the positive spleen titers were seldom greater than 1 : 2.

We have further evidence that type C virus was responsible for these positive CF reactions. We found numerous type C virus particles by electron microscopy in the spinal cord, spleen, and skeletal musculature of one experimentally paralyzed wild mouse and in the spinal cord or skeletal musculature of four experimentally paralyzed (P1) NIH Swiss mice. We demonstrated by the XC plaque test (*6*) that type C virus from the brain, spinal cord, or skeletal musculature of two paralyzed wild mice, and from the brain, spinal cord, or spleen of seven paralyzed (P1 and P2) NIH Swiss mice was infectious for NIH Swiss embryo cells. Infectious virus could not be demonstrated by the XC plaque test in the brain or cord extracts of five NIH Swiss mice, 7 weeks old, that had been inoculated intracerebrally at age 3 weeks or in ten uninoculated NIH Swiss mice which were 6 to 8 weeks old.

The main neuropathologic findings were gliosis, a nonspecific vacuolar neuronal degeneration, spongiosis (edema), and neurogenic atrophy of the hind leg musculature. These changes were especially marked in the anterior horns and adjacent white matter of the lower spinal cord. Although spongiosis and slight perivascular lymphoid cuffings were occasionally noted in the brainstem, inflammatory infiltrates, neuronophagia, and necrosis were conspicuously absent throughout the central nervous system. No obvious demyelination was noted in the brain, cord, or sciatic nerves.

Numerous type C virus particles were seen by electron microscopy in the lower spinal cord, budding from plasma membranes, and free in the extracellular space. An unusual feature was the finding of intracellular type C particles, sometimes in great number, replicating within the rough endoplasmic reticulum of anterior horn motor neurons. These intracellular replicating particles showed an aberrant cylindrical budding configuration. Virus particles were not identified within axons. In the sciatic nerves a few virus particles were noted in juxtaposition to the surface of normal appearing Schwann cells, but were not replicating at this site. Numerous type C particles were also found in the interstitium of the musculature of the paralyzed hind limb but virus was not seen deep within muscle cells and there was no evidence of virus replication or accompanying fiber degeneration. The same findings characterize the naturally paralyzed wild mice (*4*).

We have found that young adult wild mice obtained from the same trapping area as was the 1504E mouse embryo show a high incidence (about 70 percent) of gs antigen in the spleen detectable by CF. We observed an identical type of spontaneous lower limb paralysis in a wild mouse from this colony and we have now transmitted this lower limb paralysis to newborn NIH Swiss mice. The paralysis was observed 4 to 5 months after the newborn NIH Swiss mice were inoculated intracerebrally with brain and cord extracts from this paralyzed mouse. Thus, this colony of wild mice is similar to the colony of wild mice which was previously reported to be prone to lymphoma (*4*, *5*). Both are "switched-on" for type C virus activity and have the tendency to develop spontaneous lower limb paralysis.

The pathogenesis of the experimentally induced and naturally occurring neurologic disorders requires further definition. Based upon the morphologic, fine structural, and virologic findings, it appears that, although the type C virus is widespread in tissues of these paralyzed mice, it exerts its damaging influence primarily by a direct neurotropic effect on the anterior horn neurons of the lower spinal cord. The extensive replication of type C virus particles within the endoplasmic reticulum of some of these neurons, an atypical site for type C virus replication, could lead to disturbed neuronal function and eventual cell death.

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24 April 1973; revised 21 May 1973

## Growth Retardation in Young Mice Treated with dl-Methadone

Abstract. *Newborn mice injected daily for 6 weeks with* dl-*methadone in dosages of 2 to 8 milligrams per kilogram grew significantly more slowly than their saline-treated littermates. Litters given* d-*methadone, 4 milligrams per kilogram, grew normally. Concomitant treatment with naloxone, 10 milligrams per kilogram, prevented growth inhibition. A weight deficit persisted in mice observed 6 weeks after cessation of methadone treatment.*

Infants born of heroin-addicted mothers often are of low birth weight despite approximately full-term gestation (*1*). The cause for this immaturity is unknown.

