ML incidence was limited by the relatively mild induced anemia rather than by low proneness. However, in the high dose animals leukemia-proneness would be relatively low at this time. The declining ML incidence at doses above 170 r could then reflect the decreased probability that proneness present was of a sufficient level that the disease could be triggered, in spite of the increased severity of the anemia resulting from the higher x-ray doses used. Thus, the limiting factor in ML incidence at the higher dose range was the inadequacy of the leukemia-proneness.

In sum, x-rays (25 to 350 r) can render all rats leukemia-prone (*10*). Myelogenous leukemia develops only in the presence of an adequate triggering anemia. Threshold anemic severity for triggering is inversely related to leukemia-proneness. Proneness to leukemia varies according to dose and time after irradiation (up to at least 3 months). Anemia resulting from the removal of two-thirds of the blood volume was adequate to trigger ML in 100 percent of the rats exposed, from 50 to 350 r. Because the 5.7 percent ML incidence among the nonbled animals that received 25 r was probably only a fraction of the rats rendered leukemia-prone, the threshold x-ray dose for leukemogensis, that is, for leukemia-proneness, must be considerably lower than 25 r.

The applicability of this two-step model of leukemogenesis in other species remains to be established. However, the close resemblance of ML seen in rats (*11*, *12*) and the RF mouse (*13*, *14*) to the forms of the disease found in humans has been noted. In addition, the leukemogenic effect of ionizing radiation in both man and animals is well established (*15*).

Finally, should the present two-step leukemogenic mechanism prove valid for other species as well, two additional observations may be made. First, the relative persistence of the radiation-induced leukemia-proneness would suggest the possibility that the leukemogenic effect of ionizing radiation is additive. This would be of concern for those interested in radiological health standards, particularly since this study indicates that the leukemogenic threshold dose is probably below 25 r. Second, the number of leukemia-prone individuals in a given population must far exceed the number showing frank ML.

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3. Although several animals had white blood cell levels as high as 80,000 per cubic millimeter of blood, the majority had values ranging from 35,000 to 65,000, and several had values between 2,000 and 4,000. Thus, the majority of the animals had values similar in magnitude to that reported by Moloney *et al.* (*11*) in their transfer chloroleukemia study. The magnitude of leukocytosis in the rat would apear to be quite different from that in chronic ML in man [200,000 white blood cells per cubic millimeter is common—see W. Dameshek and F. Gunz, *Leukemia* (Grune & Stratton, New York, 1964)]. However, calculations show no real difference in granulocytic response. For example, the circulating white blood cells in man number approximately 8000 per cubic millimeter, of which 70 percent, or 5600, are granulocytes. In chronic ML, as many as 95 percent of the 200,000 circulating leukocytes may be granulocytes, an absolute value of 190,000. This would be a 32-fold increase over the normal level. On the other hand, the rat, a lymphoid animal, has a normal white blood cell count on the order of 10,000 per cubic millimeter, of which 25 percent, or 2500, are granulocytes. A white blood cell increase in this animal to 80,000 per cubic millimeter, of which 95 percent are granulocytes, would yield a total granulocytic count of 76,000 cells per cubic millimeter, a 29-fold increase. Thus, in chronic ML, both the human and rat show a similar level of granulocytosis.
4. Marrow imprints and smears were obtained from the femora at autopsy. Blood smears were studied at 2 to 6 months after bleeding, or, in the case of the nonbled animals, at 6 to 10 months after irradiation. All samples were stained with Wright's-Giemsa stains.
5. The green tumor was found in the lungs, liver, spleen, nasopharynx, marrow, and subcutaneous regions, and seeds of the tumor, 2 to 3 mm or less in length, were found in the retroperitoneal spaces. Smears, imprints, and sections of the seeds and marrow infiltrated with chloroma showed large, round cells having pale, vesicular, delicate nuclei.
6. Elevated basophil levels, and the presence of myeloblasts exhibiting Auer rods and polymorphonuclear neutrophilic granulocytes having the "pseudo-Pelger" type nuclear anomaly were not observed in previous models of ML rats (*11*). One case of a leukemic rat demonstrating a Pelger-Hüet nuclear anomaly has been reported [E. Hlavayova and F. Svec, *Acta Haematol.* **19**, 295 (1958)].
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10. That leukemia-proneness was not naturally present was shown when none of the bled unirradiated rats developed the disease. This would be consistent with the lack of any reported cases of spontaneously developed ML in this strain [W. C. Moloney *et al.* (*11*) and R. E. Zipf, L. Chiles, N. Miller, B. J. Katchman, *J. Nat. Cancer Inst.* **22**, 669 (1958)]. In contrast with this, the untreated RF mouse is known to develop ML spontaneously, especially during advanced age [A. C. Upton, F. F. Wolff, J. Furth, A. W. Kimball, *Cancer Res.* **18**, 842 (1958)]. It is possible that this mouse strain is naturally leukemia-prone, and the incidence of ML is a reflection of the risk of the animal becoming sufficiently anemic to trigger the disease, the probability of an anemic episode in a given animal being expected to increase with age.
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26 July 1971

