ACTA CHEMICA SCANDINAVICA 22 (1968) 562–570

Reductive Decomposition of S-Sulfoglutathione in Rat Liver

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A quantitative assay of S-sulfoglutathione, based on an electrolytic reduction followed by determination of the glutathione formed, has been worked out. By the application of this method an enzymatic activity catalyzing the reduction of S-sulfoglutathione in the presence of NADPH was discovered in rat liver homogenates. The activity was distinct from glutathione reductase, as demonstrated by inhibition experiments with sulfite and acidic glutathione derivatives. The enzyme was sensitive to "sulfhydryl reagents" and was apparently not active with NADH or ascorbate.

Little is known about the metabolism of the thiosulfate ester of glutathione, S-sulfoglutathione ($GSSO_3H$). Its presence in mammalian tissues has been demonstrated,[1,2] but its synthesis and degradation *in vivo* have not been elucidated. The present investigation was undertaken to study possible transformations of the thiosulfate moiety of $GSSO_3H$. Four types of reactions could be conceived: (1) liberation of thiosulfate by elimination, to give a dehydroalanyl residue in the peptide, or by substitution; (2) transfer of the sulfuryl group to, *e.g.*, water (hydrolysis), or of the sulfenyl group to a thiophilic acceptor; (3) oxidation to give sulfate and an acidic glutathione derivative (the sulfonic or the sulfinic acid); (4) reduction to give glutathione and sulfite. Reactions corresponding to (1) and (4) have been demonstrated for other thiosulfate esters,[3–5] and type (4) has in fact been found valid for $GSSO_3H$ in pea tissues.[6] The present paper describes an enzymatic reaction in rat liver homogenates similar to the reduction of $GSSO_3H$ in peas. A preliminary communication has previously been published.[7]

MATERIALS

Glutathione (GSH), NADPH (Type II), and NADH (Grade III) were purchased from Sigma Chemical Co.; glutathione disulfide (GSSG), and lactoylglutathione lyase (S-lactoylglutathione methylglyoxal lyase, EC 4.4.1.5), 1 mg/ml in 30 % glycerol, from C. F. Boehringer & Soehne, Mannheim, Germany; and methylglyoxal from Fluka AG, Buchs, Switzerland.

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was synthesized according to Ellman.[8]

S-Sulfoglutathione was prepared by the reaction between GSH, sulfite, and cupric ions under the conditions described by Swan.[9] A representative $GSSO_3H$ synthesis at this laboratory was carried out as follows. GSH, 500 mg (1.6 mmoles), was allowed to react with Na_2SO_3, 820 mg (6.5 mmoles), in 24 ml of a 0.05 M $CuSO_4$ solution (adjusted to pH 10 with conc. ammonia). The reaction was continued for at least 2 h, although paper electrophoresis indicated that more than 90% of the GSH had reacted after 60 min. In this particular synthesis, the reaction mixture was placed in a refrigerator overnight. The product was purified by passage through a Dowex 50 W column (H^+-form, X 12, 100–200 mesh, 2 × 10 cm) with water as the eluent. The effluent containing $GSSO_3H$ was concentrated in a rotary evaporator and treated with 2.0 g of barium acetate dissolved in 6 ml of water. The resulting precipitate was removed by centrifugation, and the barium salt of $GSSO_3H$ was precipitated from the supernatant by the addition of 5 volumes of 95 % ethanol. The barium salt was reprecipitated four times, and was then dried over silica gel in vacuum. Yield: 579 mg (60 %). The sulfhydryl liberation after the electrolytic reduction procedure described below was 1.00 mole per mole, as calculated for $C_{10}H_{15}N_3O_9S_2Ba \cdot 4\ H_2O$ (*cf.* Ref. 1). The product obtained was electrophoretically and chromatographically homogeneous. In particular, it was established that it was free from other acidic glutathione derivatives, which are resolved from $GSSO_3H$ by thin-layer chromatography (Table 1). In some syntheses, in which barium hydroxide had been substituted for barium acetate, $GSSO_3H$ partly decomposed with the concomitant formation of the sulfinic acid (GSO_2H) and GSSG. The purified barium salt of $GSSO_3H$ was free from copper as analyzed by a dithizone method.[10]

Table 1. Chromatography of glutathione derivatives on cellulose thin-layers.
Glass plates, 20 × 20 cm, covered with cellulose powder MN 300, were developed with acetic acid-butanol-water-pyridine, 3:15:12:10 (by vol.).

