TABLE II. A Comparison of Biological Activity of Chlorguanide, Certain Pteridines and Diaminopyrimidines.

Activity against		Chlor-guanide	Pteridine, 2,4-diamino-6,7-diphenyl	Pyrimidines: 2,4-diamino-5-aryloxy	Pyrimidines: 2-arylamino-4-alkylamino-alkylamino
P. gallinaceum in vivo	Potentiated by sulfonamides	Yes(6)	Yes(1)	Yes(2)	No(2)
	Inhibited by folic acid	" (7)	" (1)	" (2)	—
	Cross-resistant to chlorguanide	—	" (5)	No(5)	No(10)
Lactobacillus *in vitro*	Potentiated by sulfonamides	—	" (9)	—	—
	Inhibited by folic acid	Yes(8)	" (9)	Yes(8)	—

group in the 5-position is decreased by substitution on the 2- and/or 4-position amines. The antimalarial activity of the diaminopyrimidines with no or an alkyl substitution on the 5-position is dependent on substitutions on the 2- and 4-position amines. These findings accentuate the differences noted in Table II.

Summary. A chlorguanide-resistant strain of *Plasmodium gallinaceum* was cross-resistant to 2,4-diamino-6,7-diphenylpteridine. The same chlorguanide-resistant strain was not cross-resistant to 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine. Chlorguanide and 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine were not synergistic in their antimalarial activity.

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Chemotherapeutic Effects of a Naphthoquinonimine on Infection of Mice with Columbia-SK Group of Viruses.* (18717)

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Schnitzer, Buck, and Steiger(1) described the chemotherapeutic effects obtained with a certain naphthoquinone derivative (compound Ro 2-3532, *i.e.* 3-(1,4-dihydro-3-hydroxy-4-oxonaphthylideneamino) N - [β-(β-hydroxyethylamino)-ethyl] benzamide) in mice infected with Col-SK or MM virus. This substance is not soluble in water but may be rendered highly water-soluble through coupling with a reducing agent such as sodium bisulfite. This process is reversible by oxidation. The solubilized compound is designated as Ro 2-3532/1. Through cooperation with the above workers we were able to examine the chemotherapeutic properties of this substance in this Laboratory. The results are presented here. In chemotherapeutic studies of a neurotropic viral disease it is obviously of advantage to limit the experimental approach to efforts designed to restrain the virus from entering the central nervous system rather than to attempt to influence is intraneural spread. Hence, the Columbia-SK type of virus was chosen for this work. As is well-known, Col-SK virus—in contrast to Yale-SK, Lansing, or MEF virus—possesses a high and constant degree of infectivity in rodents by various peripheral channels of inoculation.

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1. Schnitzer, R. J., Buck, M., and Steiger, N., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 182.

Equally important is that this virus is also excreted through the alimentary tract in orally infected mice.

Materials and methods. In most of the experiments Col-SK virus was used as the test strain. On several occasions, however, the drug was also examined for its ability to prevent the infection of mice with two antigenically related viruses, *i.e.* EMC virus and F virus. The latter virus was recently isolated by Koch(2) as a primary rodent-pathogenic agent from the spinal fluid of a fatal case of undiagnosed human illness, characterized by vague central nervous system symptoms and definite interstitial myocarditis. The virus was subsequently identified by Bieling(3), through immunological methods, as belonging to the Col-SK group of viruses. We are indebted to Dr. R. Bieling, Marburg, for kindly sending us a sample of the F strain.† The choice of all 3 viruses was motivated by the fact that these agents, despite their common serological and biological properties, possess in rodents different endpoints of potency readily demonstrable in carefully conducted titration experiments. It was realized that in such chemotherapeutic experiments the reliability of the results may be decisively affected by possible variations in the purity of the drug as well as by possible fluctuations in the virulence of the virus. Chemical uniformity could be assured by using only pre-tested lots of the drug and making up the desired solutions of the soluble compound in distilled water, immediately prior to injection, so as to prevent oxidative deterioration. It is more difficult to satisfy the second consideration adequately. We decided to use in our tests only viral brains which had been harvested on the day of the experiment from carefully selected paralyzed passage mice infected intracerebrally 48 hours before. While pools of concentrated viral brain suspensions frozen and stored in dry ice, are acceptable as seed within certain limits (Schnitzer *et al.*)(1) previous experience has convinced us that such preparations not only contain appreciably less extractable infectious material, but that further deterioration of infectivity may occur irregularly and unpredictably over a period of time. That technical differences in the preparation of viral suspensions play an important part in determining their ultimate potency is also suggested by reference to the wide range in LD_{50} endpoints recorded by various workers who titrated Col-SK or MM virus intraperitoneally in mice. This range extends from $<10^{-8}$(4) or $<10^{-7.9}$(5) to values lying between 10^{-5} and $10^{-6.6}$(6-8) or between 10^{-6} and 10^{-7}(9).

