Shelanski, M. L., and Taylor, E. W. (1967), *J. Cell Biol. 34*, 549.
Shelanski, M. L., and Taylor, E. W. (1968), *J. Cell Biol. 38*, 304.
Stephens, R. E. (1968a), *J. Mol. Biol. 32*, 277.
Stephens, R. E. (1968b), *J. Mol. Biol. 33*, 517.
Tanford, C., Kawahara, K., and LaPanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Taylor, E. W. (1965), *J. Cell Biol. 25*, 145.
Tilney, L. G., Hyamoto, Y., and Marsland, D. (1966), *J. Cell Biol. 29*, 77.
Tsuboi, K. K., and Price, T. D. (1959), *Arch. Biochem. Biophys. 81*, 223.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

# Diabetogenic Action of 5-Thio-D-glucopyranose in Rats*

Daniel J. Hoffman and Roy L. Whistler

ABSTRACT: Rats intraperitoneally administered 5-thio-D-glucopyranose rapidly develop glucosuria and hyperglycemia. With a dose of 50 mg/kg the blood D-glucose rises to approximately 300 mg/100 ml within 2.5 hr, then drops to 160 mg/100 ml within 6 hr. Rats given booster doses of 5-thio-D-glucopyranose every hour maintain blood D-glucose levels of 300 mg/100 ml.

The same effect is observed in rats with ligated kidneys given a single 50-mg/kg dose of 5-thio-D-glucopyranose. Administration of insulin completely nullifies the diabetogenic effect. Livers of rats fasted for 20 hr had 50% less glycogen 2.5 hr after injection of 50 mg/kg of 5-thio-D-glucopyranose than saline-injected controls. Rats injected with 5-thio-D-glucopyranose excreted 97% of the sugar in the urine indicating little or no metabolism of 5-thio-D-glucopyranose. Investigations with rat liver slices, kidney slices, and diaphragms show a marked decrease in the uptake of D-glucose by these tissues when molarity ratios of 5-thio-D-glucopyranose/D-glucose from 0.5 to 1 are present in the initial incubation media at 37°. 5-Thio-D-glucopyranose did not inhibit the metabolism of D-glucose in kidney homogenates but did slightly inhibit glycolysis (8.3% with a molarity ratio 5-thio-D-glucopyranose/D-glucose of 0.50). After a series of three injections of 5-thio-D-glucopyranose at 2-hr intervals there is a 43% increase in the total catechol amine content of the urine. A 73% increase in blood nonesterified fatty acids of rats fasted for 16 hr is observed 0.5 hr af er a single 150-mg/kg dose of 5-thio-D-glucopyranose. The nonesterified fatty acid level then returns to a normal fasting level within 2 hr.

5-Thio-D-glucopyranose is neither a substrate nor an inhibitor of D-glucose oxidase which is used to analyze for D-glucose. 5-Thio-D-glucopyranose does not inhibit yeast hexokinase but acts as a poor substate. Kinetic measurements give a $K_m$ of $4 \times 10^{-3}$ M and a $V_{max}$ (glucose − 100) of 1.3.

5-Thio-D-glucopyranose was first prepared by Feather and Whistler (1962). Since 5-thio-D-glucopyranose differs from D-glucopyranose by having sulfur in place of oxygen in the pyranose ring it may act as an antagonist of this ubiquitous metabolite. At the time of this writing all but one publication concerning sugars with sulfur in the ring dealt solely with their chemical properties. Shankland *et al.* (1968) found that 5-thio-D-glucopyranose in molarity ratios of 5-thio-D-glucopyranose/D-glucose as low as 0.03 effectively interferes with the utilization of D-glucose for development of *Drosophila melanogaster*. Molarity ratios of 0.31 and above prevented development from the larval to the pupal stage. This inhibition was attributed to interference of enzyme(s) and/or the transport of D-glucose across cell membranes due to the structural similarity of the two sugars. In the present paper we report effects of 5-thio-D-glucopyranose in intact rats and in several isolated systems.

