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Effect of Eastern Equine Encephalomyelitis Virus Infection on Phosphate Transfer and Modification by Cortisone.* (22387)

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The biochemical approach to the study of viruses and their relation to host cell has produced numerous reports on alterations of host cell metabolism due to virus infection. The most prolific source of information has arisen from investigations concerned with effects of bacteriophage on its host cell, and these have been furthered by use of radioactive isotopes (1-3). Similar investigations in the field of animal viruses have not always been so easily resolved as those with bacteriophage, largely due to heterogeneous mixture of cells in host tissue and the difficulty in distinguishing the direct effect produced by virus on infected cells from indirect effects on neighboring cells and the organism as a whole (*e.g.,* inflammation). Anderson, *et al.*(4), using radioactive phosphate, reported that infection of monkey brain tissue with Lansing poliomyelitis virus produced significant changes in rate of phosphate turnover in infected areas of the brain, and suggested that it was due to an increased metabolic activity. Since these changes were detected in specific activities of total acid-soluble organic as well as in inorganic phosphate fractions, the authors discounted the possibility that these changes could be due to injury. No significant alterations in transfer rates from blood to spinal fluid were observed. On the other hand, Rafelson, *et al.*(5), found no changes in turnover of organic acid-soluble phosphates of day-old mouse brain tissue cultures infected with Theiler's GD VII virus which could be related to virus synthesis, although changes in other phosphate fractions attributable to virus synthesis were observed.

It therefore seemed of interest to investigate the effect of a neurotropic virus on the phosphate turnover in infected mouse brain tissue *in vivo* to determine if the effect of the virus infection was due to an actual metabolic requirement for virus synthesis or merely to an increased rate of transfer associated with inflammation.

Materials and methods. Swiss albino mice

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weighing 10 to 15 g (3 to 5 weeks old) were used, and were matched evenly for weight and age in each experiment. The animals were infected by intracerebral inoculation with New Jersey strain of Eastern equine encephalomyelitis (E.E.E.) virus‡ in doses of 0.03 ml. The infected animals were observed for 10 days after injection, although symptoms of infection usually developed within 36 to 72 hours. *Virus titrations.* At appropriate intervals following infection, mice used for determination of virus growth were sacrificed in groups of 5 by exsanguination and the brains rapidly removed, weighed and stored at −26°C until the end of the experiment. Virus concentration for each sample group was determined by 10-fold serial dilution method, employing 5 mice for each dilution per virus sample. LD_{50} was calculated by the method of Reed and Muench(6). *Phosphate determinations.* Mice were injected intraperitoneally with radioactive phosphate ($Na_2HP^{32}O_4$)§, 175 μc/dose, volume 0.1 ml 90 minutes before blood sample was taken by cardiac puncture. The animals were then sacrificed as above and brains rapidly removed. The tissues were dropped into acetone chilled with dry ice, weighed, and homogenized with cold trichloroacetic acid. The homogenates were centrifuged in the cold until a clear supernatant was obtained for analysis. Analytical procedure was that of Ernster, *et al.*(7), which permits estimation of both phosphorus content and radioactivity on same sample. Colorimetric determinations were made with Coleman Jr. spectrophotometer at 625 mμ and radioactivity (P^{32}) was measured with SC1C Tracerlab scaler. Brain tissues of 3 mice were pooled for each sample. Three aliquots were analyzed from each sample, and two samples were used for each result. Results are presented as relative specific activities (R.S.A.) of measured fractions(8). The ratios used were:

$$\text{R.S.A. of orthophosphate (O.P.)} = \frac{\text{Sp. act. brain O.P.}}{\text{Sp. act. blood O.P.}}$$

$$\text{R.S.A. of T.A.S. phosphate (T.A.S.P.)} = \frac{\text{Sp. act. brain T.A.S.P.}}{\text{Sp. act. blood O.P.}}$$

$$\text{R.S.A. of O.A.S. phosphate (O.A.S.P.)} = \frac{\text{Sp. act. brain O.A.S.P.}}{\text{Sp. act. brain O.P.}}$$

Drugs. 1. Cortisone acetate‖ (Cortone, Merck) was injected subcutaneously 2.5 mg per mouse in volume of 0.1 ml 2 hours before virus inoculations. 2. A Pseudomonas pyrogen solution¶ (Piromen, Baxter Laboratories) containing 400 μg/ml was used for intracerebral injection in volume of 0.03 ml. 3. Controls were injected with volumes of sterile 0.85% saline equivalent to appropriate active solution administered.

