation. The information concerning animals, solutions, fixation, and slide preparation is identical for the perfused heart experiments.

The rats were sacrificed by guillotining. The hearts were quickly removed and attached via the aorta to the perfusion apparatus. The time from sacrifice to initiation of perfusion ranged from 60–90 sec. The perfusion fluid was Millipore-filtered prior to use. The hearts were perfused at 10 ml/min with KHBS at 37° by a nonrecirculatory modified Langendorff technique. The hearts were not electrically stimulated.

Drug studies. The influence of various drugs on the degenerative changes of the isolated rat heart preparation was evaluated in

an attempt to select an agent(s) to prevent or inhibit the changes.

It was postulated that since the dissection procedures were carried out in less than ideal sterile conditions, bacterial contamination might be responsible for the significant degenerative changes. A combination of 373 mg/liter of penicillin (V-Cillin-K), 99 mg/liter of streptomycin sulfate (Pfizer) and 30 mg/liter of polymyxin B sulfate (Aerosporin) was used to test this hypothesis. The above combination was reported by Bakke *et al.* (5) to be necessary to permit prolonged incubation without bacterial growth, as tested by smear and culture.

In an attempt to corroborate the results of Tanz (1), 0.5 μg/ml of 9-alpha-fluorohydrocortisone was added to the rat isolated right ventricle preparation. This concentration was reported by Tanz to prevent degenerative changes in the cat papillary muscle. Tanz postulated that this steroid had a cardiac glycoside-like action and thus inhibited degeneration.

Ouabain, 0.33 μg/ml, was added to another group of ventricle preparations to determine if a cardiac glycoside, suggested above by Tanz, could inhibit degeneration. This concentration of ouabain was reported by Sanyal and Saunders (6) to increase the force of contraction of a guinea pig ventricular strip preparation by 80% of the initial force over a 1-hr period.

Tanz (2) also reported that chlortetracycline at 20 μg/ml increased the height of contraction and prevented loss of cross striations in isolated cat papillary muscle over a 6-hr period of continuous stimulation. This concentration of chlortetracycline (Aureomycin) was used on rat ventricle preparations to corroborate Tanz's result.

The 0.5-ml samples of drug and nondrug treated bathing media were collected after incubating isolated rat heart preparations for 6 hr at 37°. These samples were cultured on thioglycollate media for 12 hr at 37°. Samples treated with 9-alpha-fluorohydrocortisone, ouabain, and control samples showed marked clouding of the thioglycollate media indicating bacterial growth. Gram-negative bacilli and gram-positive cocci were isolated. Antibiotic treated samples showed no clouding of the thioglycollate media after 12-hr or at 48-hr incubation. No bacteria were found on gram-stains of antibiotic treated samples.

Results and Discussion. Histological degeneration of the heart was measured by three parameters: (i) loss of cross striations, (ii) central interstitial edema, and (iii) vacuoles in the fiber bundles.

The loss of cross striations in degenerating cardiac tissue is a well-substantiated phenomenon. Control cardiac muscle (Fig. 1) shows distinct A bands (dark lines) alternating with I bands (light lines). As degeneration proceeds (Fig. 2), the A bands lose their continuity and assume a granular appearance resulting in only a suggestion of an alternating light and dark pattern. The most marked degeneration (Fig. 3) shows that the fiber bundles completely lose the banded pattern and result in an amorphous acidophilic mass.

Central interstitial edema probably represents a second expression of tissue degeneration. Our hypothesis is that central interstitial edema is a histological manifestation of diffusion problems resulting from osmotic differences between the central and peripheral portions of the tissue. In support of this hypothesis Lussnitzer and Kelly (7) have shown that there are diffusion problems in *in vitro* heart preparations which are not present *in vivo*. They demonstrated that inadequate diffusion was manifested in *in vitro* preparations by the varying distribution of inulin-carboxyl-^{14}C in hamster hearts incubated at 25° in Krebs–Ringer buffer for 5 hr. Figure 4 represents a cross-section of an isolated rat ventricular strip incubated for 1 hr at 27° and Fig. 5 is a control. Figure 4 shows a relatively compact layer of fiber bundles along the periphery of the strip and progressive separation of these fiber bundles toward the center of the strip. This central edema is probably due to a number of factors: (i) inability of the O_2 in the bathing media to diffuse through the relatively thick preparation, resulting in central hypoxia, (ii) inability of the toxic wastes, e.g., lactic acid, to diffuse out of the central portion of the tissue, and (iii) inability of the high molecu-

