LD_{50} of *Crotalus terrificus* or *Bothrops jararaca* venom. Russell and Emery(9) found methylprednisone and hydrocortisone did not suppress the lethal activity of *Ancistrodon contortrix* venom in mice with the possible exception that hydrocortisone in dosage of 100 mg/kg body weight afforded some protection. Ganatra and associates(10) stated that hydrocortisone in dosage of 2 mg/20 g body weight significantly reduced the mortality rate of mice injected with 2 LD_{50} of viper venom as compared to antivenin. These authors commented that the doses of hydrocortisone used in this experiment were very large. There are no previously reported experiments wherein the effect of hydrocortisone on experimental cobra envenomation has been studied.

The mechanism of the lethal effects of venoms and bacterial endotoxin appear in many respects to be similar(11). Lillehei and MacLean(12) found pretreatment with hydrocortisone in massive doses protected the majority of dogs tested against endotoxin in lethal dosage. In the experiments reported here, doses of hydrocortisone utilized were much greater than those usually employed clinically. Repetition of hydrocortisone administration whenever systemic blood pressure began to fall appeared necessary for survival of animals. In preliminary experiments not recorded here, when smaller amounts of cortisone or hydrocortisone were utilized, no apparent benefit was obtained and all animals died. As in the experiments with endotoxin (12), it appears that massive doses of the substance are necessary if animals are to survive.

Summary. Hydrocortisone in large doses reduces the lethality of *Naja naja* venom for dogs.

1. Hoback, W. W., Green, T. W., *JAMA,* 1953, v152, 236.
2. Wood, J. T., Hoback, W. W., Green, T. W., *Virginia Med. Monthly,* 1955, v82, 130.
3. Wig, K. L., Vaish, S. K., *Indian Med. J.,* 1960, v35, 307.
4. Gupta, P. S., *ibid.,* 1960, v35, 387.
5. Benyajati, C., Koeplung, M., Sribhibhadh, R., *Trop. Med. & Hyg.,* 1960, v63, 257.
6. Allam, M. W., Weiner, D., Lukens, F. D. W., *Publication 44, A.A.A.S., Science,* Washington, D. C., 1956, 393.
7. Deichman, W. B., Radoniski, J. L., Farrell, J. J., MacDonald, Wm. E., Keplinger, M. L., *Am. J. Med. Sci.,* 1958, v236, 204.
8. Schottler, W. H. A., *Antihistamine, A.C.T.H., Cortisone, Am. J. Trop. Med. & Hyg.,* 1954, v3, 1083.
9. Russell, F. E., Emery, J. A., *Am. J. Med. Sci.,* 1961, v241, 135.
10. Ganatra, R. D., Dhopeshwarkar, G. A., Sheth, V. K., Lewis, R. A., *Indian J. Med. Sci.,* 1957, v11, 493.
11. Spink, W. W., *Yale J. Biol. & Med.,* 1958, v30, 355.
12. Lillehei, R. C., MacLean, L. D., *Arch. Surg.,* 1959, v78, 464.

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Multiplication and Cytopathogenicity of Mouse Hepatitis Virus in Mouse Cell Cultures.* (26986)

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Since 1951, several investigators have reported isolation of 8 viral agents which produce a focal hepatic necrosis in mice(1-8). Spontaneous disease was induced by none with the exception of the virus of Gledhill and Andrewes(1). The remainder became manifest only in mice inoculated with tissue suspensions in attempts to pass other agents. A number of differences between certain of these agents have been demonstrated which suggest the group is heterogeneous. Never-

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theless, Morris and Aulisio(7) were unable to reveal antigenic differences between the 4 strains which they studied. Additional comparative researches are clearly needed to define the relationships between these viruses.

No convenient method whereby they can be studied *in vitro* has been available. Although the strain MHV_1 has been propagated in tissue culture(9,10), no cytopathic effect (CPE) was observed. For infectivity assay, therefore, even with this strain mice were still required. Miyazaki, *et al.*(11) propagated MHVB (Buescher) in mouse liver tissue culture for 94 passages, but were able to detect viral replication only by comparative cell counts of infected and control cultures. Bang and Warwick(12) alone have observed CPE by mouse hepatitis virus. These workers found that MHV(P) strain destroyed macrophages from liver tissue grown on a collagen substitute.

