enpox remain those defined by previous observers: (1) Size of the elementary bodies, (2) their number inside cells and lying free, (3) their distribution. The method was found useful in the differential diagnosis of variola from varicella. Its application for establishing a positive diagnosis of chickenpox presents certain drawbacks: (a) Elementary bodies are not found in general in chickenpox vesicles 4-7 days old, and sometimes even in more recent lesions. (b) Varicella elementary bodies seem to lie near the limit of visibility, and their detection requires an effort on the part of the microscopist.

It should also be noted that the Guarnieri bodies in human skin and other tissues are Feulgen positive. Furthermore the Feulgen technic seems to be a method of choice for the study of Guarnieri bodies in tissue sections(8). The method was also applied with good results to smears and sections of chorioallantois cultures of smallpox virus. Rickettsiae could also be stained and differentiated from extraneous matter by the same technic.

Summary. A method for staining elementary bodies of smallpox is described. The method consists of the application of Feulgen's method for desoxyribonucleic acid to smears made of scrapings of the bottom of skin lesions. The procedure seems to afford the means for a quick diagnosis of smallpox, and allows differentiation of virus particles from extraneous material.

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Reversal of Aminopterin Inhibition in the Chick Embyro with Desoxyribosides.* (17818)

ESMOND E. SNELL AND W. W. CRAVENS.

From Departments of Biochemistry and Poultry Husbandry, University of Wisconsin, Madison.

Aminopterin (4 aminofolic acid, 4-aminopteroylglutamic acid)(1) is a highly toxic substance for certain microorganisms(1,2), mice (3), guinea pigs(4), chicks(5), rats(5,6), and man(7). In certain microorganisms (*e.g.* *Streptococcus faecalis*)(1) and to a lesser extent in certain animals (*e.g.* mice)(3), its inhibitory effects are prevented by relatively high amounts of folic acid; for this reason, and because of its close structural similarity to the vitamin, aminopterin is considered almost universally to act *in vivo* as an antagonist to folic acid. Especially in animals, however, and at relatively high levels of administration in all organisms, it has not proved possible to counteract the toxic effects of aminopterin with folic acid(3,7), *i.e.* the antagonistic relationship is not fully competitive. If, under these circumstances, the inhibited reaction could be made non-essential by furnishing its product, alleviation of the

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inhibition might be expected(8,9). Nothing is known of the processes which folic acid antagonists inhibit in animal tissues. In lactic acid bacteria, however, folic acid is thought to be essential for synthesis of thymine and purine bases, since a mixture of these substances replaces folic acid in the nutrition of several organisms(10,11), and counteracts growth inhibition of *Lactobacillus casei* by methylfolic acid(9).

The present studies were undertaken to determine the effect of aminopterin on the developing chick embryo, and by testing the effectiveness of various compounds in reversing any observed effects, to throw some light on the mechanism of action of this inhibitor in animal tissues. Since these studies were initiated or, Karnofsky, *et al.*(12) have reported that aminopterin inhibits development of the chick embryo. High levels of folic acid (20 mg per egg) did not counteract these inhibitory effects.

Experimental. The procedures followed in injecting solutions and incubating the eggs were identical to those described by Cravens and Snell(13) in similar studies with desoxypyridoxine. All injections were in a total volume of 0.1 ml. At higher concentrations, where insolubility of aminopterin and folic acid required it, these compounds were dissolved and injected as their sodium salts.

Results. Data of Table I show the effect of injection of aminopterin prior to incubation on embryonic development. In summarizing these data, no attempt has been made to distinguish infertile eggs from those in which the embryo died prior to the appearance of blood. Definite blood formation was considered evidence that the embryo was at least in the second day of development at death. Control eggs showed a mortality of 11.7% at 0-1 day of development and the bulk of these embryos were infertile as judged by the appearance of the blastodisc (14). One hundred micrograms or more of aminopterin inhibited embryonic development completely. By gross examination, such eggs could not be differentiated from infertile eggs. As the amount of inhibitor was reduced, the age at death increased for a small percentage of the embryos. Ten micrograms per egg still produced death of all embryos at some stage of development before hatching; the highest amount injected which allowed any live chicks to hatch was 5 μg per egg. Even 2 μg per egg significantly increased mortality. These results demonstrate the extreme activity of aminopterin in inhibiting embryonic development. They agree well with those of Karnofsky *et al.*(12), who found the LD_{50} of aminopterin to be 3 μg per egg when injected on the fourth day of incubation. For subsequent work, 20 μg of aminopterin was chosen as a convenient amount which consistently produced 100% mortality, but allowed development to proceed sufficiently to permit detection of any favorable effects exerted by other factors. The effect of time of injection upon toxicity of this amount of the inhibitor was determined using 10 eggs per group. At three days of incubation the inhibitory effect is almost immediate, and 100% mortality still results, while at 6 days a delayed toxicity was evident which frequently produced death 2 to 6 days after the injection. Fifty per cent of these embryos eventually hatched. When injected at 9 or more days of incubation, this amount of the inhibitor is non-toxic.