## Silkworm Bombyx mori L.: Nature of Diapause Factor

Abstract. *The diapause factor which is responsible for the induction of embryonic diapause in the silkworm* (Bombyx mori *L.*) *was isolated from the heads of female moths. It was extracted with 80 percent ethanol or 80 percent methanol and was heat-stable and not dialyzable. Since the active material was precipitated with ammonium sulfate and lost when incubated with proteolytic enzymes, it is highly probable that the diapause factor is a protein or a complex molecule containing peptide bonds. Molecular sieve techniques revealed two species of the diapause factor. The molecular weight of the smaller species lies between 5,000 and 10,000.*

"Diapause factor" (*1*) or "diapause hormone" (*2*), which originates in the subesophageal ganglion, determines embryonic diapause in the silkworm (*Bombyx mori* L.). The factor is released from the ganglion and exerts its effect on the ovary during the pupal stage of the female; diapause then occurs about 24 hours after the eggs have been laid.

In 1957, Hasegawa (*3*) reported the extraction of "diapause hormone" from the complex of brain and subesophageal ganglion in silkworm pupae. He extracted the material with 80 percent methanol, concentrated the extract, washed the concentrate with ether, and finally extracted the active material from the aqueous phase with chloroform. He did not, however, comment on the chemical nature of the factor. In the abstract of their paper on the mode of action of the diapause hormone, Hasegawa and Yamashita (*4*) mentioned that this factor might be "a kind of lipid." No further report on the chemical properties of the factor has appeared.

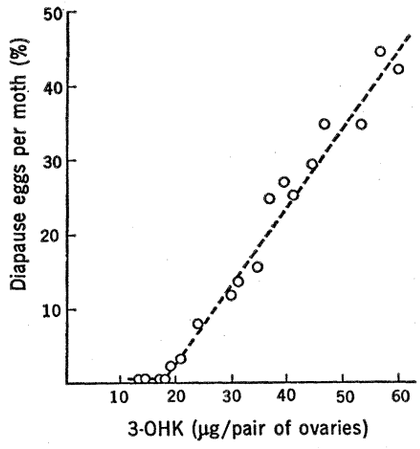


Fig. 1. Relationship between the accumulation of 3-OHK in moth ovaries and the percentage of diapause eggs laid.

Numerous investigations concerning the neurosecretory hormones of invertebrates have revealed the proteinaceous nature of many neurosecretory substances; these include, for example, "bursicon" in the pars intercerebralis of the brain and the compound ganglion in the thorax of the fly (*5*) and in the terminal abdominal ganglion in the cockroach (*6*), the hormones of the corpus cardiacum in the cockroach which induce hyperglycemia and accelerate the heartbeat (*7*), brain hormone in the silkworm (*8*), and pigment-effector hormones (*9*) and hyperglycemic hormone (*10*) in the crustacean eyestalk. With these facts in mind we have reexamined the diapause factor and now report on the chemical nature of the principle extracted from adult silkworm heads.

Heads (2000 to 4000) from female moths were used as the source of the diapause factor because large numbers of moths are easily obtained from sericultural companies (*11*). The adult head contains a complex of brain and subesophageal ganglion and is the source of the same active principle as is present in the pupal subesophageal ganglion (*12*). The heads were isolated from live moths and were stored in a deep freezer at 20°C until extracted.