Investigations to be reported here on the behavior *in vitro* of the Balb C strain(8) of virus were initiated with the purpose of developing a more convenient procedure for viral and serological assay.

Methods and materials. Virus. Balb C strain of MHV was kindly supplied as a lyophilized suspension of infected mouse brain by Dr. John B. Nelson. Stock virus was prepared in this laboratory from mouse livers of the second intraperitoneal passage. Mice were sacrificed on the 4th day and livers ground in a chilled mortar. A 10% suspension in Hanks' balanced salt solution (BSS) was passed through a Seitz S3 filter, distributed in ampoules, quickly frozen, and stored in a dry-ice chest. During storage no loss in mouse infectivity was observed over a period of 10 months.

Mice. The Harvard strain of Swiss mice was used for all experiments. Spontaneous hepatitis has not been observed in these animals.

Mouse embryo tissue culture. Fourteen to 16-day old mouse embryos were dispersed as a cell suspension in 0.25% trypsin in Hanks' BSS at 37°C for 1 hour. After centrifugation they were resuspended in medium 199 with 2% inactivated (56°C) calf serum and dispensed in tubes. Infection was usually carried out on the 3rd day. Medium 199 with 1% inactivated calf serum was used for maintenance.

Mouse kidney tissue culture. Monolayers of kidney epithelium from newborn and adult mice were kindly supplied by Sumner Berkovich. These were prepared by a modification of the method of Youngner(13) for monkey kidney cell cultures and were grown in 0.5% lactalbumin hydrolysate in Hanks' BSS with 10% inactivated calf serum. The same medium with 5% calf serum was used for maintenance.

L cells. Most experiments were carried out using L cells supplied by Dr. R. S. Chang that were grown and maintained in Eagle's minimal essential medium with 10% horse serum. Results were confirmed with another lot of L cells supplied by Dr. Monroe D. Eaton. For titrations that included 3 or 5 cultures for each dilution ten-fold serial dilutions of virus were prepared. The volume of inoculum was 0.1 ml. Incubation was at 37°C in a roller drum. Cultures were observed daily for cytopathic effect.

MHV antibody. The method of Kasel *et al.*(14), as modified by Berkovich(15) for production of viral antibodies in mouse ascitic fluid was employed. Five adult Swiss mice were inoculated intraperitoneally with 0.1 ml of a 10^{-3} dilution of the brain suspension supplied by Dr. Nelson. One of the animals died. Ten days later the remainder were inoculated with undiluted virus. Five control mice were injected with Hanks' BSS at the same time. Seven days after the second injection approximately 10^7 Ehrlich's ascites tumor cells were introduced intraperitoneally in both groups. Ten days later the serosanguinous ascetic fluid was harvested, centrifuged and stored at −15°C as the antibody solution.

Results. Viral multiplication in mouse embryo tissue culture. Mouse embryo monolayer cultures (METC) were inoculated with 0.1 ml of MHV (Balb C) as a 10% liver suspension. Undiluted culture medium harvested on the 7th day, when introduced intraperitoneally in weanling mice was followed by death with gross hepatic damage. Serial propagation in METC was then undertaken, and carried through 15 passages. Death with liver damage of intraperitoneally inoculated

