be ineffective in systems other than those associated with alterations of oxygen uptake or water transport. This question was explored by studying the effect of the hormone, given *in vivo,* on the activity of liver glutamic-pyruvic transaminase. In rats, treatment with thyroxine reduces the activity of this enzyme. Similar results were obtained in the toad.

The discrepancy between the unresponsiveness of the toad skin and bladder and the responsiveness of the liver enzyme system may reflect differences in the sensitivity of the two systems to minimal concentrations of the hormone. Alternatively the disposal of thyroxine —probably by the liver—may remove the hormone from the membrane sites of action so rapidly that its effect is negligible, while permitting the hormone to accumulate in significant quantities in the liver to reduce the level of transaminase. It is also possible that the hormone itself is not responsible for the changes in the hepatic enzyme levels, but that its metabolic product is the functioning substance. Finally, the difference may be a result of differences in the time-course of action of the hormone; however, the fact that no effect on water transfer and oxygen consumption could be found even when several intervals were studied makes this unlikely.

Summary. Thyroxine injected into intact toads *(B. spinulosus arencum)* failed to increase the water permeability and oxygen uptake of the skin and bladder, although when added *in vitro* it caused clear cut increases in these parameters. The hormone did, however, reduce the activity of the liver glutamic-pyruvic transaminase. This suggests that the failure of thyroxine, *in vivo,* to affect the metabolism and function of toad bladder or skin is not a result of end-organ unresponsiveness, but reflects some peculiarity of the metabolism or disposal of the hormone *in vivo.*

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Efficacy of N^6-(2-Hydroxyethyl)Adenine Against Coe Virus Infection In Mice. (31082)

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Coe virus, first isolated by Lennette and co-workers from patients with mild respiratory illness(1), was later identified as Coxsackie A-21 virus(2). Investigators in various parts of the world found the virus to be responsible for significant numbers of "colds," especially among military recruits. Efforts to find a suitable animal host for laboratory investigation led to successful adaptation of Coe virus to weanling mice, where infection produced muscle degeneration and flaccid paralysis(3). Coe-infected mice have subse-

quently been used in our laboratories for *in vivo* screening and evaluation of potential antiviral agents. This report summarizes studies showing that treatment of such mice with N^6-(2-hydroxyethyl)adenine (HEA) favorably influenced the course of the disease.

Materials and methods. Coe virus was obtained from Dr. E. H. Lennette, adapted to mice as previously described(3), and used as a 10% muscle suspension for infecting mice. Unless specified, mice were infected by inoculating intracerebrally (IC) approximately 10^7 plaque forming units (PFU) of virus which had been through 40-50 serial mouse passages. Virus content of mouse muscle suspensions was determined by plaque assay on ML cells as described by Buthala(4).

The above virus inoculum caused paralysis and/or death in 90-100% of infected mice within 10 days. White Swiss Webster mice, October Hills strain, weighed 11-13 g and were 3-4 weeks old at time of infection. The mice were examined on the 6th and 10th days postinoculation and scored as follows: 10 = normal appearance, 7 = slight paralysis, 5 = paralyzed, 2 = extreme paralysis, 0 = dead. The score on a given day was one-half the sum of the scores of the 20 individual mice in each group and was compared to the average obtained on the infected control groups within the same experiment. A statistical analysis of controls collected over a one-year period indicated that for significant activity (p = 95) the score for the treated group should exceed the control score by 15 units on the 6th day reading or by 20 units on the 10th day reading. Control groups were treated with the menstruum used to prepare test compounds. All samples were submitted under code so that the animals were treated and scored blind.

HEA and the xanthine oxidase inhibitor, 4-hydroxypyrazolo-(3,4-d) pyrimidine (HPP) were obtained commercially from Aldrich Chemicals, Milwaukee, Wisc. Ferrets, weighing approximately 500 g each, were obtained from Gilman Marshall, Ferret and Beagle Ranch, North Rose, N. Y.

Results. During routine screening of compounds in Coe-infected mice, HEA suspended in sterile 1% carboxymethylcellulose (CMC) menstruum was found to decrease paralysis development and incidence of death. To determine whether the activity was increased by solubility, equivalent preparations of HEA were made in CMC (suspension) and in 0.03 *N* hydrochloric acid (solution). Each of these preparations was used to treat Coe-infected mice with treatment started the day before virus inoculation. Mice were treated daily at 8:00 a.m. and 4:00 p.m. for 5 consecutive days, inoculating 0.2 ml/mouse intraperitoneally (IP) each time. Results (Table I) indicate activity, but not complete protection, with HEA at levels of 50-150 mg/kg/day either in solution or in suspension. Similar levels of activity were obtained when the compound was administered subcutaneously or orally. In subsequent studies, HEA was suspended in CMC and injected IP.