Results. To correlate virus synthesis with phosphate turnover, a number of young mice were inoculated intracerebrally with approximately 300 LD_{50} units of E.E.E. virus and sacrificed at appropriate intervals for phosphate and virus determinations.

E.E.E. virus was found to proliferate rapidly in brain tissue for the first 24 hours after inoculation (Fig. 1) and the logarithmic rate of virus increase during this period is approximately a straight line. The following period, during which animals developed symptoms (30 hours) and were dying (36 hours), showed no marked change in concentration of measurable virus in the tissues.

The rates of phosphate transfer in the ortho and total acid-soluble phosphate fractions parallel each other, with increased rates first becoming apparent at 12 to 24 hour interval and reaching their maxima at 30 hour or symptomatic period. Changes in phosphate turnovers lagged substantially behind the changes occurring in concentration of active virus in the tissues. The sharp drop in trans-

‡ This virus was obtained through the courtesy of U. S. Public Health Service Laboratory, Montgomery, Ala.

§ Radioactive $Na_2HP^{32}O_4$ was obtained from Oak Ridge, Tenn. and used without addition of carrier. Dilution of stock solution gave the specified activity on the basis of radioactive decay curve. No sample was used more than 10 days after shipment.

‖ Cortisone was obtained from Merck & Co., Rahway, N. J.

¶ Piromen was supplied by Baxter Laboratories, Morton Grove, Ill.

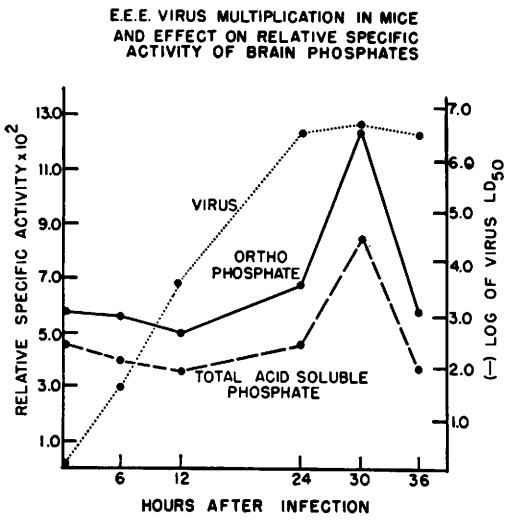

FIG. 1. Relationship of virus synthesis to R.S.A. of phosphate fractions in mouse brain following Eastern equine encephalomyelitis virus infection.

fer rate between 30 and 36 hour periods was probably due to congestion of the brain, which would tend to limit the area of tissue exposed to the P^{32}-containing blood. Marked hemoconcentration of circulating blood and apparent reduction in total blood volume were observed in moribund animals at the 36 hour period.

The organic acid-soluble phosphate (O.A.S.) fraction represents those phosphorylated compounds which are generally assumed to derive their phosphate from the orthophosphate fraction (9). If the virus infection were altering the rate of phosphate uptake on a metabolic level concerned with energy mechanisms of the tissue, it would be evident in the study of this fraction. It is apparent (Table I) that no significant change occurred in the O.A.S. fraction that could be correlated with the remarkable increase in virus concentration which occurred during the 6 to 24 hour interval. However, the R.S.A. of non-infected brain O.A.S. phosphates were again compared to those of virus infected animals to evaluate the apparent decrease noted in Table I. The brain tissues from normal and infected groups, consisting of 15 animals each, were analyzed in groups of 3. Infected animals had been inoculated with the virus 30 hours previously. The results obtained (Table II) indicate that the decrease noted above is not consistent with progression of infection; it may be more readily explained in terms of errors involved in analyses of both ortho and total acid-soluble phosphated fractions, since the O.A.S. represents the difference in both concentration and activity of these two fractions. The progressive nature of the virus infection undoubtedly provokes an increasing inflammatory response in the tissues, and the local change in vascular (blood-brain barrier) permeability associated with this response could account for the increased activity of the ortho-phosphate in brain tissues. Changes in rate of T.A.S. phosphate turnover would then be explained on the basis of increased rate of P^{32} transfer, rather than as increased rate of phosphate utilization.