Experimental work with the drug proceeded along the following basic lines of investigation: (1) evaluation of the protective effects obtainable when a single standard dose of drug (5 mg in 0.5 ml volume) is given intraperitoneally a few minutes following infection with 0.1 ml of various virus dilutions by the same route; (2) evaluation of the protective effects obtainable when a single dose of drug is given subcutaneously (5 mg) or intravenously (1 mg) a few minutes following intraperitoneal infection with 0.1 ml of various virus dilutions. Swiss albino mice weighing 9-12 g were used. To ensure an even distribution of the virus in the experimental animals, large numbers of mice were first infected at random with each successive virus dilution and then divided into equal smaller groups for subsequent treatment or for controls. The results obtained with the 3 viruses appear in

2. Koch, F., *Z. Kinderhk.*, 1950, v68, 328.

3. Bieling, R., *Transact. Austrian Soc. Microbiol.*, Salzburg, 1950.

† In preliminary experiments in this laboratory the F virus has proved to be definitely pathogenic for rhesus monkeys in serial passage, producing flaccid paralysis and typical poliomyelitic lesions in the spinal cord.

4. Jungeblut, C. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 534.

5. Weil, M. L., and Warren, J. PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 534.

6. LoGrippo, G. A., Earle, D. P., Brodie, B. B., Graef, I. P., Bowman, R. L., and Ward, R., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 528.

7. Cox, H. R., Koprowski, H., Moyer, A. W., Sharpless, G. R., and Wong, S. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 530.

8. Francis, Th., and Brown, G. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 535.

9. Findlay, G. M., and Howard, E. M., *Br. J. Exp. Path.*, 1950, v31, 45.

TABLE I. Effect of a Single Dose of Compound Ro 2-3532/1 Administered at Time of Intraperitoneal Infection of Mice with Col-SK, F, or EMC Virus.

Virus	Route of inj. Drug	Route of inj. Virus	Exp.	Virus dilutions 10^{-5} (%)	10^{-6} (%)	10^{-7} (%)	10^{-8} (%)	10^{-9} (%)	10^{-10} (%)	10^{-11} (%)	10^{-12} (%)
Col-SK	i.p.	i.p.	I-VII	16/16(100)	41/76 (54)	19/72 (26)	5/56 (9)	1/87 (1)	0/19 (0)		
	s.c. or i.v.	"		20/20(100)	19/20 (95)	15/20 (75)	8/30 (27)	0/20 (0)	0/19 (0)		
	Controls	"	Totals	30/30(100)	70/70(100)	68/70 (97)	62/69 (89)	37/60(62)	13/50(26)	1/21 (5)	
F	i.p.	"	I-IV	28/29 (96)	22/30 (73)	5/30 (17)	2/29 (7)	0/29 (0)	0/29 (0)	0/19 (0)	
	s.c.	"		10/10(100)	8/10 (80)	12/20 (60)	5/20 (25)	2/18(11)	0/20 (0)		
	Controls	"	Totals	30/30(100)	30/30(100)	40/40(100)	40/40(100)	29/40(72)	18/40(45)	7/40(17)	0/10(0)
EMC	i.p.	"	I-II	19/20 (95)	13/20 (65)	8/20 (40)	0/10 (0)	0/10 (0)			
	s.c.	"		20/20(100)	15/20 (75)	11/20 (55)	2/10 (20)	0/10 (0)			
	Controls	"	Totals	20/20(100)	16/20 (80)	13/20 (65)	5/20 (25)	1/9(11)			

Numerator = No. of mice paralyzed; denominator = No. of mice infected.
Dose of drug = 0.5 ml of 1% sol. (5 mg) i.p. or s.c.; 0.5 ml 0.2% sol. (1 mg) i.v.; dose of virus = 0.1 ml virus dilutions i.p.