Morphine and the pharmacologically related opioids can block the release of acetylcholine (ACh) from stimulated nervous tissues (*2*). Because of this property we wondered whether opioids such as methadone or levorphanol would affect limb regeneration rates and taste bud morphology in the salamander as did the inhibitors of ACh biosynthesis, hemicholinium or triethylcholine (*3*). We found virtually no limb growth occurring at the amputation site despite formation of a thick epidermal cap (*4*). Blastemal development which normally precedes differentiation appeared blocked for the entire 10-week treatment period. Opioid treatment also caused moderate taste bud atrophy. When daily treatment with the opioid was discontinued, growth rates rapidly increased and 5 weeks

Table 1. Weight of mice at 4 weeks of age after daily treatment with methadone or naloxone. Control littermates were treated with saline. Weights (in grams) are listed as means ± S.E.M. Numbers in parentheses are the number of mice in each group.

| Treatment | Dosage (mg/kg) | Treated | Control |
|---|---|---|---|
| *dl*-Methadone | 1 | 13.0 ± 0.58 (14) | 13.8 ± 0.40 (20) |
| *dl*-Methadone | 2 | 10.4 ± 0.59 (21)* | 13.3 ± 0.58 (27) |
| *dl*-Methadone | 4 | 7.7 ± 0.49 (13)* | 11.4 ± 0.59 (11) |
| *dl*-Methadone | 8 | 7.4 ± 0.56 (14)* | 12.8 ± 0.81 (16) |
| Naloxone | 10 | 14.8 ± 0.55 (15) | 15.2 ± 0.51 (12) |
| Naloxone plus *dl*-methadone | 10<br>2 | 12.4 ± 0.50 (15) | 12.3 ± 0.69 (13) |
| *d*-Methadone | 4 | 12.4 ± 0.68 (16) | 11.6 ± 0.60 (14) |

* $P < .01$.

later phalanges appeared, the normal interval for this differentiation to follow amputation. Evidently the growth inhibition was reversible. These results prompted an extension of the study to include the effect of methadone on growth of mammals.

For these studies we chose newborn mice, since they are not only quite fetal at birth (lacking taste buds, body hair, or functional eyes) but they can be injected directly, thus avoiding the placental barrier.

Young adult male and female Swiss-Webster mice were bred and the pregnant females were then isolated. From 8 to 16 pups were produced in each litter. Half of each litter were injected daily from the second day postpartum with a single dose of methadone, 1 to 8 mg/kg. The remaining half were injected with normal saline. The injections were made subcutaneously in a volume of 50 $\mu$l with a 30-gauge needle mounted on a microsyringe. The dosages of 4 or 8 mg/kg narcotized the newborn mice for nearly 1 hour, whereas the lesser amounts produced neither narcosis nor excitement. After the age of 14 days, these higher dosages of methadone only excited the mice and concomitantly elicited the Straub tail reflex. In order to minimize the killing of narcotized or excited pups, the mother was not returned to the cage until each animal in the litter appeared recovered.

Mice injected daily with methadone at 2 mg/kg showed a relative failure to gain weight during the first week of treatment. In later weeks the failure to gain weight by the group treated with methadone became more pronounced. These findings (Fig. 1) represent growth rates of mice from several litters ($N = 21$) injected with saline and experimentals from the same litters ($N = 16$) treated daily with methadone in a dosage of 2 mg/kg. The control and methadone-treated groups contained an equal distribution of male and female mice. This is of some importance, since male mice grow more rapidly than do females after the age of 4 weeks. Because some of the females became pregnant when methadone was discontinued at the end of 6 weeks, only male mice were observed for the next 6 weeks. It was noted that the weights (28.9 ± 1.86 g) of 6 males previously treated with methadone remained subnormal as compared to 11 saline-treated controls (33.9 ± 0.84 g) ($P < .01$). None of the mice exhibited signs of abstinence such as weight loss or "wet-dog shakes" when methadone was withdrawn.

Growth was not retarded in mice treated daily with methadone in a dosage of 1 mg/kg, whereas mice injected with 4 or 8 mg/kg grew less rapidly than the groups given 2 mg/kg. The decrease in weight gain appears related to the log-dose of methadone. However, mice treated concomitantly with naloxone in a daily dosage of 10 mg/kg grew normally. Naloxone, the potent antagonist which lacks agonistic activity, did not, at the dosage given, slow growth rates. Moreover, growth was not retarded by daily injection of *d*-methadone, 4 mg/kg. Evidently growth retardation by methadone is stereospecific and can be antagonized. These data are listed in Table 1.