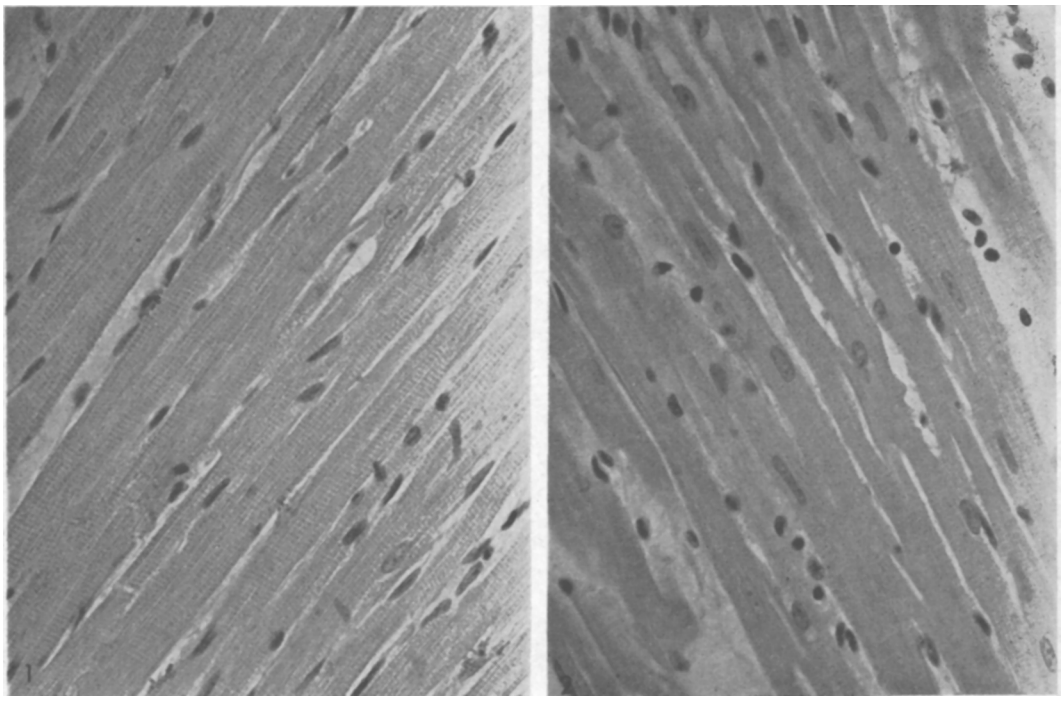

FIG. 1. Control rat right ventricle preparation, longitudinal section; vacuoles (0) cross striations intact (0), no edema (0), ×425.

FIG. 2. Rat right ventricle strip incubated for 1 hr at 27°. Loss of cross striations (+), ×425.

lar weight cellular degeneration products resulting from central necrosis from diffusing out of the central portion of the strip. The result is an increase in central osmolarity. These factors produce a high central osmotic pressure gradient sufficient to attract H_2O from the incubation solution into the central portion causing the central interstitial edema. Law (8) showed that the rat plantaris muscle incubated in oxygenated Ringer's solution developed 2 distinct zones after incubating for 1 hr at 38°. The outer zone which was approximately 12 cells in width was well preserved. "The cells of the inner zone showed not only a marked morphologic peculiarity, but were separated by far greater distances than were the cells of the outer zone." Law's photomicrographs of the rat plantaris muscle show the same type of central interstitial edema as we have observed in the incubated rat right ventricle strip and atria. Law attributed the bizonal nature of the plantaris preparations to partial or extreme hypoxia of the inner zone.