Table I. Quantitative evaluation of the data is given in Fig. 1.

It will be seen from Table I that a single dose of 5 mg of the drug administered intraperitoneally, was sufficient to protect a high percentage of the mice against infection with multiple paralytic doses of Col-SK virus injected simultaneously by the same route. Experiments in which the virus was injected intraperitoneally, but the drug was injected subcutaneously or intravenously, showed likewise definite protection, though inferior to that obtainable when both were given by the same route. This protection could be demonstrated to a similar degree in repeated experiments. No protection whatsoever was observed in mice which had been infected by the intracerebral or intramuscular route even when minimal doses of virus and maximal doses of drug were injected simultaneously at the same portal of entry. Experiments with orally infected mice fed a diet containing the drug in 2% concentration gave inconclusive results in repeated tests. Work is in progress to determine whether the drug has any effect on the fecal excretion of the virus. A clear-cut protection against multiple paralytic doses of virus was also demonstrable when the drug was used in mice infected intraperitoneally with the F virus. Again, these effects could be elicited when drug and virus were injected by the same or by separate peripheral routes. In the case of EMC virus, the effect of the drug under corresponding experimental conditions was much less marked, even when drug and virus were given by the same route. When a precise comparison of the protective action of the drug against the 3 viruses is made and the data are analyzed mathematically (Fig. 1), it will be found that the drug protected evenly against at least 1000 LD_{25}, LD_{50} or LD_{75} of Col-SK and F virus, whereas the protection observed against EMC virus extended only from 5 to about 10 LD_{25}, LD_{50} or LD_{75}.

Attempts were made in a few experiments to determine the effective quantitative range of the drug against infection with variable doses of Col-SK virus and also to investigate the time limits following infection at which drug treatment still prevented paralysis and

TABLE II. Effect of Intraperitoneal Injection of Compound Ro 2-3532/1 on Intraperitoneal Infection of Mice with Col-SK Virus.

Virus Dilutions	Relation between dose of drug and protection Dose of drug given at time of infection				Relation between interval of time and protection Interval between infection and inj. of 5 mg of drug				
	2.5 mg	1.25 mg	0.5 mg	0.25 mg	15 min.	30 min.	60 min.	120 min.	Controls
10^{-7}	3/20	1/10	8/20	10/20	11/19	10/18	11/20	17/20	30/30
10^{-8}	0/20	2/10	6/19	7/20	1/17	0/17	1/19	4/20	25/29
10^{-9}	0/18	0/10	1/19	4/19	0/15	0/19	0/13	4/20	14/30

death. In all these tests drug and virus were given by the same route, *i.e.* intraperitoneally. The results are shown in Table II. It appears from Table II that significant protection was still evident with quantities of drug ranging from one-half (2.5 mg) to one-tenth (0.5 mg) of the standard dose, provided the amount of virus did not exceed 10 LD_{50}. As far as the time limits of drug efficacy are concerned, the results suggest that the standard dose of drug (5 mg) still exerted a significant degree of protection up to and including 1 hour following intraperitoneal infection with 10 LD_{50} doses of virus. The above data are based on a comparatively small number of animals; therefore, any conclusions on these important points must be tentative at present.

