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DETOXICATION OF A-METHOPTERIN AND AMINOPTERIN BY THE ACETYLATING ENZYME OF LIVER

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SUMMARY

(1) A-methopterin has been found to lose its activity as growth inhibitor of *Streptococcus faecalis* on incubation with pigeon-liver acetylating enzymic system.

(2) The derivative formed was heat labile.

(3) Omission of any one of the essential cofactors required for acetylation of aromatic amines was found to prevent inactivation of A-methopterin.

(4) The presence of aromatic amines such as *p*-aminobenzoic acid, diminished the formation of the inactive derivative from A-methopterin possibly through competitive inhibition.

(5) Bioautography of the product formed on incubating A-methopterin with the pigeon-liver acetylating system showed the presence of only one inactive derivative.

(6) On elution and rechromatography after heat treatment the inactive derivative was reconverted to a compound producing a no-growth zone in the bioautogram having the same R_F as A-methopterin (R_F 0.67).

(7) The evidence strongly suggests that A-methopterin is acetylated by the pigeon-liver enzymic system, and this could explain the observed competitive inhibition produced by A-methopterin on the acetylation of sulphanilamide.

(8) The above observations are discussed in relation to differences in acetylating capacity reported to occur between different species and to the varying susceptibility of different species to the toxic effects of antifolics. It is proposed that acetylation is the mechanism by which A-methopterin and aminopterin are detoxified in the liver.

INTRODUCTION

A-methopterin (4-amino-10-methylpteroylglutamic acid) and aminopterin (4-aminopteroylglutamic acid) are analogues of folic acid having considerable importance in the chemotherapy of leukaemia[1]. These compounds are also known to inhibit selectively the growth of several experimental tumours but their effectiveness as chemotherapeutic agents in cancer is, however, limited as they produce only temporary remissions on the growth of malignant cells[2]. The mechanisms by which the leukaemic cells acquire resistance to these drugs are not clearly established. Among the factors controlling the effective concentration of these drugs in tissues, reactions responsible for their detoxication must play a significant role. Studies by JACOBSON[3,4] have

shown that aminopterin, on incubation with various tissues including leukaemic cells, loses its activity as an inhibitor of actively dividing cells. In extension of our results reported earlier[5] the present paper describes findings showing that the acetylating enzyme system of pigeon liver converts A-methopterin into a derivative which is inactive as a growth inhibitor of *S. faecalis*.

MATERIALS AND METHODS

Materials

A-methopterin and folinic acid were obtained from Lederle Laboratory, American Cyanamid Co., aminopterin from L. Light and Co., folic acid, USP quality, from Tanabe Seiyaku (Japan), CoA from Sigma Chemical Co., ATP from Z.W. Waldhoff, sulphanilamide, B.P. quality, from Polsha Chemical Co. and cysteine from E. Merck.

Metabolic experiments

Swiss mice, weighing 20–30 g were kept in metabolic cages. Materials were injected intraperitoneally in a total volume of 0.4 ml. Urine was collected for 24 h, in a flask containing 1.0 ml of toluene. Determination of the total, and free aromatic amine was carried out as follows:

The volume of the collected urine (1–2 ml) was made to 10.0 ml and a portion (2.0 ml) was treated with 2 N HCl to make the urine 0.2 N. It was then heated at 100° for 1 h to hydrolyse the combined aromatic amine. Free aromatic amine was determined in a similar aliquot without acid- or heat-treatment. The assay of the aromatic amine was carried out according to BRATTON AND MARSHALL[6]. The difference between the total and free aromatic amines was taken to be the acetylated amine. It is known that folic acid is degraded to an aromatic amine on heating with acid[7]. Therefore in experiments in which folic acid was injected, it was necessary to remove undegraded folic acid and related compounds from urine prior to the determination of the total aromatic amine. This was done by treating an aliquot of the urine sample with 2.0 ml of 10 % $ZnSO_4$ solution and 2.0 ml of 0.5 N NaOH. After 2 min, the $Zn(OH)_2$ gel was removed by centrifuging and the free and combined aromatic amines were estimated in the supernatant. Preliminary experiments had shown that the $Zn(OH)_2$ gel adsorbed only the undegraded folic acid. The free, or combined, aromatic amine remained in the supernatant quantitatively.