The antiviral activity of HEA was not enhanced by pretreating for 3 days prior to in-

TABLE I. Comparative Activity of HEA in Suspension and in Solution.

Virus: Coe IC

Treatment: IP, 2×/day for 5 days, starting one day before virus inoculation.

Evaluation: Each mouse scored on days 6 and 10 postinoculation. For significant activity (P=95): Δ6-day=15 or Δ10-day =20.

Sample	Preparation	Dosage (mg/kg/day)	Score 6-Day	Score 10-Day	Evaluation
Control			24	13	
HEA	Suspension	50	39	26	Active
"	Solution	50	42	32	"
"	Suspension	100	44	34	"
"	Solution	100	49	40	"
"	Suspension	150	55	28	"
"	Solution	150	57	51	"

TABLE II. Four Year Summary of HEA Activity in Mice.

Virus: Coe IC

Treatment: 100 mg/kg/day IP, 2×/day for five days, starting one or two days before virus inoculation.

Scores: For significant activity, Δ6-day = 15 or Δ10-day = 20.

Paralysis scores: Mean Δ values		10-Day survivors/total mice	
6-Day	10-Day	Treated	Control
23	25	420/870 (48%)	186/1633 (11%)

fection, by inoculating virus IP rather than IC, by mixing compounds and virus before inoculating IP, or by more frequent treatment of infected animals. Adenine itself was inactive in Coe-infected mice and was also ineffective in reversing the antiviral activity of HEA.

Data collected over a 4-year period (Table II) showed that HEA was active not only on the basis of paralysis scores, but also on the completely objective basis of survivors. Statistical analysis indicated that these treated and control survival values were significantly different at >99.99% confidence level.

TABLE III. Effect of HEA on Virus Titer in Mouse Muscle.

Virus: Coe IC

Treatment: 100 mg/kg/day IP, 2×/day, starting one day before virus inoculation.

Titers: Virus content of gluteal muscles from individual mice was determined by plaque assay in ML cells. Titers, expressed as $\log_{10}$ PFU/ml of 10% muscle suspension, represent mean values for 5-10 mice/group.

Inoculum (PFU/mouse)	Treatment	Muscle titer on indicated day post-inoculation Day 2	Day 3	Day 6
3×10^3	Control	3.2	4.3	4.0
3×10^3	HEA	0	2.6	3.5
3×10^4	Control	4.9	5.5	
3×10^4	HEA	3.1	4.6	
1.5×10^6	Control	6.3		
1.5×10^6	HEA	5.8		

Muscle was harvested from HEA-treated mice which had been inoculated with various levels of Coe virus. Virus content of these muscles was determined and compared to controls run in parallel (Table III). With a relatively low virus inoculum (3,000 PFU), less virus was recovered from treated than from control animals at 2 or 3 days post-inoculation; by the 6th day, there was little difference between the groups. As the level of challenge virus was increased, differences between treated and control groups became less.

During toxicity studies of HEA, crystalline deposits, later identified as N^6-(2-hydroxyethyl)-2,8-dioxyadenine, were observed in the kidneys of rats and dogs.* Accumulation of this metabolite in the kidney appeared to be a major cause of HEA toxicity. Treatment with HEA in combination with the xanthine oxidase inhibitor, HPP, resulted in a significant reduction of crystal formation in the kidneys of these animals(5). In other studies, it was found (Table IV) that simultaneous administration of HPP markedly decreased the mouse toxicity of HEA, even at a 20:1 weight ratio of HEA:HPP. Thus, most mice receiving 400 or 200 mg/kg/day of HEA alone died within a week, while nearly all survived in groups receiving these levels of HEA mixed with 20 mg/kg/day of HPP. Weight in these latter groups remained nearly constant during treatment but increased rapidly after treatment was stopped. This weight picture was comparable to that observed in mice receiving 100 mg/kg/day HEA alone, *i.e.*, slight weight loss during treatment, but weight gain thereafter. These results substantiate the reversibility of HEA toxicity by HPP and also indicate that toxicity resulting

TABLE IV. Effect of Xanthine Oxidase Inhibitor (HPP) on Mouse Toxicity of HEA.

Treatment: IP, 2×/day from day 1 through day 5 with HEA alone or with a mixture of HEA and HPP.

Treatment	Dose (mg/kg/day)	Day 1 Avg wt (g)	Day 4 ΔWt (avg)	Day 4 Dead/ Total	Day 7 ΔWt (avg)	Day 7 Dead/ Total	Day 11 ΔWt (avg)	Day 11 Dead/ Total
HEA	400	11.0	−2.3	4/10		10/10		
HEA + HPP	400 + 20	11.0	−0.3	0/10	+3.0	3/10	+5.3	3/10
HEA	200	11.4	−2.6	0/10	−2.4	8/10	+0.1	8/10
HEA + HPP	200 + 20	11.5	+0.6	0/10	+3.3	1/10	+4.8	1/10

* These crystals were first noted during toxicity studies by Dr. J. E. Gray, of our Pathology Dept. The compound was chemically characterized by Dr. D. L. Smith, Dept. of Physical and Analytical Chemistry.

when HEA was given alone was reversible, since surviving mice showed weight gain and apparent good health after cessation of treatment.

