# Effect of 2,6-diaminopurine on Virus of Russian Spring Summer Encephalitis in Tissue Culture.* (19004)

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The destructive effect of Russian Spring Summer Encephalitis (RSSE) virus infection on several transplantable tumors in mice has been reported(1,2). The mice eventually succumb to systemic injury from the virus after their tumors have been destroyed. Hence one phase of our program includes a search for agents with viricidal activity, which might be employed therapeutically after the oncolytic action of the virus is complete.

Compounds structurally related to the components of nucleic acid have been shown to interfere with the growth of micro-organisms (3-9). Since an infecting virus and its host cells are presumed to be in competition for nucleic acid metabolites such as purines and pyrimidines(10), or the enzymes utilizing them, a rational approach to the treatment of virus infection is to employ potential antimetabolites of nucleic acid. Thompson *et al.* (11-12) reported that the growth of vaccinia virus *in vitro* is inhibited by 2,6-diaminopurine (DAP).† That compound accordingly was employed in our study. The results indicate that DAP inhibits the multiplication of RSSE in tissue culture. Since no agent therapeutically active against this virus has been hitherto reported, the results of the study are presented herewith.

*Methods.* RSSE virus multiplies readily in a Maitland type tissue culture. This technic lends itself well to the assay of the effect of a compound on the proliferation of the virus as well as of the tissue. The cultures were set up in 50 cc Erlenmeyer flasks containing 0.5 cc of 10% horse serum, 4.5 cc Tyrode's solution, and about 100 mg of minced tissue. Two types of mouse tumors were used; the Crocker Sarcoma 180 and the Carcinoma 1025. Both minced mouse and chick normal embryonic tissues were employed. The tumor tissue was cut into 2 mm cubes and 10 pieces added to each flask. Tumor fragments from flasks inoculated with virus were implanted into mice immunized against RSSE virus during the course of the experiment as a test of tissue viability (bioassay). Unless otherwise stated, 0.1 cc of a 10% suspension of infected mouse brain was the inoculum. It was added at approximately the same time as the other tissue culture components, including the test compound. Penicillin and streptomycin were always added to prevent bacterial contamination. The contents of each flask were thoroughly mixed and incubated at 37°C. Control flasks containing no virus were run concurrently with each experiment. At 24, 48, and 72 hour intervals, designated flasks were removed from the incubator and the super-

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

1. Moore, Alice E., *Cancer,* 1949, v2, 525.

2. Moore, Alice E., *Cancer,* 1951, v4, 375.

3. Wooley, D. W., *J. Biol. Chem.,* 1944, v152, 225.

4. Hitchings, G. H., Falco, E. A., and Sherwood, M. E., *Science,* 1945, v102, 251.

5. Roblin, R. O., Jr., Lampen, J. O., Engligh, J. P., Cole, Q. P., and Vaughan, J. R., *J. Am. Chem. Soc.,* 1945, v67, 290.

6. Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., *J. Biol. Chem.,* 1948, v174, 765.

7. Klotz, I. M., and Mellody, M., *J. Bact.,* 1948, v56, 253.

8. Hitchings, G. H., Elion, G. B., Falco, E. A., Russel, P. B., Sherwood, M. B., and VanderWerff, H., *J. Biol. Chem.,* 1950, v183, 1.

9. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 689.

10. Evans, E. A., Jr., *Bact. Rev.,* 1950, v14, 210.

11. Thompson, R. L., Wilken, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russel, P. B., *Science,* 1949, v110, 454.

12. Thompson, R. L., Price, M. L., Minton, S. A., Jr., Elion, G. B., Hitchings, G. H., *J. Immunol.,* 1950, v65, 529.

† Obtained through the courtesy of Burroughs-Wellcome Laboratories.

TABLE I. Effect of Different Concentrations of DAP on Multiplication of RSSE Virus in Tumor Tissue Culture.

| Hr in tissue culture | No DAP | Molar concentrations of 2,6-diaminopurine $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-7}$ |
|---|---|---|---|---|---|---|
| 24 | 5.5* | 3.5 | 3.5 | 3 | 5.5 | 7 |
| 48 | 6.5 | 1.5 | 1 | 3 | 5.5 | 5.5 |
| 72 | 3 | NV† | NV | .5 | 4 | 3.5 |

* Titers expressed as reciprocal of $LD_{50}$.
† NV designates no virus.

TABLE II. Effect of Adding DAP 24 Hr After the RSSE Inoculum.

| | Hr after virus inoculation 24 hr | 48 hr | 72 hr |
|---|---|---|---|
| S-180 + virus | 6.5* | 7 | 5.5 |
| + DAP + virus | — | 3 | .5 |

* Titers expressed as the reciprocal of $LD_{50}$.

natant fluids tested for virus content by intracerebral inoculation of 0.03 cc into 3-4 week old mice. Titers were calculated in terms of the 50% endpoint by the method of Reed and Muench(13).

*Results.* Each of the experiments reported is representative of 3 or more studies.

