The Effect of Some Heterocyclic Disulfides and Thiones on the Carbohydrate Metabolism of Ehrlich Ascites Tumor[1]

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The effect of six thione-forming disulfides and five corresponding thiones on carbohydrate metabolism of Ehrlich ascites tumor has been studied by manometric and isotopic methods. The effects observed confirm previous observations in that basic disulfides are strong inhibitors of glycolysis and respiration, while acidic disulfides, as well as most of the thiones, have little or no effect. Two thiones however affect carbohydrate metabolism. 5-Cyano-(2-thiopyridone) appears to prevent the aerobic utilization of exogenous glucose, whereas benzothiazole-2-thione causes a partial reversal of the Crabtree effect.

We have recently studied the effect of some thione-forming disulfides on the carbohydrate metabolism of Ehrlich ascites tumor cells,[2,3] and their interaction with Ehrlich ascites cells and homogenates.[4-6] In our continuing study of the effect of disulfides and thiols on cell metabolism, we are now presenting the results obtained with several thione-forming disulfides and the corresponding thiones. These disulfides have the property of reacting with thiols in an essentially complete and irreversible manner, as exemplified by 2,2′-dithiobis(isonicotinic acid). The products are a disulfide and a heterocyclic thione (in this case 4-carboxy-2-thiopyridone). The thione nature of these compounds is evidenced by their uv spectra.[7]

Materials and Methods.—Swiss mice, bearing 5- to 10-day old ascites tumor, were sacrificed by cervical fracture. The fluid was collected, pooled, heparinized (50 USP units/ml), and used immediately. Manometric determinations were carried out in a conventional Warburg apparatus at 37°. Readings were taken at 5-min intervals for 1 hr or more after introduction of the compound under study. The detailed procedure for the manometric experiments, as well as the determination of solubilities, was given previously.[2]

The isotopic experiments were performed as described elsewhere;[6] the experiments with IV and V were performed three times, those with VIII twice, all with closely agreeing results. The result of one individual experiment with each compound is reported in Figures 1, 2, and 3.

Results

Each disulfide used (Table I) reacted quantitatively with cysteine to give the corresponding thione.[4,5,7,8] The thiones were stable to air oxidation with the exception of the amino derivative II, which was readily oxidized to the disulfide,[7] and could therefore not be studied under the present experimental condi-

