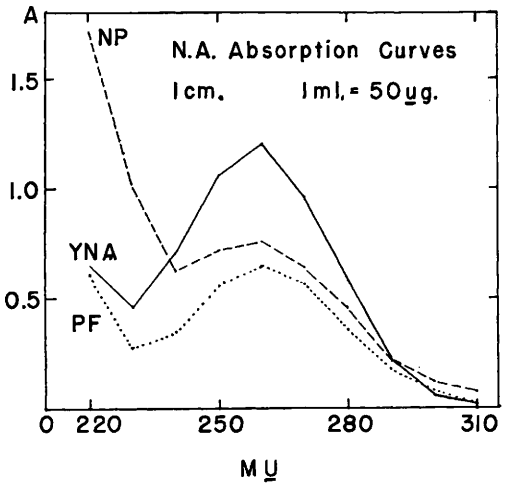

FIG. 1. Absorption spectra. NP = natural nucleoprotein complex; YNA = yeast nucleic acid; PF = protein-free endotoxin.

this physical procedure did not differ statistically among themselves in toxicity, but all of them were invariably more toxic than the product obtained by extraction of gonococcal cells at an alkaline pH. It is believed these methods will be useful in biological studies where cell-free, chemically unchanged fractions are desired. 2) Phenol-water mixtures are suitable for removal of the protein component from nucleoprotein complex of *N. gonorrhoeae* endotoxin. The protein-free endotoxin contains mainly polysaccharide and NA. The dried product is more toxic than those obtained by other methods. It dissolves readily in water resulting in a slightly opalescent solution. This fraction should be useful in studies where portein-free material is desired.

ADDENDUM: While this paper was in press we were able to remove all of the NA from the endotoxin. The method employed, chemical nature and other properties of the product will be described in subsequent publication.

We wish to thank Mr. Harold Russell for skillful technical assistance.

1. Tauber, H., Garson, W., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 669.

2. ———, *ibid.*, 1958, v97, 886.

3. Described by Cornfield, J., Mantel, N., *J. Am. Stat. Assn.*, 1950, v45, 181; Irwin, J. O., Cheeseman, E. A., *J. Hyg.*, 1939, v39, 574.

4. Westphal, O., Luderitz, O., and Bister, F., *Z. Naturforsch.*, 1952, v7, 148.

5. Dische, Z., *Methods of Biochem. An.*, 1955, v2, 321.

Received September 22, 1958. P.S.E.B.M., 1958, v99.

Effect of 4-Amino Folic Acid Antagonists on Biological Acetylations.* (24461)

WILLARD JOHNSON, GEORGE CORTE AND ROLAND JASMIN
Research Laboratories, Frank W. Horner Ltd., Montreal, Canada

Previous studies have shown that p-aminosalicylic acid (PAS) inhibits acetylation of isoniazid (INH), and produces a marked increase in plasma levels of INH when the 2 drugs are administered in combination(1). The synergistic effect of the INH-PAS combination observed in tuberculosis therapy has been attributed, in part, to inhibition of INH acetylation by PAS(2). It is conceivable that similar events may occur when multiple antimetabolite combinations are employed in cancer chemotherapy. Thus, apart from the anticancer effect contributed by each member of the combination by virtue of its antimetabolite activity, secondary factors may be present to further increase the over-all effect(3). For example: 6-amino-nicotinamide (6-AN), a carcinostatic antimetabolite (4-7) is a competitive inhibitor of arylamine acetylation, and is itself acetylated in rabbits(1,8). Administration of this substance in combination with a second antimetabolite having similar properties with respect to acetylation could give rise to increased plasma and cellular concentrations of either substance, due to inhibition of acetylation. The end result could be

* Presented in part before the Can. Fed. of Biol. Soc. Kingston, Canada, June 9, 1958.

TABLE I. Inhibition of Sulfanilamide (SA) and Isoniazid (INH) Acetylation in Pigeon Liver Extracts.