The effects of several compounds injected in an attempt to counteract the inhibitory action of aminopterin are shown in Table II. All injections were made just prior to incubation. The compounds tested included folic acid, vitamin B_{12}, thymidine, hypoxanthine desoxyriboside, thymine, and hypoxanthine. An enzymatic digest of desoxyribonucleic acid,† and concentrates of the *Leuconostoc*

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TABLE I.
Effect of Aminopterin on Hatchability of Eggs.

Substance injected	Amt inj. μg	No. of eggs	% embryos dying at 0-1 day	2-5 days	6-20 days	% hatch
Aminopterin	1000	31	100			
"	500	15	100			
"	200	15	100			
"	100	15	100			
"	50	30	96.7	3.3		
"	20	115	73.9	22.6	3.5	
"	10	15	53.4	33.3	13.3	
"	5	30	36.7	23.3	16.7	23.3
"	2	15	26.7	13.3	6.7	53.3
Water (.1 cc)		129	11.6	6.2	11.6	70.5
None		128	11.7	4.7	2.3	81.3

citrovorum factor‡ were also tested. None of these substances showed any significant effects on hatchability or mortality at the levels tested when injected without aminopterin.

Folic acid, vitamin B_{12}, and concentrates of the *Leuconostoc citrovorum* factor (LCF) were ineffective in overcoming effects of the inhibitor, both when injected singly and together. Thymdine in amounts of one or 2 mg per egg partially counteracted the inhibitory effects of aminopterin. Not only were some live chicks obtained, but consistent and pronounced shift toward longer survival of those embryos which died was evident. Thymine at equivalent levels and at higher levels (not recorded) was completely without ecect. Although hypoxanthine desoxyriboside alone was ineffective in overcoming aminopterin inhibition, it exerted a markedly favorable effect when injected together with thymidine. Hypoxanthine plue thymidine was no more effective than thymidine alone; thymine plus hypoxanthine desoxyriboside was ineffective. This pronounced action of the desoxyribosides in counteracting the effects of aminopterin is underscored by the high hatch obtained when an enzymatic digest of purified desoxyribonucleic acid, prepared in such a manner as to contain the desoxyribosides, was injected. It is of interest that combinations of thymidine with either folic acid or LCF were not significantly more active than thymidine alone, however, when all 3 substances were injected together, an apparent improvement in hatchability resulted.

Discussion. Inhibition of growth by any antivitamin results from failure of one or more catalytic reactions for which the corresponding vitamin (or a coenzyme derived from it) is essential. It has been generally assumed in such cases(8,16), that a metabolically inactive complex is formed between antivitamin and a specific protein which prevents formation of a metabolically active complex between the vitamin and this protein. Where such an inhibitor-protein complex is readily dissociable, inhibition can be alleviated by sufficient amounts of the appropriate vitamin. Where it is relatively undissociated, no reason to expect a competitive type of reversal exists(16). In the latter case, growth should still be possible if the *products* of the blocked reaction are supplied in a utilizable form(8,9).

The results of the above experiments are most readily interpreted in terms of this hypothesis. Since the pronounced inhibitory effects of aminopterin upon growth of the chick embryo cannot be reversed by folic acid, it may be assumed that complexes formed between aminopterin and the folic acid receptors

† This digest was the $MgSO_4$ concentrate of enzymatically hydrolyzed desoxyribonucleic acid prepared by the method of Klein(15). It contains the mixed desoxyribonucleosides derived from the nucleic acid.

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‡ We are indebted to Dr. C. A. Baumann and to Dr. T. R. Wood for concentrates of this factor..

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TABLE II.
Effect of Various Compounds on the Inhibitory Action of Aminopterin.

Compound injected, amt.*	No. eggs	% embryos dying 0-5 days	% embryos dying 6-20 days	% hatch
None	128	16.4	2.3	81.3
Water (.1 cc)	129	17.9	11.6	70.5
Aminopterin, 20 μg	115	96.5	3.5	0
" "				
+ Folic acid, 1 mg	15	100	0	0
+ Thymidine, 1 "	49	77.6	12.2	10.2
+ " 2 "	9	77.8	11.1	11.1
+ Thymine, .52 "	10	100	0	0
+ Hypoxanthine desoxyriboside, 1 mg	10	100	0	0
+ LCF,† 33.3 μg	10	100	0	0
+ Vit. B_{12}, 1 μg	10	100	0	0
+ Thymidine, 1 mg + folic acid, .1 mg	20	85	10	5
+ " 1 " + " " .5 "	20	60	20	20
+ " 1 " and Hypoxanthine desoxyriboside, 2 mg	19	36.8	31.6	31.6
+ Thymidine, 1 mg and Hypoxanthine, 1.1 mg	10	90	10	0
+ Thymine, .52 mg and Hypoxanthine desoxyriboside, 2 mg	10	100	0	0
+ Thymidine, 1 mg + LCF† 66.6 μg	10	80	10	10
+ " 1 " + " 66.6 " + Folic acid, .5 mg	10	50	10	40
+ " " .5 " + LCF† 6.7 μg + Vit. B_{12}, 1 μg	10	100	0	0
+ Desoxyribonucleic acid digest, 5 mg	10	50	0	50

* Thymidine (m.p. 185-186°, $[\alpha]_D^{25°}$ (1% solution in NaOH) = 31.2°) and hypoxanthine desoxyriboside, $[\alpha]_D^{25°}$ (1% solution in water) = −20°) were isolated from sodium desoxyribonucleate. Other pure compounds were obtained from commercial sources.