It seemed next of interest to determine whether or not drug-protected animals had acquired any immunity towards reinfection with Col-SK or with F virus. In these tests a total of more than 300 surviving drug-treated mice were challenged, 2 weeks after their initial injection, with a moderate dose (10^{-5} *i.p.*) of the homologous virus. Suffice it to say, that all these animals, without exception, developed paralysis like the corresponding controls. Thus, there was no question that drug treatment had failed to leave the protected animals with any appreciable immunity. The following experiments deal with efforts to analyze the probable mode of action of the drug. From the outset, it seemed unlikely that the drug should be capable of modifying the contact between virus and susceptible cells. Experiments in which the

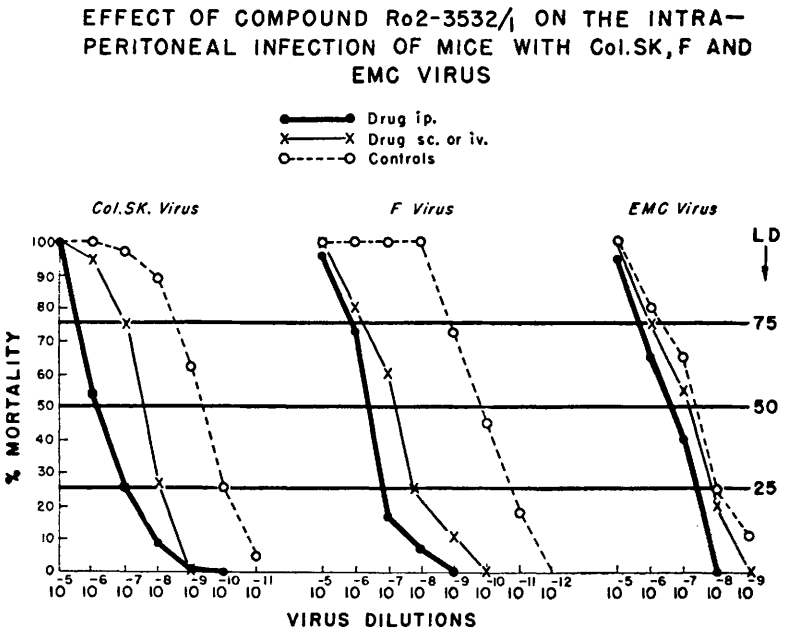

FIG. 1.

drug was used to study a possible hemagglutination-inhibitory action on sheep red cells failed to provide a clear answer, however, because the drug exercised a marked destructive effect upon the erythrocytes. Experiments were then designed to investigate the fate of the virus in the drug-treated animals. Groups of mice which had received small doses of virus (10^{-6} or 10^{-7}) and the standard dose of drug (5 mg), both by intraperitoneal injection, were killed at intervals ranging from 2 hours to 24 hours. The peritoneal cavity was washed out with 1 ml of saline and the washings were transferred to new mice by intramuscular injection. At the same time 0.25 ml of blood was collected from the killed animals and injected intramuscularly into new mice. Control mice, which had received virus but no drug, were killed at the same intervals and their peritoneal washings and blood were transferred to new mice in the same way. The results of several tests may be summarized by saying that in the case of untreated control animals live virus could be recovered regularly at all intervals from the peritoneal cavity and at the later stages also from the blood, whereas in the case of the drug-treated mice virus was only sporadically found in the peritoneal cavity at the early intervals and the virus either failed to appear later in the blood stream or the viremia was definitely delayed. Our data are in essential agreement with the basic information collected by Evans and Chambers(10) on the mechanism of infection with MM virus following peripheral infection of hamsters. They also offer a clue as to why the drug-treated surviving animals had remained fully susceptible to reinfection.

