TABLE II

5-(1-METHYL-2-THIOALKYLETHYLIDENE)-RHODANINES (II)

R	M.p.,[a] °C.	Yield,[b] %	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Inhibition, % *A. niger* at 250 p.p.m.
CH_3	110–110.5	48	$C_7H_9ONS_3$	38.33	38.73	4.13	4.22	100[g]
C_2H_5	127–128	31	$C_8H_{11}ONS_3$	41.17	41.45	4.75	5.07	47
C_3H_7	90.5–91.5	31[c]	$C_9H_{13}ONS_3$	43.69	44.01	5.30	5.47	64
$(CH_3)_2CH$	112–112.5	21	$C_9H_{13}ONS_3$	43.69	44.03	5.30	5.38	49
C_4H_9	106.5–107.5	44[d]	$C_{10}H_{15}ONS_3$	45.94	46.03	5.78	5.42	15
$(CH_3)_2CH—CH_2$	112–112.5	21	$C_{10}H_{15}ONS_3$	45.94	46.12	5.78	5.56	14
$(CH_3)_3C$	127.5–128	30	$C_{10}H_{15}ONS_3$	45.94	46.23	5.78	6.10	39
C_5H_{11}	91–91.5[e]	14	$C_{11}H_{17}ONS_3$	47.96	47.99	6.22	6.02	20
C_6H_{13}	91.5–92	76[f]	$C_{12}H_{19}ONS_3$	49.79	49.52	6.62	6.68	20

[a] Except as noted all compounds crystallized from cyclohexane or benzene–cyclohexane as yellow needles. [b] Except as noted yields are for material melting within at least five degrees of the analytical sample. [c] For crude product, m.p. 79–81°. [d] Crude product m.p. 93–95°. Yellow flakes. [f] M.p. 83–85°. [g] P.p.m. causing % inhibition 250, 100; 100, 100; 50, 64; 25, 28.

servation of Davies and Sexton[10] that sulfur *per se* makes no contribution to fungistic activity.

Experimental

Alkyl Acetonyl Sulfides.[8,9]—These were prepared by the following general procedure: To a solution prepared by dissolving 7.7 g. of sodium in 200 ml. of ethanol, one-third mole of mercaptan was added. The mixture was stirred and chilled in an ice-bath while 33.3 g. of chloroacetone was added dropwise over a period of 20 minutes with stirring (precipitation of sodium chloride). The mixture was refluxed for two hours with vigorous stirring. The salt was removed by filtration. Fractionation yielded the mercaptan as a yellow-greenish or greenish liquid.

Fungistatic Testing.—These tests measured the inhibition in the rate of radial growth of *Aspergillus niger* produced when the culture medium contained a known concentration, usually 250 parts per million (p.p.m.), of the rhodanine derivative. The procedure was essentially that of Leonard and Blackford.[11]

(10) W. H. Davies and W. A. Sexton, *Biochem. J.*, **40**, 331 (1946).

(11) J. M. Leonard and V. L. Blackford, *J. Bact.*, **57**, 339 (1949).

DURHAM, N. C.

[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Isolation and Identification of a Naturally Occurring Analog of Methionine[1a]

BY ROBERT A. MCRORIE,[1b] GEORGE L. SUTHERLAND,[1c] MARGARET S. LEWIS, A. D. BARTON,[1d] MARGARET R. GLAZENER AND WILLIAM SHIVE

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A heat-labile compound more effective in preventing the toxicity of sulfanilamide than molar equivalents of methionine for *Escherichia coli* has been isolated from cabbage juice and identified as a 3-amino-3-carboxypropyldimethylsulfonium salt, $(CH_3)_2S^+—CH_2—CH_2—CH(NH_2)—COOH$. The enhanced activity of this methionine analog and its wide distribution in plants suggest an important role in the storage or transfer of methyl groups.

During the course of a survey of the effects of natural materials on the reversal of sulfonamide toxicity for *E. coli* in the basal medium employed for the assay of vitamin B_{12},[2] cabbage juice was observed to exert an effect on aseptic addition which was markedly decreased when the sample was heated prior to assay or autoclaved in the assay medium. This loss in activity was far in excess of that to be expected from destruction of methionine, *p*-aminobenzoic acid or vitamin B_{12} which support growth under the conditions employed.