Enzymic preparations

An acetone-powder of pigeon liver was prepared in the usual way[8] and stored at −15° until required. Crude enzymic preparations consisted of 5 % extracts of the powder in 0.2 M NaF solution. Acetone fractionation of the acetylating enzyme system was carried out according to the method of CHOU AND LIPMANN[9]. In experiments in which acetate effect was demonstrated, the acetone-precipitated fractions of the enzyme were dialysed for 30 h, with continuous stirring, against two changes of cold, distilled water (3 l each).

Assay of A-methopterin and aminopterin

These substances were assayed by inhibition analysis using *S. faecalis* as the test organism. The medium was that described in *Methods of Vitamin Assay*[10]. Inhibition of growth of the organism by A-methopterin was determined in the system containing

0.8 mμg of folic acid per ml of the medium. The test solutions were so diluted that they contained approx. 0.5–0.8 mμg of A-methopterin per ml of the assay system. The diluted solutions were kept at about 2° until assay. Each determination was carried out in duplicate at three different levels of concentration of the inhibitor. Since it was found that inactivation of A-methopterin occurred rapidly, the zero-time samples were autoclaved at 10 lb pressure for 10 min. The sterilization of the test samples was carried out using a sintered-glass bacteriological filter (Jena).

Bioautographic technique

3.0 ml of absolute alcohol were added to the reaction mixture cooled in ice. The precipitate formed was removed by centrifuging. It was washed once with 3.0 ml of alcohol. The combined supernatants were then concentrated to dryness *in vacuo* under cold conditions.

Chromatography: The yellow residue obtained was dissolved in 200 μl of 0.05 M phosphate buffer (pH 7.4) and portions (5 μl) were spotted in duplicate on Whatman No. 3MM filter paper (25 × 55 cm). The control spot consisted of standard A-methopterin (100 mμg/5 μl). The chromatogram was developed with 5 % Na_2HPO_4 at pH 6.5 using descending conditions at 20°. The solvent front was allowed to advance to 40 cm. Throughout the procedure the samples were protected from light.

The inhibitory zones were located by bioautography[11]. The developed chromatogram was cut into strips and placed in contact with the surface of solid medium suitable for growth of *S. faecalis* on specially prepared Perspex plates. The plates were then incubated at 32° for 18–20 h and at the end of this period the inhibition zones could be detected clearly on removal of the strips.

The solid basal medium for growth of *S. faecalis* was prepared as follows: All the constituents, except glucose, were autoclaved (10 lb for 10 min) with 1.2 % Difco Bacto Agar and folic acid (15 mμg/ml). Glucose solution was sterilised separately and added to the medium to a concentration of 4 %. The mixture was cooled to 45°. It was then seeded with 7.0 ml of a suspension of an 18-h culture of *S. faecalis*. The medium (350 ml) was then quickly poured into a plate (Perspex I.C.I., 27 × 25 × 1.8 cm). Initial experiments had established that the Perspex plates could be effectively sterilized by swabbing with alcohol. When the chromatographic strips were subjected to heat-treatment, they were first sprayed with water and then heated for 30 min at 100°.

RESULTS

Effects of A-methopterin on the urinary excretion of the aromatic amine formed from injected folic acid

The results given in Table I demonstrate the effect of A-methopterin on the excretion of the aromatic amine in the urine of mice injected with folic acid. Injection of 0.5 μmole of folic acid produced considerable increase, above the control, in the excretion of the aromatic amine as has been reported in rats by DINNING *et al.*[12]. In the experiments described in Table I, it is evident that injections of small quantities of A-methopterin did not produce any effect on the total aromatic amine excreted, but decreased considerably the excretion of the combined form of the amine formed from folic acid. Results of experiments in which *p*-aminobenzoylglutamic acid was injected, show more directly that A-methopterin brings about considerable inhibition

of the acetylation of this compound. (*p*-Aminobenzoylglutamic acid is the amine expected to be formed from folic acid.)

