inoculated into new-born mice. The specimen secured on the 7th day, however, was found to contain virus, all 10 of a litter dying or being sacrificed when showing obvious illness. Three of them were proven, on histologic examination, to have typical lesions of myocardium, liver and pancreas. A fourth was frozen, and after 10 days storage in the deep freeze, proved infective when injected intraperitoneally in 10^{-1} dilution into 0-day sucklings. All the animals died on the 3rd day. Three examined histologically proved to have characteristic pancreatitis and hepatitis.

This experiment proves that Connecticut No. 5 virus may be excreted in the urine of infected adult mice. The optimal period and concentration remain to be determined.

Exp. III. That the lesions produced by inoculation of urine from infected mice are due to the Conn. No. 5 virus and not to non-specific toxic substances is indicated by the fact that the addition of immune anti-Conn. No. 5 serum to the infected urine for one hour before inoculation confers at least partial protection.

Infected urine + Conn. #5 antiserum	.02 cc	3/8
" " + normal mouse serum	"	8/8
" " + anti-Ohio R serum	"	8/8

The 3 deaths in the first group occurred within the first 2 days after injection and were probably non-specific. Similar early deaths have occurred in mice inoculated with normal urine.

Conclusion. Infant mice infected with the Conn. No. 5 strain of Coxsackie virus eliminate virus in the urine. The infectious titer of urine obtained on the 2nd day after intraperitoneal inoculation is $10^{-5.6}$. Adult mice with pancreatic disease caused by Conn. No. 5 virus may also excrete virus in the urine.

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Production of Cytoplasmic Azurophilic Inclusions in Connective Tissue Cells by Suspending Agents.* (18796)

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During recent studies on the action of cortisone acetate (Cortone-Merck) on acute localized antigen produced inflammation in the mouse(1), it was observed that cytoplasm of fibroblasts of loose connective tissue of treated mice contained acidophilic inclusions (2,3). The purpose of this investigation was to obtain information on the origin and nature of the inclusions and on the mechanism underlying their formation.

TABLE I. Changes in Percentage Distribution of Fibroblastic Inclusions in Cells of Loose Connective Tissue of Intact Mice Produced by Cortone (Merck).

	No. of animals	Mean % of fibroblasts containing cytoplasmic inclusions and S. E. 4 hr	24 hr	48 hr	96 hr	6 days
1 mg Cortone s.c. in 2 cc suspending agent	20	28 ± 9.87	40.6 ± 6.28	36.6 ± 5.58	34.6 ± 4.59	26.5 ± 5.63
2 mg Cortone s.c. in 4 cc suspending agent	20	36.5 ± 7.15	52.5 ± 4.47	48 ± 4.87	42.6 ± 5.68	28.2 ± 4.65
1 mg crystalline cortisone acetate s.c. in .2 cc saline	20	0	0	0	0	0