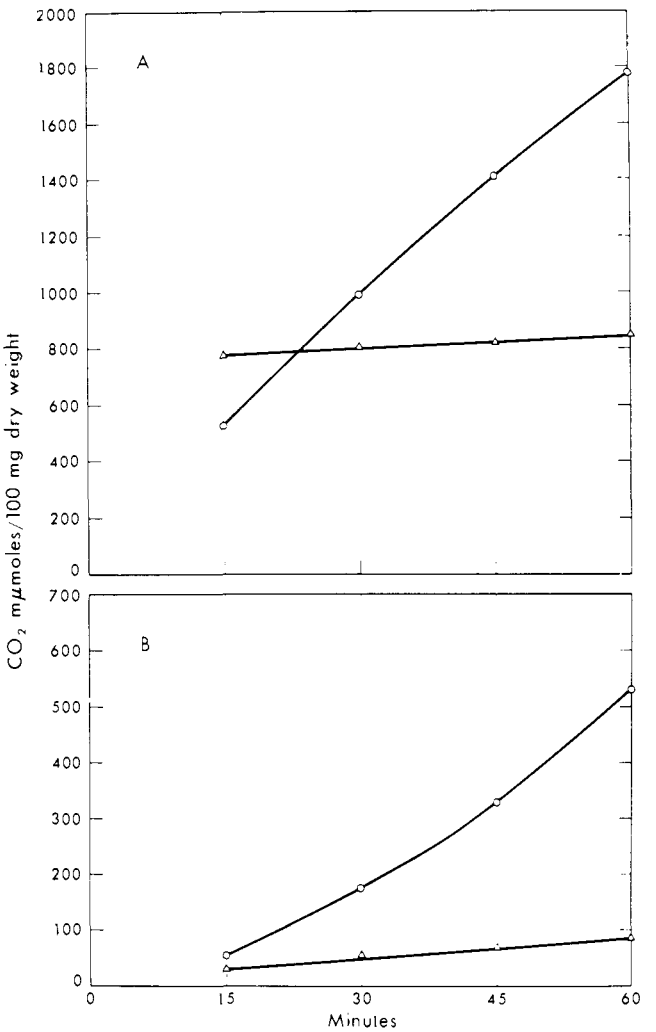

Figure 1.—Effect of 5-cyano-2-thiopyridone (IV) on aerobic production of $^{14}CO_2$ from [^{14}C]glucose by Ehrlich ascites tumor cells. Each flask contained 30 μmol of [^{14}C]glucose (1 μCi) and about 50 × 10^6 washed Ehrlich ascites cells (about 15 mg dry weight) in Krebs–Ringer phosphate buffer (pH 7.2). Total volume per flask was 3.0 ml. Incubations were carried out at 37°. $^{14}CO_2$ was collected and counted as reported previously:[6] (○) $^{14}CO_2$ production by washed Ehrlich ascites cells; (△) $^{14}CO_2$ production in the presence of 3 μmol of IV. (A) Evolution of $^{14}CO_2$ from 1-[^{14}C]glucose (O_2–CO_2, 95:5). The figures plotted were obtained by subtracting the values for $^{14}CO_2$ recovery using 6-[^{14}C]glucose from those obtained using 1-[^{14}C]glucose. (B) Evolution of $^{14}CO_2$ from 6-[^{14}C]glucose (O_2–CO_2, 95:5).

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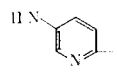
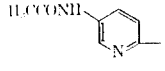
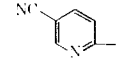
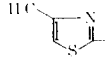
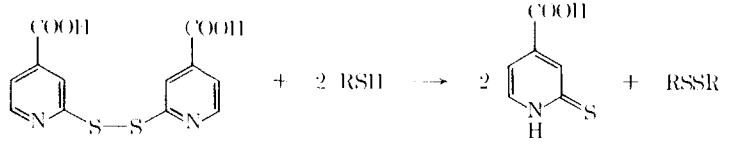
(8) The reaction of XI with thiols, which we have not reported previously, leads also quantitatively to the thione V.

TABLE I[a]

R	Thiones R—C(=S)—NH							Disulfides RSSR					
	Compound	Ref prepn	Concn, 10^{-3} M	% change Q_{O_2}	% change Q_{O_2}(G)	% change $Q^{O_2}_{CO_2}$	% change $Q^{N_2}_{CO_2}$	Compound	Ref prepn	Concn,[b] M	% change Q_{O_2}	% change Q_{O_2}(G)	% change $Q^{O_2}_{CO_2}$	% change $Q^{N_2}_{CO_2}$
	I	*b*	5	+20	0	+20	−5	VII	*h*	5×10^{-3}	+10	+10	+15	0
	II	*c*						VIII	*i*	5.1×10^{-3}	−90	+40[c]	−80	−95
	III	*d*	1	0	+15	+5	0	IX	*d*	2.8×10^{-3}	−60[d]	+5	+15	−10
	IV	*e*	1	+10	+45[f]	+5	+5	X	*i*	1.5×10^{-3}	−50[d]	+10	+5	−15
	V	*g*	1	−5	+30	+125	+40	XI	*g*	7×10^{-3}	−5	0	0	+10
	VI	*g*	1	0	0	+15	0	XII	*l*	3.5×10^{-3}	90	−25	−20	−90

[a] Experiments were carried out at 37° for 1 hr, except as noted. Q values represent μl mg of dry weight per hour. Q_{O_2} = rate of O_2 uptake in air; Q_{O_2}(G) = rate of O_2 uptake in air in the presence of 0.05 M glucose; $Q^{O_2}_{CO_2}$ = rate of CO_2 evolution in O_2:CO_2 (95:5) in the presence of 0.05 M glucose; $Q^{N_2}_{CO_2}$ = rate of CO_2 evolution in N_2:CO_2 (95:5) in the presence of 0.01 M glucose. The results reported are the average of two to four closely agreeing experiments, and express the per cent difference between the control and the flask containing the inhibitor. [b] H. H. Fox and J. T. Gibas, *J. Org. Chem.*, **23**, 64 (1958). [c] This thione is readily oxidized by air[7] and could not be used in the present experiments. [d] W. T. Caldwell and E. C. Kornfeld, *J. Amer. Chem. Soc.*, **64**, 1695 (1942). [e] H. Forrest and J. Walker, *J. Chem. Soc.*, 1939 (1948). [f] This stimulation of Q_{O_2}(G) brings the rate of O_2 uptake close to that obtained in the absence of added glucose. [g] Commercial products. [h] The concentrations of VII, IX, X, XI, and XII are their solubilities in buffer under the conditions used (see Materials and Methods). [i] D. B. Grassetti and J. F. Murray, Jr., *Anal. Chim. Acta*, **46**, 139 (1969). [j] In the first hour, a 30–50% stimulation, and in the second hour a 30% inhibition are observed. [k] A gradual inhibition takes place, which becomes almost complete after 60–90 min. [l] E. B. Buchman, A. O. Reims, and H. Sargent, *J. Org. Chem.*, **6**, 764 (1941).