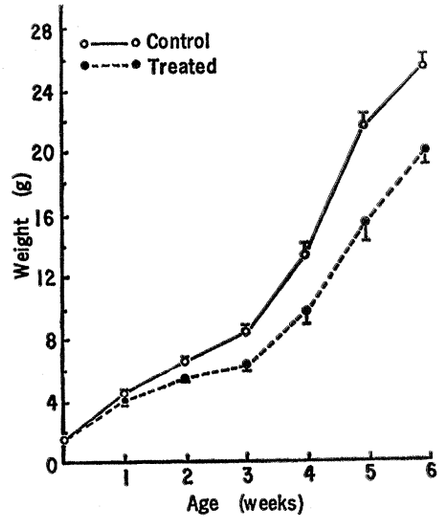


Fig. 1. Growth rates of newborn mice injected with normal saline (control, $N = 21$) or with *dl*-methadone, 2 mg/kg ($N = 16$). Treated and control groups derive from the same litters. The horizontal bar indicates S.E.M. Group weights are significantly different ($P < .01$) at each weekly interval.

The causes of the growth inhibition induced by methadone are unknown. Quite possibly, reduced food intake is a factor. This possibility (*5*) is difficult to evaluate, since equal pair feeding of breast-fed animals poses experimental difficulties. Gastrointestinal disturbances may also be involved, since opioids generally increase gut tone. However, the factor of food intake was found (*3*) unimportant in studies of cholinolytic drugs on the growth of the salamander limb; the amputated limb regenerated at a normal rate in the starved animal. Repression of anabolic factors such as growth hormone has not been ruled out and remains still another possibility. However, no tolerance seemed to develop to the growth inhibition during the 6 weeks of treatment, as might occur if a central mechanism were involved.

The growth retardation induced by methadone may be related to the inhibition of ACh release which morphine-like compounds manifest. Whether the ACh blockade is peripherally or centrally mediated is unknown. Regardless of the exact mechanism, it is clear that methadone treatment retards development of young mice and prevented embryonal cell growth in the salamander (*3*).

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5. In recent experiments we injected nursing mouse mothers subcutaneously with 100 $\mu$c of tritiated leucine, part of which was subsequently secreted into their milk. Pups treated daily with *dl*-methadone in a dosage of 2 mg/kg for the first 3, 5, 7, or 14 days of life were allowed to nurse from their mothers for 24 hours after injection. The total

radioactivity in the body of each pup was then calculated from the radioactivity found in an aliquot of whole body homogenate (prepared in 10 volumes of 0.1*N* HCl). The amount of radioactivity in pups treated for 5 or more days was found equal to that in their saline-treated littermates. In another experiment half of each of several litters were treated with methadone for the first 5 days of life, and then the drug was discontinued. When these mice reached the age of 4 weeks they weighed the same as their untreated littermates. Evidently the initial anorectic effect of methadone, to which tolerance rapidly occurs, is not the cause of the growth retardation seen in the chronically treated young mice.

6. Dr. S. Mulé kindly provided *d*-methadone.
7. This study was supported by grants DA-00020-6 from PHS and from the Dysautonomia Foundation, Inc.

17 May 1973

## Pentapeptide (Pepstatin) Inhibition of Brain Acid Proteinase

Abstract. *The pentapeptide pepstatin obtained from culture filtrates of actinomycetes completely inhibited brain acid proteinase (cathepsin D) at exceedingly low concentrations. Among the brain enzymes tested, the effect is specific for acid proteinase because addition of 1000-fold higher concentrations was without effect on neutral proteinase, aminopeptidase, and arylamidases. Pepstatin also inhibits pepsin as tested with hemoglobin or with* N-*acetylphenylalanyl-*L-*diiodotyrosine as substrate. Pepstatin must be regarded as the most powerful agent yet described that inhibits intracellular acidic proteolytic enzyme in brain.*

Cathepsin D or acid proteinase is one of the major intracellular enzymes involved in the breakdown of proteins. This enzyme is of considerable clinical interest because its activity is elevated in a number of degenerative conditions, including muscular dystrophy, denervation atrophy, inflammatory and allergic conditions (*1*), experimental allergic encephalomyelitis, and multiple sclerosis (*2, 3*). Thus agents modifying the activity of intracellular proteolytic enzymes may have great potential in treatment of degenerative conditions. Our recent finding of endogenous factors present in brain extracts influencing protein breakdown (*4*) indicated the need for studying the effect of other naturally occurring inhibitors of acid proteinase. One inhibitor of great promise is a pentapeptide (pepstatin), obtained from the culture filtrates of actinomycetes (*5*), which inhibits pepsin and some intracellular proteinases present in liver and uterus (*6*). We now report the effect of added pepstatin on some typical intracellular proteolytic enzymes extracted from brain and tumor tissue, as compared to the effect of this inhibitor on extracellular enzymes active at acid and neutral *p*H.