Inhibition of acetylation by A-methopterin in relation to sulphanilamide concentration

Table II shows the relationship of the sulphanilamide concentration to the inhibition produced by varying amounts of A-methopterin on the excretion of the acetylated form of this amine in the urine of mice. Increasing inhibition of the

TABLE I

EFFECT OF A-METHOPTERIN ON THE URINARY EXCRETION OF THE AROMATIC AMINE FORMED FROM INJECTED FOLIC ACID

Expt. No.	*Compound injected (μmoles)*	*A-methopterin (μg)*	*Aromatic amine excreted (μmoles)*		*Acetylation (%)*	*Inhibition (%)*
			Free	*Total*		
1	None (6 animals)	None	0.023	0.029	20.7	—
2	Folic acid (8 animals) (0.5)	None	0.165	0.213	22.5	—
3	Folic acid (6 animals) (0.5)	6	0.193	0.210	8.1	64.0
4	Folic acid (6 animals) (0.5)	12	0.173	0.192	9.9	56.0
5	*p*-Aminobenzoylglutamic acid (0.5) (2 animals)	None	0.29	0.43	32.5	—
6	*p*-Aminobenzoylglutamic acid (0.5) (2 animals)	6	0.22	0.30	26.6	18.1
7	*p*-Aminobenzoylglutamic acid (0.5) (3 animals)	12	0.28	0.34	17.6	45.8

TABLE II

INHIBITION OF ACETYLATION OF SULPHANILAMIDE BY A-METHOPTERIN IN MICE

Expt. No.	*Sulphanilamide injected (μmoles)*	*A-methopterin (μg)*	*Sulphanilamide excreted (μmoles)*		*Acetylation (%)*	*Inhibition (%)*
			Free	*Total*		
	None	None	0.023	0.029	20.7	—
2	0.2 (23 animals)	None	0.185	0.256	27.7	—
3	0.2 (6 animals)	6	0.187	0.228	18.0	35.0
4	0.2 (8 animals)	12	0.222	0.265	16.2	41.5
5	0.2 (7 animals)	24	0.195	0.227	14.1	49.1
6	0.5 (13 animals)	None	0.239	0.338	29.3	—
7	0.5 (6 animals)	6	0.244	0.325	24.9	15.4
8	0.5 (12 animals)	12	0.267	0.339	21.3	27.3

acetylation of a particular concentration of sulphanilamide was obtained with higher concentrations of A-methopterin. It is also evident that increasing concentrations of sulphanilamide lower the inhibition produced by a particular concentration of A-methopterin. Thus 6 μg of A-methopterin produced 35 and 15% inhibitions when 0.2 μmole and 0.5 μmole of sulphanilamide were injected respectively.

The mechanism of acetylation of aromatic amines by the acetylating enzyme system in extracts of pigeon liver has been extensively studied by LIPMANN and his colleagues[13]. It has been shown that enzymic extracts of pigeon liver require acetate, ATP and CoA as cofactors for acetylation of aromatic amines. Fig. 1 shows that the inhibition of acetylation of sulphanilamide by A-methopterin increases on additions up to 25 μg of the inhibitor.

Effect of various compounds on the inhibition of acetylation of sulphanilamide by A-methopterin

The effects of addition of CoA, cysteine, ATP, folic acid and folinic acid on the inhibition produced by A-methopterin on acetylation of sulphanilamide by pigeon-liver extract are given in Table III. Among the cofactors required for the acetylating system, only added ATP was able to reverse partially (20 %) the inhibitory effect of A-methopterin. Folic acid (100 μg) had no action on the acetylation of sulphanilamide nor on the inhibition produced by A-methopterin on this system. Folinic acid at levels of 20 μg inhibited the acetylation of sulphanilamide and enhanced the inhibition produced by A-methopterin. It is also evident (Table III) that aminopterin inhibits the acetylation of sulphanilamide to a lesser extent than A-methopterin.

