15. Nurnberger, J. I., Gordon, M. W., in Waelsch: *Ultrastructure and Cellular Chemistry of Neural Tissues,* Hoeber, N. Y., 1957, 100.

16. Gray, D. E., DeLuca, H. A., *Am. J. Physiol.,* 1956, v184, 301.

17. Gosselin, R. G., Gabourel, J. D., Kalser, S. C., Wills, J. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 533.

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Metabolic Alteration of 2-Methylthio-4-Amino-5-Hydroxymethylpyrimidine (Methioprim).*† (25205)

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Amethopterin-resistant mutants of *Bacillus subtilis* and mutants of *Escherichia coli* resistant to purine antagonists show increased sensitivity to the inhibitory effect of methioprim (2 - methylthio - 4-amino-5-hydroxymethylpyrimidine) when compared to the parent organisms, *i.e.,* collateral sensitivity(1). Significant growth suppression of subcutaneous tumors (Ehrlich carcinoma, clone 2 and Krebs) is also observed with this compound(2). Furthermore, chromatography of urine from dogs and rats receiving methioprim reveals, in both cases, the excretion of an additional ultraviolet-absorbing substance with anti-bacterial activity similar to methioprim‡. The present study reports that rat liver readily metabolizes methioprim to at least 3 products.

Materials and methods. Rat liver incubations. Adult male Sprague-Dawley rats were sacrificed by decapitation. Livers were immediately excised and placed in chilled bath of Krebs phosphate buffer, pH 7.4(3). Slices weighing 70-100 mg were cut with a Stadie-Riggs microtome. Three slices were placed in each 20 ml beaker with 3 ml of Krebs buffer solution to which methioprim (20 μmoles/ml) was added. The buffer-substrate mixtures and control beakers were incubated under atmosphere of 95% O_2-5% CO_2 at 36.5°C with constant shaking in Dubnoff metabolic incubator. At end of 5 hours, the reaction was stopped and protein precipitated by rapidly heating in boiling water bath for 5 minutes followed by cooling. The supernatant fluid was collected by centrifugation for analysis of metabolic products. Aliquots of supernatants of incubation mixtures and controls were applied to large sheets of Whatman 3 MM paper and ascending chromatograms were developed. Initial separation of components of incubation mixtures was carried out using top layer of solvent prepared by mixing n-butyl alcohol (500 ml): water (500 ml); glacial acetic acid (16 ml). Chromatograms were examined for metabolic products by using ultraviolet light, and various spray reagents and by bioautographic technic using microbiological assay described below. *Microbiological assay.* Methioprim and some closely related compounds suppress growth of amethopterin-resistant mutants of *Bacillus subtilis* to a greater extent than the parent strain(1). Such compounds have no effect on growth of these strains when 2 - methyl-4-amino-5-aminomethylpyrimidine (the pyrimidine moiety of thiamine) is present in growth medium. For routine purposes, an agar-diffusion method and a chemically-defined medium(4) were used. An amethopterin-resistant mutant of *Bacillus subtilis,* designated as strain 6051/A, was homogeneously seeded into medium with and without a supplement of 2-methyl-4-amino-5-aminomethylpyrimidine. Similar platings of the parent *B. subtilis* 6051 were also made. Either paper discs impregnated with test solutions or strips of paper chromatograms were placed on the

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‡ R. Guthrie, unpublished data.

surface of assay plates and incubated at 25°C up to 3 days. Zones of inhibition were noted. Presence of methioprim (concentrations as low as 10^{-7} M can be detected) or similar compounds may be ascertained easily by characteristics described above. *Chemicals.* 2-Methylthio-4-amino-5-formylpyrimidine was prepared by the method of Okuda and Price (5), 2-thio-4-amino-5-pyrimidinecarboxylic acid and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid by the method of Ulbricht and Price(6). 2-Methylthio-4-hydroxy-5-hydroxymethylpyrimidine was prepared by reduction of corresponding carbethoxypyrimidine with lithium aluminum hydride in ether. The yield in this solvent was low; however, recently, Hoover(7) reported synthesis of this compound in good yield by use of dimethyl ether of diethyleneglycol as solvent. 2-Methylsulfonyl-4-amino-5-formylpyrimidine was prepared by the method of Nairn(8). 2-Thio-4-amino-5-hydroxymethylpyrimidine and methioprim were kindly supplied by Dr. Stanton Harris and Dr. James Sprague, Merck and Co. The other compounds were obtained from commercial sources.

Results. Initial chromatograms of incubation mixtures revealed 4 new components designated as follows according to behavior under ultraviolet light: Component I, Rf 0.95—fluorescent; Component II, Rf 0.82—ultraviolet-absorbing; Component III, Rf 0.75—fluorescent; and Component IV, Rf 0.25—ultraviolet-absorbing. Components I, II and IV inhibited the assay organism *B. subtilis* 6051/A. When amounts of methioprim equivalent to incubation concentrations were chromatographed as a control, an ultraviolet-absorbing contaminant was observed with the same Rf value as Component IV. The nature of this compound will be discussed below. As measured microbiologically 25-30% of methioprim was metabolized.