On the basis of measurements by bioautograph techniques,[3] the loss in activity appeared to be due to the destruction of a single compound which differed in R_f from all compounds known to be active in the assay as shown in Fig. 1. An effect of the active principle in reversing the toxicity of sulfanilamide for *E. coli* was noted only under conditions of limiting methionine biosynthesis. The ability of the compound to replace only methionine among the various reversing agents for sulfonamide inhibitions in *E. coli* suggested that the active principle was probably closely related biochemically if not structurally to methionine. The same zone which gave biological activity on paper chromatograms gave a positive ninhydrin reaction (yellow turning to purple on standing or after prolonged heating), a weak positive test for sulfur with the iodine–azide reagent,[4] and a negative nitroprusside test for

(1) (a) Preliminary reports covering portions of this work have been presented: W. Shive, R. A. McRorie, G. L. Sutherland, M. S. Lewis, M. Rupp, J. L. Reger and F. Armstrong, 121st Meeting, American Chemical Society, Milwaukee, Wis., March, 1952; W. Shive, *International Review of Vitamin Research*, **23**, 329 (1952); (b) Eli Lilly and Co. Research Assistant; (c) Eli Lilly and Co. Postdoctorate Research Fellow, 1949–1951; (d) Eli Lilly and Co. Postdoctorate Research Fellow, 1948–1949.

(2) W. Shive, *Ann. N. Y. Acad. Sci.*, **52**, 1212 (1950); W. Shive, E. R. Alexander and M. S. Lewis, in preparation.

(3) W. A. Winsten and E. Eigen, *Proc. Soc. Exp. Biol. Med.*, **67**, 513 (1948).

(4) E. Chargaff, C. Levine and C. Green, *J. Biol. Chem.*, **175**, 67 (1948).

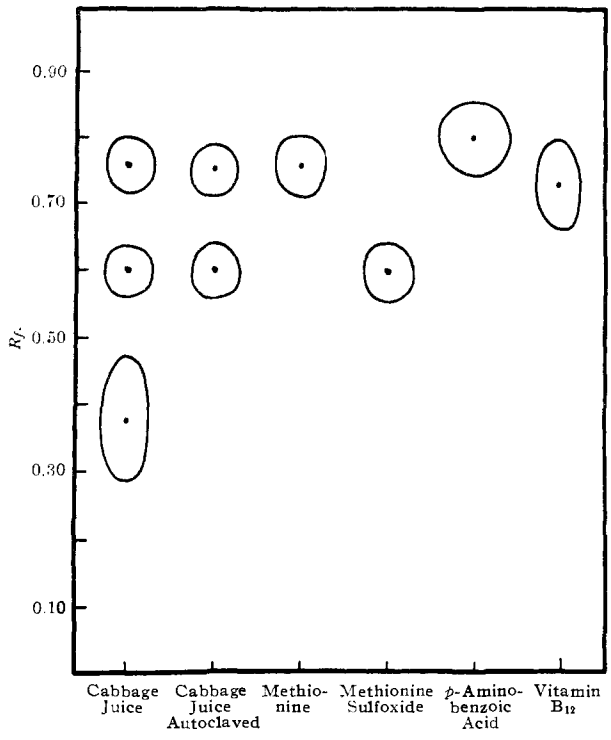

Fig. 1.—Bioautograph of cabbage juice and other compounds reversing sulfanilamide toxicity for *E. coli* (Texas); solvent, 65% pyridine (ascending).

–SH or –S–S– groups. However, no inactivation or change in R_f was observed on treatment with peroxide or peroxide and molybdate under conditions which oxidize methionine to the sulfoxide and sulfone, respectively.[5]

These results suggest that the compound might be a derivative of methionine with a modified sulfur atom. Since concentration procedures indicated that a strongly basic group was present in the molecule, sulfonium derivatives of methionine were considered, and the methylsulfonium derivative of methionine synthesized. The probable identity of this synthetic compound with the natural was ascertained by paper chromatography in a number of different solvents and development of bioautographs to obtain the same R_f values for the natural and synthetic factors. Both the natural and synthetic factors decomposed on heating in aqueous solution to form a compound which had the same R_f values as homoserine in a number of different solvents.