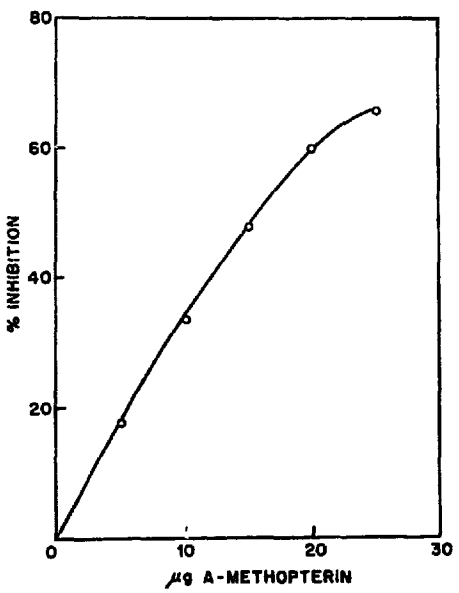

Fig. 1. Inhibition of acetylation of sulphanilamide by various quantities of A-methopterin. Conditions of the experiment as in Table III.

Effect of A-methopterin on one of the enzyme components (acetate thiokinase) of the acetylating system

Experiments designed to locate the inhibitory effect of A-methopterin on the overall acetylating enzyme system were carried out by using pigeon-liver extracts fractionated according to CHOU AND LIPMANN[9]. In this procedure the acetate thiokinase (Fraction A-40) catalyses the formation of acetyl-CoA from acetate, ATP and CoA. The arylamine acetylase (Fraction A-60) catalyses the transfer of the acetyl group from acetyl-CoA to the acceptor amine. Results given in Table IV show that A-methopterin does not inhibit the acetyl-CoA formation as assayed by acethydroxamic acid method[9]. It may be inferred from these observations that A-methopterin inhibits only the arylamine acetylase component of the acetylating enzyme system.

A-methopterin as a competitive inhibitor of acetylation of sulphanilamide

The kinetics of the inhibition produced by A-methopterin on the acetylation of sulphanilamide were studied by plotting the results according to LINEWEAVER AND BURK[14]. It is clear from Fig. 2 that A-methopterin competitively inhibits the acetylation of sulphanilamide.

Inactivation of A-methopterin and aminopterin by the acetylating enzyme system of pigeon liver

The possible formation of acetyl derivatives of A-methopterin and aminopterin was studied by incubating these compounds with crude enzymic extracts of pigeon-liver acetone powder under suitable conditions. The changes in activity of these compounds as inhibitors of growth of *S. faecalis* were then measured by the assay of the system for A-methopterin or aminopterin before and after incubation. Results given in Table V show that more than 40 % of added A-methopterin is inactivated by incubation with the acetylating enzyme system of pigeon liver for 180 min. Under similar conditions the activity of aminopterin diminished by 24 %. It is also evident from the results that, on heating the incubated mixtures, the original inhibitory

TABLE III

EFFECT OF VARIOUS COMPONENTS ON A-METHOPTERIN INHIBITION OF ACETYLATION OF SULPHANILAMIDE

Incubation system contained: Na_2HPO_4, 0.02 M; $NaHCO_3$, 0.028 M; sodium acetate, 0.02 M; ATP, 0.004 M; sulphanilamide, 1 μmole; CoA, 12.5 μg; Mg^{2+}, 0.0055 M; cysteine, 0.005 M; crude enzymic extract, 60 mg; volume, 3.0 ml; incubation for 90 min at 37°.