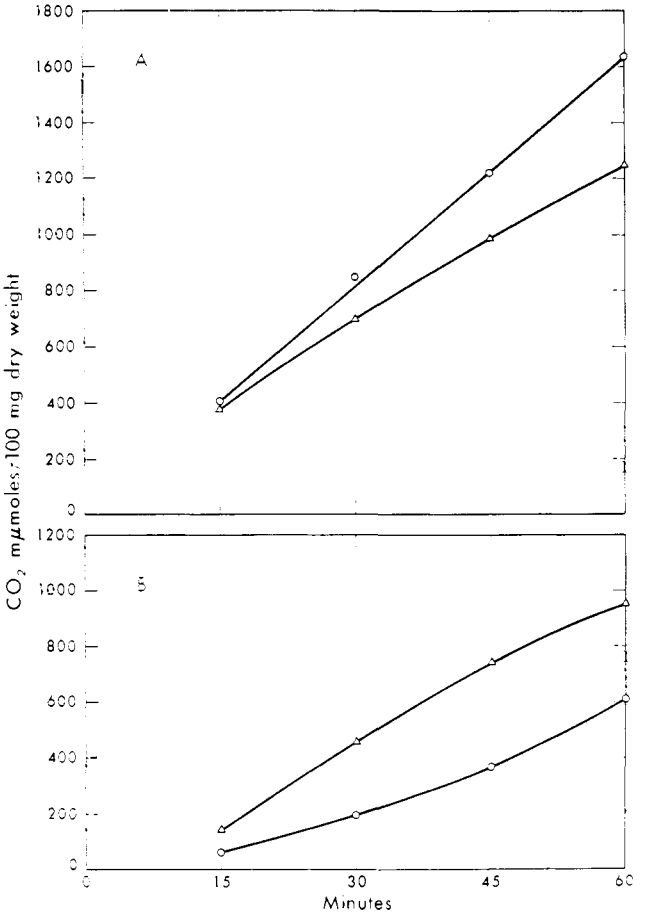


Figure 2.—Effect of benzothiazole-2-thione (V) on aerobic production of $^{14}CO_2$ from [^{14}C]glucose by Ehrlich ascites tumor cells. See Figure 1 for experimental conditions. (○) $^{14}CO_2$ production by washed Ehrlich ascites cells; (△) $^{14}CO_2$ production in the presence of 3.0 μmol of V. (A) Evolution of $^{14}CO_2$ from 1-[^{14}C]glucose (O_2–CO_2, 95:5). The figures plotted were obtained by subtracting the values for $^{14}CO_2$ recovery using 6-[^{14}C]-glucose from those obtained using 1-[^{14}C]glucose. (B) Evolution of $^{14}CO_2$ from 6-[^{14}C]glucose (O_2–CO_2, 95:5).