The natural factor was isolated as the crystalline bromide which had the same X-ray diffraction pattern as synthetic 3-amino-3-carboxypropyldimethylsulfonium bromide. The isolation of the natural factor was hampered by a gradual loss of activity. Even synthetic material in the solid state gradually decomposes.

Details of the microbiological activity of the compound are presented elsewhere.[6]

Experimental

Assay.—*E. coli* (Texas) was used as the assay organism in the medium adapted for assay of vitamin B_{12}.[2] In some assays, L-glutamic acid was replaced by twice as much DL-glutamic acid since the methionine contamination of some samples of the natural isomer gave high blank readings. DL-Methionine was used as a standard at levels of 0–75 mμM per 10 ml. Samples for assay were sterilized by filtration and added aseptically after autoclaving the basal medium. After the factor had been concentrated several fold, dilutions in sterile water could be added directly to the sulfonamide-containing medium without obtaining contaminated cultures in the short incubation period. Assays were incubated 14–16 hours at 37°. In initial assays, samples were added before and after autoclaving. After the alcohol precipitation steps in the concentration, only the heat-labile activity remained, and subsequent assay for heat stable activity was unnecessary. A typical response curve is shown in Table I.

TABLE I

GROWTH RESPONSE OF *E. coli* (TEXAS) TO CABBAGE JUICE AND METHIONINE

Additions per 10 ml. medium	Galvanometer reading[a]	
Cabbage juice, ml.	Aseptic	Autoclaved
0	11	11
0.003	15	11
.01	18	12
.03	28	12
.10	47	16
Methionine, mμ M		
5		13
10		20
15		27
20		34
50		42
75		45

[a] Distilled water reads 0; an opaque object, 100.

Extraction Procedure.—Vegetables were diced and ground in a meat grinder, and the juice was removed by squeezing in a towel or juice press. After the fine pulp had settled, decantation through cheesecloth gave a clear filtrate. The relative activity of vegetable juice extracts tested was: parsley > cabbage > turnip greens > turnips > pepper, carrot, onion or lettuce. No appreciable activity could be detected in extracts of yeast, liver or spleen. Cabbage was selected for further study as the most readily available active source. A typical 50-lb. batch of cabbage yields 7–10 liters of juice containing 50 mg. per ml. of dry solids and having an activity equivalent to 150–200 γ DL-methionine per ml., although great variations in activity were observed for different batches of juice.

Concentration Procedure. Exchange on IRC-50 Resin.—(A) Filtered juice was percolated over IRC-50 resin (200 g. resin/liter of juice) which had been converted to the barium form by treatments with barium acetate. The column was washed with water and eluted with 1 N hydrochloric acid. Successive 100-ml. fractions were collected, and essentially all activity was removed just after the eluate became acid. Excess barium was removed from the active fractions by precipitation with dilute sulfuric acid, and the precipitated barium sulfate was removed by centrifugation. After neutralization, the active fractions were evaporated to dryness *in vacuo*. The concentrate, although containing inorganic salts, was twice as active as the original solids of cabbage juice and contained 79% of the original activity.

(B) An alternate step involved the conversion of the resin to the ammonium form with ammonium hydroxide and eluting with 1 N acetic acid. Active fractions were combined and dried *in vacuo*, and excess ammonium acetate was removed by sublimation under reduced pressure at 40–50°. The residue was 3 to 4 times as active as the original cabbage juice solids and contained 67% of the original activity. This modification yields more stable preparations and requires less manipulation.

Alcohol Fractionation and Chromatography on Alumina.—Solids (16 g.) from either of the above concentration procedures were suspended in 80 ml. of methanol with stirring. Insoluble material (4 g.) was removed by centrifugation, washed with two 10-ml. portions of methanol and discarded.