*Effect of different concentrations of DAP.* Initially, different concentrations of DAP were added to the culture flasks containing RSSE virus with Sarcoma 180 as the tissue source to determine whether the agent was viricidal. It was found that a $10^{-3}$ M concentration inhibited the multiplication of virus only. At a $10^{-2}$ M concentration, both tumor growth and virus multiplication were stopped, whereas at a concentration of $10^{-5}$ M neither was affected. As a result of the data summarized in Table I, a concentration of $10^{-3}$ M of DAP was chosen for more detailed study of virus multiplication *in vitro*.

*Effect of adding DAP after virus.* When DAP is added to the tissue culture 24 hours after the virus inoculation, at the time when the tissue is infected and the virus is actively multiplying, the inhibitory effect is marked. As shown in Table II, the virus titered $10^{-6.5}$ when the DAP was added. Twenty-four hours later the titer had fallen, indicating that viral multiplication had halted. Only a small amount of virus could still be detected after 48 hours.

*Recovery of virus from DAP-treated tissue cultures.* Attempts were made to recover the virus after it had been in contact with DAP. When fresh tumor tissue was added to the treated cultures daily, or when the washed, previously infected tissue was placed in fresh Tyrode's solution with horse serum, no virus could be detected by intracerebral inoculation in mice. This was the case even though from 66% to 100% of the tissue was viable on bioassay and virus was present up to 48 hours. Clearly, the effect of DAP on the virus was irreversible under the circumstances employed.

*Effect of adenine and guanine on the viricidal action of DAP.* Inhibition of the growth of *L. casei* by DAP has been reported(6). The inhibition could be reversed by either adenine or guanine at low concentration of DAP. The effect was reversed by adenine but not by guanine at higher concentrations. The inhibition of vaccinia virus *in vitro* by DAP is prevented by adenine in equimolar concentration, but guanine is ineffective in this system(12). A similar effect has been observed in the *in vitro* inhibition of RSSE multiplication by DAP. When added to the tissue cultures simultaneously with DAP and RSSE, adenine, in equimolar concentrations, prevented the inhibitory action of the compound, but guanine failed to do so. The results of a typical experiment are presented in Table III.

Twenty-four hours after the virus had been exposed to DAP, equimolar concentrations of adenine and of guanine were added to each of 2 sets of culture flasks. As shown in Table IV, within 48 hours after the addition of adenine, the virus began to recover its ability to multiply. Guanine, however, proved to be ineffective in reversing the inhibitory effect.

*Effect of 8-azaguanine.* Since DAP was active in inhibiting the multiplication of RSSE, the effects of some structurally related compounds were ascertained. The results

13. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

TABLE III. Effect of Adenine and Guanine on the Inhibitory Action of DAP in Tumor Tissue Culture.

| | Hr in tissue culture | | | | | |
|---|---|---|---|---|---|---|
| | 24 hr | | 48 hr | | 72 hr | |
| | % growth | Titer* | % growth | Titer | % growth | Titer |
| S-180 | 100 | — | 100 | — | 100 | — |
| + virus | 66 | 5 | 30 | 4.5 | 10 | 3.5 |
| + DAP | 100 | — | 83 | — | 0 | — |
| + virus | 100 | 3.5 | 66 | 1 | 12 | NV† |
| + Aden. | 100 | — | 83 | — | 0 | — |
| + Aden. + virus | 83 | 5 | 66 | 5.5 | 83 | 3 |
| + Guan. | 100 | — | 66 | — | 33 | — |
| + Guan. + virus | 66 | 3.5 | 100 | 1.5 | 0 | 10-0‡ |

* Expressed as the reciprocal of $LD_{50}$.
† NV indicates no virus detected.
‡ 1 of 3 mice inoculated with undiluted supernatant died of encephalitis.

TABLE IV. Partial Reversal of the Action of DAP on Multiplication of RSSE in Tumor Tissue Culture by Adenine.

| | Titers* | | |
|---|---|---|---|
| | 24 hr | 48 hr | 72 hr |
| S-180 + virus | 6.5 | 7 | 5.5 |
| + DAP + virus | 2 | NV‡ | NV |
| + virus + aden.† | — | 1.5 | 3.5 |
| + guan.† | — | .5 | .75 |

* Expressed as the reciprocal of $LD_{50}$.
† Adenine and guanine added 24 hr after 2,6-diaminopurine.
‡ NV indicates no virus detected.

obtained with 8-azaguanine‡ are typical and indicate that the compounds have no power to inhibit the multiplication of the virus. At the concentration used ($10^{-3}$ M), 8-azaguanine exerted little effect on the growth of the sarcoma.

*Tissue other than Sarcoma 180.* In all the preceding experiments, Sarcoma 180 was the source of tissue. However, tumor from the culture flasks, both control and experimental, did not always give 100% growth on bioassay. In this system, one could not be certain that DAP itself had no effect on the viability of the tumor tissue. It was therefore necessary to employ a tissue that would be less sensitive to DAP than is the Sarcoma 180. It has been found that Carcinoma 1025(14) in tissue culture is relatively resistant to the action of DAP. Accordingly the studies were repeated with this tumor. Minced mouse and chick embryonic tissues were also used. The results are presented in Table V, including those obtained employing DAP and RSSE in a tissue-free medium. It is evident that the DAP had no effect on the virus *per se*, but does interfere with its ability to propagate in any form of tissue culture employed.

