ance of cell borders, "macula-densa-like" apparently syncytial structures arise, in which the nuclei are often actually enlarged and, in some places, deformed (PAS stain × 400).

FIG. 8. Higher magnification of another region of the kidney shown in Fig. 5. Here the PAS-positive, small, hyalin granules within the tubular epithelium are clearly visible. Note also that the brush-border tends to accumulate in clumps or is almost completely shed off (PAS stain × 720).

FIG. 9. Proximal convoluted tubules from the inner, not atrophic, stratum of the cortex shown in Fig. 5, 7 and 8. Unlike in the region shown in Fig. 7 (which is photographed at the same magnification), the tubules in this stratum (chiefly composed of spiral segments) are normal, the brush-border is well developed, the cytoplasm abundant and the stroma hardly detectable (PAS stain × 400).

tense and rather selective atrophy of the outer cortex was equally evident in the intact and in the hypophysectomized rats receiving subcutaneous NaH_2PO_4. Presumably, this change, unlike the nephrocalcinosis and the hyalin-cast formation, is not significantly dependent upon any of the pituitary hormones.

Summary. 1) Experiments on rats indicate that oral administration of excess sodium phosphate causes intense calcification in the "zona intermedia" of the kidney in intact but not in hypophysectomized rats. 2) The "granuloma-pouch" technic permits the subcutaneous administration of as much as 10 ml of 5% NaH_2PO_4 per rat daily. (Normally, this amount could not be given subcutaneously because of its topical necrotizing effect.) When so administered, the phosphate solution causes a generalized nephrocalcinosis throughout the kidney and this is also virtually abolished by hypophysectomy. Apparently, hypophyseal hormones play an important conditioning rôle in the pathogenesis of this kind of nephrocalcinosis. 3) Repeated injections of hypertonic phosphate solutions, into subcutaneous granuloma-pouches, cause the formation of numerous hyalin casts and protein-precipitates in the kidney and this change was likewise totally abolished by hypophysectomy. When thus administered, phosphate solutions can also induce a pronounced and highly selective atrophy of the entire outer renal cortex, but this change is essentially of the same severity in the presence and in the absence of the hypophysis.

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Mechanism of Heparin Protection Against a Histamine Releasor (48/80).* (22522)

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It was previously reported that fibroblasts ingest a variety of metachromatic substances which are deposited in their cytoplasm in granular forms(1,2). Since the recognition of mast cells is dependent primarily on a specific (metachromatic) staining of their cytoplasmic granules, rather than any definitive shape or appearance of the cell(3), the granulated fibroblasts are analogous to tissue mast

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cells and may be described as "quasi-mast cells" to indicate this superficial resemblance. It was postulated that micellophagosis, *i.e.*, the fibroblastic ingestion of mucopolysaccharides, may play an important role in local detoxification by the absorption and intracellular degradation of conjugates of (tissue) mucopolysaccharides with noxious agents(2). When the conjugate is metachromatic and initially deposited in the cytoplasm as granules, this intermediate phase of detoxification would be characterized by the appearance of fibroblasts having metachromatic granules in their cytoplasms.

The phenyl alkyalmine, 48/80, has been found to induce acute intoxication in laboratory animals following parenteral injection (4), as well as definite cytological changes in the mast cells of the loose connective tissue (5). It was of interest to investigate the possibility that the acidic mucopolysaccharides of ground substance could modify the toxicity of this compound by complexing with the amine and thereby initiating micellophagosis of the toxic agent.