For the bioassay of the diapause factor, use was made of the fact that the accumulation of 3-hydroxykynurenine (3-OHK) in the ovary is under the control of this factor. The amount of 3-OHK increases in the ovaries only from day 3 to day 6 after pupation, and the rate of accumulation is strongly accelerated by the diapause factor (*13*). Therefore, day-4 pupae, conditioned to lay nondiapausing eggs, were injected with the samples containing the diapause factor; the pupae were killed after 24 hours for estimation of the amount of 3-OHK. The quantity of 3-OHK was determined by procedures already described (*13*). We routinely used a group of 10 to 15 pupae for a given concentration of the sample. We allowed some of the injected pupae to develop into adults and to lay eggs so that we might observe the presence or absence of diapause in them. Moths emerging from pupae which had been injected with diapause factor laid diapause eggs (brown) mixed with nondiapause eggs (light yellow) and "semidiapause" eggs (light brown). The larvae hatched from semidiapause eggs later than normal controls, and the embryos often failed to complete normal development. Fukuda and Kohno (*14*) suggested that these semidiapause eggs were derived from ovaries which had matured under the influence of insufficient amounts of the diapause factor.

Table 1. Thermostability of the diapause factor at three different hydrogen ion concentrations. Control animals were injected with distilled water. Unheated samples are indicated by incubation times of 0.

| Incubation time (min) | Temperature (°C) | 3-OHK (micrograms per pair of ovaries) |
|---|---|---|
| | *pH 2.0* | |
| 0 | | 38.7 |
| 30 | 100 | 39.1 |
| 90 | 100 | 42.5 |
| 360 | 45 | 41.2 |
| | *pH 7.6* | |
| 0 | | 38.2 |
| 30 | 100 | 41.1 |
| 90 | 100 | 41.9 |
| 360 | 45 | 40.5 |
| | *pH 10.2* | |
| 0 | | 40.7 |
| 30 | 100 | 45.2 |
| 90 | 100 | 41.2 |
| 360 | 45 | 41.6 |
| Control | | 15.5 |

In our experiments, the semidiapause eggs which hatched later than the control eggs (nondiapause eggs) were scored as 50 percent. To show the relationship between the accumulation of 3-OHK and the percentage of diapause eggs, we injected pupae with serially diluted diapause factor. The percentage of the diapause eggs increased almost linearly with the increasing concentration of the diapause factor (Fig. 1). Although the factor causing the accumulation of 3-OHK may not be identical with that responsible for the determination of diapause, a close parallelism between these two phenomena was observed. Therefore, the activity of the diapause factor can practically be estimated by measuring the content of 3-OHK in the ovaries.

To determine the best extractant for

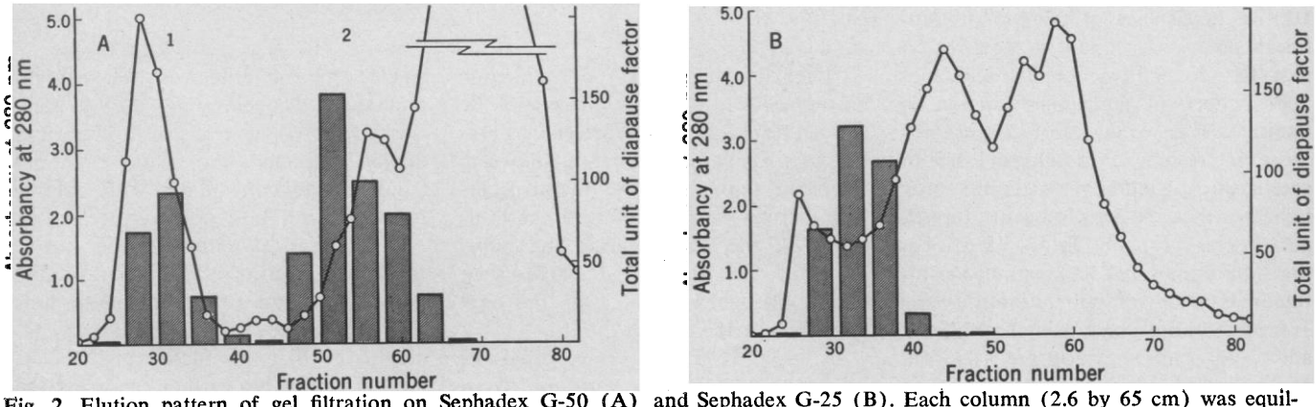


Fig. 2. Elution pattern of gel filtration on Sephadex G-50 (A) and Sephadex G-25 (B). Each column (2.6 by 65 cm) was equilibrated with distilled water in the cold room. The flow rate was 46 ml/hour; the fraction size was 5 ml. Contents of four tubes were combined and used for bioassay of the principle.