Additional tests were run using the HEA: HPP combination in Coe-infected mice to determine whether the higher permissible dosages of HEA would result in greater antiviral activity and to establish whether antiviral activity and toxic effect of HEA could be separated. Results (Table V) again showed that HPP reduced the toxicity of HEA, since it was possible to test HEA at 200 mg/kg/day, a level usually lethal. However, there was no apparent increase in antiviral activity at this level. It did appear that the toxic effect was separable from the antiviral effect, since there was no decrease in activity when using the combination, whereas there was a sharp decrease in toxicity. Neither HPP nor the dioxy derivative of HEA showed any antiviral activity.

TABLE V. Effect of HPP on Activity of HEA.

Virus: Coe IC

Treatment: IP, 2×/day for 5 days, starting one day before virus inoculation; 3 control and 2 treated groups of 20 mice/group.

Evaluation: For significant activity, Δ6-day = 15 or Δ10-day = 20.

Treatment	Dose (mg/kg/day)	6-Day Score	6-Day Δ	10-Day Score	10-Day Δ
Control		30		6	
"		28		0	
"		32		4	
Avg control		30		3	
HEA	50		24		19
"	50		13		22
"	100		31		38
"	100		21		20
HEA + HPP	100 + 20		14		23
" "	100 + 20		21		20
" "	200 + 20		20		18
" "	200 + 20		27		30
HPP	20		3		1

TABLE VI. Effect of a Single HEA Injection on Coe Infection in Mice.

Virus: Coe IC

Treatment: A single IP injection of 75 mg/kg HEA at indicated intervals before and after virus inoculation.

Treatment time (hr)	No. of mice	Mean score Δ6-Day	Mean score Δ10-Day	Activity
−72	40	−2	−3	—
−48	40	8	12	—
−24	80	26	36	+
− 2	80	25	36	+
+ 2	80	24	29	+
+17	80	18	28	+
+24	80	9	22	±
+48	60	−6	7	—

A single IP injection of HEA at a level of 75 mg/kg was found to show significant antiviral activity in Coe-infected mice (Table VI). Time of treatment could be varied from at least 24 hr before to 17 hr after intracerebral inoculation of virus, with resultant activity. Since the acute LD_{50} of HEA in mice is approximately 700 mg/kg by the IP route, these results provided further evidence that antiviral activity was not dependent on toxicity.

Twice daily IP treatment with HEA temporarily inhibited formation of viral hemagglutinins in the lungs of mice infected with the PR-8 strain of influenza A virus. HEA treatment at 10-100 mg/kg/day decreased the level of PR-8 hemagglutinins in lungs harvested 24 hr postinoculation, but there was little difference between treated and control groups by 48 hr. HEA was further evaluated against influenza in ferrets, where the disease is more comparable to that in man. Lungs were examined at 3 and at 4 days after IN inoculation with PR-8 virus. No significant differences were found in lung lesion scores, hemagglutination titers or mouse infectivity titers between control animals and those treated twice daily with 100 mg/kg/day of HEA. In other experiments, HEA did not prolong survival time nor increase the number of survivors in mice infected with lethal strains of influenza, herpes simplex or vaccinia viruses.

Discussion. Our primary interest in Coe virus concerns its role as a causative agent in human upper respiratory disease. In order to test for compounds potentially active in man against this virus, a suitable laboratory animal host was needed. Because of their availability, cost and convenience, mice were the animals of choice for *in vivo* testing of potential antiviral agents. Effective operation of a high volume screen requires definitive endpoints in the test system with no ambiguity in evaluation of test results. After con-

siderable effort, Coe virus was successfully adapted to mice with infection accompanied by an easily observed and readily measured disease syndrome, *i.e.*, muscle degeneration followed by flaccid paralysis(3). Although there is no assurance that an agent effective in preventing muscle degeneration by Coe virus in mice would also ameliorate a respiratory infection by the same virus in man, there is perhaps some precedent in the case of certain thiosemicarbazones, highly active against neurovaccinia infections in mice and also capable of preventing generalized infection with the closely related smallpox virus in man(6).