Methods. Protection study. Mice 2 to 3 months old and ranging in weight from 21 to 25 g were challenged intraperitoneally with various doses of 48/80‡ contained in a volume of 0.25 ml. Both C-57 and CBA mice were used in this study since no marked differences in their respective reactions to the 48/80 were noted. A 48/80 LD_{50} of 4.0 $\pm$ 0.3 μg/g of mouse was established. Additional mice were pretreated with various amounts of heparin (117 I.U./mg)‡ injected in a volume of 0.25 ml by the intraperitoneal route. Thirty minutes later these animals were challenged with standard doses of 48/80 and the survivals noted. Fifty to 115 mice were used per 48/80 challenge group and the amount of heparin necessary to protect 50% of the mice against the respective challenge doses of 48/80 was calculated by probit analysis. The mucopolysaccharides, chondroitin sulfate, and hyaluronic acid were prepared as saline solutions for comparison in activity with heparin. *Tissue Study.* CBA mice, 8 to 10 weeks old, were injected subcutaneously on the dorsal surface with 1.0 cc of air to form an air pouch in the loose subcutaneous connective tissue. Test substances, in a volume of 0.2 cc, were injected into the bubble formed within the loose subcutaneous connective tissue. At the appropriate time the animals were sacrificed and the connective tissue bubble dissected free of the surrounding tissues. The ventral surface of it was exposed and a portion removed, spread on a glass microscope slide, air-dried, and stained with May Gruenwald-Giemsa stain in the usual manner(6). Similar samples were removed and stained with Azure A for comparison. Test substances were suspended in isotonic saline and injected in 0.2 cc volumes containing a) 500 μg heparin, b) 100 μg 48/80, c) a mixture of 500 μg heparin and 100 μg 48/80, and d) a saline control. Ten animals were used for each group and were sacrificed 30 minutes following injection and tissue samples were obtained. The tissues were examined with the aid of oil immersion magnification, and the following types of cells counted: a) agranular fibroblasts, b) fibroblasts with a few cytoplasmic granules, and c) cells, some of which were of uncertain origin, whose cytoplasm contained numerous metachromatic granules. No distinction was made between intact and degranulating mast cells. Five hundred cell counts per tissue spread for each of the 10 samples of the 4 groups were performed.

Results and observations. Preliminary observations showed that the 50% lethal dose of 48/80 varied markedly with the route of injection (Table I). For any one route, mice

TABLE I. Effect of Injection Route and Treatment on Toxicity of 48/80.

Variation in 48/80 toxicity relative to inj. route		Effect of some mucopolysaccharides on 48/80* toxicity	
Route	LD_{50}, μg/g	Polysac. (200 μg)	Survivors
Subcut.	17.6	Hyaluronic acid	2/20
Intraper.	4.0	Chondroitin sulfate	0/20
Intrav.	1.5	Heparin	19/20
		Saline	0/20

* 7.2 μg/g, i.p.

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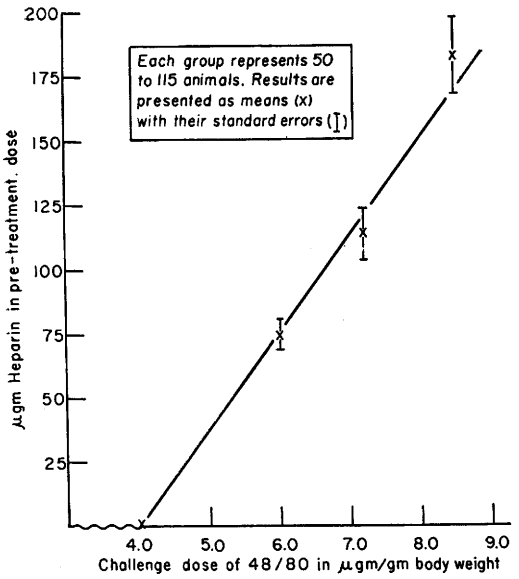

FIG. 1. Dose response curve illustrating protective effect of heparin pretreatment in mice against subsequent challenge with varying amounts of 48/80 in which the ordinate represents the heparin dose sufficient to protect 50% of the mice against a given challenge dose of 48/80.

were separated into groups of 10 animals each and injected with graded doses of the amine contained in 0.25 ml saline. The numbers of survivors 24 hours later were noted and the LD_{50} calculated(7). The results as shown in Table I could be interpreted to indicate that tissue constituents may bind with the toxic amine and thereby account for the differences noted in its toxicity relative to the route of administration.

Groups of 10 mice each were then pretreated with intraperitoneal injections of saline containing 200 μg hyaluronic acid, chondroitin sulfate, or heparin, and challenged 30 minutes later with a lethal dose of 48/80 (7.2 μg/g). The experiment was repeated and the accumulated results, presented in Table I, indicated that only heparin offered a marked and (what has since been found to be) a reproducible type of protection against the toxic effects of the amine.