The presence of vacuoles in the fiber bundles is a third expression of tissue degeneration. Whether the vacuoles represent the accumulation of cellular breakdown products and/or the accumulation of exogenous fluid is not known at the present time. The contents of the vacuoles were investigated by staining with oil red O for lipid and with periodic acid Schiff (PAS) for glycogen. These stains failed to demonstrate either substance to be present in the vacuoles. Figure 6 shows vacuoles and edema in a preparation incubated for 6 hr at 37°. Figure 7, a control, shows no vacuoles or edema.

Effects of tissue thickness. A comparison of a thick tissue slice represented by a right ventricular strip (Fig. 4) and a thin tissue slice, represented by an auricular appendage (Fig. 8), reveals that the histological signs of tissue degeneration are much less marked in the thin preparation. The remainder of the auricle with the exception of the auricular appendage, shows degenerative changes identical to those in the ventricular strip. The re-

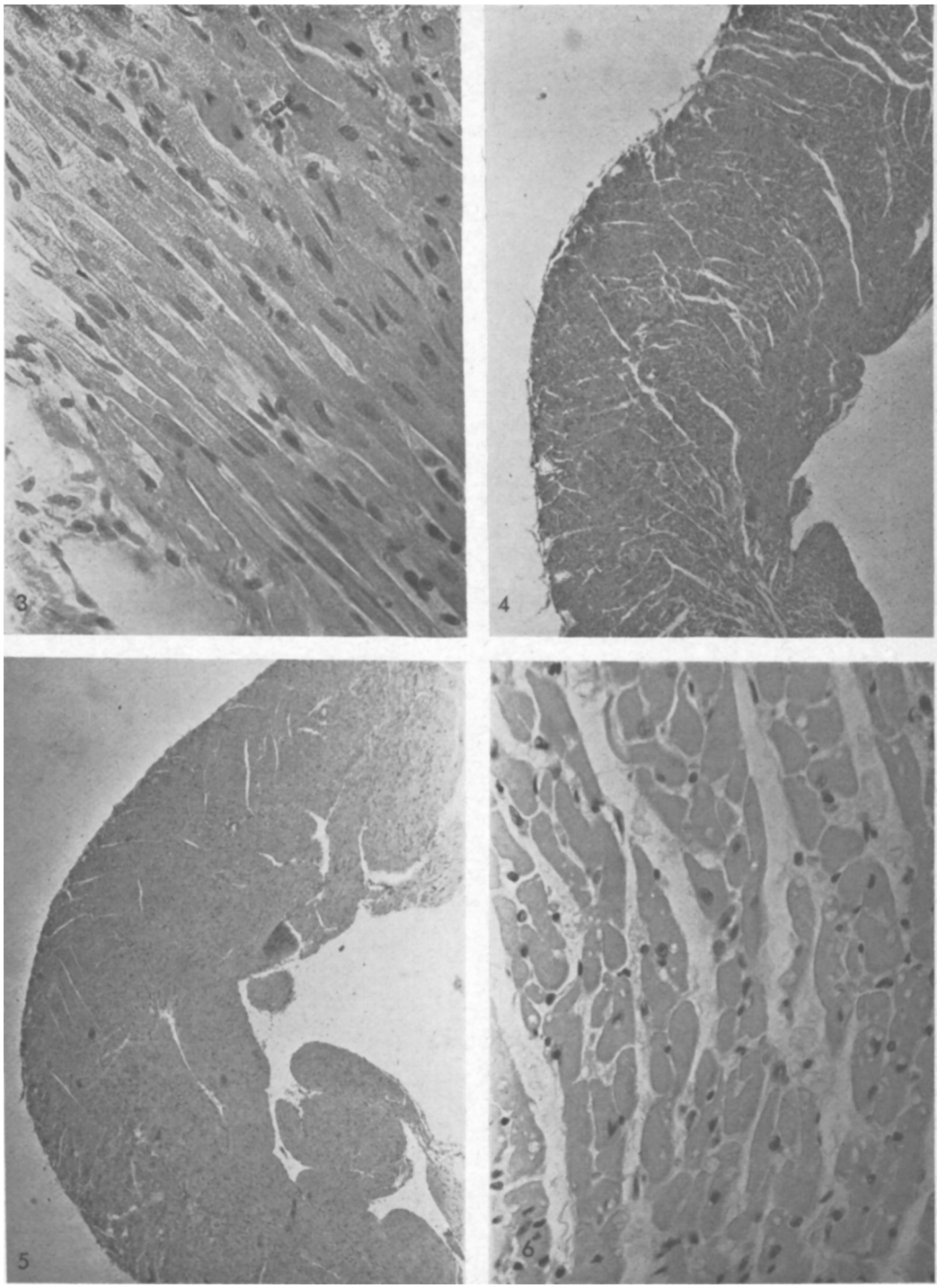

FIG. 3. Rat right ventricle strip incubated for 6 hr at 37°. Loss of cross striations (++), ×425.

FIG. 4. Rat right ventricle strip incubated for 1 hr at 27°. Central interstitial edema (++). Note: Relatively compact periphery (outer ⅔) and fiber separation centrally (inner ⅓), ×100.

FIG. 5. Control rat right ventricle strip. Note: Uniform compactness, ×100.

FIG. 6. Rat right ventricular strip incubated for 6 hr at 37°. Vacuoles (++++), central interstitial edema (++++), ×425.

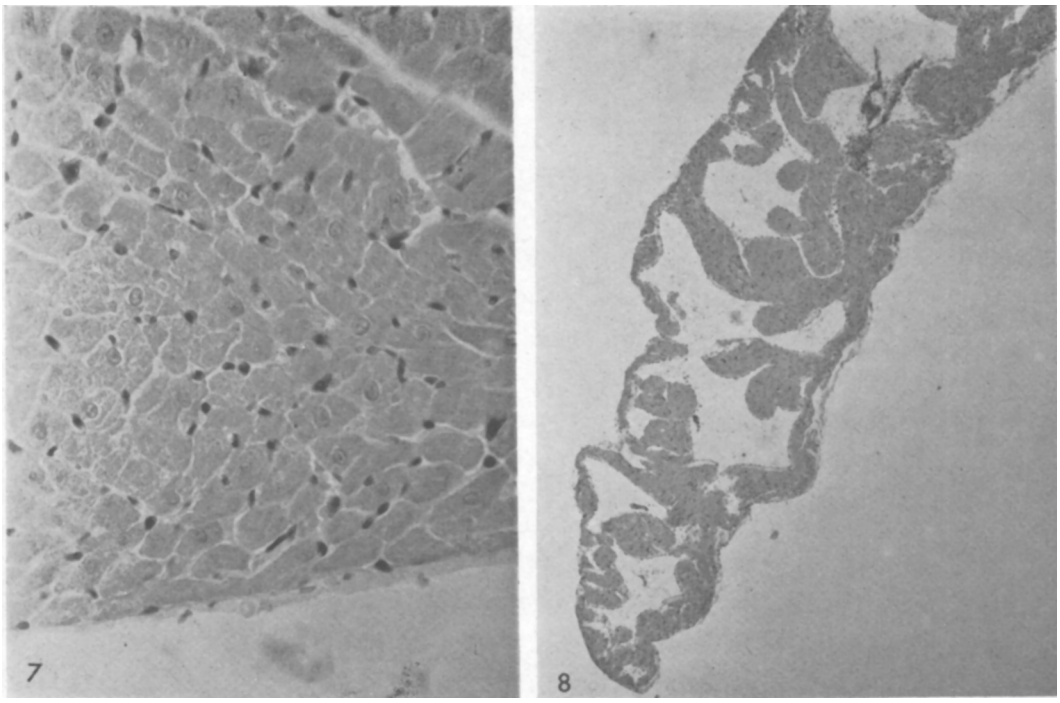

FIG. 7. Control rat ventricular strip, cross section; vacuoles (0), central interstitial edema (0), ×425.