## Experimental Section

### *Materials*

Crystalline 5-thio-D-glucopyranose was prepared by the method of Rowell and Whistler (1966): mp 135–136°, $[\alpha]_D^{20}$ +188° (*c* 1.56, water).

Wistar rats from our colonies, maintained on Purina Lab-Block feed, were given water *ad libitum*. Experimental rats were injected with various volumes of 5-thio-D-glucopyranose solutions. Control rats received intraperitoneal injections of equivalent volumes of isotonic saline. Blood was obtained by cardiac puncture unless otherwise stated.

Regular iletin insulin was purchased from Eli Lilly and Co., Indianapolis, Ind. Yeast hexokinase and ATP[1] were obtained from Calbiochem.

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[1] ATP is the dipotassium salt of adenosine triphosphate.

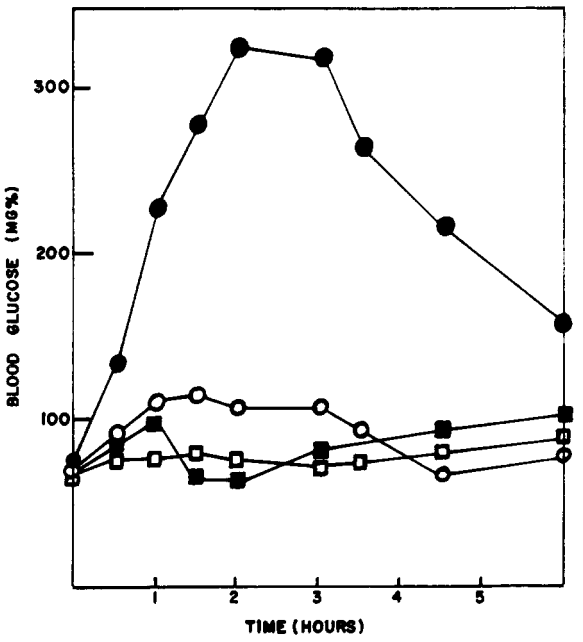


FIGURE 1: Effect of 5-thio-D-glucopyranose on the blood D-glucose level. Six rats were used in each group. (●—●) Given 50 mg/kg of 5-thio-D-glucopyranose in saline; (○—○) given 50 kg/kg of D-glucose in saline; (□—□) given saline only; (■—■) given 0.3 unit of insulin simultaneously with 50 mg/kg of 5-thio-D-glucopyranose in saline.

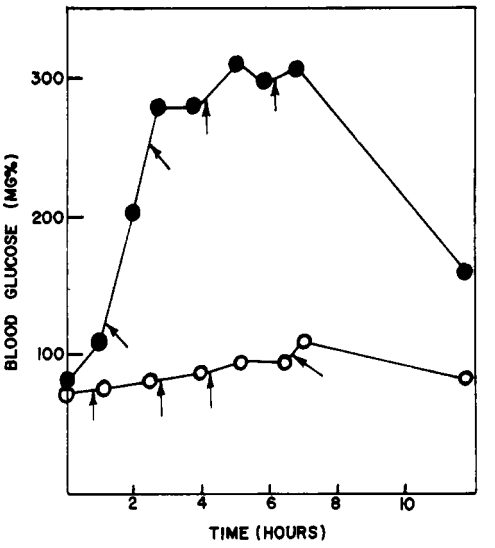


FIGURE 2: Maintenance of high blood D-glucose levels with booster doses of 5-thio-D-glucopyranose. Eight rats were in each group. The arrows denote the time of intraperitoneal injections. (●—●) Received 25 mg/kg of 5-thio-D-glucopyranose in saline; (○—○) saline alone.