Protection. A quantitative study of the dose response relationship between heparin pretreatment doses and standard 48/80 challenge doses (6.0, 7.2 or 8.5 μg/g) was then performed. All of these 48/80 doses were rapidly lethal to non-heparinized (saline) control mice. The results in terms of the 50% protective doses of heparin were plotted against their respective 48/80 challenge doses, as shown in Fig. 1. The straight line, obtained by the method of least squares, suggests that the increased tolerance of the heparin-treated mice may be due to a direct interaction of these two drugs *in vivo*. When solutions of the two drugs are mixed together, a cloudy solution results. Mota, *et al.*(5) have reported 48/80 inhibition of heparin anticoagulant activity *in vitro*.

Fibroblastic granulopoiesis. Experiments were then performed to determine the cellular responses involved in this heparin-enhanced tolerance to 48/80. It was noted that in mice pretreated with 500 μg of heparin intravenously and then injected subcutaneously with 100 μg 48/80, the fibroblasts in the injection area often had numerous small metachromatic granules in their cytoplasm, which suggested that cellular mechanisms were involved in the enhanced tolerance of heparin-treated mice to 48/80. When a mixture of heparin and the amine was injected, many of the cells were found to contain metachromatic granules in their cytoplasms. An experiment was then carried out to determine the requirements for this granulopoietic response. Four groups of animals were used as noted above. In the saline controls (Fig. 2, 3) the pouching and injection procedures caused a slight degranulation of some mast cells with a subsequent uptake of the shed granules by nearby fibroblasts(8) (Table II) during the 30-minute interval between time of injection and sampling of the tissue. The injection of heparin alone did not markedly alter the relative distribution of the cells, and the appearance of the tissue was essentially the same as those of the controls, except for the strong metachromasia of the ground substance due to the presence of the injected mucopolysaccharide. On the other hand, 48/80 caused a widespread disruption of mast cells (Fig. 4, 5), as previously reported(5). The increase in granulated fibroblasts (Table II) appeared to be partially due to the increased number of shed mast cell granules