FIG. 8. Rat auricular appendage incubated for 1 hr at 27° shows (0) edema and (0) vacuoles, ×100.

sults of the thin vs thick preparation study are quantitatively represented in Table I. These results are consistent with the hypothesis that the ventricular strip and most of the atria are too thick for adequate exchange of O_2 and metabolites. The ventricular strip and atrium have become centrally necrotic probably due to hypoxia. Our studies show that the average thickness of the well-preserved peripheral zone in an incubated rat heart preparation is 16–20 cells wide (Figs. 4 and 10).

TABLE I. The Effect of Tissue Thickness on the Degenerative Changes of Isolated Rat Heart Strips Incubated 6 hr at 27°.

Degenerative parameter	Auricular appendage	Right ventricular strip
Loss of cross striations	—[a]	+
Vacuoles	0	++
Central interstitial edema	0	+++

[a] Auricular appendage sections showed only cross cut fibers. Cross striations could not be observed or evaluated.

TABLE II. The Effect of the Type of Preparation on the Degenerative Changes of Isolated Rat Heart Ventricular Strips.[a]

Degenerative parameter	Perfused	Incubated
Loss of cross striations	0	+
Vacuoles	0	+++
Central interstitial edema	++++	++++

[a] Temperature 37°; treated 1 hr.

Effects of incubation vs perfusion. Perfusion of the whole rat heart resulted in a reduction of most degenerative changes which were seen in incubated right ventricular strips and atria. The quantitative comparisons are shown in Table II. These results are supported by Weissler *et al.* (9), who have shown that the isolated rat heart perfused at 32° with 5% albumin in Krebs–Ringer's bicarbonate solution showed no electron microscopically observable degeneration over a

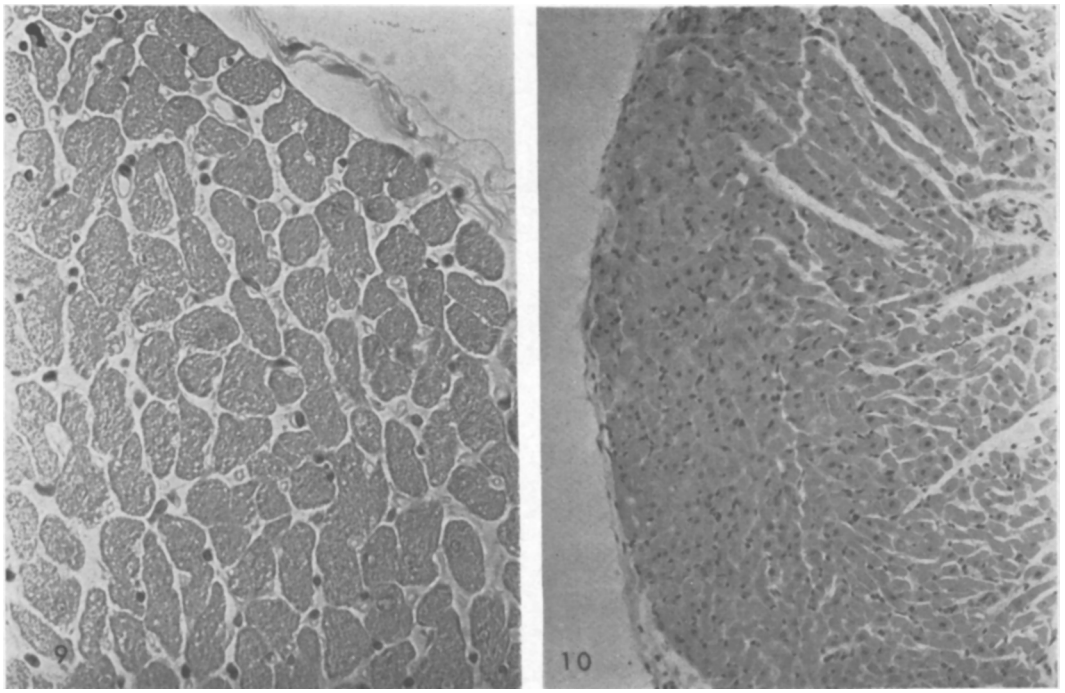

FIG. 9. Whole rat heart preparation perfused 1 hr at 37°. Diffuse interstitial edema (++++) extending to the edge of the preparation. Note: (0) vacuoles, ×425.