(5) C. E. Dent, *Biochem. J.*, **43**, 169 (1948).

(6) R. A. McRorie, M. R. Glazener and W. Shive, in preparation.

Isopropyl alcohol (500 ml.) was added to the combined supernatant and washings and placed in the cold for 3–4 hours to complete precipitation. The precipitate (1–2 g.) was removed by centrifugation and discarded. All activity was absorbed on alumina when the centrifugate was percolated through a column prepared as a slurry in methanol (6 g. of alumina per g. of solids). The column was washed successively with 300-ml. portions of methanol, 75% methanol and 50% methanol. Elution was accomplished with water. The active aqueous fractions were combined and filtered through Celite to remove suspended alumina. The solids at this stage were purified 100-fold over those obtained from elution of the IRC-50 resin column. The yield was 60–70% of the total activity of the starting eluate.

At this stage in the concentration, a comparison with synthetic 3-amino-3-carboxypropyldimethylsulfonium salts[7] on paper chromatographs gave similar R_f values in various solvents as shown in Table II.

TABLE II

COMPARISON OF R_f VALUES OF CABBAGE JUICE FACTOR AND 3-AMINO-3-CARBOXYPROPYLDIMETHYLSULFONIUM SALTS

Solvent	R_f values[a] Natural	Synthetic
Butanol–water–acetic acid (4:1:1)	0.10	0.12
2,6-Lutidine 65%	.08	.08
Pyridine 65%	.38	.37
1-Butanol–pyridine–water (5:5:2)	.02	.02
1-Butanol–pyridine–water (1:4:1)	.05	.04
Ethylene glycol monobutyl ether–ammonia–water (4:1:1)	.30	.30
t-Butyl alcohol saturated with water	.32	.30
1-Butanol–ethyl-2 *N* hydrochloric acid (4:1:2)	.14	.14

[a] Ninhydrin and bioautographs.

Precipitation as Phosphotungstate and Crystallization as Bromide.—Samples of the synthetic methylsulfonium derivative of methionine could be precipitated with phosphotungstate and converted to halides by quaternary ammonium halides; so this method was applied to the natural concentrate. A similar procedure has been used recently for the separation of the methylsulfonium derivative of methionine prepared chemically from the methionine in casein hydrolysates.[8]

The active fractions eluted from the alumina column were adjusted to *p*H 4 and precipitated in the cold with excess phosphotungstic acid. After standing overnight the precipitate was recovered by centrifugation, washed with water and decomposed by stirring with four times its weight of tetraethylammonium bromide in 50% aqueous acetone for 1 hour. After centrifugation, the supernatant was concentrated to a sirup *in vacuo* and precipitated by the addition of absolute ethanol. The precipitate was washed with several portions of absolute ethanol to remove excess tetraethylammonium bromide and dissolved in a few drops of water. Absolute ethanol (10 ml.) was added, and the solution was allowed to stand. White platelets of the bromide salt separated on standing in the cold for 2–3 days and after drying decomposed at 139°. The crystalline factor was concentrated approximately 1000-fold over the original cabbage juice. A synthetic sample of the methylsulfonium bromide derivative of methionine decomposed at 140°, and a mixture of the natural product with the synthetic material decomposed at 139–140°.

A comparison of the X-ray diffraction patterns[8a] is indicated by the interplanar spacings of the synthetic and natural salts shown in Table III.

Degradation Product of the Methylsulfonium Derivative of Methionine.—Methioninemethylsulfonium iodide (500 mg.) was autoclaved in 100 ml. of water for two hours. The solution contained a compound which gave the ninhydrin reaction and had migration characteristics identical with homoserine as indicated in Table IV. The water was removed *in vacuo*, and the resulting yellow viscous liquid slowly crystallized after standing at 4°. The crystals were washed with dry 1-butanol, chloroform and carbon tetrachloride. The needle-like crystals melted at 179–181°.

Anal. Calcd. for $C_4H_8NO_2I$: N, 6.12. Found: N, 6.11.