*Discussion.* The activity of 2,6-diaminopurine in the inhibition of Russian Spring Summer Encephalitis virus in cultures of normal and neoplastic tissues has been established. The mechanism of the action is still not clear. It is only when virus multiplies in the tissue that inhibition takes place. In the rat DAP has been shown to be a precursor of nucleic acid guanine(15), and to be highly toxic. Hence the viral inhibitory effect may be related to a block at some stage of nucleic

TABLE V. Effect of DAP on Multiplication of RSSE in Different Tissues.

| | Titers* | | |
|---|---|---|---|
| | 24 hr | 48 hr | 72 hr |
| S-180 + virus | 5.5 | 6.5 | 3 |
| + DAP | 3.5 | 1.5 | NV† |
| 1025 + virus | 7 | 6 | 6 |
| + DAP | 3 | 3.5 | 4.5 |
| Mouse embryo + virus | 5.5 | 7 | 7 |
| + DAP | 5.5 | 5 | 5 |
| Chick embryo + virus | 7 | 6.5 | 5.5 |
| + DAP | 5 | >6 | 3.5 |
| No tissue + virus | 2 | .5 | NV |
| + DAP | 2 | .5 | NV |

* Expressed as the reciprocal of $LD_{50}$.
† NV indicates no virus detected.

‡ Obtained through the courtesy of the American Cyanamid Co.

14. Berger, R. E., personal communication.

15. Bendich, A., and Brown, G. B., *J. Biol. Chem.*, 1948, v176, 1471.

acid metabolism. Since the virus in a tissue-free medium is not affected by DAP, the action is not a direct one. The data presented suggest that DAP interferes with some metabolic process in tissue related to nucleic acid synthesis, and one that is necessary for virus multiplication.

Biesele(16) has reported that DAP, in the concentration used in these studies, preferentially injures Sarcoma 180, as compared to mouse embryonic skin, in a roller tube tissue culture. Hence, it is necessary to exclude the possibility that the results presented here were due to death of the tumor cells caused by DAP and consequent removal of any medium capable of permitting viral multiplication. Four facts provide evidence against this possibility. (1) Although on bioassay most of the tumor is killed in the virus-infected control flasks without DAP, nevertheless the virus can be recovered in high titer. Hence very slight tissue growth permits viral multiplication. Extensive tissue growth in the DAP-treated flasks yielded no virus. (2) Such virus as may survive 48 hours of contact with DAP has lost its capacity to multiply when put into fresh media containing actively growing tissue. (3) Although the Carcinoma 1025 is relatively resistant to the action of DAP, inhibition of viral multiplication also occurs in this tissue. (4) DAP inhibits RSSE *in vivo*(17).

*Summary and conclusions.* (1) The multiplication of the virus of Russian Spring Summer Encephalitis in tissue culture is inhibited by 2,6-diaminopurine. (2) The action can be blocked as well as reversed by the addition of equimolar concentration of adenine. Guanine is not effective.

16. Biesele, J. J., Berger, R. E., Wilson, A. T., Hitchings, G. H., and Elion, G. B., *Cancer,* 1951, v4, 186.

17. Moore, A. E., and Friend, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 153.

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## Effect of 2,6-diaminopurine on the Course of Russian Spring-Summer Encephalitis Infection in the Mouse.* (19005)

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The compound 2,6-diaminopurine (DAP) has been shown(1) to be concerned in the synthesis of polynucleotide guanine. It also has been proved to have certain types of biological activity. It prolongs survival time of mice with transplantable leukemia(2). It is seriously injurious to the cells of Sarcoma 180 in tissue culture(3) in concentrations which have no effect on normal embryonic cells growing at the same rate although it exerts no demonstrable effect on the same tumor *in vivo*(4). It has also been shown to have an injurious action on the hematopoietic system of dogs(5) and swine(6) and chick embryo(7). It is known to inhibit the multiplication of vaccinia virus *in vitro*(8) while having no effect *in vivo*. The demonstration that it inhibits multiplication of Russian Spring Summer Encephalitis virus (RSSE) in

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

1. Bendich, A., *et al., J. Biol. Chem.,* 1950, v185, 423.

2. Burchenal, J. H., *et al., Cancer,* 1949, v2, 119.

3. Biesele, J. J., *et al., Cancer,* 1951, v4, 186.

4. Stock, C. C., *et al., Annals of N. Y. Acad. Science,* 1950, v52, 1360.

5. Phillips, F. S., and Thiersch, J. B., *Fed. Proc.,* 1949, v8, 325.

6. Cartwright, G. E., *et al., J. Lab. and Clin. Med.,* 1950, v35, 518.

7. Karnofsky, D. A., *et al.,* PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 447.

8. Thompson, R. I., *et al., Science,* 1949, v110, 454.