Data presented in Tables I and II show conclusively that treatment with HEA decreased the amount of paralysis and the number of deaths in Coe-infected mice. In some experiments, more than half of the treated mice remained in apparent good health but a variety of treatment routes and schedules never gave complete protection. This is probably due to slight differences in the way individual mice react to Coe infection and to HEA. The fact that antiviral activity was not significantly increased with the higher levels of HEA tolerated in the presence of HPP (Table V) may further indicate that there is a delicate balance between resistance and susceptibility to the infection, that this varies among individuals within a group of 20 mice, and that the observed levels of protection represent the maximum obtainable with this compound.

Variations were also found in levels of virus recovered from the muscle of individual Coe-infected mice, but average titers were lower in those treated with HEA than in controls (Table III). Partial inhibition of virus multiplication by HEA, permitting mobilization of the host's own defense mechanism, is probably sufficient to obtain significant protection, since inoculation of control mice with 10^6 rather than 10^7 PFU of virus produced little overt illness. The situation is again reminiscent of the thiosemicarbazones, where treatment of vaccinia-infected mice caused only a 1 or 2 log drop of virus titer in the brain, but resulted in nearly all animals remaining healthy(7).

The lower levels of viral hemagglutinins found in early harvests of lungs from PR-8-infected, HEA-treated mice compared to untreated controls was of interest and indicated a possible effect of HEA on the host rather than a specific anti-Coe effect. In this case, the transient inhibition was not sufficient to alter significantly the course of the disease, perhaps due to the fact that this strain of virus was rapidly lethal for mice, thus overwhelming natural host defenses. Preliminary studies on mechanism of HEA antiviral action indicated that the compound was not virucidal and that it did not enhance antibody or interferon production in mice.

The 2,8-dioxy derivative of HEA isolated from kidneys and urine of the treated animals presumably was produced *in vivo* by xanthine oxidase and was analogous to the crystalline product found by Philips *et al*, in kidneys of mice and rats treated with large doses of adenine(8). Elion *et al* observed that HPP markedly decreased the metabolic oxidation of 6-mercaptopurine to 6-thiouric acid, resulting in potentiation of the antitumor properties of 6-mercaptopurine(9). More recently, HPP under the generic name of allopurinol, has shown promise in treatment of gout by inhibiting production of uric acid(10). The fact that HPP markedly decreased the toxicity of HEA for mice (Table IV), indicated that the derivative produced by xanthine oxidase was a major source of HEA toxicity. Evidence that toxicity could be reduced without concomitant loss of antiviral activity (Table V) and that HEA showed activity at levels only about one-tenth the lethal dose (Table VI), indicated that antiviral activity of the compound was not dependent on its toxicity.

The number of agents which have shown any activity in animals against human respiratory viruses is vanishingly small. None have shown remarkable activity. Any active agent is of potential interest, since it is conceivable that minimal animal activity may translate to outstanding clinical utility. Infecting virus levels are much lower in nature and most infections are self-limiting. Several workers have demonstrated the feasibility of producing a uniform, mild, cold-like illness in human volunteers inoculated intranasally

with Coe virus(11,12,13). Therefore, it was considered worthwhile to test HEA for its ability to prevent or modify an experimental Coe infection in man. Preliminary results of HEA treatment of such an experimental Coe infection were recently reported(14) and will be described later.

Summary. The compound N^6-(2-hydroxyethyl)adenine (HEA) was effective in favorably modifying experimental Coe virus infection in mice. HEA showed antiviral activity when a single injection, approximately one-tenth the lethal dose, was given from 24 hours before to 17 hours after virus inoculation. The toxicity, but not the antiviral activity of HEA, was partly reversed by simultaneous treatment with a xanthine oxidase inhibitor.

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Alkaline Phosphatases of the Chick. Partial Characterization of the Tissue Isozymes.* (31083)

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Evidence obtained from *in vivo* studies with diseased and injured bone(1,2) on the effect of biochemical inhibitors(3), immunochemical and histochemical demonstrations(4, 5,6) suggested that serum alkaline phosphatase is derived from bone. Observations from liver studies suggested a hepatogenous origin of the increased serum alkaline phosphatase found in jaundice and hepatic duct ligation (7,8). It has also been concluded that whereas the major fraction of the serum alkaline phosphatase originates from bone, a small portion may be produced by liver cells(9,10). By observing the enzyme content in the serum and other tissues after fasting and fat injection(11) and also the inhibitory effect of this enzyme *in vitro*(12), an intestinal origin of serum alkaline phosphatase has been generally accepted. A diverse origin was also suggested after carbohydrate ingestion(13). Water dosing elevates the serum alkaline phosphatase(14,15).

In lieu of the diverse results obtained under a variety of nutritional and physiological conditions, studies were initiated whereby changes in the alkaline phosphatase level of the chick were observed under more normal conditions. The source of the serum enzyme was sought during these investigations.

In recent years, chromatographic techniques have become available to study the difference between functionally similar enzymes(16,17,18). The combined use of chromatographic techniques and isotopic Ca^{45}

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