Isolation and identification of metabolic products. Component I: This compound appears in low yield and has not been identified. It does not react with ninhydrin or dihydropyrimidine spray reagents. Its Rf value and/or ultraviolet spectrum differs from all the following compounds: 2-thio-4-amino-5-pyrimidinecarboxylic acid, 2-methylthio-4-amino-pyrimidine, 2-methylthio-4-amino-5-methylpyrimidine, 2-thio-4-amino-5-hydroxymethylpyrimidine, 2-methylsulfonyl-4-amino-5-formylpyrimidine, 2-methylthio-4-hydroxy-5-hydroxymethylpyrimidine, uracil, 5-hydroxymethyluracil, cytosine, and 5-hydroxymethylcytosine.

TABLE I. Comparison of Component II with Synthetic 2-Methylthio-4-amino-5-formylpyrimidine.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Melting point	192 uncorr	192-93 uncorr
Nitrogen	Positive	Positive
M.P. of semicarbazone	272 uncorr	270-72 uncorr
Infrared spectrum and ultraviolet spectrum	In both cases no discernible difference	
Molar extinction coeff.	Both 1.14×10^4 at 316 mμ	
Rf values†	In both cases no discernible difference	
Microbiol. assay*	Inhibitory*	Inhibitory*

* Microbiological assay: Inhibits *B. subtilis* 6051/A. Growth inhibition reversed by 2-methyl-4-amino-5-aminomethylpyrimidine.

† Solvents (see text for ratios)

1. n-butyl alcohol—glacial acetic acid-water — Rf = 0.82
2. ethanol—NH_4OH — Rf = 0.79
3. n-propyl alcohol—water — Rf = 0.85

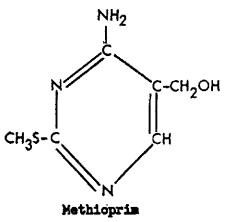
Component II: This component was eluted from initial chromatograms with 95% ethanol and purified further by successive ascending chromatography in ethanol(1): 1N NH_4OH (1), and then n-propyl alcohol(1): water(1). Purified Component II was recrystallized from absolute ethanol. On the basis of series of tests listed in Table I, it was concluded that Component II was identical to 2-methylthio-4-amino-5-formylpyrimidine.

Component III: Isolation of this compound was achieved with identical procedure used for component II. Table II shows comparative tests made with 2-methylthio-4-amino-5-pyrimidinecarboxylic acid. It was concluded that component III and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid were identical.

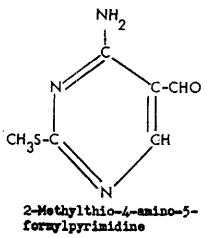
Component IV: This compound was also eluted from initial chromatograms with 95% ethanol and purified by successive ascending chromatography with n-butyl alcohol saturated with water, and then n-propyl alcohol

(6): HCl(2): water(2). The properties of this material are listed in Table III. Several stock samples of methioprim contained a small concentration of an ultraviolet-absorbing contaminant with an Rf value similar to Component IV. After reviewing the method for preparation of methioprim(6) it was shown that the contaminant was formed in the reduction of corresponding carbethoxypyrimidine to methioprim with lithimum aluminum hydride. Purification of this yellow-colored, toluene-insoluble substance by chromatography on a cellulose column eluted using top layer of a mixture of n-butyl alcohol (500 ml) : glacial acetic acid (16 ml) : water (500 ml) and analysis has led to the conclusion that it is an aluminum containing derivative of methioprim arising from incomplete hydrolysis of the reduction intermediate. The properties of this material are also listed in Table III.

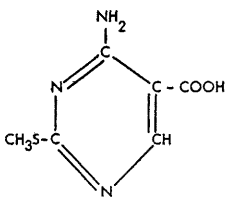
Discussion. The present data demonstrate that the metabolism of methioprim by rat liver slices involves an oxidation of the 5-hydroxymethyl group to 5-formyl and 5-carboxylic acid derivatives as well as an unidentified ultraviolet-absorbing material. This sequence is shown in the following diagram:



Methioprim



2-Methylthio-4-amino-5-formylpyrimidine



2-Methylthio-4-amino-5-pyrimidine-carboxylic acid

Despite precise knowledge as to the nature of the unidentified product, it does not appear to be a decarboxylated or deaminated derivative of 2-methylthio-4-amino-5-pyrimidinecarboxylic acid.

The catabolism in the rat of nucleic acid pyrimidines, uracil and thymine, has been shown by several investigators(9,10,11) to proceed *via* reduction to corresponding dihydropyrimidines prior to ring cleavage yielding *beta*-alanine and *beta*-aminoisobutyric acid respectively. Existence of an oxidative mechanism was substantiated by the observation that incubation of radiothymine with rat liver slices also gave rise to 5-hydroxymethyluracil and uracil-5-carboxylic acid(12). The oxidative metabolism of uracil and thymine in microorganisms has been reported in a soil bacterium to involve only a limited attack of the 6-C

TABLE II. Comparison of Component III with Synthetic 2-Methylthio-4-amino-5-pyrimidinecarboxylic acid.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Nitrogen	"	"
Melting point	220-30 dec.‡	219-228 dec.‡
Ultraviolet spectrum	In both cases no discernible difference	
Ultraviolet nature	Fluorescent	Fluorescent
Rf values†	In both cases no discernible difference	
Microbiological assay*	Negative	Negative

* Microbiological assay: No inhibition of *B. subtilis* 6051 or *B. subtilis* 6051/A.