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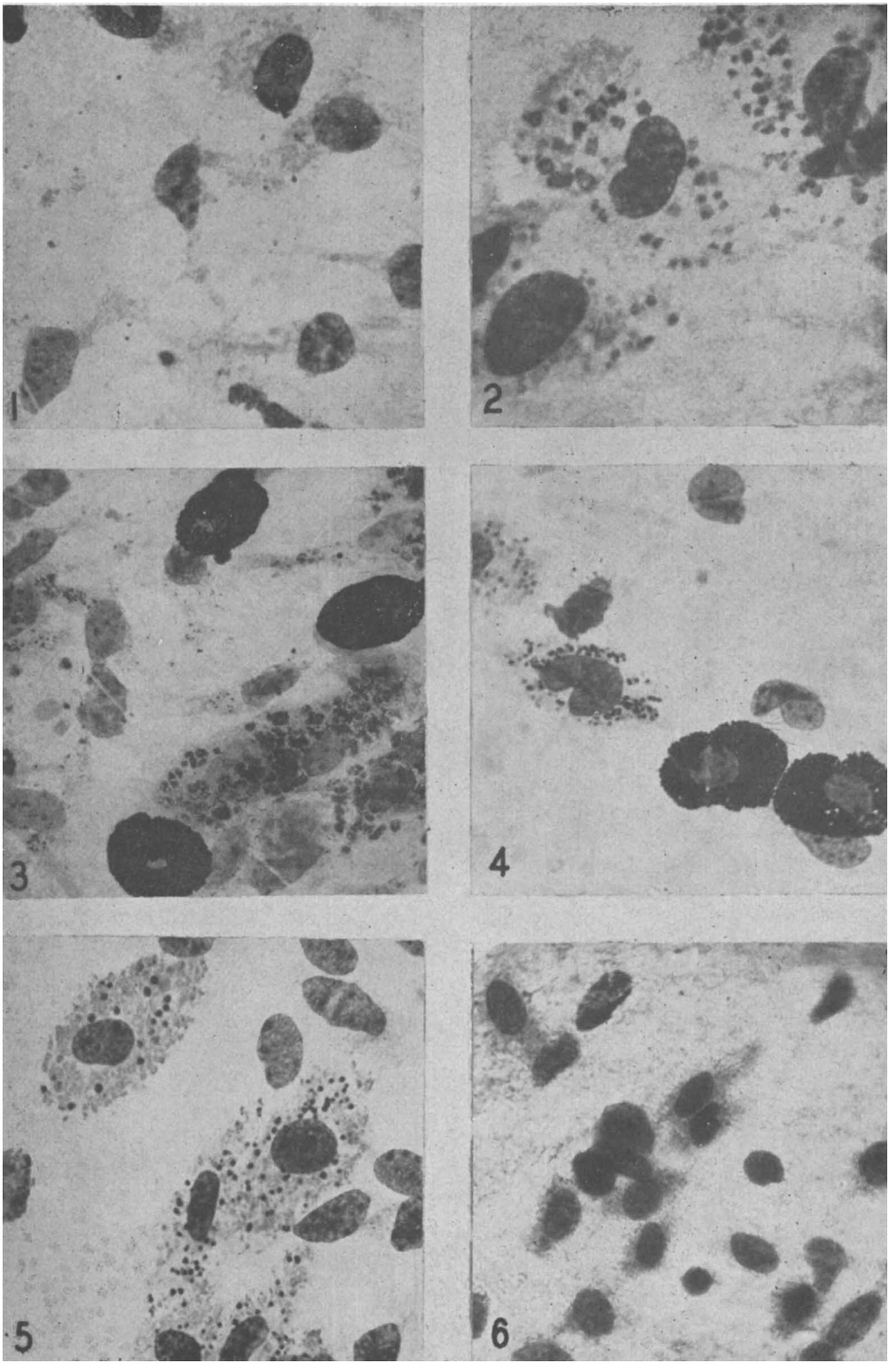
1
2
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Photomicrographs #1-6, Showing Spreads of Loose Connective Tissue of Non-sensitized Intact Animals. The magnification of Fig. 1, and 3-6 is ×720; Fig. 2 is ×830.

FIG. 1. Normal connective tissue.

FIG. 2. Connective tissue 4 hr after s.c. injection of 1 mg cortisone.

FIG. 3. Connective tissue 24 hr after s.c. injection of 1 mg cortisone.

FIG. 4. Connective tissue 48 hr after s.c. injection of 1 mg cortisone.

FIG. 5. Connective tissue 96 hr after s.c. injection of 1 mg cortisone.

FIG. 6. Connective tissue from the area of cortisone injection (4 hr after 1 mg).

Since Cortone is an aqueous suspension with stabilizing and suspending agents, the present work included a study of the role of both the hormone, and the suspending vehicles in respect to their capacity of inducing cellular inclusions.

Materials and methods. About 200 CBA mice of equal sex distribution, 16 weeks of age and of an average weight of 20 g were used. The animals were kept under optimal laboratory conditions with free access to food (dog chow supplemented with calf meal and vitamins) and drinking water.

The cellular inclusions were studied with the aid of the connective tissue spread method (1). The tissue sheets taken either from the site of injection or from other subcutaneous sites were expanded on slides to a thin cuticle, quickly air-dried and stained with May-Grünwald Giemsa. In some of the spreads, a differential cell count was performed. A total of 800 cells was counted in each of several spreads from the same animal, and the percentage of cells containing inclusions was determined for each cell type. The compounds employed for injection were Cortone (Merck) and the various components of the cortisone acetate suspending vehicle. Pectin, methylcellulose, polyvinyl alcohol and spinal cord lipids were other macromicellar materials studied for comparison with the components of cortisone suspending vehicle. The injections were made into the subcutis of the shaved abdomen if not otherwise indicated. The exact procedures employed are described below with the results.