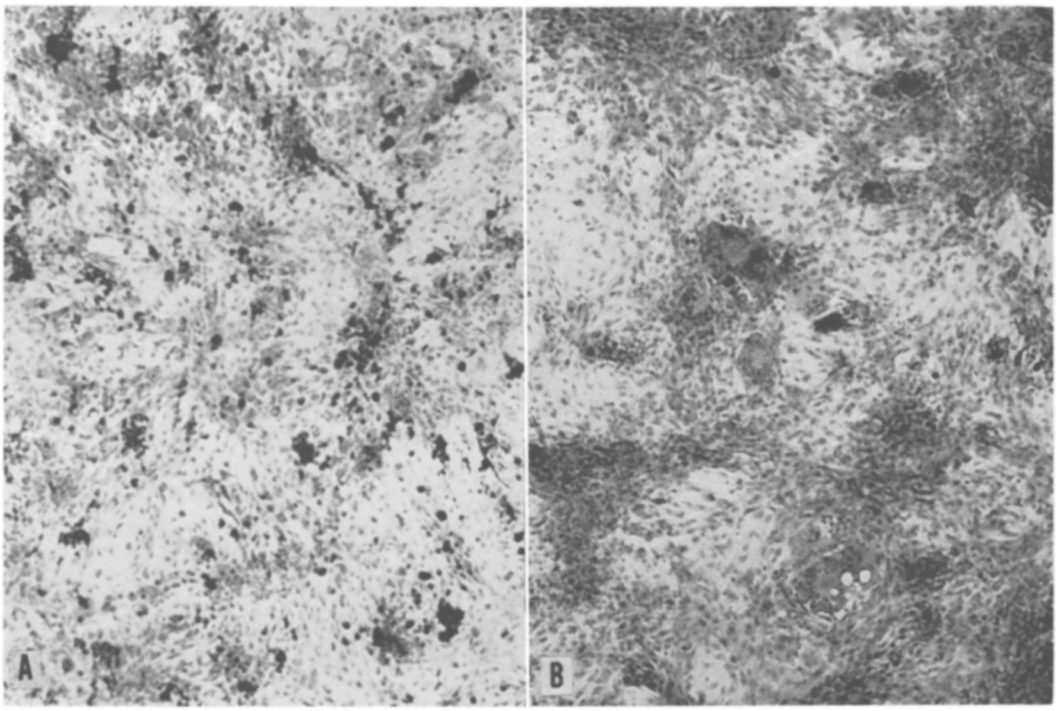


FIG. I. Changes in mouse embryo tissue culture infected with mouse hepatitis virus. Hematoxylin and eosin stain.
a. Uninfected METC. Magnification × 61.
b. METC 72 hrs after infection with MHV, 14th passage. Magnification × 61.

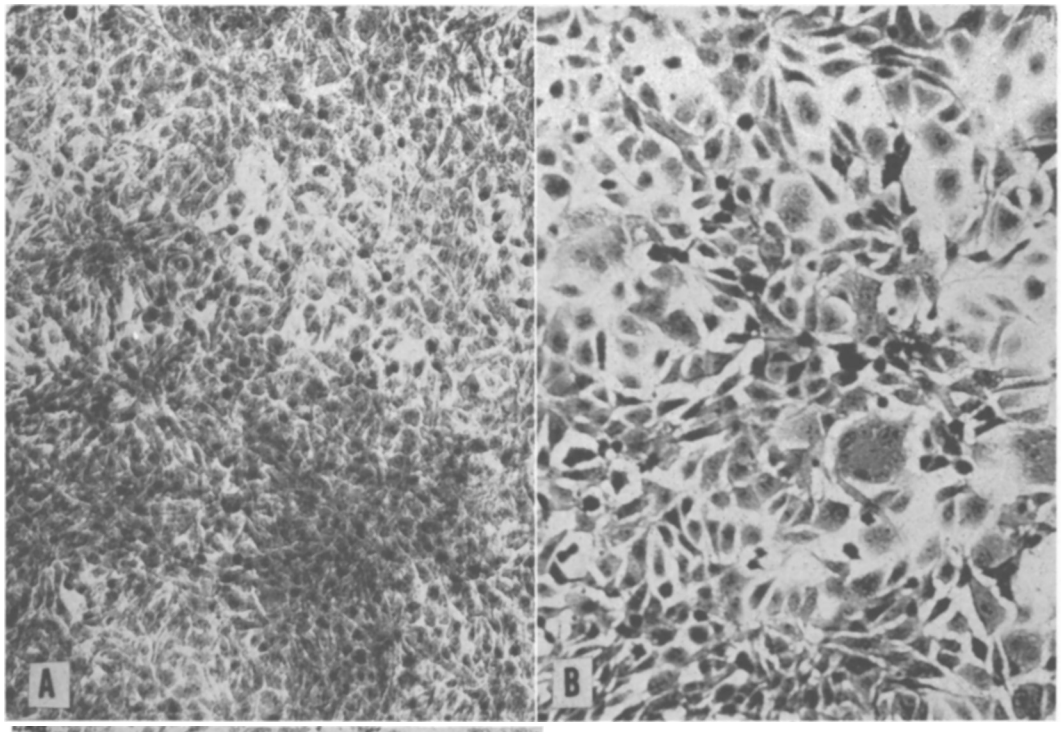
weanling mice was observed with medium harvested from the 3rd, 7th, 11th, and 15th tissue culture passages. Mouse infectivity titer of the liver suspension used to initiate the first passage was $10^{-2.5}LD_{50}$/ml. Titer of fluid from the 15th cell culture passage was $10^{-5}LD_{50}$/ml. Since the original inoculum in this passage had been diluted more than 10^{-20}, replication of the virus in tissue culture was demonstrated.