Expt. No.	*Supplements added*	*Acetylation (%)*	*Inhibition (%)*
1	None	73.0	—
	A-methopterin (15 μg)	41.0	43.8
	Aminopterin (50 μg)	54.0	26.0
2	None	76.0	—
	Folinic acid (20 μg)	57.0	25.0
	A-methopterin (15 μg)	44.0	42.1
	Folinic acid (20 μg) + A-methopterin (15 μg)	34.0	55.3
3	None	79.8	—
	ATP (16.5 mg)	79.0	—
	A-methopterin (15 μg)	33.8	58.2
	A-methopterin (15 μg) + ATP (16.5 mg)	49.0	38.0
4	None	92.0	—
	Folic acid (100 μg)	91.0	—
	A-methopterin (15 μg)	53.0	42.4
	A-methopterin (15 μg) + folic acid (100 μg)	45.6	50.0
5	None	86.6	—
	CoA (12.5 μg)	86.6	—
	A-methopterin (15 μg)	43.3	50.0
	A-methopterin (15 μg) + CoA (12.5 μg)	43.3	50.0

TABLE IV

EFFECT OF A-METHOPTERIN ON ACETATE THIOKINASE (A-40)

Complete system contained: 0.01 M Tris buffer (pH 8.3); ATP, $4 \cdot 10^{-3}$ M; sodium acetate, $5.6 \cdot 10^{-2}$ M; NaF, $3.3 \cdot 10^{-2}$ M; Mg^{2+}, $5.6 \cdot 10^{-3}$ M; cysteine, $5.6 \cdot 10^{-3}$ M; NH_2OH, $4.2 \cdot 10^{-1}$ M; 0.3 ml of fraction A-40 and 0.15 ml of heated 10% extract of pigeon liver. Incubation for 90 min at 37°.

Expt. No.	*Component*	*Acethydroxamic acid formed (μmoles)*
1	Complete system	0.32
2	Complete system + 15 μg A-methopterin	0.32

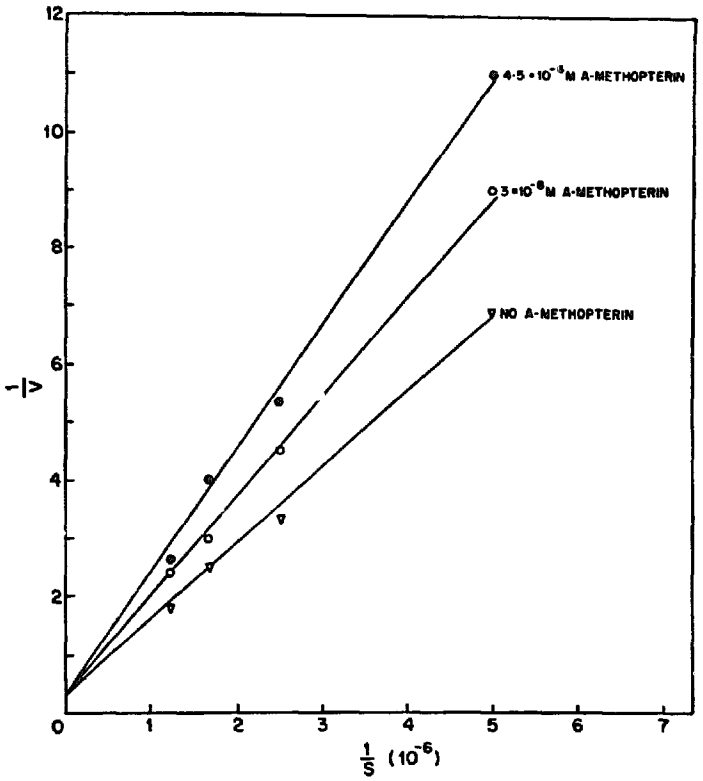

Fig. 2. Competitive inhibition of acetylation of sulphanilamide by A-methopterin. Conditions of the experiment as in Table III.