TABLE III

INTERPLANAR SPACINGS IN 3-AMINO-3-CARBOXYPROPYLDIMETHYLSULFONIUM BROMIDE

Synthetic, Å.	Isolated, Å.	Order of intensity
4.57	4.56	2
4.02	4.02	3
3.69	3.63	1
3.14	3.10	7
3.00	2.97	6
2.73	2 68	4
2.57	2.60	5

TABLE IV

R_f VALUES OF HEAT DEGRADATION PRODUCT OF METHIONINEMETHYLSULFONIUM IODIDE IN VARIOUS SOLVENTS

Solvent	R_f values[a] Degradation product[b]	Homoserine
Pyridine 65%	0.58	0.58
2,6-Lutidine 65%	.28	.28
95% Ethanol–ammonium hydroxide (95:5)	.36	.36
1-Butanol–95% ethanol–ammonium hydroxide (8:1:3)	.14	.14
1-Butanol–acetic acid–water (4:1:1)	.23	.23
Isobutyric acid 85%	.34	.33
95% Ethanol–acetic acid (95:5)	.21	.21

[a] Ninhydrin, single and mixed chromatograms. [b] Autoclaved 20 min. in aqueous solution.

A sample of homoserine lactone hydroiodide prepared by treating homoserine with hydriodic acid and similarly purified melted at 178–180° and showed no depression in melting point on mixing with the lactone hydriodide of the degradation product of the methylsulfonium derivative of methionine.

Anal. Calcd. for $C_4H_8NO_2I$: N, 6.12. Found: N, 5.89.

Discussion

Although methylsulfonium salts of methionine have been prepared previously[7] and have been shown to replace methionine for the rat,[9] in creatine biosynthesis in rat liver slices[10] but not in homogenates,[11] and as a sole sulfur source for *Proteus morganii*,[12] this study presents the first evidence for the natural occurrence of this unique amino acid. Since it is three times as active as methionine on a molar basis, as shown in Fig. 2, in the sulfonamide system where the incorporation of a single carbon unit is blocked, it apparently serves not only as a precursor of methionine but also as a precursor of some other essential cell constituent, possibly by incorporation of a labile methyl group. Choline, betaine, sarcosine and dimethyl-β-propiothetin (a possible product of methionine methylsulfonium salts) are not effective alone or in combination with methionine in resolving the difference in activity between methionine and its methylsulfonium analog. This specificity suggests the possibility of a rather unique role of the sulfonium derivative. Subsequent to the preliminary reports of

(7) G. Toennies and J. J. Kolb, THIS JOURNAL, **67**, 849 (1945).

(8) T. F. Lavine and N. F. Floyd, *Federation Proc.*, **12**, 236 (1953).

(8a) We are indebted to Dr. S. H. Simonsen of the Department of Chemistry for the X-ray diffraction data.

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(10) P. Handler and M. L. C. Bernheim, *ibid.*, **150**, 335 (1943).

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(12) F. P. Meyers and J. R. Porter, *J. Bact.*, **50**, 323 (1945).

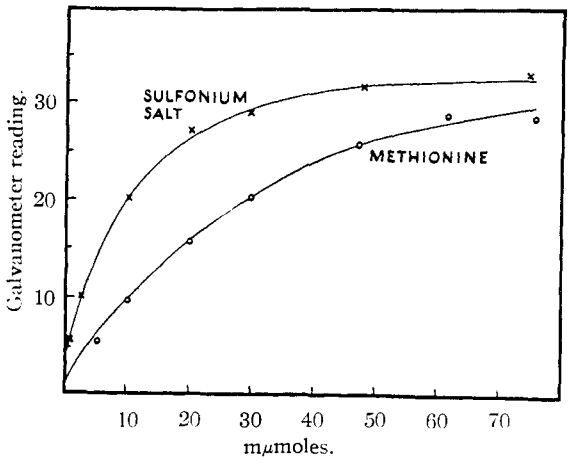

Fig. 2.—Growth response of *E. coli* (Texas) to L-methionine and L-methioninemethylsulfonium iodide.