Inhibitor	Inhibitor conc., M/l × 10^{-5}	Inhibition, % SA	INH
A-methopterin (4-amino-N^{10}-methyl-PGA)	5	99	
	2	73	56
	1	62	
Aminopterin (4-amino-PGA)	25	89	
	5	58	
	4		50
Folic acid (PGA)	100	0	3
2,4-Diaminopteridine	"	0	8
2,4-Diamino-6,7-diphenylpteridine	"	50	50
Para-aminobenzoylglutamic acid (PABGA)	"		39

Incubation mixture: Potassium phosphate buffer, pH 7.4, .02 M; potassium acetate, .02 M; potassium citrate, .02 M; sodium ATP, .004 M; SA or INH, 8×10^{-4} M; pigeon liver extract, 1 ml (equivalent to 60 mg of acetone powder); acetylation inhibitors, as indicated; total volume, 3 ml. Incubation for 30 min. at 37°C.

augmented carcinostasis, increased host toxicity, or both. It was therefore of interest to test the effects on arylamine acetylation of various carcinostatic antimetabolites which may be employed in combination with 6-AN. In the course of these investigations it was found that A-methopterin and aminopterin markedly inhibited acetylation of sulfanilamide and INH in a non-competitive manner. This inhibition was due, in part, to an effect on acetate activation.

Materials and methods. Procedures employed in the sulfanilamide and INH acetylation studies were as previously described(8,1). Acetyl-Coenzyme A formation in pigeon liver extracts was measured by the method of Chou and Lipmann(9) and hydroxamate formation according to Lipmann and Tuttle(10). The methods for *in vivo* acetylation studies were as previously described(11). Aminopterin and A-methopterin (Methotrexate) were generously supplied by Dr. J. M. Ruegsegger of Lederle Lab. Div., Am. Cyanamid Co. These substances were used without further purification. 2,4-Diaminopteridine and 2,4-diamino-6,7-diphenylpteridine were obtained through the courtesy of Dr. E. Rohrman, The Eli Lilly Research Laboratories, Indianapolis, Ind.

Results. A-methopterin and aminopterin at 10^{-3} M inhibited completely acetylation of sulfanilamide and INH. From Table I it can be seen that the concentration required for 50% inhibition was, in the case of A-methopterin, less than 10^{-5} M, and for aminopterin, 5 x 10^{-5} M. Folic acid (PGA) was not inhibitory at 10^{-3} M, and also failed to reverse the inhibition caused by A-methopterin and aminopterin. Furthermore, acetylation was inhibited only 50% by 10^{-3} M 2,4-diamino-6,7-diphenylpteridine and not at all by 10^{-3} M 2,4-diaminopteridine. These results indicated that the 4-amino group of A-methopterin and aminopterin was directly involved in the inhibitory effect, and that the 2-amino group, which is also present in folic acid, was inactive. It was also apparent that the pteridine moiety, alone, did not account for the whole inhibitory effect. No diazotizable amine was produced when A-methopterin or aminopterin was incubated with the complete acetylating system in absence of sulfanilamide, thus indicating that the observed inhibition was not partly due to an artifact arising from cleavage of the molecule with consequent release of para-aminobenzoylglutamate (PABGA). Moreover, the latter at 10^{-3} M inhibited INH acetylation by only 39% (Table I). Para-aminobenzoic acid (PABA) at the same concentration was previously shown to inhibit INH acetylation by 41% (1).

The nature of the inhibition by A-methopterin was investigated by using varying concentrations of sulfanilamide and A-methopterin. The results, plotted by the method of Lineweaver and Burk(12) indicated that the inhibition was non-competitive (Fig. 1). Attempts to reverse the inhibition by addition of cysteine or excess Coenzyme-A were unsuccessful. Citrovorum factor, not being readily available, was not tried.