*Methods*

*D-Glucose and 5-Thio-D-glucopyranose Analysis.* D-Glucose was determined with a D-glucose oxidase preparation (Glucostat) from Worthington Chemical Co. Method 1B of the Glucostat procedures was employed for blood samples. 5-Thio-D-glucopyranose was neither a substrate nor an inhibitor of this D-glucose oxidase preparation. 5-Thio-D-glucopyranose was assayed by paper chromatography in 1-butanol–ethanol–water (40:11:19) as the irrigant on Whatman No. 1 paper. Detection was by the silver nitrate reagent of Trevelyan *et al.* (1950), with quantitation *via* a Chromoscan densitometer (National Instrument Laboratories Inc., Rockville, Md.).

*Tissue Incubations.* The effect of 5-thio-D-glucopyranose on D-glucose uptake by liver, kidney, and diaphragm tissues of fed rats was examined. Liver and kidney slices were prepared with a Stadie-Riggs tissue slicer. About 200 mg of tissue slices or of hemidiaphragm were incubated in 4.2 ml of Krebs bicarbonate buffer (Umbreit *et al.*, 1957). Initial D-glucose concentration was 6.29 mM and that of 5-thio-D-glucopyranose 0, 3.06, or 6.12 mM. D-Glucose utilization at 37° was determined after 2 hr.

*Kidney Homogenates.* A 10% kidney homogenate in cold isotonic KCl was prepared with a Kontes tissue homogenizer. The homogenate (0.30 ml) was then added to the medium of LePage (1950) to give a total volume of 3.00 ml. The initial D-glucose concentration was 10 mM with either 0, 5.0, or 10 mM 5-thio-D-glucopyranose. Controls were treated with 1.0 ml of 5% trichloroacetic acid and incubated. After incubation at 37° for 1 hr, protein was precipitated with 1.0 ml of cold 20% trichloroacetic acid, neutralized with KOH, centrifuged, and diluted to 10 ml with distilled water. Aliquots were then assayed for D-glucose by the Glucostat method and for lactate (Baker, 1961).

*Kinetic Measurements with Yeast Hexokinase.* Kinetic measurements of the phosphorylation of 5-thio-D-glucopyranose by ATP and yeast hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) were performed with A Radiometer TTTI automatic titrator. To each assay mixture was added 5.0 ml of a solution $4.0 \times 10^{-3}$ M in ATP and $8.0 \times 10^{-3}$ M in $Mg^{2+}$ and 1.0 ml of the desired substrate concentration. The pH was adjusted to 7.5 with NaOH and then 30 μl (4.2 units)[2] of yeast hexokinase was added. The pH was readjusted to 7.5 and the rate of proton liberation was measured at pH 7.5.

## Results

*Intraperitoneal Injection of 5-Thio-D-glycopyranose.* Female rats (150–165 g) given a single dose (50 mg/kg) of 5-thio-D-glycopyranose in isotonic saline rapidly developed glucosuria and hypergylcemia. Blood D-glucose increased four- to fivefold above normal in 2 hr (Figure 1). Only a slight rise in the blood D-glucose was observed when an equivalent dose of D-glucose was administered.

Insulin (0.3 unit) given simultaneously with a 50-mg/kg dose of 5-thio-D-glycopyranose completely nullified the hyperglycemic effect of this sugar.

The drop in the blood D-glucose level after 3 hr is due to loss of 5-thio-D-glucopyranose in the urine. Analysis indicated that 97.3 ± 5.7% of the administered 5-thio-D-glucopyranose was recovered unaltered

[2] One unit of yeast hexokinase is the amount that phosphorylates 1.0 μmole of D-glucose in 15 min at 30°.

TABLE I: Effect of 5-Thio-D-glucopyranose upon the Uptake of D-Glucose in Various Rat Tissues.

| Tissue[a] | 5-Thio-D-glucopyranose[b] (mM) | D-Glucose Uptake (μmoles/g per hr) |
|---|---|---|
| Liver | | −1.40 |
| | 3.06 | −7.90 |
| | | −1.97 |
| | 6.12 | −10.9 |
| Kidney | | +9.90 |
| | 3.06 | +6.39 |
| | | +10.1 |
| | 6.12 | +3.01 |
| Diaphragm | | +11.1 |
| | 3.06 | +2.70 |
| | | +8.55 |
| | 6.12 | +4.20 |

[a] Six rats were used; each supplying two tissue samples. [b] The initial media contained 6.29 mM D-glucose.