† Solvents (see text for ratios)

1. n-butyl alcohol—glacial acetic acid-water — Rf = 0.75
2. ethanol—NH_4OH — Rf = 0.32
3. n-propyl alcohol—water — Rf = 0.60

‡ Uncorr.

TABLE III. Comparison of Component IV with Impurity Isolated from Stock 2-Methylthio-4-amino-5-hydroxymethylpyrimidine.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Lithium	Negative	Negative
Aluminum	Positive	Not determined
M. P.	200-210 dec uncorr	200-210 dec uncorr
Acidity and basicity	Amphoteric	Amphoteric
Ultraviolet absorption		
.1N HCl	Max 245-250 mμ	Max 245-250 mμ
.1N NaOH	Max 284 mμ	Max 284 mμ
Treatment with warm acid	Yields methioprim	Not determined
Physical nature	Hygroscopic	Not isolated as a solid

position of the pyrimidine ring(13). Others (14,15) have obtained barbituric acid from uracil oxidation by strains of Nocardia, Corynebacteria, and Mycobacteria.

The biological oxidation of the hydroxymethyl group to an acid is not limited to pyrimidines. Pyridoxine is converted to 4-pyridoxic acid(16) and other data implies that pantothenyl alcohol gives rise to pantothenic acid (17).

It is of interest to note that the magnitude of antibacterial activity as measured in this study declines in sequence with the oxidation of methioprim *i.e.* $-CH_2OH > -CHO >$ COOH (inactive).

Summary. Rat liver slices convert methioprim to 2-methylthio-4-amino-5-formylpyrimidine and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid. Another unidentified ultraviolet-absorbing material is also produced. The antibacterial activity of methioprim is greater than the 5-CHO derivative, whereas the 5-COOH derivative is inactive.

1. Guthrie, R., Loebeck, M. E., Hillman, M. J., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 792.

2. Holland, J. F., Guthrie, R., Sheehe, P., Tieckelmann, H., *Cancer Research,* 1958, v18, 776.

3. Krebs, H. A., *Biochim. et Biophys. Acta,* 1950, v4, 249.

4. Guthrie, R., *J. Bact.,* 1949, v57, 39.

5. Okuda, T., Price, C. C., *J. Org. Chem.,* 1958, v23, 1738.

6. Ulbricht, T. L. V., Price, C. C., *idem.,* 1956, v21, 567.

7. Hoover, J. R. E., Fisher, J., Presented at A.C.S. Regional Meeting, Delaware Valley, Philadelphia, 1958.

8. Nairn, J. G., Ph.D. Thesis, Dept. of Chemistry, University of Buffalo, Buffalo, N. Y., 1958.

9. Fink, R. M., McGaughey, C., Cline, R. E., Fink, K., *J. Biol. Chem.,* 1956, v218, 1.

10. Fink, K., Cline, R. E., Henderson, R. B., Fink, R. M., *idem.,* 1956, v221, 425.

11. Cannelakis, E. S., *idem.,* 1957, v227, 329.

12. Fink, K., McGaughey, C., Henderson, R. B., *Fed. Proc.,* 1956, v15, 251.

13. Wang, T. P., Lampen, J. O., *J. Biol. Chem.,* 1952, v194, 785.

14. Hayaishi, O., Kornberg, A., *idem,* 1952, v197, 717.

15. Lara, F. J. S., *J. Bacteriol.,* 1952, v64, 279.

16. Huff, J. W., Perlzweig, W. A., *Science,* 1944, v100, 15.

17. Rubins, S. H., Cooperman, J. M., Moore, M. E., Scheiner, J., *J. Nutrition,* 1948 v35, 499.

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Ascending Infection as a Mechanism in Pathogenesis of Experimental Non-Obstructive Pyelonephritis.* (25206)

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Experimental pyelonephritis has generally been produced by methods involving intra- or extra-renal obstruction to the flow of urine with subsequent injection of bacteria into the blood stream. Ureteral ligation(1,2), electrocauterization(3), renal massage(4), or the scars of antecedent staphylococcal infection (5) have resulted in the localization of bacteria in the traumatized kidney with subsequent development of acute pyelonephritis. In addition, *S. aureus* in rabbits and mice(6), *P. acruginosa* in mice(7), *S. fecalis* in rats (8) and *C. renale* in mice(9) have, after intravenous injection, produced infections of kidney in the absence of induced urinary obstruction. The experimental models involving obstruction and hematogenous dissemination have been widely studied. In the absence of a clear demonstration that infection of the kidney may ascend directly from the bladder, the concept has become increasingly accepted that the hematogenous route is the principal one for the spread of infection to the kidneys, although many investigators have indicated the lack of definitive experimental informa-

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