TABLE V

INACTIVATION OF A-METHOPTERIN AND AMINOPTERIN BY ACETYLATING ENZYMIC SYSTEM OF PIGEON LIVER

Incubation system as in Table III. 13.2 μg of A-methopterin (Expts. 1 and 2) or aminopterin (Expt. 3) added instead of sulphanilamide.

Expt. No.	*System*	*Sample (ml)*	*Time of incubation (min)*	*Growth of S. faecalis*	
				Klett units (660 mμ)	*Expressed as per cent of control in absence of added inhibitor*
1	Crude enzyme system	0.6	0	76	42.0
	Crude enzyme system	0.6	180	118	65.6
	Autclaved system*	0.6	180	87	48.0
	Crude enzyme system	0.8	0	50	27.8
	Crude enzyme system	0.8	180	91	50.0
	Autoclaved system*	0.8	180	56	30.8
2	Crude enzyme system	0.4	0	75	43.0
	Crude enzyme system	0.4	180	125	71.8
	Crude enzyme system + 13.2 μg *p*-amino benzoic acid	0.4	0	87	49.7
	Crude enzyme system + 13.2 μg *p*-amino benzoic acid	0.4	180	107	61.5
3	Crude enzyme system	0.8	0	152.5	78.8
	Crude enzyme system	0.8	180	164.5	84.5
	Autoclaved system*	0.8	180	153.5	79.3
	Crude enzyme system	1.0	0	85.0	43.8
	Crude enzyme system	1.0	180	108.5	56.0
	Autoclaved system*	1.0	180	86.0	44.6

* Autoclaved at 10 lb pressure for 10 min.

activity of A-methopterin and aminopterin can be recovered. Addition of an acetylable amine such as *p*-aminobenzoic acid to the incubation system containing A-methopterin, diminished the extent of inactivation of A-methopterin by 26 %.

TABLE VI

EFFECT OF OMISSION OF COMPONENTS OF THE ACETYLATING SYSTEM ON THE INACTIVATION OF A-METHOPTERIN

Incubation system as in Table V. Purified fractions (0.4 ml of each) used instead of crude enzymic extracts.

Expt. No.	*System*	*Sample (ml)*	*Time of incubation (min)*	*Growth of S. faecalis*	
				Klett units (660 mμ)	*Expressed as per cent of control in absence of added A-methopterin*
1	Complete system (Fractions A-40 + A-60 + cofactors)	0.6	0	79	36.4
	Complete system (Fractions A-40 + A-60 + cofactors)	0.6	180	109	50.0
		0.8	0	43	20.0
		0.8	180	70	32.4
	A-40 omitted	0.6	0	78	36.6
	A-40 omitted	0.6	180	76	35.0
		0.8	0	44	20.0
		0.8	180	44	20.0
2	Complete system (Fractions A-40 + A-60 + cofactors)	0.6	0	70	35.0
		0.6	180	98	49.0
		0.8	0	30	15.0
		0.8	180	70	34.7
	A-60 omitted	0.6	0	70	35.0
		0.6	180	66	33.0
		0.8	0	32	16.0
		0.8	180	32	16.0
3	Complete system (Fractions A-40 + A-60 + cofactors)	0.6	0	90	45.5
		0.6	180	132	65.7
		0.8	0	49	24.4
		0.8	180	91	45.2
	Acetate omitted	0.6	0	91	45.5
		0.6	180	91	45.5
		0.8	0	52	25.7
		0.8	180	49	24.5
4	Complete system	0.6	0	101	52.5
		0.6	180	129	67.0
		0.8	0	62	33.0
		0.8	180	87	46.0
	ATP omitted	0.6	0	92	48.0
		0.6	180	84	44.5
		0.8	0	63	33.5
		0.8	180	61	33.0
5	Complete system	0.6	0	40	20.0
		0.6	180	70	35.2
		0.8	0	20	10.2
		0.8	180	51	25.2
	CoA omitted	0.6	0	35	17.8
		0.6	180	33	17.0
		0.8	0	24	12.5
		0.8	180	23	12.0

Components of the acetylating enzymic system required for the inactivation of A-methopterin

It is clear from results given in Table VI that the activity of A-methopterin as a growth inhibitor decreased by 23 % when it was incubated in presence of the two purified active protein fractions and all the essential cofactors of the acetylating enzymic system. In experiments in which ATP, CoA, acetate or any one of the protein fractions were omitted in turn, the potency of A-methopterin was found to be the same before and after incubation.