Histopathologic lesions in mice produced by the original virus and the 15th METC passage were identical and consisted of the same type of focal necrosis with scant cellular reaction.

To determine whether the agent present after 2 mouse and 15 METC passages was antigenically identical with that in the original seed a neutralization test using ascitic fluid from mice immunized with the seed virus was carried out. A 1:10 dilution of heated immune ascitic fluid neutralized $10^{3}LD_{50}$ of MHV while a similar dilution of heated ascitic fluid from control animals failed to neutralize $10^{2}LD_{50}$. It is concluded therefore that the agent propagated is antigenically similar to the Balb C strain.

Cytopathic effect in METC was not observed until the 7th passage when vacuolization, eosinophilic cytoplasmic inclusions, and multinucleate giant cell formation were noted. The extent of these changes in the several subsequent passages varied, but they were easily and consistently recognized following the 11th passage.

The cytopathic effect in METC of the agent in the 14th passage is illustrated in Fig. I. By 48 to 72 hours giant cells are present which exhibit a central area of homogenous, intensely eosinophilic masses surrounded by 5 to 100 nuclei within a cytoplasmic sheet which is frequently vacuolated. Many cells are subsequently lost from the sheet, but are partly replaced by secondary growth. These changes are not followed by complete destruction of the cell sheet which survives for approximately the same length of time as in uninoculated cultures. As incubation is con-



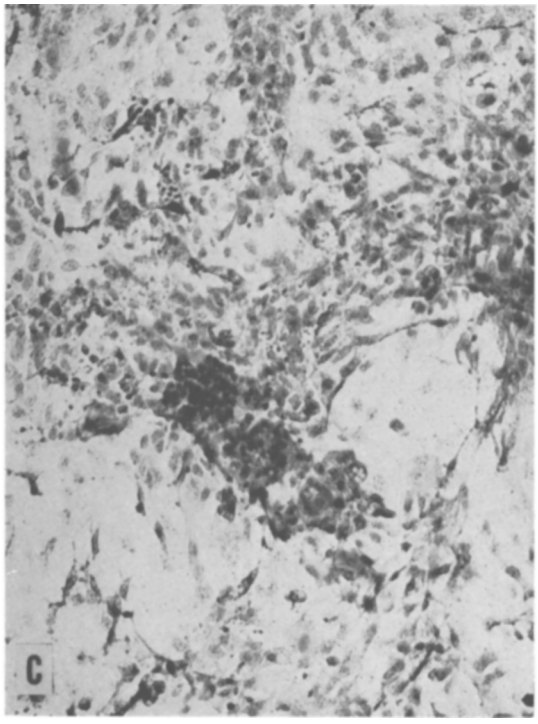


FIG. II. Sequential changes in L cell cultures infected with MHV (9METC and 6 L cell passages). Hematoxylin and eosin stain.

a. Uninoculated L cell culture. Magnification × 61.

b. L cell culture infected with MHV after 48 hours. × 153.

c. Infected L cell culture after 96 hrs. Giant cells have disappeared and regrowth of cell sheet has begun. × 61.

tinued giant cells become fewer and the cell sheet appears to be less extensively involved. Giant cells have been observed, however, as late as 28 days after infection.

Although vacuolation and giant cell formation may be discerned in unstained cultures, they do not alone provide reliable criteria for viral multiplication. Staining must be carried out in cultures employed for titrations of viral infectivity or neutralizing antibodies to detect CPE of minimal extent at the end point.

Hemadsorption tests were done with negative results using guinea pig and chick erythrocytes and infected cell cultures.

Viral multiplication in mouse kidney tissue cultures. Attempts to demonstrate MHV replication in kidney epithelial cells from newborn and adult mice following inoculation of 10% liver suspension were unsuccessful. In contrast inoculation of these systems with virus derived from a single passage in METC resulted in multiplication in mouse kidney epithelial cells with associated, although mini-

mal CPE, essentially similar to that in METC.