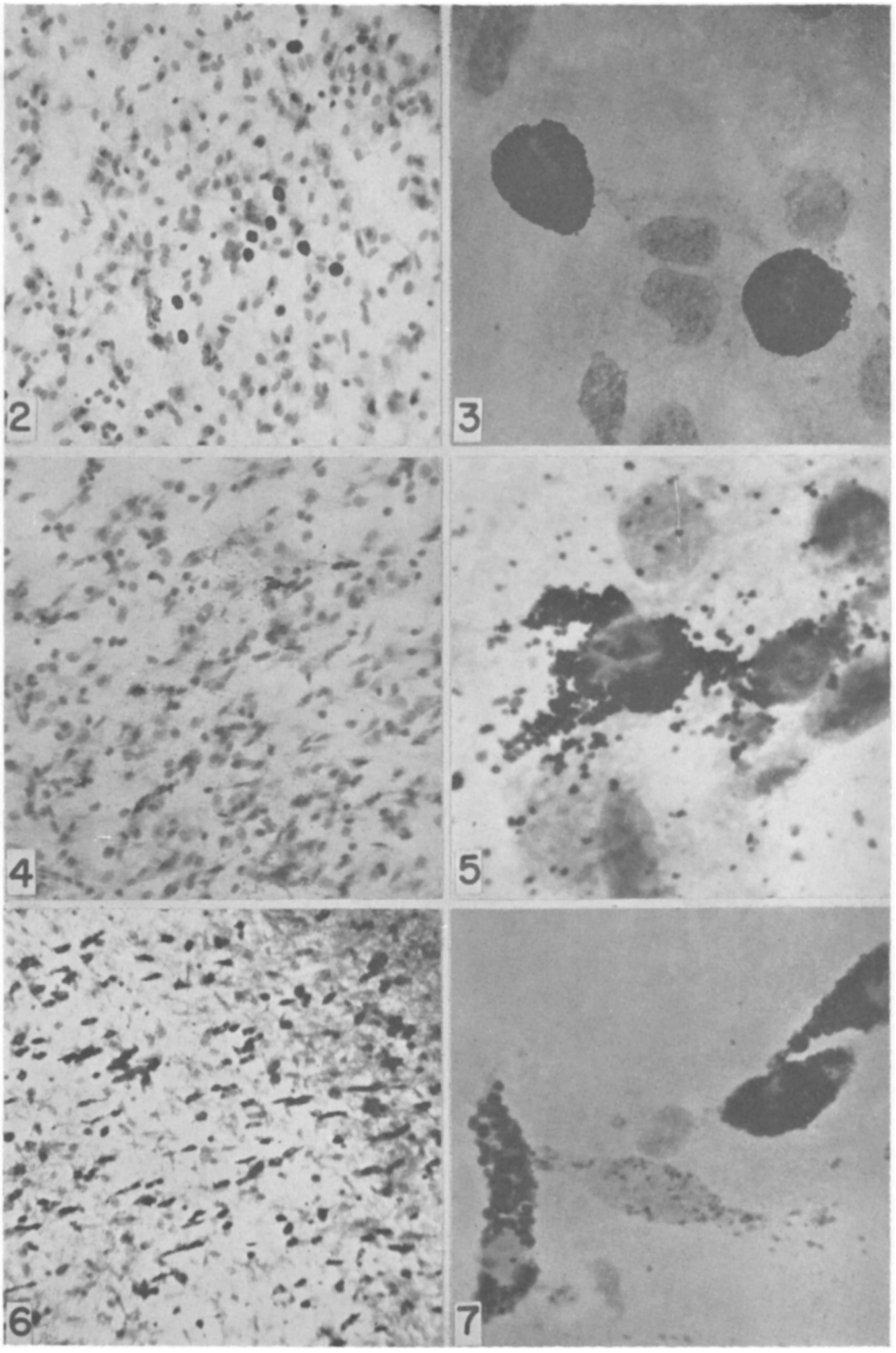

FIG. 2-7. Air-dried spreads of mouse loose connective tissue obtained 30 min. after local injection of test materials. Stained with May Gruenwald-Giemsa stain.

FIG. 2. Saline control tissue (× 150).

FIG. 3. Same as Fig. 2 with increased magnification showing intact mast cells of control tissue (× 1550).

FIG. 4. Tissue after 48/80 injection. Note widespread degranulation of mast cells (× 150).

FIG. 5. Same as Fig. 4 with increased magnification showing mast cell response to 48/80 injection (× 1550).

FIG. 6. Tissue after 48/80-heparin injection. Note increased concentration of cells exhibiting dense metachromatic cytoplasms as compared to Fig. 2 and 4 (× 150).

FIG. 7. Same as Fig. 6 with increased magnification showing the cytoplasmic granulation of fibroblasts in response to treatment. Note fibroblasts in center with few granules, in contrast to heavily granulated cells in lower left and upper right of field (× 1550).

available for fibroblastic uptake(8). The tissues from animals injected with the 48/80-heparin complex contained an increased concentration of cells with metachromatic granules in their cytoplasms (Fig. 6, Table II). Fig. 7 shows the morphology of some of these cells with the cytoplasmic granulation induced by heparin-48/80 treatment. The granules in some cells were coarse as in mast cells; in some they were rather fine, and in others there was a mixture of both types of granules. The elongated cytoplasm of many of these quasi-mast cells contrasts markedly with the rounded-up appearance of the typical connective tissue mast cell in the mouse. However, variant morphological types are not infrequently observed in this animal.

It is obvious from Fig. 6 that a local injection of the heparin-48/80 complex will induce an increased *concentration* of metachromatically granulated cells in the loose connective tissue. The *percentage distribution* of these cells in the tissue, as well as their general morphology, suggests that they originated from agranular fibroblasts (Table II).

TABLE II. Percentage Distribution* of Granulated Cells in Loose Connective Tissue following Subcutaneous Injection of Saline, Heparin, 48/80 or Heparin-48/80 Complex.