Detection of the inactive derivative of A-methopterin by bioautography

Bioautography was employed in attempts to detect the inactive derivatives of A-methopterin formed on incubation with the acetylating enzyme system. It is clear from the results illustrated in Fig. 3 that only one no-growth zone is detected in the incubated mixture having the same R_F (R_F 0.69) as A-methopterin. On heating the chromatographic strip prior to bioautography, however, two no-growth zones having R_F 0.69 and R_F 0.83 respectively could be observed. The no-growth zone with R_F 0.83 appeared only after heating, indicating that this derivative was initially inactive as a growth inhibitor of *S. faecalis*. Experiments in which the portion of the chromatogram corresponding to R_F 0.83 was eluted, heated and again subjected to bioautography, produced a no-growth zone (R_F 0.67) having the same R_F as A-methopterin.

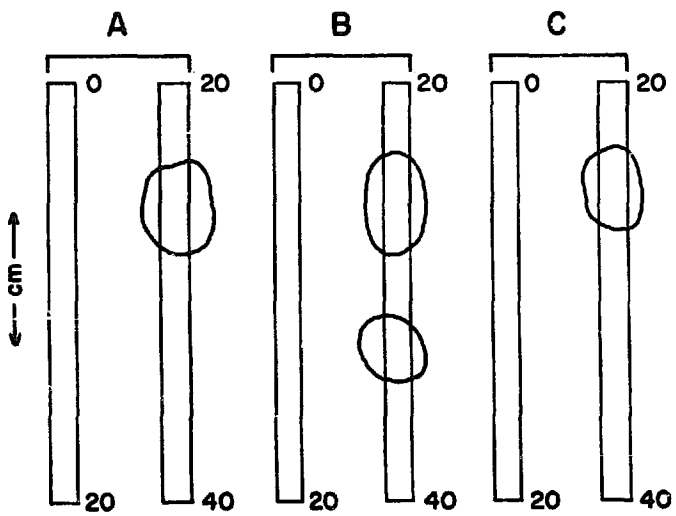

Fig. 3. A drawing of a bioautogram demonstrating the heat-labile derivative of A-methopterin. Incubation mixture as in Table V. A, incubated mixture; B, incubated mixture after heat treatment; C, standard A-methopterin. For bioautographic technique, see MATERIALS AND METHODS.

DISCUSSION

Studies reported in this paper demonstrate that A-methopterin and aminopterin lose their activity as inhibitors of growth of *S. faecalis* when incubated with enzyme systems of liver which catalyse the acetylation of aromatic amines. The inactivation of A-methopterin occurred only in presence of all the essential factors required for acetylation. Omission of cofactors like ATP or CoA prevented the inactivation of A-methopterin. Experiments with the system in which the acetylating enzyme had been resolved into two active protein fractions, further demonstrated that both the protein components were required for the activity of the system. It is apparent from

