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Thiocyanate Formation by Extracts of *Escherichia coli* and of Liver.* (24560)

J. W. HYLIN, HILDEGARDE FIEDLER† AND JOHN L. WOOD
Division of Chemistry, University of Tennessee Medical Units, Memphis

Previous work(1,2,3) has demonstrated the presence of a desulfurase enzyme system in *E. coli* which cleaves β-mercaptopyruvate to free sulfur and pyruvate at pH 7.4. This system appears to be similar to that found in liver although comparison of the properties of the bacterial and mammalian enzymes has not been reported. The present work shows that *E. coli* extracts incubated with β-mercaptopyruvate at pH 8.0 or above do not release free sulfur but transfer it to an acceptor in the environment in a manner analogous to the liver system(4). When cyanide is present, the transferred sulfur readily reacts to produce thiocyanate.

Materials and methods. Escherichia coli (wild type) was grown in the liquid medium of Monod and Williams(5) with addition of 1 g of yeast extract per liter. Ten liters of this medium were inoculated with 100 ml of a 2-day culture and the mixture was incubated at room temperature with vigorous aeration. After 36 hours the cells were collected in a Sharples centrifuge, washed once with distilled water and lyophilized.

Bacterial enzyme preparation. Two g of dried cells and 20 g of alumina were ground in a ball mill for 4 hours at room temperature. One hundred ml 0.01 M K_2HPO_4 were then added and grinding continued an additional 30 minutes. The creamy mixture was centrifuged at 3000 x g to remove the alumina and larger particles and then at 20,000 x g for 1 hour. The supernatant liquor was diluted to 100 ml with distilled water and 5 ml of M $MnCl_2$ were added to remove nucleic acids. The resulting precipitate was removed by centrifugation; excess Mn++ was removed by passing the supernatant through a column of Dowex 50 cation exchange resin, NH_4+ form. This preparation was without thiosulfate transsulfurase activity.

Rat liver preparation. A lyophilized enzyme preparation, made from rat liver acetone powder by the method of Meister *et al.* (1), was purified as described by Kun(6). This material exhibited both thiosulfate and β-mercaptopyruvate transsulfurase activities. *Ammonium β-mercaptopyruvate.* This substrate was prepared by the method of Parrod as modified by Kun(7). *Pyruvate assay.* Pyruvic acid, enzymatically produced from β-mercaptopyruvate, was determined by the method of Friedmann and Haugen(8). *Standard enzyme assay.* The enzyme (0.3 ml) was mixed with 0.5 ml of substrate (84 μmoles) and one ml of 0.2 M "Bis"‡ buffer was added to give a final pH of 9.1. This mixture was incubated for 15 minutes at 37°. Two-tenths ml of KCN solution (28 μmoles) were added and incubation continued for another 15 minutes. Enzyme action was stopped with 0.5 ml of 40% formaldehyde and the volume was made up to 5 ml with water. One ml of Goldstein's reagent(9) was added and the color produced was determined photometrically. The substrate for determination of thiosulfate transsulfurase was $Na_2S_2O_3$ and for β-mercaptopyruvate transsulfurase was NH_4 β-mercaptopyruvate.

Results. The *E. coli* enzyme system was found to be relatively stable at 100° for 5

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† Present address: Inst. for Clin. Chem., Univ. of Munich.

‡ Bis = bis(hydroxymethyl)aminomethane or 2-amino-2-methyl-1,3 propanediol.

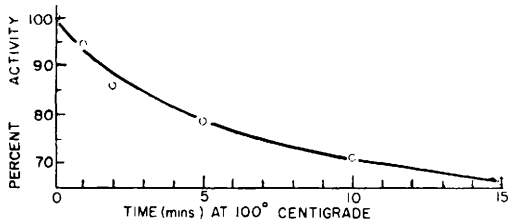

FIG. 1. Effect of heating on *E. coli* enzyme.

minutes (Fig. 1); these conditions completely inactivated the transsulfurases of rat liver. Mild acid hydrolysis of the bacterial preparation also completely destroyed enzyme activity.

Table I shows the bacterial enzyme, unlike liver β-mercaptopyruvate transsulfurase, to be somewhat insensitive to inactivation by sulfhydryl reagents. In keeping with these observations, the rat liver enzyme was inactivated by air oxidation in presence of copper at pH 6.8 (Table II). These activities were restored completely, or in part, by addition of β-mercaptoethanol which reduces disulfide linkages to sulfhydryl. The enzyme substrate, β-mercaptopyruvate, was not as effective. *E. coli* transsulfurase was not affected by air oxidation.

TABLE I. Effect of Sulfhydryl Reagents and Cyanide on β-Mercaptopyruvate Transsulfurase Activity.