Compound	R_F-Value
GSH	0.35
GSSG	0.32
GSO_2H	0.17
GSO_3H	0.19
GSO_2SH	0.25
$GSSO_3H$	0.30

Glutathione sulfonic acid (GSO_3H) was prepared by hydrogen peroxide oxidation of GSH (*cf.* Ref. 11), and was purified by ion-exchange chromatography on Dowex 50 W and precipitation of the barium salt as described for $GSSO_3H$. The sulfinic acid and the barium salt of the thiosulfonic acid (GSO_2SH) were synthesized according to Eriksson and Sörbo.[12] Before use, the barium salts of glutathione derivatives were converted into the sodium form by treatment with an equimolar amount of sodium sulfate.

Rat livers (Wistar, male and female) were kept frozen and were thawed before homogenization in 0.14 M KCl with a teflon-glass Potter-Elvehjem homogenizer. Unless otherwise stated, the enzyme preparation was a 20 % (w/v) homogenate, centrifuged at 1000 *g* for 15 min.

METHODS

The standard assay of $GSSO_3H$ destruction was based on the determination of remaining $GSSO_3H$ after incubation. The reaction mixture of a total volume of 2 ml contained: 0.2 ml of the centrifuged rat liver homogenate; 3 mM $GSSO_3H$ (sodium salt); 2 mM NADPH; and 45 mM phosphate buffer, pH 7.5. The reaction was started by the addition of the enzyme or the substrate and was run at 30°C. When the incubation was carried out

in a nitrogen atmosphere, Warburg flasks were employed; otherwise stoppered Hagedorn tubes were used. In either case the reaction vessels were agitated in a thermostated water bath. The reaction was stopped by the addition of 1 ml of 10 % metaphosphoric acid. After centrifugation, 1.5 ml of the supernatants was applied to Dowex 50 W (H^+) columns (0.9 × 2.5 cm), which were eluted with water. The effluents were collected in 10 ml volumetric flasks, containing sufficient 20 % metaphosphoric acid to give a final acid concentration of 3.5 %. The 10 ml samples were then placed in cathode chambers and were reduced by the electrolytic procedure of Dohan and Woodward.[13] The anode vessel contained 4 % sulfosalicylic acid. After 45 min (40 mA, *ca.* 22 V) the reduction was completed, and 2 ml of the solution was then bubbled with nitrogen for 15 min to remove sulfite, which otherwise interferes with the sulfhydryl determination. An aliquot (usually 0.2 ml) of the sulfite-free sample was then transferred to a cuvette containing 2 ml of 0.6 mM DTNB in 0.2 M phosphate buffer, pH 8.0, plus a sufficient amount of the buffer to give a final volume of 3 ml. The sample was read at 412 nm against a blank composed of 2 ml of the reagent plus 1 ml of buffer. Calibration with cysteine and GSH gave identical results, when the presence of 3 % GSSG in the GSH preparation (as determined with glutathione reductase and NADPH — conditions as described for the mixed disulfide of coenzyme A and GSH [14]) was taken into account. We obtained an apparent extinction coefficient of 14 $mM^{-1}cm^{-1}$ (*cf.* Ref. 8).

It was also possible to determine GSH in the reduced sample with lactoylglutathione lyase, which offers a more specific determination. The results were in accordance with those obtained by the sulfhydryl determination, but were less reproducible and often lower by about 10 %. The lactoylglutathione lyase assay (*cf.* Ref. 15) was based on the thiolester absorption at 240 nm,[16] which was measured in a system consisting of 2.5 ml of 0.2 M phosphate buffer, pH 6.8; 0.5 ml of the reduced sample; and 20 μl of lactoylglutathione lyase. The extinction was read, and 20 μl of 0.1 M methylglyoxal was added. The increase of the extinction was determined and corrected for the absorption of methylglyoxal.

When it had been established (*cf.* Table 5) that $GSSO_3H$ was reduced to GSH, it was sometimes found more convenient to determine the formation of GSH instead of the decomposition of $GSSO_3H$. In this case, however, it was necessary to perform the reaction anaerobically, since otherwise the GSH formed was rapidly oxidized. The sulfhydryl analysis was then carried out directly on a nitrogen-bubbled supernatant of the acid denatured reaction mixture. The values were corrected for the sulfhydryl content of the homogenate.

$NADP^+$ was determined spectrophotometrically at 327 nm as the cyanide complex, which was assumed to have an extinction coefficient of 5.9 $mM^{-1}cm^{-1}$.[17] An aliquot (0.2 ml) of the supernatant after the metaphosphoric acid denaturation was added to 1.8 ml of 0.2 M Na_2HPO_4 in a cuvette. The extinction was determined before and after the addition of 0.5 ml of 1 M KCN. A correction for the absorption of the KCN solution was applied.