these observations that loss of inhibitory activity of A-methopterin was not due to its non-specific adsorption on proteins. It is also significant, that requirements for acetate could be demonstrated after extensive dialysis of the enzymic extracts. These observations and the presence of amino groups in A-methopterin capable of becoming acetylated suggest that A-methopterin is inactivated through the formation of an acetyl derivative. By using the technique of bioautography, it was possible to detect the formation of only one derivative of A-methopterin in the incubation mixtures. This derivative was completely inactive as a growth inhibitor of *S. faecalis* and it was unstable at high temperature. Its formation could be demonstrated only if the chromatographic strips had been heated prior to bioautography. This treatment resulted in a clear no-growth zone having an R_F (0.83) distinct from the R_F (0.69) of A-methopterin. By eluting the particular portion of the chromatogram and subjecting it to a second bioautographic procedure, it was shown that the derivative of A-methopterin formed on incubation was reconverted to A-methopterin on heating. These observations are also in harmony with the fact that the loss of inhibitory activity on the growth of *S. faecalis* observed on incubating A-methopterin with an acetylating system could be completely recovered by autoclaving the mixtures before analysis.

JOHNSON *et al.*[15] demonstrated recently, that aminopterin and A-methopterin inhibit the acetylation of sulphanilamide in rabbits *in vivo* as well as in preparations of pigeon liver. Present studies have confirmed these findings in mice and in extracts of pigeon liver. It is also apparent from the results that A-methopterin does not affect the conversion *in vivo* of folic acid into *p*-aminobenzoylglutamic acid but brings about a considerable decrease in the urinary excretion of the combined form of this aromatic amine. Inhibition of the acetylation of sulphanilamide by A-methopterin could not be reversed by addition of extra cysteine or CoA which suggests that it was not due to inactivation of essential thiol groups. Acetylation of A-methopterin by similar enzymes which acetylate aromatic amines in liver would conceivably lead to a competition between the two substrates for the aryl amine acetylase and can explain many of the observations described here. Thus the inhibition, by A-methopterin, of the acetylation of sulphanilamide *in vivo*, can be expected to decrease as observed, with increasing concentrations of sulphanilamide. The results of experiments *in vitro* have also shown that A-methopterin is a competitive inhibitor of acetylation of sulphanilamide in extracts of pigeon liver. It may be recalled that according to JOHNSON *et al.*[15] the inhibition by A-methopterin of the acetylation of sulphanilamide follows the kinetics of a non-competitive inhibitor. The reason for this difference is not clear. It may be mentioned that in the present studies chromatographically pure A-methopterin has been used. Observations showing that the presence of an amine, capable of acetylation (*p*-aminobenzoic acid), decreases the inactivation of A-methopterin are in agreement with the mutually competing effects of the two substrates for the formation of their respective acetyl derivative by the pigeon-liver enzyme. Folic acid which has an amino group in the second position of the molecule did not inhibit acetylation of aromatic amines. It is interesting to note that attempts to chemically acetylate folic acid have been found to produce the corresponding derivative at the N^{10} position[16], which indicates that the 2-amino group of the pteridine moiety is considerably resistant to acetylation. Thus it is possible that aminopterin and A-methopterin are inactivated through the formation of the corresponding 4-acetylamino derivative.

The above mechanism can also explain some of the earlier observations on the effects of antifolics in animals. It had been observed by FOUNTAIN *et al.*[17] that the concentration of A-methopterin, detected in the liver and kidneys of mice which had been injected with A-methopterin, increased several fold when the samples were boiled before assay. Since both these organs contain the acetylating enzyme system, the increased activity observed on boiling the tissues could have resulted from the breakdown of a heat-unstable acetyl derivative of the type suggested by the present experiments. Studies relating to the acetylation of sulphanilamide in various animal species have shown that the guinea-pig is less active than the rabbit[18], and the dog is reported to be very poor in its acetylating capacity[19]. Studies on the toxicity of aminopterin by MINNICH *et al.*[20] have also indicated significant differences between species. It was found *e.g.*, that among three species tested, the rabbit was the most resistant to the drug, the guinea-pig showing less resistance than the rabbit. The dog was the least resistant to aminopterin toxicity. Apparently the susceptibility to the toxic action of aminopterin in the above species can be correlated inversely with their capacity to acetylate aromatic amines. Possibly inactivation by acetylation is the mechanism by which A-methopterin and aminopterin are detoxified in the liver.

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