† 66.6 μg of this concentrate was equivalent to approximately 1 ml of reticulogen (Lilly) in growth-promoting power for *Leuconostoc citrovorum*. Concentrates of this factor counteract the inhibitory effect of aminopterin for *L. citrovorum* (2).

are much less dissociable than those formed with folic acid itself. Since aminopterin inhibition is partially counteracted by thymidine, and more effectively counteracted by thymidine *plus* hypoxanthine desoxyriboside, these compounds would appear to be products, the formation of which is normally catalyzed by folic acid or its derivatives, and is prevented in the embryo by aminopterin at the levels employed here. The failure of hypoxanthine desoxyriboside to show any effect in the absence of thymidine is readily explicable if it is assumed that the first product to become limiting because of the aminopterin-induced folic acid deficiency is thymidine, the second, hypoxanthine desoxyriboside (or its nutritive equivalent), etc. The failure of mixtures of these two products completely to counteract aminopterin inhibition may result from partial failure of other metabolic reactions for which folic acid is required. This explanation of the observed facts implies that at concentrations of aminopterin considerably lower than 20 μg per egg, thymidine should be relatively more effective in counteracting aminopterin inhibition, while at considerably higher concentrations of aminopterin, mixtures of thymidine and hypoxanthine desoxyriboside might be relatively much less effective in counteracting inhibition than was found under the conditions used here. These implications have not been checked experimentally.

Previous work with bacteria had implicated folic acid in the synthesis of products related to thymidine and hypoxanthine desoxyriboside. In otherwise complete media, for example, the folic acid requirement of *Streptococcus faecalis* and of *Lactobacillus casei* is eliminated by addition of thymine (or

thymidine) and any one of several purine bases to the medium(11); these same products counteract the inhibitory effects of methylfolic acid for *L. casei*(9). The present work demonstrates the existence of closely similar mechanisms in higher animals. Differences in detail exist, however. Thus, while thymine and hypoxanthine are effective in the cited bacterial systems, they are ineffective in the animal system, where the corresponding desoxyribosides show activity. A somewhat closer parallel to the results obtained in the chick embryo is provided by a strain of *Escherichia coli,* in which the inhibition of growth produced by aminopterin was counteracted by thymidine, but not by vitamin B_{12}, thymine, hypoxanthine or hypoxanthine desoxyriboside(17). Similarly, with *Leuconostoc citrovorum,* thymidine counteracts aminopterin inhibition(2) and purine bases are required in the medium to obtain this response(18). With this organism, however, concentrates of the *L. citrovorum* factor are highly active in counteracting aminopterin inhibition(2). Current evidence indicates that this factor is a metabolically active form of folic acid(2,19); however, at the levels available for testing, it was ineffective in counteracting aminopterin inhibition of embryonic growth under our conditions. Larger amounts might prove effective.

The decreased toxicity observed for aminopterin with increasing age of the embryo is similar to that observed previously for desoxypyridoxine(13), and may have a similar explanation. It was pointed out earlier that the specific aminopterin-protein complex presumably formed in the embryonic tissue must be relatively undissociable. Since the amount of this specific protein (enzyme) present in the embryo increases with embryonic development, an increase in the amount of aminopterin required for inhibition, *i.e.* an apparent decrease in inhibitory activity, might be expected. Ackermann and Potter(17) have discussed the occurrence of inhibitors of this nature, where the magnitude of the inhibitory effect is dependent upon the amount of enzyme present in the system.

Summary. Aminopterin, injected prior to, or early in, the incubation period, is highly toxic to the chick embryo. Ten micrograms or more of this compound produce 100% mortality; 100 μg or more per egg completely inhibit embryonic development. The inhibitor becomes relatively less toxic as embryonic development progresses; at nine days or more, twenty micrograms is non-toxic. The same amount injected at three days results in almost immediate embryonic death.

Thymidine partially counteracted the inhibitory effects of aminopterin. A combination of hypoxanthine desoxyriboside and thymidine was more effective than thymidine alone. In the absence of thymidine, hypoxanthine desoxyriboside was without effect. An enzymatic digest of desoxyribonucleic acid also counteracted aminopterin inhibition partially. Thymine, hypoxanthine, folic acid, vitamin B_{12}, and concentrates of the *Leuconostoc citrovorum* factor, alone and in various combinations, were ineffective in counteracting aminopterin inhibition.

These experiments indicate that aminopterin acts in animal tissues in part by preventing synthesis of thymidine and one or more of the purine desoxyribosides, and that these compounds are immediately essential for embryonic growth. Since aminopterin is thought to act by creating a deficiency in folic acid, the necessity for folic acid in synthesis of these compounds in animal tissues is implied.

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