Treatment group	Fibroblasts	Sparsely granulated fibroblasts	Heavily granulated cells
	%		
Saline	90.1 ± 1.4 (87.2-93.0)	4.3 ± .6 (3.0-5.6)	5.7 ± 1.1 (3.4-7.9)
Heparin	87.0 ± 2.2 (82.2-91.7)	5.9 ± 1.0 (3.7-8.1)	7.1 ± 1.4 (4.2-10.0)
48/80	72.8 ± 2.0 (68.6-77.0)	21.1 ± 1.9 (17.0-25.2)	6.1 ± 1.2 (3.5-8.7)
Heparin-48/80	6.5 ± 1.8 (2.8-10.3)	16.4 ± 2.0 (12.2-20.6)	77.1 ± 2.9 (71.1-83.1)

* Results presented as percentage of means and stand. errors with 95% confidence limits on 500 cell counts per tissue spread. Nine to ten spreads examined for each group.

The fate of these granulated cells was obscured by the resultant inflammatory reaction to the injection material, but observations made at intervals suggested that much of the material was digested by the cells. Twenty-four hours after injection, the number of granulated cells, as well as the number of granules per cell, appeared to be markedly decreased.

Discussion. The ubiquitous distribution of loose connective tissue throughout the body as "packing material" of the blood vessels implies its importance as an intermediary in the transportation of metabolites to and from parenchymal cells. The presence of the macromolecular acidic mucopolysaccharides in the ground substance of this tissue suggests that it may have a physiological role as a "buffering" or detoxifying system for noxious amines, thereby enabling the tissue to maintain the local environment within a physiological range. This particular function of the acidic mucopolysaccharides could be analogous to actions of cation exchange resins.

The binding of the toxic phenyl alkylamine, 48/80, by heparin and its sequestration in granular form by fibroblasts of the tissue has been presented as a model of this local detoxifying response of the loose connective tissue. The beneficial results arising from experimentally increasing the tissue concentration of heparin prior to challenge with an otherwise lethal dose of 48/80 supports this concept. Unpublished results indicate that other sulfated polysaccharides such as dextran sulfate are also effective. Conversely, heparin treatment seems to enhance tolerance of mice to other noxious basic substances such as polymyxin B or stilbamidine(9).

Comparison of the granulated fibroblast to tissue mast cells would appear to be rather inopportune since knowledge concerning the

cellular precursor(s) as well as the function of the mast cells is largely speculative. However, certain similarities between these cells are apparent, as noted in the results. It is noteworthy that morphological similarity can be enhanced if the mice are treated with hydrocortisone before injection of the heparin-48/80 complex(9). The hormone treatment induces a rounding-up of the fibroblasts(6) and the quasi-mast cells produced from these cells are therefore without cytoplasmic processes.

Chemically, the similarity is limited to the presence of heparin in the granules of both types of cells(10), since the mast cell granule has been reported to contain also histamine (11), 5-OH tryptamine(12), chymotryptic activity(13), etc.(14,15), in contrast to the phenyl alkylamine content of the heparin-48/80 cell. However, granulated fibroblasts can also be induced by heparin complexes with other substances such as protamine (basic protein)(16), polymyxin B (basic polypeptide), or stilbamidine (diamidine)(9). In addition, numerous other metachromatic polysaccharides can be ingested by fibroblasts and give rise to cytoplasmic granules(9). It is of interest that injury to these cells can initiate a shedding of the granular material similar to mast cell degranulation(9).

From the results presented, it seems probable that metachromatically granulated cells may arise and persist for varying periods of time in the tissues as a result of fibroblastic uptake of material not readily digested by the cell. The presence or persistence of such material would be a function of the rates of cellular uptake and disposal which could be considered as relative to the metabolic state of the cell as well as to the "foreignness" of the ingested material. In this manner, tissue obtained from various pathological states may give the appearance of an increased concentration of "mast cells" largely as a result of present and past states of interaction between connective tissue cells and a noxious environment.

Summary. 1. Mice pretreated with various amounts of heparin exhibit resistance, relative to heparin dose, to the toxic effects of subsequent injections of otherwise lethal doses of 48/80. There is a straight-line relationship between the 50% protective heparin pretreatment doses and the various 48/80 challenge doses. 2. Fibroblasts exposed to the heparin-48/80 complex rapidly sequester and deposit this material as metachromatic granules in their cytoplasm. 3. The possible significance of these results is discussed.

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