the presence of the methylsulfonium derivative of methionine in cabbage juice, an intermediate, a sulfonium derivative of methionine which is probably S-adenosylmethionine, has been reported to be involved in the biological transfer of the methyl group of methionine to certain other products containing biologically active labile methyl groups.[13] However, since some organisms requiring methionine do not respond to its methylsulfonium derivative,[6] and since it has been found to be inactive in the biosynthesis of creatine by liver homogenates which can synthesize creatine from methionine,[11] no universal role can be attributed to the compound. By the action of certain organisms upon the methylsulfonium derivative of methionine, dimethyl sulfide is released.[14] In the present investigation, homoserine was identified as a product of decomposition of the methylsulfonium derivative heated in aqueous solution. A characteristic odor of dimethyl sulfide was also obtained under these conditions.

It is interesting that the occurrence and heat-lability of the methylsulfonium derivative parallels closely the occurrence and heat-lability of vitamin U, an unidentified factor in cabbage and other vegetable juices which is reported to reduce the incidence of histamine-induced ulcers in guinea pigs and to be beneficial in treatment of peptic ulcers in human beings.[15]

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AUSTIN 12, TEXAS

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Mannich Reactions of Pyrimidines. I. 2,6-Dimethyl-4-hydroxypyrimidine[1]

BY H. R. SNYDER AND HAROLD M. FOSTER[2]

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2,6-Dimethyl-4-hydroxypyrimidine reacts with formaldehyde and piperidine to yield bis-(1-piperidylmethyl) and tris-(1-piperidylmethyl) derivatives. A similar condensation was observed with morpholine in place of piperidine, but not with dimethylamine. The structure of the bis-(1-piperidylmethyl) derivative was established as 2-bis-(1-piperidylmethyl)-methyl-4-methyl-6-hydroxypyrimidine by reductive cleavage of the pyrimidine ring to yield *n*-butyramide, and by hydrogenolysis of the carbon to nitrogen bond of the Mannich base to yield 2-isopropyl-4-methyl-6-hydroxypyrimidine. Products of the reactions of the bis-(1-piperidylmethyl) Mannich base with methyl iodide and with acetic anhydride are described. Evidence for the formulation of the tris-(1-piperidylmethyl) derivative as 2-bis-(1-piperidylmethyl)-methyl-4-(1-piperidyl-2-ethyl)-6-hydroxypyrimidine is given. An improved procedure for the synthesis of 2,6-dimethyl-4-hydroxypyrimidine is described.

Numerous aldol type condensations in the pyrimidine series involving either methyl substituents or the nucleus have been observed. In general, simple methylpyrimidines undergo reaction at the methyl group,[3] whereas methylpyrimidines which have any combination of two or more hydroxy, thio, or amino substituents undergo nuclear condensation.[4-8] 4 - Methyl - 6 - hydroxypyrimidine[9] and 2-hydroxy-4,6-dimethylpyrimidine,[10] two methylpyrimidines which have but a single ring-activating substituent, react with aromatic aldehydes to yield styryl derivatives.

In the present work, the reactivity of 2,6-dimethyl-4-hydroxypyrimidine (I) in the Mannich reaction was studied. This pyrimidine yielded no solid products when treated with formaldehyde and dimethylamine under a variety of conditions. On the other hand, 2,6-dimethyl-4-hydroxypyrimidine reacted with piperidine and formaldehyde in benzene–ethanol solution to yield two Mannich bases. The material which was isolated in the greater amount has the composition calculated for $C_{18}H_{30}N_4O$, and proved to be a bis-(1-piperidylmethyl) derivative (II) of I. The other base has the composition calculated for $C_{24}H_{41}N_5O$, and is a tris-(1-piperidylmethyl) derivative (III) of I. When 0.05 mole of I was treated with approximately 0.1 mole each of piperidine and formaldehyde, II was isolated in yields of about 40%. When the reaction was scaled up to twice this size, the yield of II fell to about 25%; III was isolated in yields of

(1) Abstracted in part from the Thesis submitted by H. M. Foster to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1953.
(2) National Science Foundation Fellow, September, 1952, to July, 1953.
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