FIG. 10. Rat right ventricular strip incubated for 1 hr at 37°. Central interstitial edema (+++++). Note the compact peripheral area and the central fiber separation, ×100.

period of 90 min unless both O_2 and glucose were removed from the perfusion media. The marked interstitial edema seen in the perfused heart (Fig. 9) probably has a different etiology than that seen in the incubated preparation (Fig. 10). The perfusion (hydrostatic) pressure was approximately 50 mm Hg, which was sufficient to overcome the osmotic pressure contributed by the perfusing media. The high perfusion pressure forces fluid across the capillary membrane into the interstitial spaces and results in edema. There was uniform distribution of the interstitial edema in the perfused heart (Fig. 9) in contrast to the central localized edema of the incubated preparation (Fig. 10). This interstitial edema explains partially the increase in total water content seen with the perfused hearts. We were able to show that 10 freshly dissected hearts had a mean water content of 76.9 ± 0.53% (± SD). Perfusion of 13 hearts for 3 hr resulted in a significant increase in water content to 80.2 ± 0.32% ($p < .001$).

Effects of duration of incubation and temperature of environment. There was a progressive increase in all three parameters of tissue degeneration with an increase in duration of incubation or environmental temperature. Quantitative results are shown in Tables III and IV. These results are consistent with a degenerative process in that the degree of change increases with duration of treatment and temperature.

Effects of drugs. Antibiotic treatment of isolated rat heart preparations with penicillin, polymyxin B, streptomycin, and chlortetracycline failed to support the hypothesis that bacterial contamination was responsible for the degenerative changes. Comparable degenerative changes were seen in antibiotic treated preparations with no evidence of bacterial growth in the incubation media and in non-antibiotic treated preparations with obvious bacterial contamination of the incubation media. From these studies we concluded that the degenerative changes probably were not due to bacterial contamination.

TABLE III. The Effect of Duration of Incubation at 27° on Rat Right Ventricle Strips.

	Time						
	(min)				(hr)		
Degenerative parameter	0	5	15	30	1	2	6
Loss of cross striations	0	0	0	0	+	+	+
Vacuoles	0	++	++	++	++	++	++
Central interstitial edema	0	+	+	+++	+++	+++	+++

9-Alpha-fluorohydrocortisone (0.5 μg/ml) was reported by Tanz (1) to prevent degenerative changes in the cat papillary muscle. This concentration failed to reduce degenerative changes in rat heart preparations and higher concentrations (20 μg/ml) were equally disappointing.

Chlortetracycline at 20 μg/ml did not prevent loss of cross striations in rat heart preparations. These results failed to support Tanz's (2) observations on isolated cat papillary muscle.

Ouabain, 0.33 μg/ml, had no effect in decreasing histologically observable degenerative changes in isolated rat heart preparations.

Summary. Histological evaluation of isolated rat heart preparations has shown that: Incubated isolated right ventricular strips and atria undergo observable degeneration after relatively short periods of time and at temperatures of 27° or higher. The degree of degeneration is a function of: (a) duration of incubation; (b) incubation temperature; and (c) thickness of the soaked preparation. Cardiac degeneration is not prevented by penicillin, streptomycin, polymyxin, chlortetracycline, 9-alpha-fluorohydrocortisone, or ouabain in the doses employed. The perfused whole heart is superior to the incubated isolated tissue preparation. The perfused heart shows few signs of degeneration at 37° for prolonged periods.

TABLE IV. The Effect of Temperature of Incubation on Rat Right Ventricle Strips (treated 1 hr).

	Temp.		
Degenerative parameter	9°	27°	37°
Loss of cross striations	0	+	+
Vacuoles	0	++	+++
Central interstitial edema	0	+++	++++

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