TABLE II: Effect of 5-Thio-D-glucopyranose on Glycolysis by Kidney Homogenates.[a]

| Initial Media (mM) | | | |
|---|---|---|---|
| D-Glucose | 5-Thio-D-glucopyranose | Lactate Produced (μmoles) | D-Glucose Metabolized (μmoles) |
| 10 | 0 | 5.69 ± 0.62[b] | 3.90 ± 0.22 |
| 10 | 5 | 5.22 ± 0.80 | 4.11 ± 0.14 |
| 10 | 10 | 4.64 ± 0.56 | 3.12 ± 0.26 |

[a] All results from six incubations of a single pooled sample from two rats. [b] Standard error of the mean.

in the urine 24 hr after injection; thus little or no metabolism of 5-thio-D-glucopyranose occurred. If a high blood level of 5-thio-D-glucopyranose is maintained by booster injections of 5-thio-D-glucopyranose (25 mg/kg), a hyperglycemic condition persists until 5-thio-D-glucopyranose injections cease (Figure 2). Comparable effects were observed in rats having ligated kidneys and given a single 50-mg/kg dose of 5-thio-D-glucopyranose. The blood D-glucose level of saline-injected controls varied between 50 and 86 mg per 100 ml while those injected with 5-thio-D-glucopyranose maintained blood D-glucose levels between 300 and 340 mg per 100 ml for at least 10 hr.

*The Effect of 5-Thio-D-glucopyranose on Liver Glycogen Levels.* Twelve male rats (160–190 g) were fasted for 20 hr. Six received intraperitoneal injections of 1.0 ml of saline and six received injections of 5-thio-D-glucopyranose (50 mg/kg) in 1.0 ml of saline. Liver samples were quickly removed 2.5 hr after intraperitoneal administration and analyzed for glycogen according to Good *et al.* (1933). The livers of rats given saline had 1.35 ± 0.22% glycogen, but those administered 5-thio-D-glucopyranose had only 0.68 ± 0.15% glycogen.

*The Effect of 5-Thio-D-glucopyranose on D-Glucose Uptake by Liver, Kidney, and Diaphragm Tissue.* 5-Thio-D-glucopyranose significantly inhibited D-glucose uptake in liver, kidney, and diaphragm tissue (Table I). In liver there was a net D-glucose output in all instances and this output was enhanced by 5-thio-D-glucopyranose. Both kidney and diaphragm tissue showed a net uptake of D-glucose which was inhibited 35–76% by 5-thio-D-glucopyranose.

*The Effect of 5-Thio-D-glucopyranose upon Glycolysis in Kidney Homogenates.* Since D-glucose uptake was inhibited in various tissues by 5-thio-D-glucopyranose, inhibition either of D-glucose metabolism or of D-glucose transport across cell membranes must occur. To determine whether glycolysis is inhibited by 5-thio-D-glucopyranose, kidney homogenates were incubated at 37° for 1 hr and the media was assayed for D-glucose utilization and for lactate production (Table II). Glycolysis was inhibited 18.5% at a molarity ratio 5-thio-D-glucopyranose/D-glucose of 1.0 and 8.3% at a molarity ratio of 0.50. This low inhibition of glycolysis suggests that this is not the main action of 5-thio-D-glucopyranose.