To evaluate the role of inflammation in alteration of phosphate transfer, 6 groups of

TABLE I. Comparison of Virus Concentration with R.S.A. of Organic Acid-Soluble Phosphate Fraction in Mouse Brain after Infection with Eastern Equine Encephalomyelitis Virus.

Time after infection (hr)	Virus titer	Organic acid-soluble phosphates ($\times 10^1$)
0	$10^{-1.0}$	6.44 (5.65, 7.22)
6	$10^{-1.7}$	4.93 (4.7, 5.15)
12	$10^{-3.6}$	4.12 (3.17, 4.52)
24	$10^{-6.6}$	4.17 (3.98, 4.35)
30	$10^{-6.7}$	4.01 (3.60, 4.42)
36	$10^{-6.5}$	3.24 (2.67, 3.75)

TABLE II. Comparison of R.S.A. of O.A.S. Phosphates from Brain Tissues of Non-Infected Mice with Those from E.E.E. Virus-Infected Animals.

Group* No.	Non-infected (R.S.A. $\times 10^1$)	Virus† (R.S.A. $\times 10^1$)
1	6.22	6.68
2	6.35	5.92
3	7.61	6.76
4	6.66	—
5	5.56	7.59
Avg	6.48	6.73

* Each group consisted of brains from 3 mice.
† Brains obtained from mice infected 30 hr previously with E.E.E. virus.

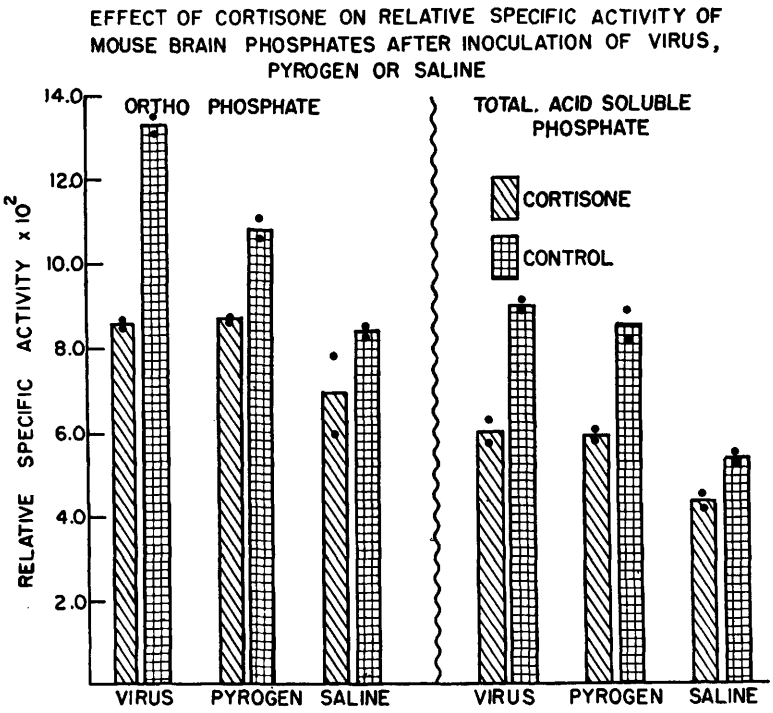

FIG. 2. Effect of cortisone on R.S.A. of mouse brain phosphates after inoculation of virus, pyrogen, or saline.