The sum total of these experiments seemed to suggest that the drug exercised a prompt and profound antiviral action *in vivo* as the result of direct contact with the virus, but the data, by themselves, are hardly sufficient to prove the point. Unfortunately, the problem cannot be approached to complete satisfaction by studying the action of the drug on the virus in *in vitro* prepared mixtures because it is difficult or impossible to separate virus from the drug by physical methods such as dialysis, sedimentation or filtration. However, a simple method which seemed adequate enough to yield reliable information was utilized in some experiments. It consisted in setting up mixtures of 0.5 ml virus dilutions (10^{-2} or 10^{-4}) in combination with 0.5 ml drug solutions (5% or 0.5%), letting these mixtures stand in the icebox for various lengths of time (0 minutes, 30 minutes, 60 minutes) and then making ten-fold serial dilutions of these mixtures which were carried into final concentrations of the drug beyond its known effective range but which were still within the range of expected viral infectivity. These mixtures were then injected, in 0.1 ml volume, into mice by the intramuscular route to test for the presence of viable virus. Control mixtures were set up with distilled water and handled the same way. The results showed that a virus dilution of 10^{-2} was not influenced by a 5% drug concentration, except after 60 minutes contact, when some mice survived with 10 LD_{50} doses of virus (10^{-7}); however, a virus dilution of 10^{-4} in combination with a 0.5% drug concentration apparently had suffered a definite loss of infectivity at the 30 and 60 minute intervals since mice survived with 100 LD_{50} doses of virus (10^{-6}, 10^{-7}). Curiously enough, the amount of virus which could be suppressed *in vitro* was of the same order as that which responded to *in vivo* administration of the drug. These results would indicate that comparatively small doses of the drug are capable of inactivating, in a more or less fixed ratio, moderate though not large amounts of virus upon direct contact *in vitro*.

Discussion. It appears from our data that we were able fully to confirm the observations reported by Schnitzer *et al.*(1), namely, that reproducible chemotherapeutic effects may be obtained in mice with compound Ro 2-3532/1 following intraperitoneal infection with multiple paralytic doses of Col-SK virus. These protective effects, however, being limited to the extraneural phase and early intervals of the infection are probably more chemoprophylactic than chemotherapeutic in the true sense of the word. Our experiments show, in addition, that the drug exerts a similar definite

10. Evans, C. A., and Chambers, V. C., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 436.

protective action on the corresponding infection with F virus. On the other hand, the results obtained against infection with EMC virus are so slight that one must question the significance of such minimal protection. The magnitude of the chemotherapeutic effects against Col-SK and F virus seems to be quite constant, depending critically upon the quantitative relationships between virus and drug and approximating occasionally the "all or nothing" type of a chemical reaction. This remarkable regularity in the chemotherapeutic system makes it possible to predict, with fair accuracy, the outcome of an experiment. Expressed in LD_{50} of Col-SK virus, which the drug-treated animals tolerated without developing paralysis, the protection obtained in our experiments was significantly in excess of that recorded by the original investigators. Obviously, this difference was caused by the use of more virulent virus preparations in our hands. It is difficult, however, to explain satisfactorily the fixed maximum level of virus which the drug brought under control, irrespective of the prevailing endpoints of infectivity. One possible assumption would be that during storage of frozen virus a certain proportion of active virus particles is converted into inactive virus particles, both functioning as physical or chemical substrate in association with the drug. A similar observation relating to discordance between viral mass and viral infectivity—albeit within a much lower order of concentration—was recently reported for the Lansing virus(11). The absence of any convincing action of the drug on EMC virus, which is in marked contrast with its pronounced action on Col-SK and F virus, requires some comment. Since EMC and Col-SK virus are antigenically very closely related one may have to look for physical rather than chemical differences between the two viruses for an explanation. Measurements of size for the three viruses in this group, *i.e.* Col-SK, MM and EMC, have been estimated as follows by various authors: Col-SK virus 9-14 mμ (by ultrafiltration), 20-35 mμ (by electronmicroscopy), 130 S (sedimentation constant by ultracentrifugation); MM virus 11-16 mμ (by ultrafiltration), 10-20 mμ (by electronmicroscopy), 125 S (sedimentation constant by ultracentrifugation); EMC virus 35 mμ (by electronmicroscopy), 153 S (sedimentation constant by ultracentrifugation). No corresponding figures are available for the F virus. The data suggest that EMC virus is slightly larger than Col-SK or MM virus, a fact which may be of some significance in connection with a possible adsorption of the drug on the virus particle.