Inhibition by pepstatin was studied on representative brain enzymes, some of which had already been purified (*7*). Acid proteinase was purified from an acetone-dried powder prepared from the crude mitochondrial fraction of pig brain, enriched in lysosomes, as follows: the enzyme was extracted with acetone-formate buffer (0.2*M*, 20 percent acetone by volume) and the supernatant was subjected to Sephadex gel filtration and chromatography on diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex. Acid proteinase exists in isoenzymatic forms, with molecular weights from approximately 27,500 to 48,000 (*4, 7, 8*). One component with a molecular weight of 27,500 giving a single band on sodium dodecyl sulfate–disc gel acrylamide electrophoresis was selected for our study. The purified enzyme can be lyophilized and is stable when stored at 0°C. Neutral proteinases are labile and were prepared (fresh for each series of experiments) by extraction of rat brain with ten volumes of 0.32*M* sucrose; the extracts were then subjected to Sephadex gel filtration and DEAE-cellulose chromatography (*8*). Aminopeptidase showing a marked specificity for leucylglycylglycine (Leu-Gly-Gly) and an arylamidase specific for monoacylated naphthylamides were prepared from the sucrose supernatants by chromatography on DEAE-cellulose and DEAE-Sephadex with elution by a salt gradient different from that used for the other proteolytic enzymes (*9*). Crystalline pepsin and trypsin were obtained from Worthington (Freehold, N.J.). Pepstatin (isovaleryl-L-valyl-L-valyl-L-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) was a gift from Dr. H. Umezawa (Banyu Pharmaceutical Co., Osaka).

The following methods of analysis were used. Acid proteinase and pepsin were measured with denatured hemoglobin in 0.05*M* citrate buffer at *p*H 3.2, the reaction was terminated by addition of 6 percent trichloroacetic acid, and the acid soluble fraction was quantified by the Folin procedure (*10*); in addition, pepsin was assayed with a synthetic substrate *N*-acetylphenylalanyldiiodotyrosine (APDT) in 10 m*M* HCl, *p*H 2.0, with a modified ninhydrin procedure (*11*); neutral proteinase was determined with denatured hemoglobin by a standard quantitative ninhydrin procedure (*8*); aminopeptidase was assayed with Leu-Gly-Gly by the ninhydrin procedure of Yemm and Cocking (*12*); arylamidase was assayed with arginyl-β-naphthylamide; and trypsin was assayed with benzoylarginylnaphthylamide by a coupled diazonium procedure (*9*).

Pepstatin at exceedingly low concentrations (in the nanogram range) inhibited purified and crude brain proteinase preparations (Fig. 1A). Crude enzyme was prepared from normal whole brain, and also from a sample of human astrocytoma, by extraction with ten volumes of a hypotonic buffer, 10 m*M* tris-HCl, *p*H 7.6, and was

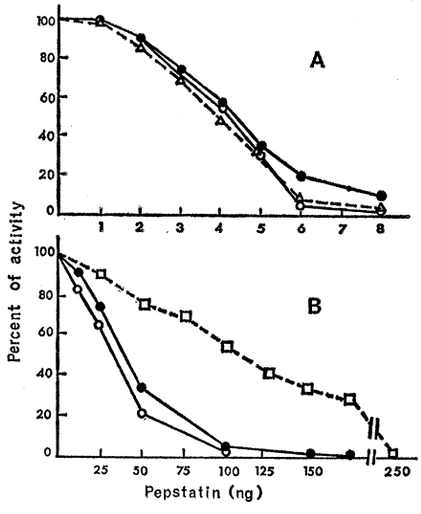


Fig. 1. Inhibition of brain acid proteinase (A) or pepsin (B) with increasing concentrations of pentapeptide (pepstatin). The incubations with hemoglobin as substrate were done as follows: 5.0 mg (●) or 0.6 mg (○) of hemoglobin in 1 ml of 50 m*M* citrate buffer (*p*H 3.2) containing 25 μg of purified porcine acid proteinase or 5 μg of pepsin was incubated for 1 hour at 37°C. When crude enzyme was prepared by hypotonic extraction of rat brain, the mixture was incubated with 100 μg of enzyme protein and 5.0 mg of hemoglobin (△). Inhibition of pepsin by pepstatin was measured with the synthetic substrate *N*-acetylphenylalanyl-L-diiodotyrosine (□), in 1 ml of 10 m*M* HCl (*p*H about 2.0) and incubated for 45 minutes at 37°C. Inhibition is expressed as the percentage of activity remaining after addition of pepstatin relative to that in absence of inhibitor.