Results. Cortisone acetate was injected subcutaneously in either 1 mg or 2 mg doses (Table I). Spreads were taken from the subcutaneous area of injection at 2, 4, 24, 48, 96 hours and 6 days after treatment. Single subcutaneous injections of either dose of Cortone were followed by accumulation of azurophilic cytoplasmic inclusions (Table I, Fig. 2 to 5), in fibroblasts of loose connective tissue in the injected area. The number of these inclusions varied from a few to 100 or more and their size averaged between 0.5 and 2 μ. There were marked variations in the shape of these inclusions within single cells (Fig. 2-5). The number of fibroblasts affected by 1 mg or 2 mg of Cortone was negligible 2 hours after injection, rose sharply in the subsequent 2 hours and moderately from the 4th to the 24th hour. At this time, the phenomenon reached its peak with the involvement of nearly 50% of the fibroblasts at the site of injection. After 24 hours the number of affected fibroblasts decreased, but so slowly that even after 6 days the incidence was still more than one-half of that found at 24 hours. Crystalline cortisone acetate (1 mg) suspended in saline did not produce the azurophilic inclusions at any of the periods studied. It did, however, damage fibroblasts which were in the area of deposition of the crystals. (Fig. 6).

The various components of the cortisone suspending agent were tested for their capacity to induce cytoplasmic inclusions. At least 5 animals were used in each group listed in Table II. Spreads of loose connective tissue were taken 6 hours after administration from the area of injection. The approximate number of cells containing cytoplasmic inclusions is indicated by pluses in Table II. As depicted in Table II, a cortisone acetate free solution containing all other components of Cortone (Components A, B, and C) was as effective in producing topical cellular inclusions as Cortone. On the other hand, no inclusions appeared after Component A (saline and benzyl alcohol) and only a comparatively small percentage of fibroblasts was affected when the injected solution contained "Tween 80" (Component B). This leaves carboxy-

TABLE II. Cellular Inclusions in Loose Connective Tissue of Intact Mice Produced by the Cortisone Acetate Suspending Agent and Other Colloidal Agents. (6 hr after s.c. injection.)

Cortisone acetate (in suspending agent) 1 mg in .2 cc	3+	Pectin .5% in .2 cc saline	3+
Crystalline cortisone (suspended in saline) 1 mg in .2 cc	—	Methylcellulose .5% in .2 cc saline	—
Cortisone suspending agent. Comps. A, B, and C,* .2 cc	3+	Polyvinyl alcohol .5% in .2 cc saline	—
Comp. A, .2 cc	—	Spinal cord lipids .5% in .2 cc saline	—
Comps. A and B, .2 cc	+	Saline sol., .2 cc	—
Comp. C, .2 cc	3+		

* Comp. A—saline sol. with 1.5% Benzyl alcohol; Comp. B—saline sol. with .4% Tween 80 (Polyoxyethylene sorbitan Monooleate); Comp. C—saline sol. with .5% sodium carboxymethyl cellulose.

TABLE III. Distribution of Fibroblast Inclusions in Mice Receiving .1 cc Cortone, Cortone Suspending Agent or Its Components. (Spreads taken 6 hr after injection.)

	s.c. injection					i.p. injection		
	Injection area		Incision area	General		Incision area	General	
Component tested	Intact	Adrex	Adrex	Intact	Adrex	Adrex	Intact	Adrex
Saline Sol.	0	0	0	0	0	0	0	0
Comp. A	0	0	0	0	0	0	0	0
A + B	+	+	0	0	0	0	0	0
C	3+	3+	+	0	0	0	0	0
A + B + C	3+	3+	+	0	0	0	0	0
Cortone + Comp. A + B + C	3+	3+	2+	+	+	2+	+	+

5 animals used in each experimental group. For constituents of components tested, see Table II. 1+ = 1% to 10%. 2+ = 10% to 40%. 3+ = more than 40%.

methylcellulose (Component C) as the major responsible factor. Carboxymethylcellulose differed from Cortone in that it produced a localized inflamed area in which azurophilic inclusions were found in macrophages as well as in fibroblasts. When carboxymethylcellulose was given along with cortisone acetate (Cortone) the local inflammatory reaction was inhibited and inclusions were confined to topical fibroblasts since macrophages were absent. Of the other macromicellar components tested, only Pectin was as effective in producing cellular inclusions (in fibroblasts as well as macrophages) as carboxymethylcellulose (Table II). No effect on connective tissue cells was observed in animals treated with methylcellulose, polyvinyl alcohol or spinal cord lipids.