Inhibitor (5×10^{-3} M)	% inhibition *E. coli*	Rat liver
None	0	0
Iodosobenzoate	13	31
Iodoacetamide	15	24
p-Mercurichlorobenzoate	13	83
Cyanide	28	0

TABLE II. Effect of Cu^{2+} and Air on β-Mercaptopyruvate Transsulfurase Activity.

Treatment	Addition after treatment	% inhibition Rat liver*	*E. coli*†
none	none	0	0
	β-MP‡	29	0
	-ME§	8	3
Cu^{2+}	none	16	2
	β-MP	37	0
	-ME	8	7
Cu^{2+} and air¶	none	75	7
	β-MP	87	2
	-ME	59	3

* Cu^{2+} conc. was 5×10^{-5} M.
† *Idem* 3.3×10^{-5} M.
‡ β-Mercaptopyruvate, 42 μmoles.
§ β-Mercaptoethanol, 84 " .
¶ Aeration was accomplished by blowing a slow stream of water-saturated air through solution for 5 min.

Iodine in the acid pH range oxidizes the sulfhydryl group to disulfide. Table III shows that rat liver β-mercaptopyruvate transsulfurase is inactivated by treatment with iodine at pH 4.5 but is less sensitive than is the accompanying thiosulfate transsulfurase. The bacterial enzyme was not tested since it becomes inactive at acid pH. Iodination at pH 7.4 has the greatest inactivating effect upon the bacterial enzyme. This enzyme system, in contrast to the 2 liver transsulfurases, thus appears to be independent of sulfhydryl group function.

Bacterial transsulfurase is sensitive to cyan-

TABLE III. Effect of Iodine on Transsulfurase Activities.

	% inhibition						
	Rat liver*						*E. coli*
	Thiosulfate‡		Thiosulfate + β-mercaptoethanol		β-Mercapto-pyruvate§		β-Mercapto-pyruvate
μmoles I_2†	pH 4.5	pH 7.4	pH 4.5	pH 7.4	pH 4.5	pH 7.4	pH 7.4
0	0	0	0	0	0	7	0
.1	0	0	0	0	0	3	0
.2	8	0	0	0	1	12	3
.3	91	6	4	2	6	12	23
.4	99	10	27	0	25	46	70
.5	100	6	83	0	31	32	84
.6	100	76	100	0			86

* Protein content of enzyme solution 1.8 mg/ml.
† After treatment with iodine, enzyme activities were determined by standard assay.
‡ Substrate for "rhodanese" activity.
§ Addition was made in 2 equal parts, the second immediately before incubation to reverse oxidation.

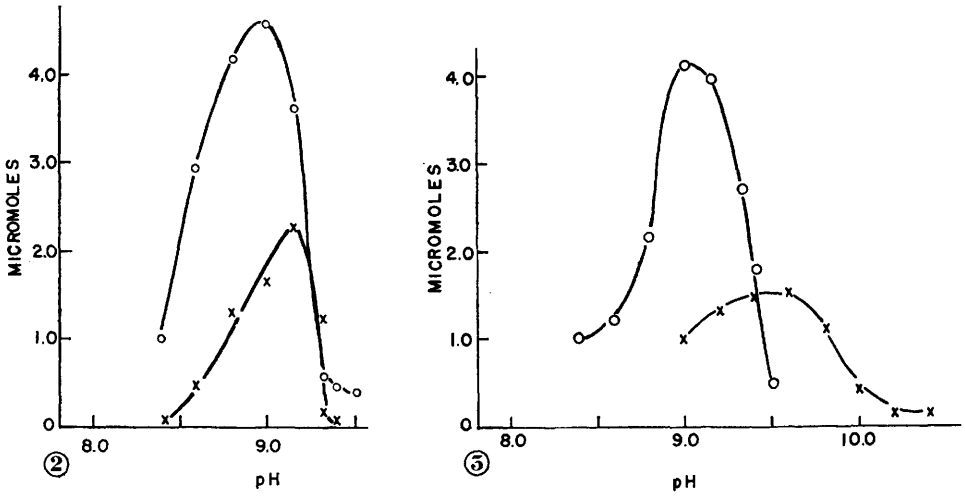

FIG. 2. pH optima of rat liver enzyme. ○——○ = pyruvate, ×——× = KSCN.
FIG. 3. pH optima of *E. coli* enzyme. ○——○ = pyruvate, ×——× = KSCN.

ide, resembling in this aspect thiosulfate transsulfurase(10). Similar inhibition by cyanide has been related by Sorbo(10) to breaking of essential disulfide bonds in thiosulfate transsulfurase.