6 animals each were treated as follows: 3 groups of mice were injected subcutaneously with cortisone, while 3 remaining groups received control injections of 0.85% saline. Two hours later, 6 animals from the cortisone-treated and 6 animals from saline control groups were injected intracerebrally with 30,000 LD_{50} units of E.E.E. virus. At the same time, 6 cortisone-treated and 6 control animals received an intracerebral inoculum of the Pseudomonas pyrogen. The remaining 6 cortisone-treated and 6 saline controls received a control intracerebral injection of 0.03 ml saline. Tissues were obtained for phosphate analysis 28 hours after infection.

Fig. 2 demonstrates quite clearly that increased permeability of the blood-brain barrier to phosphate may be initiated by various agents and is therefore of a non-specific nature. It is interesting that all effects observed in the orthophosphate fraction are reflected in the total acid-soluble phosphate determinations. The hormone treatment effectively modified the increased rate of phosphate uptake in brain tissue injured by either virus infection, pyrogen, or saline injections. Rate of uptake in tissues from animals pretreated with cortisone and subsequently inoculated intracerebrally with virus or pyrogen, showed an R.S.A. no greater than tissue from untreated controls which had received only an injection of saline. It is doubtful, therefore, that increased rate of phosphate transfer serves any useful purpose for synthesis of the virus, but rather, demonstrates the loss of integrity of the blood-brain barrier coincident with the development of inflammation in these tissues.

However, the possibility that the reduced transfer of phosphate might affect the rate of virus synthesis was not wholly disqualified by these experiments. Mice were therefore separated into 2 groups, one of which received 2.5 mg of cortisone subcutaneously. The other group was injected with an equal volume of saline. Two hours later, the animals in each group were injected with approximately 300 LD_{50} units of the virus. Brain tissues from 5 animals of each group were obtained at times noted in Fig. 3 and prepared

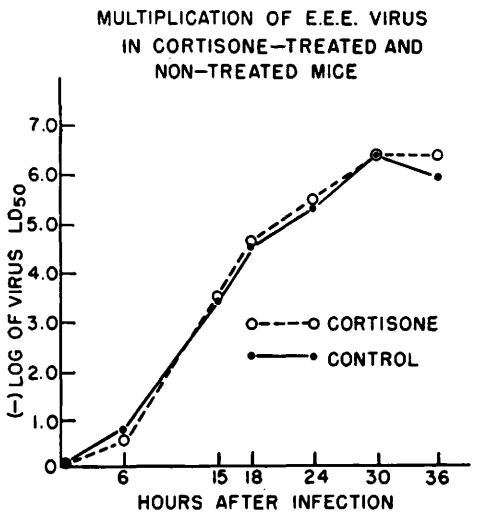

FIG. 3. Synthesis of Eastern equine encephalomyelitis virus in mouse brain of cortisone- and non-treated mice.

for comparative virus determinations. The results show that growth rate of E.E.E. virus in the C.N.S. tissues of the mouse after cortisone treatment is apparently equal to virus growth in those tissues of untreated controls. They suggest that cortisone asserted little or no direct influence on intracellular development of this virus or its ability to infect other susceptible cells of the mouse C.N.S. under the conditions of this experiment.

Discussion. E.E.E. virus infection of mouse C.N.S. tissues was found to enhance the specific activity of brain phosphates following P^{32} administration. Anderson, *et al.*(4), have reported that poliomyelitis virus infection has a similar effect in monkey brain. When our results were analyzed in terms of their *relative specific activities,* the virus effect on phosphates consisted essentially of an enhanced transfer rate of orthophosphate from blood to brain. It has been previously pointed out that a rapid exchange of phosphate normally occurs between the inorganic and acid-soluble phosphates in tissue(9). On this basis, any alteration in transfer rate of P^{32}-labeled inorganic phosphate would be reflected in specific activity of organic phosphates.