In 120 mice, Cortone and the components of the cortisone suspending vehicle were tested for their capacity to produce cellular inclusions in areas remote from the site of injection. To exclude endogenous adrenal cortical secretion, which could be responsible for a possible generalized effect on the fibroblasts, 5 of 10 animals in each group were adrenalectomized prior to injection with the test component preparation. The components were injected subcutaneously or intraperitoneally into groups of 60 mice each. Connective tissue spreads were taken from various locations after 6 hours, as follows: (a) from the area of injection in subcutaneously injected mice; (b) from the incision area for adrenalectomy in adrenalectomized mice; and (c) from subcutaneous areas distant from the injection zone in the subcutaneously injected intact and adrenalectomized animals. In adrenalectomized subcutaneously injected mice, the cytological phenomena were qualitatively the same as in intact animals (Table III). The incidence of topical fibroblastic inclusions was high after subcutaneous injection of any solution containing carboxymethylcellulose, much lower when the solution contained only "Tween 80" and zero when it contained neither of these 2 agents. When carboxymethylcellulose was given with cortisone ace-

tate (Cortone), fibroblasts in sites other than the local injection area contained azurophilic inclusions.

The number of fibroblasts affected when Cortone was administered to the adrenalectomized animals was smaller than that seen in intact mice in spreads taken from distant areas of the subcutis. Intraperitoneal administration of Cortone also produced generalized fibroblastic inclusions. No generalized inclusions were found after "Tween 80" or carboxymethylcellulose administration. In Cortone injected mice the incidence of distant fibroblasts containing inclusions was lower after intraperitoneal than after subcutaneous injection.

Morphological characteristics of the inclusions. Irregularly shaped small dark blue inclusions appeared in the cytoplasm of fibroblasts about one-half hour after injection. Their number and size increased greatly during the following 3-4 hours with accompanying changes in coloration. After 4 hours, the inclusions were large azurophilic elements with jagged edges. They differed from intracellular granules, *i.e.* those in mast cells, by their variable size, shape, number and staining characteristics; from the inclusions characteristic of phagocytes by their homogeneity and shape and by the absence of a surrounding vacuole. During the first hours the affected fibroblasts maintained their normal shape (Fig. 3). However, later the inclusions increased in number and size and the cells tended to assume a more rounded shape. After 24 hours there was a slow decrease in the number and size of inclusions in affected cells (Fig. 5).

Preliminary observations indicated that the azurophilic inclusions were not modified mitochondria. Also, it was apparent that they were not composed entirely of lipid since they did not disappear when treated for prolonged periods with high concentrations of ethanol.

Comment and summary. The evidence presented indicates that various suspending agents produce characteristic changes in fibroblasts and macrophages of loose connective tissue. Similar alterations occurred in endothelial system. The azurophilic inclusions were so named because they stain with the azur component of the polychrome dyes used. The data indicate that administration of cortisone acetate in conjunction with the suspending agents tends to increase the number of affected fibroblasts in areas remote from the site of injection.

The number of azurophilic inclusions in each fibroblast and their persistence for many days following a single injection suggests that the function of this cell may be altered. This possibility should be taken into account in evaluating the action of Cortone on connective tissue reactions. Further, since the suspending agents also affect endothelial cells it is conceivable that they (as well as cortisone itself) could be responsible for modifying capillary permeability as described by Menkin(4). Also, such agents might modify the rate of repair following traumatic or chemical injury by altering the fibroplastic activity of fibroblasts.

The alterations in the affected cells did not appear to be typically degenerative since their nuclei were not adversely affected except in the very central portion of the site of injection. The azurophilic material did not appear to be phagocytized substance but rather altered cytoplasm.

Although the presence of azurophilic inclusions has not been described by other authors, intravenous injection of polyvinylpyrrolidon for prolonged periods produced alterations in reticulo endothelial cells of spleen, lymph nodes, liver, etc., which were similar to those found in lipoid storage diseases(5).

In view of the extent and duration of the cytological alterations described here and the widespread use of suspending agents, further investigation of their possible harmful tissue effects should be pursued.

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