The bacterial and liver β-mercaptopyruvate transsulfurases evidently catalyze the same reaction, namely conversion of β-mercaptopyruvate to pyruvate and an unidentified reactive form of sulfur. Formation of thiocyanate from β-mercaptopyruvate can be visualized as a 2-step process. The first step consists of conversion of β-mercaptopyruvate to pyruvate accompanied by a transfer of sulfur to the environment. In the absence of an acceptor, free sulfur separates(1,2). If the environment contains a component reactive with sulfur, a valence change may occur, or elemental sulfur may be fixed in compounds such as polysulfides. The second step in thiocyanate formation is not so clearly defined but is the slower reaction. If elemental sulfur is fixed in the polysulfide form, it can readily react with cyanide; free sulfur in the solid phase produces no thiocyanate within the time period of the standard assay procedure. The pH optimum for the first of the above steps was determined for the rat liver enzyme (Fig. 2) and for the bacterial enzyme (Fig. 3). Also shown in the figures is the pH optimum of the 2 steps combined.

The reaction of the second step has not been established to be enzyme-catalyzed. It is possible that an enzyme-sulfur complex exists since the pH optimum for thiocyanate formation in the presence of cyanide is 9.6 for the *E. coli* system and 9.1 for the liver system. Alternatively, polysulfide formation with different types of sulfhydryl groups on compounds in the environment may account for the differences in optimum pH of the 2 systems.

Summary. The properties of enzyme systems of *E. coli* and of rat liver, which degrade β-mercaptopyruvate, have been compared. The bacterial system was much more stable to heat and less susceptible to oxidation. These characteristics have been related to lack of dependence upon sulfhydryl group. At the pH optimum for pyruvate formation, the enzyme activity may be characterized as a transsulfurase rather than a desulfurase. When cyanide is the sulfur acceptor, thiocyanate formation occurs. The pH optimum for *E. coli* system is 9.6 and for rat liver system 9.1.

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Enhancement of Hematopoietic Action of Hydrocortisone by Cobalt in Adrenalectomized Rats. (24561)

JAMES W. FISHER* (Introduced by W. F. Cantrell)
Dept. of Pharmacology, University of Louisville, School of Medicine, Louisville, Ky.

We have recently reported that adrenalectomy interferes with the hematologic response to cobalt in the rat although rats subjected to a sham operation respond to cobalt as readily as normal rats(1). Further analysis of this effect seemed to require experiments in which adrenalectomized rats were treated with both cobalt and adrenal hormones. Several reports indicating that adrenocortical hormones stimulate erythropoiesis in the bone marrow of the adrenalectomized and intact rat(2-5) and induce an elevation in total circulating red cell volume in the normal rat(6) suggested that the hormones of the adrenal cortex should be investigated in this way. The present report describes the results of concurrent administration of cobalt and hydrocortisone to adrenalectomized rats in which it was found that these substances together stimulate erythropoiesis considerably more effectively than hydrocortisone alone.

Materials and methods. Male albino rats of the Wistar (Purdue) strain were studied in 2 experiments: (I), 8 control rats, 8 adrenalectomized rats receiving 2 mg hydrocortisone per kg daily and 8 adrenalectomized rats receiving a combination of 2 mg hydrocortisone and 2.5 mg elemental cobalt per kg daily for a period of 28 days after operation. (II) 8 control rats, 8 adrenalectomized rats receiving 1 mg hydrocortisone per kg daily, and 8 adrenalectomized rats receiving a combination of 1 mg hydrocortisone and 2.5 mg elemental cobalt per kg daily for a period of 28 days after operation. Erythrocyte counts, hemoglobin and hematocrit determinations were made at 10, 20 and 28 days. The hydrocortisone and cobalt injections were started on the day following the operation. Total red cell volumes were determined at 28 days. Cobalt chloride† was given as an intraperitoneal injection daily at a dosage of 2.5 mg elemental cobalt (10 mg $CoCl_2 \cdot 6H_2O$) per kg of body weight. The hydrocortisone acetate‡ was suspended in 0.9% NaCl solution and injected subcutaneously daily at various sites under the skin of the back. Blood was obtained for erythrocyte, hemoglobin and hematocrit determination by anesthetizing the rat with ether and clipping the tail. Erythrocyte counts were done with U.S. Certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Van Allen hematocrit tubes (1.6% aqueous sodium oxalate as diluent) which were spun for 30 minutes at 3,000 rpm with a radius of 17 cm. Hemoglobin determinations were made on the Klett-Summerson colorimeter by the acid hematin method(7). Body weights were determined at the time of the hematologic studies. Red cell volume was

* Public Health Service Fellow of Nat. Heart Inst. in Dept. of Pharmacology, Univ. of Louisville. Present address: Dept. of Pharmacol., Univ. of Tenneseee, Memphis. Supported by grants from Heart Assn. of Louisville and Jefferson County and Research Division of Lloyd Bros., Cincinnati, O.

†Analytical grade $CoCl_2 \cdot 6H_2O$ with maximum limits of impurity for lead of 0.005%.

‡ Hydrocortisone acetate supplied through courtesy of Upjohn Co., Kalamazoo, Mich.