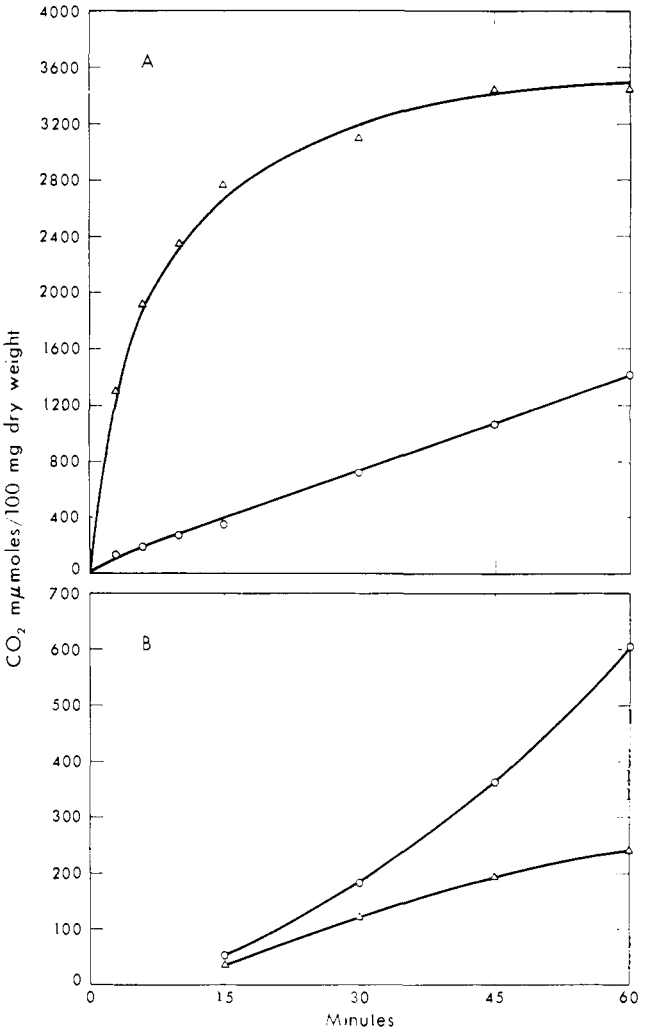


Figure 3.—Effect of 2,2'-dithiobis(5-aminopyridine) (VIII) on aerobic production of $^{14}CO_2$ from [^{14}C]glucose by Ehrlich ascites tumor cells. See Figure 1 for experimental conditions. (○) $^{14}CO_2$ production by washed Ehrlich ascites cells; (△) $^{14}CO_2$ production in the presence of 1.5 μmol of VIII. (A) Evolution of $^{14}CO_2$ from 1-[^{14}C]glucose (O_2–CO_2, 95:5). The figures plotted were obtained by subtracting the values for $^{14}CO_2$ recovery using 6-[^{14}C]glucose from those obtained using 1-[^{14}C]glucose. (B) Evolution of $^{14}CO_2$ from 6-[^{14}C]glucose (O_2–CO_2, 95:5).

tions. Results of the manometric experiments are reported in Table I.

Thiones.—Whereas thiones I, III, and VI have marginal effects, IV and V elicited a significant metabolic response. Compound IV caused a stimulation of Q_{O_2} (G), and had no effect on the other properties studied (Table I). This stimulation corresponds to an apparent reversal of the Crabtree effect amounting to about 70%. Further studies with 1-[^{14}C]glucose and 6-[^{14}C]glucose as substrates were undertaken. The aerobic metabolism of glucose by Ehrlich ascites cells *via* the hexose monophosphate (HMP) pathway in the presence of IV, as judged from the production of $^{14}CO_2$ from 1-[^{14}C]glucose, after an initial stimulation, soon comes to a standstill (Figure 1A). The conversion of the C-6 of glucose to CO_2 can be used as a measure of oxidation *via* the Krebs cycle. Results presented in Figure 1B suggest that IV exerts an inhibitory activity on this cycle. A more detailed study of the metabolic effects of this interesting compound is in progress and will be reported elsewhere.

Thione V, while stimulating Q_{O_2} (G), also stimulates aerobic and anaerobic glycolysis. The stimulation of Q_{O_2} (G) corresponds to an apparent reversal of the Crabtree effect[9] of about 50%, as measured manometrically. Results of experiments using the ^{14}C-labeled glucoses (Figure 2) show that V caused a slow inhibition of the HMP pathway under these conditions (Figure 2A), and a moderate stimulation of the Krebs cycle (Figure 2B). Thus, the observed manometric stimulation of Q_{O_2} (G) is confirmed by the isotopic experiments and appears to be due to a partial reversal of the Crabtree effect.

Disulfides.—Disulfides VII and XI have little effect on the manometric characteristics of Ehrlich ascites cells. Whereas this lack of effect may be due to the relative insolubility of compound XI, this is not the case of compound VII.