*Blood Nonesterified Fatty Acid after 5-Thio-D-glucopyranose Injection.* Female rats (110–145 g), fasted for 16 hr, were given either a single 2-ml dose of 5-thio-D-glucopyranose (150 mg/kg) or isotonic saline. After injection, each rat was decapitated at a specific time and its blood was collected, heparinized, and centrifuged at 3000 rpm for 5 min, and the plasma was assayed for nonesterified fatty acid according to Dole (1956). A rapid increase (73% in 0.5 hr) in the blood nonesterified fatty acid occurred in rats treated with 5-thio-D-glucopyranose (Figure 3). This is also characteristic of the diabetic state.

*The Effect of 5-Thio-D-glucopyranose on Urinary Catecholamine Levels.* Ten male rats (150–185 g) were each given three 10-mg intraperitoneal injections of 5-thio-D-glucopyranose in 2-hr intervals. Ten controls were given the same series of isotonic saline injections. Their urine was collected for a total of 10 hr in bottles containing 1 ml of 2 N HCl. The total catecholamine content of the urine was determined by the fluorescence assay of Udenfriend (1962). The 5-thio-D-glucopyranose treated rats had 43% more urinary catecholamines than the saline-treated controls. This can be correlated with the increased glycogen breakdown observed in rats treated with 5-thio-D-glucopyranose.

*5-Thio-D-glucopyranose as a Substrate for Yeast Hexokinase.* Kinetic measurement of the rate of phosphorylation of 5-thio-D-glucopyranose by yeast hexokinase showed it to be a poor substrate for this enzyme. The enzyme has a $K_m$ of $4 \times 10^{-3}$ M and a $V_{max}$ of 1.3 (D-glucose = 100) with 5-thio-D-glucopyranose.

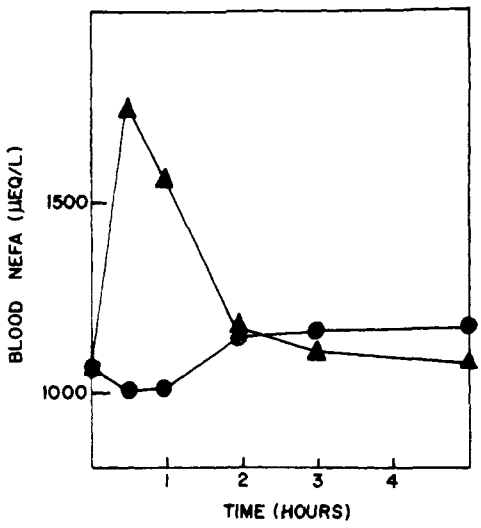


FIGURE 3: Effect of 5-thio-D-glucopyranose on blood nonesterfied fatty acid levels. Each group had six rats that were fasted for 16 hr. (▲—▲) Received 150 mg/kg of 5-thio-D-glucopyranose in saline; (●—●) saline alone.

Taking $8 \times 10^{-6}$ M as the $K_m$ for D-glucose, 5-thio-D-glycopyranose has a phosphorylation coefficient (Sols and Crane, 1954) of $2.6 \times 10^{-5}$. This indicates 5-thio-D-glucopyranose is of poor physiological suitability as a substrate for yeast hexokinase. Further studies showed that 5-thio-D-glycopyranose is not an inhibitor of D-glucose phosphorylation by yeast hexokinase.

To show that 5-thio-D-glucopyranose is phosphorylated by yeast hexokinase a media containing 0.8 mmole of 5-thio-D-glucopyranose, 0.7 g of ATP, 0.1 g of magnesium chloride hexahydrate, and 50 units of yeast hexokinase at pH 7.5 were incubated at 35° for 24 hr. Paper chromatography of the media on Whatman No. 1 paper (1-butanol–ethanol–water, 40:11:19) using the silver nitrate detection reagent showed a spot with an $R_{glucose}$ of 0.22 after 2-hr incubation. Besides being a reducing compound, the spot also gave positive tests for phosphate (Bandurski and Axelrod, 1951) and SH (Marvel, 1951).