Paper electrophoresis and thin-layer chromatography were carried out as previously described.[18]

RESULTS AND DISCUSSION

Assay of $GSSO_3H$. It was necessary to develop a procedure for the quantitative determination of $GSSO_3H$ before the present investigation could be carried out. Thus, we attempted to convert $GSSO_3H$ into GSSG (which can readily be assayed with glutathione reductase) by treatment with GSH under different conditions

$$GSSO_3^- + GSH \rightarrow GSSG + HSO_3^-$$

However, this could not be quantitatively achieved. Therefore, it was decided to reduce $GSSO_3H$ and determine the GSH formed. Although the reduction could be carried out with sodium borohydride, it was not possible to determine

GSH by the DTNB method, since sulfite interfered. Neither was the lactoylglutathione lyase assay suitable because of its low sensitivity in combination with the dilution of the sample, caused by the addition of a large volume of acid to destroy the borohydride remaining. The use of a more concentrated acid decreased the yield of thiol.

Using the electrolytic reduction method of Dohan and Woodward,[13] Sörbo has found that thiosulfate esters interfere with the determination of disulfides.[19] The application of this method to the $GSSO_3H$ assay was indeed found to give a quantitative reduction. In order to determine GSH by the enzymatic method, however, it was necessary to substitute the sulfosalicylic acid (which has a strong UV absorption) in the cathode chamber for metaphosphoric acid. By nitrogen bubbling through the reduced sample it was possible to eliminate sulfite, after which GSH could be determined with DTNB.

The conditions were worked out for a $GSSO_3H$ content not exceeding about 3 μmoles in the reduction vessel. With this amount 40 min was sufficient for the reduction (Fig. 1). It was established that a tenfold excess of GSH did not interfere in the assay, when the acid denaturation was performed within 10 min after the addition of GSH.

The discrepancy between the lactoylglutathione lyase assay and the DTNB method for GSH determination (*cf.* Table 2) was variable (mostly less than 15 %). As yet we have not been able to solve this inconsistency, which was not encountered when measurements were made on pure GSH samples. It could not be explained by sulfite remaining in the sample, since prolonged nitrogen bubbling did not lower the value obtained with the DTNB

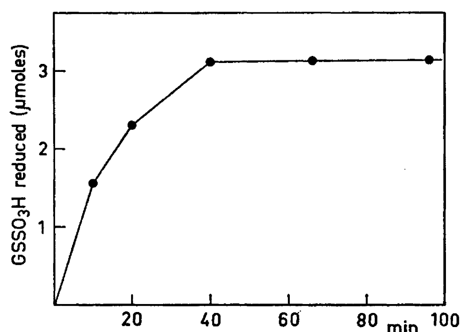

Fig. 1. Electrolytic reduction of $GSSO_3H$ in the presence of biological material.
$GSSO_3H$ was added to 5 ml of human plasma, which was then treated with 2 ml of metaphosphoric acid and centrifuged. A portion of the centrifugate was subsequently chromatographed on a Dowex 50 W (H^+) column and reduced by the electrolytic method as described in Methods. Samples were withdrawn from the reduction vessel at different times and were analyzed by the DTNB method.

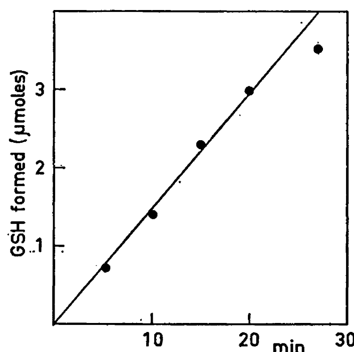

Fig. 2. Enzymatic reduction of $GSSO_3H$ as a function of time.
The incubations were carried out anaerobically as described in Methods, and the activity was measured by the GSH formation.

method. As the two methods gave essentially the same result, the DTNB assay, which is more reproducible and has a higher sensitivity, was usually employed.

It should be pointed out that the assay in combination with the ion-exchange chromatography on Dowex 50 W is generally applicable to any of the four types of $GSSO_3H$ destruction outlined in the introduction. In types (1), (2), and (4) the peptide moiety will not pass the ion-exchanger unless an incorporation of a component containing a very acidic group such as phosphoryl or sulfuryl takes place. In the latter case interference will be obtained only if the compound contains a sulfhydryl group or can be reduced to a thiol. If thiosulfate should be liberated, it will decompose into sulfur and sulfite, the latter of which will be removed by the nitrogen bubbling. In type (3) the acidic products will not react positively in the GSH determination.