Compound VIII inhibits Q_{O_2} and glycolysis, and causes a 30–50% stimulation of Q_{O_2} (G), which is followed by inhibition after 1 hr. Isotopic experiments carried out under aerobic conditions (Figure 3) showed that VIII causes a large stimulation of the HMP pathway (Figure 3A), and a moderate inhibition of

(9) H. G. Crabtree, *Biochem. J.*, **23**, 536 (1929).

the Krebs cycle (Figure 3B). The stimulation of Q_{O_2} (G) is thus not due to a reversal of the Crabtree effect, but is presumably associated with the large stimulation of the HMP pathway. Previously we have observed that 2,2'-dithiodipyridine also stimulates the HMP pathway,[6] although to a lesser extent than VIII. With both disulfides, the initial stimulation is followed by an inhibition.

Disulfides IX and X, at the low concentrations permitted by their limited solubility (about 10^{-5} M), inhibit Q_{O_2} (only in the absence of added glucose), and have no effect on glycolysis.

Compound XII is a strong inhibitor of Q_{O_2} and of $Q_{CO_2}^{N_2}$, while Q_{O_2} (G) and $Q_{CO_2}^{O_2}$ are relatively unaffected.

In general, the results obtained with disulfides confirm our previous observations of metabolic effects with structurally related analogs.[3]

Among the thiones studied in this report, 5-cyano-(2-thiopyridone) (IV), which appears to prevent utilization of exogenous glucose *via* the Krebs cycle and the HMP pathway, is unique. This effect is probably not due to any metabolic conversion to its corresponding disulfide X, or to hydrolysis of CN to COOH, in either the thione or the disulfide, since these have been shown not to cause such effects.[3] Thione V, which reverses the Crabtree effect, presumably acts as the thione rather than the disulfide, because the solubility of the disulfide XI is too low to elicit metabolic effects.

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Cofactor Inhibitors of Thymidylate Synthetase. Piperidine and Tetrahydroquinoline Analogs of Tetrahydrofolic Acid[1,2]

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The inhibition of the enzymes thymidylate synthetase and dihydrofolate reductase was examined with a series of pyridine and quinoline compounds and their reduced derivatives. Condensation of ethyl *p*-aminobenzoate or ethyl *p*-aminobenzoylglutamate with pyridine-2-carboxaldehyde followed by reduction of the Schiff base gave the corresponding secondary amines, **3a** and **3b**. Catalytic reduction of **3a** and **3b** yielded ethyl *p*-*N*-(2-piperidylmethyl)aminobenzoate **4a** and the glutamate **4b**. Condensation of **4a** or **4b** with 5-formyluracil gave 2-*p*-carbethoxyphenyl-3-(5-uracil)octahydroimidazo[1,5-*a*]pyridine **5a** and the corresponding glutamate analog **5b**. The synthesis of analogous series (**11**–**13**) starting from quinoline-2-carboxyaldehyde utilized the same procedure. Similarly, starting with pyridine-3-carboxyaldehyde, ethyl *p*-*N*-(3-pyridylmethyl)aminobenzoate (**7a**), the glutamate **7b**, and the 3-piperidyl analog **8** were prepared. Enzyme inhibition studies revealed the saponification product of **7b** to have highest activity against the synthetase [(**7b** salt) (DL-THFA) = 0.12 for 50% inhibition] while **12** was most inhibitory against the reductase [(**12**)/(DHFA) = 8 for 50% inhibition].

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridine 5'-phosphate using N_5,N_{10}-methylenetetrahydrofolic acid as the cofactor in the 1-C transfer.[3] This observation has led to the investigation of analogs of this cofactor for inhibition of the enzyme.[2,4,5] These studies have examined the structural features essential for binding of folate analogs to dihydrofolate reductase and thymidylate synthetase.

Previous studies from these laboratories have shown that the pyrimidine ring moiety of the pteridine system is not essential for binding to thymidylate synthetase and in fact may contribute little to binding.[2a,c] The common features for inhibition were found in models containing the analogous N_5, N_8, and N_{10} of the cofactor.

The purpose of this study is the design and synthesis of models to examine the requirements for both the analogous N_5 and N_8 in the cofactor. Therefore 2- and 3-substituted pyridines, piperidines, quinolines, and tetrahydroquinolines were prepared containing the methylaminobenzoate or methylaminobenzoylglutamate groups. In addition, the 2-piperidyl (**4**) and 2-tetrahydroquinolyl (**12**) derivatives were condensed to give analogs (**5** and **13**) of the proposed intermediate in the enzymatic reaction.[6]

The synthesis of compounds in the 2- and 3-piperidine

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