When one contrasts the chemotherapeutic effects produced by compound Ro 2-3532/1 with the protective effects obtainable through the use of Burnet's RDE (receptor destroying enzyme) preparations from *Vibrio cholerae* cultures(12) against the same infection(13, 14), it becomes evident that one is confronted with very different mechanisms of operation. In the case of the drug, all available evidence points to the fact that such protective action as is demonstrable in these tests lasts only as long as the virus has not begun to multiply on susceptible cells. In other words, since there is nothing to suggest that the drug will effectively compete with virus attachment to cells, or retard intracellular growth, one is led to the assumption that infection is aborted by some direct action of the compound on "resting" virus which is in process of transit from a suitable peripheral portal of entry to its ultimate locus of multiplication. In keeping with this assumption is the rapid sterilization of the infected primary site of infection by the drug and the absence of any demonstrable immunity in the drug-treated surviving animals. In other words, one is dealing essentially with a disinfectant, but a disinfectant which has the unique ability of rendering virus non-infectious *in vivo* on an apparently quantitative basis, without causing injury to tissues and without impairment of its antiviral action by body fluids. In the case of RDE treat-

11. Bennett, B. L., and Salk, J. E., *J. Immunol.*, 1951, v66, 277.

12. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Br. J. Exp. Path.*, 1946, v27, 228.

13. Verlinde, J. D., and De Baan, P., *Ann. Inst. Past.*, 1949, v77, 632.

14. Jungeblut, C. W., *Bull. N. Y. Acad. Med.*, 1950, v26, 571.

ment, the inhibition of hemagglutination *in vitro* suggests a blocking action on cell receptors rather than any form of direct interaction between Col-SK virus and RDE principle. This concept is further supported by the initial presence of live virus at the portal of entry of the infected animal which has received RDE by the same route, as well as by the fact that a substantial degree of immunity to reinfection can be demonstrated in a majority of the surviving animals. It seems plausible that further experiments in which the cell-protective action of a potent enzymatic principle, such as RDE, is combined with the direct antiviral action of a colloidal dye of the Ro 2-3532 type, should permit the realization of even more powerful chemotherapeutic effects over a longer range of the infectious process.

Summary and Conclusions: The chemotherapeutic effect of a certain napthoquinonimine (compound Ro 2-3532/1) was studied on the peripheral infection of mice with Col-SK virus, F virus and EMC virus. It was found that a well tolerated single dose of 5 mg of the drug, administered intraperitoneally, was sufficient to protect a high percentage of the mice against infection with multiple paralytic doses of Col-SK or F virus injected simultaneously by the same route. Experiments in which the virus was injected intraperitoneally but the drug subcutaneously showed likewise a definite degree of protection though inferior to that observed when both were given by the same route. Significant protection could also be obtained up to and including 1 hour after intraperitoneal infection with small doses of virus. Experiments in which the drug was tested for its chemotherapeutic action against EMC virus showed only a minimum degree of protection considered to be insignificant. Some attempts were made to analyze the probable mode of action of the drug on Col-SK virus. It is concluded that protection occurs not because of cell-receptor blockade but that the virus is rendered non-infectious by some form of direct interaction between virus and drug.

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Chemotherapeutic Effect of 2-hydroxy-1,4-naphthoquinonimine on Infections of Mice with Col SK Virus. (18718)

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These studies were prompted by the observations of Woods and Rusoff(1) and Bieter and Wright(2) according to which the tetrazo dyestuff trypan red exerted a marked prophylactic effect on the intra-abdominal infection of mice with MM virus. These findings could not be reproduced in our experiments thus confirming the work of Hammon, Aird and Sather(3). Nevertheless, experimental work with various azo dyestuffs was included in the screening program with Col SK and MM virus which has been carried out in this laboratory. These azo dyestuffs, most of them derived from sulfonamides, also failed to show any protective effect on the infection with the above viruses. It was, however, observed that compounds of a series of 2-hydroxy-1,4-naphthoquinonimines possessed more or less marked activity, if administered to mice previously infected with MM- or Col SK-virus. A group of 160 compounds of this type has been tested during recent years and 67 members of the series were found to be active in the viral infection. In the present note we shall limit ourselves to the description of the chemotherapeutic property of one representa-

1. Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, v82, 297.

2. Bieter, R. N., and Wright, H. N., *Poliomyelitis* 1949, p. 276. (J. B. Lippincott Co., Philadelphia)

3. Hammon, W. McD., Aird, R. B., and Sather, G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 511.