the principle, female moth heads were extracted with chloroform; petroleum ether; acetone; a mixture of choloroform and methanol (2 : 1 by volume), which is the extractant used for the isolation of total lipid from animal tissues (*15*); distilled water; 40 percent ethanol; 80 percent ethanol; or 80 percent methanol. The extracts thus obtained were dried in a vacuum. The lipid residue remaining in the evaporation flasks was removed by centrifugation and discarded because it showed no appreciable activity. The yellow oily materials prepared by extraction with chloroform, petroleum ether, acetone, or the mixture of chloroform and methanol were dissolved in sesame oil, whereas the extracts with water, 40 percent ethanol, 80 percent ethanol, or 80 percent methanol were dissolved in distilled water so as to contain the yield from 400 heads per milliliter. Little or no active principle as evidenced by accumulation of 3-OHK was extracted with choloroform, ether, acetone, or the mixture of chloroform and methanol. These extracts showed no appreciable effect in producing diapause eggs. Extracts in water and 40 percent ethanol had some activity; extracts in 80 percent ethanol or 80 percent methanol had the highest activity. Extraction with 80 percent ethanol or with 80 percent methanol yielded 25.5 or 27.0 percent of diapause eggs, respectively, and 40.4 or 38.9 $\mu$g of 3-OHK per pair of ovaries, respectively. Therefore, 80 percent ethanol or 80 percent methanol was used for extraction in later experiments.

Stability with respect to heat at three different hydrogen ion concentrations was examined. Methanolic extract of the diapause factor was dried in a vacuum and dissolved in buffer solution at *p*H 10.2 (0.05*M* glycine-NaOH), *p*H 7.6 (0.05*M* tris-HCl), or *p*H 2.0 (0.05*M* glycine-HCl) and heated at 100°C for 30 or 90 minutes or at 45°C for 360 minutes. The activity was not reduced by the treatments (Table 1).

Next, crude extract of the diapause factor was subjected to fractional precipitation with ammonium sulfate. The precipitate at every step was dialyzed for 24 hours against distilled water. The active principle was recovered in the precipitate produced by ammonium sulfate at saturations of 50 to 100 percent.

Gel filtration was performed on Sephadex G-50 and G-25. In the bioassay, one unit was defined as the amount of the factor causing a twofold accumulation of 3-OHK relative to that in control animals (water injection). The samples were diluted to the concentration which, when injected, could accelerate the accumulation of 3-OHK in the ovaries two- to fourfold relative to controls. Two peaks with diapause factor activity were observed in the elution profile of Sephadex G-50 chromatography (Fig. 2A): one peak was in a high-molecular-weight fraction (peak 1) which was excluded completely from the gel; the other one was in the low-molecular-weight fraction (peak 2). In the bioassay of peak 2, the responses of 3-OHK accumulation showed almost a linear relation to the concentration of the sample (Fig. 3). The peak 2 obtained from Sephadex G-50 was further applied to the Sephadex G-25 column (Fig. 2B). The diapause factor activity was excluded from Sephadex G-25, suggesting that the molecular weight of the principle would be above 5,000. On the other hand, the retention on the Sephadex G-50 column shows a molecular weight under 10,000. Therefore, the molecular weight of the diapause factor lies between 5,000 and 10,000. The fact that peak 1 from Sephadex G-50 filtration was excluded even from Sepharose 6B suggests quite a large molecular size.