Viral multiplication in L cells. Addition of the virus in the form of 10% liver suspension or the fluid from a single passage in METC to cultures of L cells was followed by loss of the agent as determined by inoculation of weanling mice. Harvest from the third METC passage did survive or multiply in L cells. Following the 15th direct METC passage, the virus induced CPE upon first passage in L cells.

Fig. II illustrates sequentially the changes that occur in stained L cell cultures. Eosinophilic intracytoplasmic masses and multinuclear giant cell formation are present, as well as focal degeneration and cell depletion. Four to 6 days after infection giant cell formation becomes scanty and regrowth of the cell sheet begins. CPE is easily recognized in unstained L cell cultures (Fig. III). It provides a reliable criterion of viral activity in titrations of infectivity and in neutralization tests. The changes are maximal by the 5th day at the titration end point and by the 3rd day with a larger inoculum.

The 15th METC passage gave a titer of $10^{-5.2}ID_{50}$/ml in L cells as compared with a titer of $10^{-5.3}LD_{50}$/ml in suckling mice.

In Vitro neutralization. Neutralization of CPE in L cell cultures by mouse MHV immune ascitic fluid was demonstrated. Inactivated immune fluid prevented CPE in a dilution of 1:128. Higher dilutions were not tested. Unheated immune fluid exhibited the same neutralizing capacity. Control ascitic fluid inhibited CPE only in a dilution of 1:4.

Growth curves. To determine the characteristics of MHV multiplication in tissue culture growth curves were carried out in METC and L cell cultures. Virus carried through 9 METC and 6 L cell passages was used in these experiments. Results are summarized in Fig. IV. Maximal titer as determined in L cell cultures was obtained in METC on the 3rd day, but a constant production of virus was maintained over a period of 28 days. In L cells a chronic infection was also established although virus after an initial increase dropped for a time to an un-

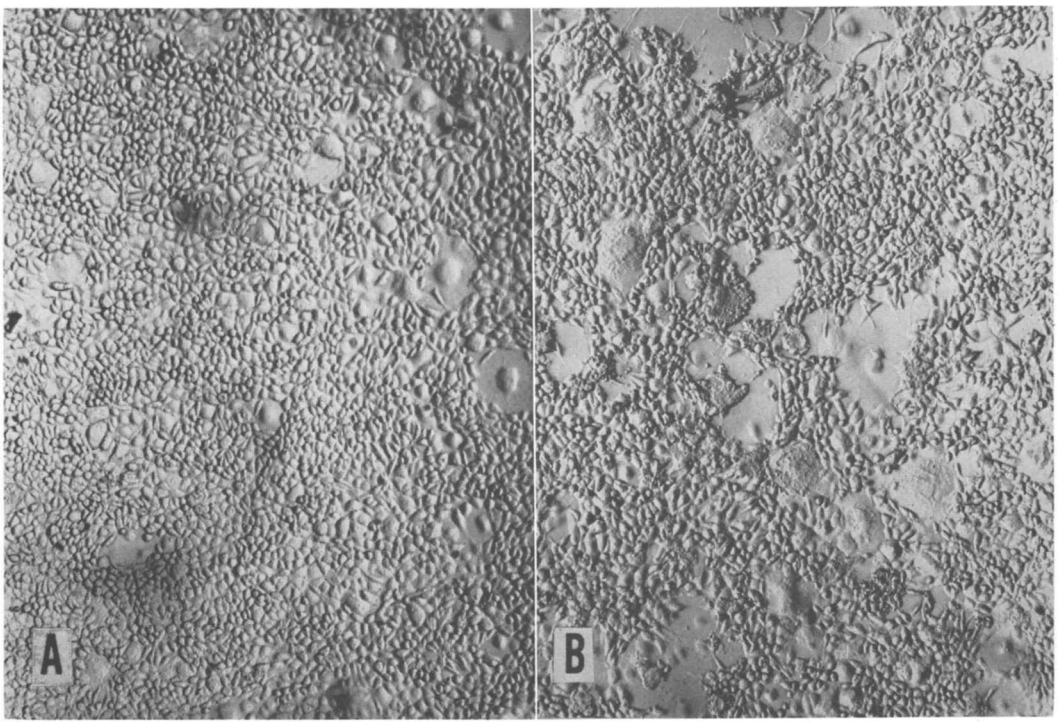


FIG. III. Unstained L cell cultures. Magnification × 61.
a. Uninfected.
b. 72 hrs after infection with MHV, 15 METC passage × 61.