## Discussion

It is apparent that 5-thio-D-glucopyranose has a pronounced physiological effect when administered orally or intraperitoneally to rats. The immediate effect is a prompt rise in blood D-glucose to a level which is roughly maintained but not increased by subsequent doses of 5-thio-D-glucopyranose. Simultaneously with increase in blood D-glucose a complimentary glucosuria develops. As 5-thio-D-glucopyranose is removed, unaltered, from the blood by urinary excretion, blood D-glucose levels return to normal. Coincidental with blood D-glucose rise there is an increase in blood nonesterified fatty acid which reaches a maximum within a 0.5 hr after administration of 5-thio-D-glucopyranose and returns to normal within 2 hr (Figure 3).

D-Mannoheptulose (Simon and Kraicer, 1967) also has a diabetogenic action. Its hyperglycemic action has been attributed to the inhibition of insulin release (Simon *et al.*, 1963). A dose of 2 g/kg increased the blood D-glucose level of rats in 2 hr to 250 mg/100 ml which then dropped to normal levels. D-Mannoheptulose differs from 5-thio-D-glucopyranose in that it has no effect upon D-glucose uptake by various tissues (Chernick *et al.*, 1962), it inhibits hexokinase (Coore and Randel, 1964), and stimulates liver and muscle glycogen deposition 4 hr after administration.

From the data obtained with kidney and diaphragm tissue sections (Table I) and kidney homogenates (Table II) the conclusion could be drawn that 5-thio-D-glucopyranose inhibits membrane transport of D-glucose. However, in liver tissue sections (Table I), the effect of 5-thio-D-glucopyranose seems to be upon D-glucose production. A possible conclusion is that 5-thio-D-glucopyranose in some way inhibits glycogen synthesis or stimulates glycogen breakdown. This would lead to a net overproduction of D-glucose by the liver. A liver glycogen depletion was observed in fasted rats given 5-thio-D-glucopyranose.

In considering the action of 5-thio-D-glucopyranose it is interesting to note a finding of Dr. H. R. Kaback.[3] He found that 5-thio-D-glucopyranose, supplied by us, inhibited transport of methyl α-D-glucopyranoside through a cell membrane preparation of *Escherichia coli* by a noncompetitive inhibition of the phosphoenolpyruvate–phosphotransferase system (Kaback, 1968). It is recognized that this transport inhibition in a bacterial is not directly translatable to mammalian cells.

While the data showing that 5-thio-D-glucopyranose increases blood D-glucose, blood nonesterified fatty acid and urinary catecholamine excretion in rats is interesting, precise interpretation of the effects are not possible at this time, and further work is clearly indicated.

## References

Baker, S. B. (1961), Standard Methods of Clinical Chemistry, New York, N. Y., Academic, p 167.

Bandurski, R. S., and Axelrod, B. J. (1951), *J. Biol. Chem. 193*, 404.

Chernick, S. S., Scow, R. O., Simon, E., and Stricker, F. A. (1962), *Proc. Soc. Exptl. Biol. Med. 109*, 589.

Coore, H., and Randle, P. (1964), *Biochem. J. 91*, 56.

Dole, P. U. (1956), *J. Clin. Invest. 35*, 150.

Feather, M. S., and Whistler, R. L. (1962), *Tetrahedron Letters No. 15*, 667.

Good, C. A., Kramer, H., and Somogyi, M. (1933), *J. Biol. Chem. 100*, 485.

Kaback, H. R. (1968), *J. Biol. Chem. 243*, 3711.

LePage, G. A. (1950), *Cancer Res. 10*, 77.

Marvel, C. S. (1951), *Anal. Chem. 23*, 823.

Rowell, R. M., and Whistler, R. L. (1966), *J. Org. Chem. 31*, 1514.

[3] Personal communication presented with permission of H. R. Kaback, National Heart Institute, Laboratories of Biochemistry, National Institutes of Health, Bethesda, Md. 20014.