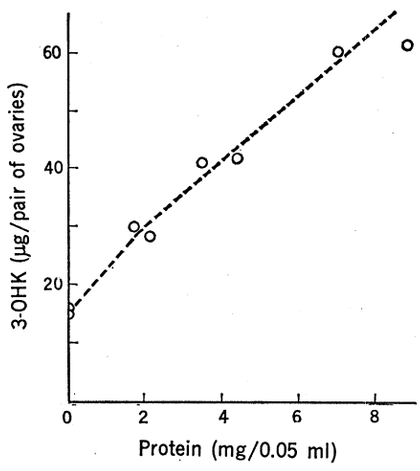


Fig. 3. Relationship between the accumulation of 3-OHK in the ovaries and protein concentration of peak 2 from Sephadex G-50 column. Protein was determined by the method of Lowry *et al.* (*18*) with bovine serum albumin used as a standard. Each pupa received routinely 0.05 ml of the test solution.

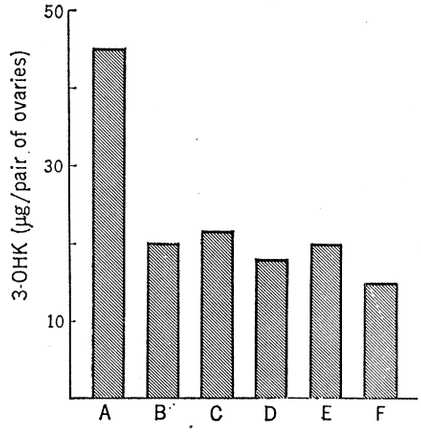


Fig. 4. Inactivation of the diapause factor by proteolytic enzymes: *A*, buffer only; *B*, pronase; *C*, nagarse; *D*, trypsin; *E*, $\alpha$-chymotrypsin; and *F*, control (water injection). Details of the treatments are given in the text.

The active fraction obtained from gel filtration of Sephadex G-25 followed by filtration on G-50 was lyophilized and was then dissolved in 0.1*M* tris-HCl buffer (*p*H 7.6) containing 0.01*M* $CaCl_2$. The solution was incubated with four proteolytic enzymes, namely pronase (streptomyces proteinase, Kaken Kagaku), nagarse (crystalline subtilisin, Nagase Sangyo), trypsin (crystallized twice, Worthington Biochemical), and $\alpha$-chymotrypsin (crystallized three times, Worthington Biochemical), the final concentration of the enzymes being 0.025 percent. After incubation at 37°C for 6 hours, the tubes were heated at 100°C for 30 minutes to inactivate these enzymes (insensitivity of the diapause factor to heat had already been indicated); the tubes were centrifuged, and the supernatant was used for bioassay. The diapause factor was inactivated almost completely by all proteolytic enzymes (Fig. 4).

Although the high stability of the diapause factor in heat and in extreme hydrogen ion concentration is inconsistent with the nature of ordinary proteins, the characteristics of the diapause factor nevertheless suggested that the molecule has peptide bonds, and the molecular weight is in a range of 5,000 to 10,000. Since some proteins, such as gonadotropic hormones (*16*) and ribonuclease (*17*), exhibit quite unusual thermostability, the possibility of proteinaceous nature cannot be excluded. The active principle which exhibited the higher molecular weight in the Sephadex G-50 column may either be considered to be the aggregated state or a complex consisting of the lower-molecular-weight species and a high-molecular-weight substance or substances.

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19. We thank Dr. S. Fukuda for valuable suggestions, Dr. C. D. Turner for his help in the preparation of the manuscript, and K. Soma for his assistance in rearing the silkworms.


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22 June 1971


# Neuronal $GM_1$ Gangliosidosis in a Siamese Cat with β-Galactosidase Deficiency


Abstract. *A juvenile Siamese cat with severe, progressive motor disability was shown to have extensive neuronal degeneration caused by accumulation of $GM_1$ ganglioside. Tissues from brain and kidney were markedly deficient in β-galactosidase activity. The disease in this cat is thought to be inherited as an autosomal recessive trait, and is strikingly similar to juvenile $GM_1$ gangliosidosis of children.*


Inherited defects of sphingolipid metabolism cause devastating diseases in man. At least five such diseases are due to abnormalities in ganglioside metabolism, in which specific lysosomal hydrolases appear to be deficient or absent. Remarkable progress in unraveling these complex diseases has been made by the study of human patients (*1*), despite its limitations. However, suitable animal models are badly needed for intensive study of pathogenetic mechanisms and potential curative measures. Diseases analogous to the human gangliosidoses with respect to histology have been described for a few animal species (*2*), but the underlying biochemical defects in these diseases have not been well characterized. In this report we describe preliminary clinical, pathological, genetic, and biochemical studies of a disease in Siamese cats which shows striking similarities to juvenile $GM_1$ gangliosidosis (*3*) of children.