Phosphate turnover in degenerating nerve tissue has been observed to be higher than in either normal or regenerating tissue(10), and similarly, P^{32} has been found to accumulate to a greater extent in traumatic, purulent, or ischemic brain lesions than in control tissue (11). A non-specific inflammatory substance (Piromen) was found to substitute for the virus infection in provoking an increased rate of phosphate transfer from blood to brain tissue. Inhibition of this effect by cortisone pretreatment in both virus-infected and pyrogen-treated mice is indicative of a common effect induced by these two different agents. The antiphlogistic effect of adrenal cortical extract and of cortisone has been demonstrated with regard to suppression of alterations in blood-brain barrier permeability(12, 13), as well as of cellular infiltration into virus-infected brain tissue(14).

Anderson, *et al.*(4), suggested that their observed increases in phosphate turnover rates of virus-infected brain tissue must be related to increased metabolic activity within the cell. However, the present study failed to show any correlation between rate of virus growth and turnover rate of organic acid-soluble phosphates. In fact, the only significantly increased activity noted in phosphates depended on increased rate of orthophosphate transfer, and this was shown to follow rather than precede appearance of measurable virus in the tissue. These results suggest that the observed association of increased virus concentration and an altered rate of phosphate transfer is not necessarily indicative of increased metabolic requirements of the virus-infected cell. Cortisone inhibition of the virus effect on rate of phosphate transfer without alteration of rate of virus production implies that the measurable changes demonstrated in this study are evidence of pathology (*i.e.,* inflammation), rather than metabolism.

Summary. Infection of mice with virus of Eastern equine encephalomyelitis induced an enhanced rate of phosphate transfer from blood to brain tissues, which could be markedly reduced by pretreatment of the infected animals with cortisone. Non-specific alteration of phosphate transfer to brain tissue could also be suppressed to a similar degree

by cortisone treatment. Comparison of the E.E.E. virus growth in both cortisone-treated and untreated animals showed no effect of cortisone on rate of virus proliferation. The effect of this neurotropic virus infection on rate of phosphate transfer from blood to brain acid-soluble phosphates appeared to be due to inflammation, rather than to a specific metabolic requirement for virus synthesis.

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Electrophoretic Distribution of Serum Proteins in Rabbit, Guinea Pig and Rat Following BCG Administration. (22388)

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Pulmonary tuberculosis in humans has been shown to produce an increase in the alpha-1, alpha-2, and gamma globulin concentration of the serum protein (1-4). Electrophoretic studies of tuberculous guinea pig and rabbit sera revealed an increased total protein and gamma globulin with a decrease in the albumin concentration(5-7).

In an attempt further to understand biochemical factors of host resistance to tuberculosis, a study of serum protein composition in natively resistant (rat) and susceptible (guinea pig and rabbit) animals was undertaken. It also seemed important to study the effect of an immunizing strain (BCG) on the serum protein pattern of susceptible and resistant animals.

Methods. Eight male albino rabbits (avg wt 1100), 8 male albino guinea pigs (550 g) and 8 male albino rats (360 g, Sprague-Dawley) were fasted for 16 hours and bled by cardiac puncture to obtain normal sera. The sera were separated and stored at –20°C until chemical and physical determinations could be made. The animals were then inoculated intraperitoneally with a suspension of tubercle bacilli (BCG).* The rabbits received 6.0 mg; the guinea pigs, 3.0 mg; and the rats, 2.0 mg (wet wt) of the organism suspended in 1.0 ml of saline. The rabbits and guinea pigs were maintained on a diet of rabbit chow pellets (containing no antibiotics) and the rats were fed laboratory Purina chow (no antibiotics). All diets were supplemented with greens and water *ad libitum.* Four weeks following the inoculation of BCG, all of the above animals were again fasted for 16 hours and bled by cardiac puncture. The sera were stored at –20°C. The analyses on control and experimental sera were performed in duplicate at the same time. Electrophoresis of the serum protein was done on

* Culture obtained from Dr. S. R. Rosenthal, Tice Laboratory, University of Illinois, Chicago.