The hydroxamic acid method of Chou and Lipmann(9) was used to study the effect of A-methopterin on activation of acetate to acetyl-S-CoA. As shown in Table II, acetate activation was inhibited only 33-36% by 10^{-4} M A-methopterin, at which concentration complete inhibition of sulfanilamide acetylation occurs. It is thus apparent that inhibi-

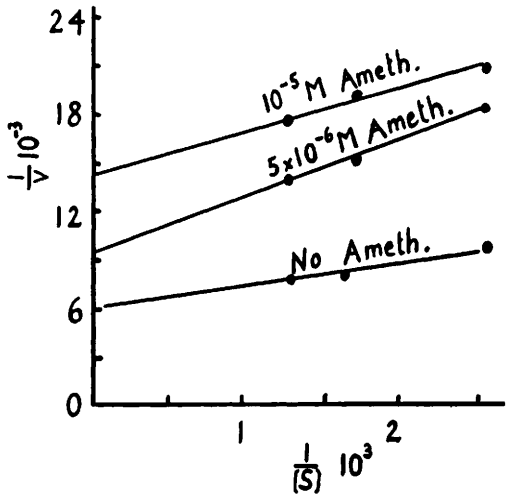

FIG. 1. Non-competitive inhibition of sulfanilamide acetylation by A-methopterin (Ameth.). Velocity (V) expressed as μg of sulfanilamide acetylated in 30 min., and sulfanilamide concentration (S) as molarity. Other conditions are given under "*Materials and methods.*"

tion of acetate activation can account for but a part of the total inhibitory effect of A-methopterin on acetylation.

The experimental data presented in Table III suggest that A-methopterin also modifies the acetylation of sulfanilamide *in vivo*. When A-methopterin was administered to rabbits conjointly with sulfanilamide mean blood levels of free sulfanilamide were markedly higher, at 2, 4, and 6-hour intervals after dosage, than when sulfanilamide alone was given. Furthermore, there was a concomitant decrease in plasma levels of acetylated sulfanilamide. In Exp. I, Table III, the dose of A-methopterin (25 mg/kg) has been calculated as the equivalent of the concentration required for 50% inhibition *in vitro*. Fountain *et al.*(13) have reported that administration of A-methopterin to mice resulted in retention of the compound in the liver for 3 weeks. To test the possibility that A-methopterin might be similarly retained by rabbit liver and lead to prolonged inhibition of acetylating ability, a rabbit was given 50 mg/kg of A-methopterin 18 hours prior to a single dose of 200 mg/kg of sulfanilamide. No impairment of acetylation was observed, and the high dose of A-methopterin appeared to have no deleterious effect on the rabbit. It is conceivable that resistance of the rabbit to the toxic effects of folic acid antagonists(14), may be due to ability to acetylate the 4-amino group of these compounds. However, no evidence for this has yet been obtained.

Discussion. The 4-amino derivatives of folic acid have been shown to inhibit conversion of folic acid to citrovorum factor (CF), and to interfere with utilization of preformed CF(15). The toxic effects of these analogues are blocked by CF administration, or prior injection of large amounts of folic acid. The present studies indicate that the 4-amino folic acid analogues are potent non-competitive inhibitors of arylamine acetylation, an activity which is apparently quite unrelated to the antimetabolite function of these compounds.

TABLE II. Effect of A-methopterin on Acetate Activation in Pigeon Liver Extract.

Exp.	A-methopterin	Acethydroxamate formed (μmoles)	% inhibition
1	0	3.68*	
	10^{-4} M	2.47	33
2	0	3.62	
	10^{-4} M	2.34	36

Incubation mixture: As in Table I, except that 0.5 M hydroxylamine, pH 7.4, replaced SA or INH; and 1 ml of pigeon liver extract (equiv. to 120 mg of acetone powder) was used. Incubation, 1 hr at 37°C.

* When acetate was omitted 0.53 μmoles of acethydroxamate were formed.

TABLE III. Effect of A-methopterin on Plasma Levels of Sulfanilamide in Rabbits.

Exp.	No. of rabbits	Drug dose (mg/kg)* Sulfa.	A-meth.	Sulfanilamide in blood (mg/100 ml)† Hr after dosing 2 Free	2 Acet.	4 Free	4 Acet.	6 Free	6 Acet.
1	3	400		14.0	10.6	9.4	8.1	7.2	8.1
		"	25	23.1	6.8	15.4	6.1	10.4	6.2
2	3	200		5.0	3.7	3.8	5.2	2.4	9.3
		"	35	7.8	3.6	6.3	4.7	4.0	5.4

* Drugs were given orally as a suspension in water. † Mean values.