Table 1. Gangliosides in brain. Values are expressed as micrograms of NANA per gram wet weight of tissue.

| Total ganglio-sides | Distribution of gangliosides | | | |
|---|---|---|---|---|
| | $GM_1$ | $GD_{1A}$ | $GD_{1B}$ | $GT_1$ |
| | *Diseased cat* | | | |
| 2265 | 1411.1 | 231.0 | 151.8 | 188.0 |
| | *Control cat* | | | |
| 946 | 170.3 | 350.0 | 104.1 | 141.9 |

Table 2. Enzyme activity in brain and kidney. Activities are expressed as nanomoles of substrate cleaved per milligram protein per hour.

| Source | β-Galactosidase | Arylsulfatase-A |
|---|---|---|
| | *Diseased cat* | |
| Brain | 38 | 110 |
| Kidney | 20 | 223 |
| | *Control cat* | |
| Brain | 256 | 95 |
| Kidney | 105 | 183 |

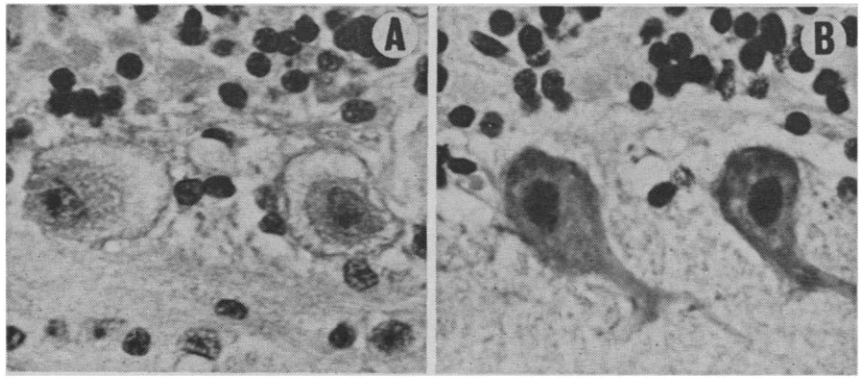


Fig. 1. Purkinje cells of diseased cat (A) and normal sibling (B). Sections of cerebellum are stained with hematoxylin-eosin (× 500).

A male Siamese cat developed normally until the age of 4 months, when he showed weakness and incoordination of his hind legs. General ataxia appeared and progressed until the animal was totally incapacitated at the age of 6 months when he was killed humanely. No gross lesions were found at necropsy. Histopathological examination of sections stained with hematoxylin-eosin revealed that neurons throughout the central nervous system, as well as those in sympathetic ganglia and retina, were enlarged and rounded, and had lost Nissl bodies from the cytoplasm (Fig. 1). Margination and chromatolysis of neuronal nuclei were frequent. In frozen sections the cytoplasm of neurons stained intensely with the periodic acid Schiff stain but weakly with oil red O and Sudan black B stains. Increased numbers of glial elements were evident throughout the brain, and there was occasional neuronophagia. Lesions outside the nervous system were limited to the spleen, where a few macrophages had foamy, vacuolated cytoplasm.

Gangliosides were extracted from brain by the method of Suzuki (*4*). The total amount of ganglioside extracted from brain was determined by measuring the *N*-acetylneuraminic acid (NANA) content, with the use of the resorcinol method as modified by Mietinen and Takki-Luukkainen (*5*). Individual gangliosides were separated by ascending thin-layer chromatography with a propanol : water (73 : 27 by volume) solvent system. After 6 hours of migration, gangliosides were located with resorcinol reagent, and the NANA content of each fraction was measured (*4*). Recovery of NANA from gangliosides exceeded 95 percent. Brain tissue from a normal Siamese cat, 6 months