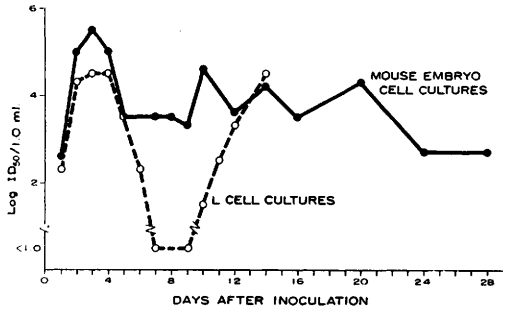


FIG. 4. Growth Curve of Mouse Hepatitis Virus in Mouse Embryo and L Cell Cultures as Determined by Assay in L Cell Cultures

detectable level only to reappear after the cell sheets had again grown out.

Discussion. Interest in mouse hepatitis viruses has derived largely from the activation of such latent agents in the attempt to transmit human hepatitis and leukemia. The discovery of these agents has also afforded a convenient model for laboratory study of problems relating to a viral hepatitis such as the effect of diet(16) and of the mechanism of transaminase elevation(17). The availability of a convenient *in vitro* method for detection of at least one member of the group should facilitate such studies.

It is of interest that a single passage of the virus under study in a susceptible cell system rendered it capable of multiplication in another system consisting of cells in which initially replication did not occur. Further investigation of this phenomenon seems desirable as the results may suggest procedures whereby adaptation of virus to an initially resistant cell can be accomplished.

Summary. The data presented indicate that MHV (Balb C) multiplies in mouse embryo cell cultures and, after passage in that system, in mouse kidney epithelium and L cells. In all these systems a cytopathic effect was observed after serial passage. These observations provide convenient techniques for studying *in vitro* this and perhaps other strains of mouse hepatitis virus.

1. Gledhill, W. W., Andrewes, C. H., *Brit. J. Exp. Path.*, 1951, v32, 559.

2. Jordan, J., Mirick, G. S., *Bull. Johns Hopk. Hosp.*, 1951, v89, 326.

3. Nelson, J. B., *J. Exp. Med.*, 1952, v96, 293.

4. Buescher, E. L., *Annual Historical Report of 406 General Med. Lab., U. S. Army*, 1952, 46.

5. Stanley, N. F., Dorman, D. C., Pansford, J., *Austral. J. Exp. Biol. Med. Sci.*, 1953, v31, 147.

6. Braunsteiner, H., Friend, C., *J. Exp. Med.*, 1954, v100, 665.

7. Morris, J. A., Aulisio, C. G., *Fed. Proc.*, 1954, v13, 506.

8. Nelson, J. B., *J. Exp. Med.*, 1955, v102, 581.

9. Gompels, A. E. H., Niven, J. S. F., *J. Path. Bact.*, 1953, v66, 567.

10. Starr, T. J., Pollard, M., PROC. SOC. EXP. BIOL. MED., 1959, v100, 97.

11. Miyazaki, Y., Katsuta, H., Aoyama, Y., Kawai, K., Takaoka, T., *Jap. J. Exp. Med.*, 1957, v27, 381.

12. Bang, F., Warwick, A., *Virol.*, 1959, v9, 715.

13. Youngner, J. S., *Monolayer Tissue Cultures*, PROC. SOC. EXP. BIOL. MED., 1954, v85, 202.

14. Kasel, J. A., Lieberman, R., Smith, H. A., *Virol*, 1959, v9, 702.

15. Berkovich, S. Unpublished data.

16. Ruebner, B., Bramhall, J. L., *Gastroent.*, 1960, v39, 335.

17. De. Ritis, F., Coltorti, M., Giusti, G., *Science*, 1956, v124, 32.

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Urinary Mucopolysaccharide Excretion in the Sex-Linked Form of the Hurler Syndrome.* (26987)

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Hurler's syndrome (lipochondrodystrophy, gargoylism) is an hereditary aberration of the metabolism of mucopolysaccharides in human

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