The mechanism of action is not fully known. The inhibitory effect on acetyl-CoA formation (Table II) was too weak to account for inhibition of sulfanilamide acetylation by A-methopterin *in vitro*. Furthermore, it is unlikely that an effect on acetate activation is responsible for inhibition of sulfanilamide acetylation in rabbits, since acetyl-CoA can be generated at normal rates from other metabolic precursors. It is possible however that these substances may displace acetyl-CoA from the acetylating enzyme. A further possibility is that inhibition of acetylation may be only one instance of a more generalized phenomenon. Fountain *et al.*(13) observed that, following a parenteral dose of A-methopterin in mice, a more or less constant amount of the drug was retained in the liver for at least 3 weeks. The authors suggested that the retained drug was probably concentrated in the enzymes associated with folic acid and CF. It is now apparent that the folic acid antagonists have a high affinity for enzymes associated with arylamine acetylation, and probably others.

It is not possible to say whether, at the dosage levels presently employed therapeutically, sufficient concentrations of the folic acid antagonists can accumulate in the cell to inhibit acetylation processes. Such a possibility should not be lost sight of, particularly in the event that aminopterin or A-methopterin is administered in combination with a drug known to be acetylatable, such as 6-aminonicotinamide. Indeed, one might expect that simultaneous administration of 6-aminonicotinamide and a 4-amino folic acid antagonist would result in carcinostatic potentiation.

Summary. 1) Acetylation of sulfanilamide and of isoniazid in pigeon liver extracts was markedly inhibited (non-competitively) by 4-amino analogues of folic acid. A-methopterin (10^{-5} M) and aminopterin (5×10^{-5} M) inhibited acetylation by 62 and 58%, respectively. Folic acid (10^{-3} M) was not inhibitory, and failed to reverse the inhibition by A-methopterin. 2,4-Diamino-6,7-diphenylpteridine (10^{-3} M) gave 50% inhibition of acetylation and 2,4-diaminopteridine (10^{-3} M) was inactive. A-methopterin, administered to rabbits conjointly with sulfanilamide, produced a marked increase in plasma level of free sulfanilamide, and a concomitant decrease in acetyl-sulfanilamide. 2) The possible significance of these results with regard to combination chemotherapy of cancer was discussed.

1. Johnson, W. J., Corte, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 446.

2. Mandel, W., Cohn, M. L., Russell, W. F., Jr., Middlebrook, G., *ibid.*, 1956, v91, 409.

3. Veldstra, H., *Pharmacol. Rev.*, 1956, v8, 339.

4. Shapiro, D. M., Dietrich, L. S., Shils, M. E., *Cancer Research*, 1957, v17, 600.

5. McCell, J. D., Rice, W. B., Adamkiewicz, V. W., *Can. J. Biochem. and Physiol.*, 1957, v35, 795.

6. Halliday, S. L., Sloboda, A., Will, L. W., Oleson, J. J., *Fed. Proc.*, 1957, v16, 190.

7. Herter, F. P., Frick, H. C., Hyman, G. A., Findlay, C. W., Jr., Shapiro, D. M., *Proc. Am. Assn. Cancer Res.*, 1958, v2, 307.

8. Johnson, W. J., *Can. J. Biochem. and Physiol.*, 1955, v33, 107.

9. Chou, T. S., Lipmann, F., *J. Biol. Chem.*, 1952, v196, 89.

10. Lipmann, F., Tuttle, L. C., *Biochim. et Biophys. acta*, 1950, v4, 301.

11. Johnson, W. J., *Can. J. Biochem. and Physiol.*, 1955, v33, 523.

12. Lineweaver, H., Burk, D., *J. Am. Chem. Soc.*, 1934, v56, 658.

13. Fountain, J. R., Hutchison, D. J., Waring, G. B., Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 369.

14. Dinning, J. S., Keith, C. K., Davis, P. L., Day, P. O., *Arch. Biochem.*, 1950, v27, 89.

15. Nichols, C. A., Welch, A. D., PROC. SO. EXP. BIOL. AND MED., 1950, v74, 403.

Received September 25, 1958. P.S.E.B.M., 1958, v99.