Shankland, D. L., Stark, J. H., and Whistler, R. L. (1968), *J. Insect Physiol. 14*, 63.
Simon, E., and Kraicer, P. E. (1957), *Arch. Biochem. Biophys. 69*, 592.
Simon, E., Kraicer, P. E., and Shelesynak (1963), *Nature 197*, 1264.
Sols, A., and Crane, R. K. (1954), *J. Biol. Chem. 210*, 581.
Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1957), Manometric Techniques, Minneapolis, Minn., Burgess, p 149.
Udenfriend, S. (1962), Fluorescence Assay in Biology and Medicine, Molecular Biology, New York, N. Y., Academic, p 139.

# Studies on Oxidative Phosphorylation. XVI. Sulfhydryl Involvement in the Energy-Transfer Pathway*

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ABSTRACT: The effect of organic mercurials was tested on the following energy-linked reactions in bovine heart submitochondrial particles: (a) reduction of nicotinamide–adenine dinucleotide by succinate coupled to the aerobic oxidation of ascorbate–*N,N,N',N'*–tetramethyl-*p*-phenylenediamine, (b) nicotinamide nucleotide transhydrogenase reaction (reduced nicotinamide–adenine dinucleotide → nicotinamide–adenine dinucleotide phosphate) driven by the energy from the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine, and (c) energy-driven intravesicular acidification measured by the bromothymol blue color change. Reaction a was inhibited about 90% by 10 $\mu$M or less of mersalyl or mercuriphenylsulfonate. The energy-linked transhydrogenase reaction (reaction b) was also highly sensitive to mercurials under conditions in which the energy-independent reverse reaction (reduced nicotinamide–adenine dinucleotide → nicotinamide–adenine dinucleotide phosphate) showed minimal sensitivity. The bromothymol blue response coupled to the aerobic oxidation of reduced nicotinamide–adenine dinucleotide or succinate was inhibited by low concentrations of mersalyl which did not affect the respiratory activity. When ascorbate–toluylene blue or adenosine triphosphate was used to initiate the bromothymol blue response, the inhibitory effect of mersalyl was enhanced by the presence of bovine serum albumin in the reaction medium. The results indicate that the mercurial acts by inhibiting the generation of nonphosphorylated high-energy intermediates and point to the involvement of sulfhydryl groups in the early reactions of oxidative phosphorylation.

There are several reports in the literature which point to the involvement of SH groups in the energy-transfer pathway of oxidative phosphorylation. Organic mercurials and other thiol binding agents have been shown to uncouple oxidative phosphorylation (Fluharty and Sanadi, 1960; Fletcher and Sanadi, 1962) and inhibit respiratory stimulation by ADP (Fonyo and Bessman, 1966) in whole mitochondria. In submitochondrial particles prepared from rat liver, *p*-hydroxymercuribenzoate inhibits phosphorylation coupled to the aerobic oxidation of ferrocytochrome *c* (Cooper and Lehninger, 1956), abolishes [$^{32}$Pi]ATP exchange (Cooper and Lehninger, 1957), activates latent ATPase, and inhibits dinitrophenol-stimulated ATPase (Kielley, 1963). These particulate preparations show low P/O and poor respiratory control (Bronk and Kielley, 1958) presumably due to labilization of some of the terminal steps of the energy-transfer system (Lee and Ernster, 1966).

We have examined the effect of thiol binding agents on several energy-linked reactions in submitochondrial particles in an attempt to localize the site of sulfhydryl involvement on the energy-transfer pathway. The following reactions were studied: (a) reduction of NAD by succinate driven by the aerobic oxidation of ascorbate–tetramethyl-*p*-phenylenediamine described by Packer (1963) and Vallin and Low (1964), (b) energy-linked nicotinamide nucleotide transhydrogenase reaction driven by the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine (Danielson and Ernster, 1963), and (c) energy-dependent production of proton gradient measured by the absorbance change of bromothymol blue (Chance and Mela, 1967). The results presented in this communication indicate that these three reactions

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