From the foregoing discussion it is clear that although the assay worked out is applicable to most of the conceivable transformations of $GSSO_3H$, it cannot be applied to the determination of the $GSSO_3H$ content of biological material. Even if the lactoylglutathione lyase were absolutely specific for GSH — it reacts with the naturally occurring homoglutathione [20] and some other thiols related to GSH (*cf.*, *e.g.*, Ref. 21) — the assay would not be specific for $GSSO_3H$, as the mixed disulfide of coenzyme A and GSH, for instance, will pass the ion-exchanger as well as be reduced to GSH. Nevertheless, an upper limit of the $GSSO_3H$ content may be obtained. Thus, we have found apparent values of 0.40 and 0.35 μmole/g wet weight for rat liver and rat small intestine, respectively. Determinations on calf lens [1] and rat small intestine [2] have previously given values of 0.1 and 0.16 μmole/g, respectively. If the latter determination has been performed with a good recovery, we may conclude that there is present, at least in the small intestine, a considerable amount of other acidic GSH derivatives, which can be reduced to GSH.

Enzymatic reduction of $GSSO_3H$. When $GSSO_3H$ was incubated with a rat liver homogenate in the absence of any added cofactor, it was found to be essentially stable. The addition of NADPH, however, caused a striking decomposition of $GSSO_3H$ to occur (Table 2). It was found that boiling destroyed the activity, whereas dialysis for 15 h against 0.01 M phosphate, pH 7.5, did

Table 2. Enzymatic reduction of $GSSO_3H$.

The reduction system contained in 2 ml: 0.5 ml of a 20 % rat liver homogenate (not centrifuged); 5.2 μmoles of $GSSO_3H$; 4.5 μmoles of NADPH; and 40 mM phosphate buffer, pH 7.5. The reaction was run for 60 min at 30°. Determinations of $GSSO_3H$ were carried out as described in Methods.

Experiment	$GSSO_3H$ remaining (μmoles)		$GSSO_3H$ decomposed (μmoles)
	DTNB method	lactoylglutathione lyase method	DTNB method
1. (no NADPH)	5.18	4.30	0.0
2.	1.18	0.87	4.0
3. (enzyme boiled for 3 min)	5.07	4.73	0.1

Table 3. Intracellular distribution of $GSSO_3H$ reducing activity.
7 ml of a 20 % rat liver homogenate in 0.14 M KCl was centrifuged at 80 000 *g* for 60 min. The precipitate was washed once with 0.14 M KCl, centrifuged, and then suspended in 0.14 M KCl to give a final volume of 7 ml. Samples (0.5 ml) of the different fractions were incubated for 20 min under the conditions described in Table 2. $GSSO_3H$ was determined by the DTNB method.

Fraction	$GSSO_3H$ decomposed (μmoles)
Homogenate	2.3
Supernatant	2.4
Precipitate	0.0

not cause any change. Centrifugation studies indicated that all activity was confined to the soluble fraction of the homogenate (Table 3). When the reaction was carried out under the conditions described in Methods, its progress was linear for 20 min (Fig. 2). In the assay system described in Table 2 (where too much enzyme was added to allow the reaction to be zero order), NADH or ascorbate could be substituted for NADPH, giving a $GSSO_3H$ destruction somewhat less than 50 % of that with NADPH. When the reaction proceeded linearly, however, these cofactors gave a very low activity (Table 4). We believe that the stimulative effect in the former case was due to a transhydrogenase activity in the homogenate.

Table 4. Cofactor requirement of the $GSSO_3H$ reduction.
The reaction conditions were as described in Methods, except for the cofactor concentrations which were 2.12 mM for NADH and NADPH, and 21.2. mM for ascorbate. The reaction was allowed to proceed for 15 min in a nitrogen atmosphere. GSH was determined by the DTNB method.

Cofactor	GSH formed (μmoles)
NADPH	1.83
NADH	0.11
Ascorbate	0.01

As the NADPH-dependent destruction of $GSSO_3H$ thus demonstrated showed similarities with the S-sulfoglutathione reductase in peas,[6] it was of interest to investigate whether GSH was a product of the reaction. It was found, however, that under conditions where 2.5 μmoles of $GSSO_3H$ was consumed only 0.6 μmole of GSH appeared. Since it was known that GSH can be rapidly oxidized in rat liver homogenates,[22] we decided to perform the reaction anaerobically. Good agreement between $GSSO_3H$ consumption and GSH formation was thus obtained (Table 5). Determination of $NADP^+$ (Table 5) also fitted to the formula:

$$GSSO_3^- + NADPH + H^+ \rightarrow GSH + HSO_3^- + NADP^+$$

Table 5. Stoichiometry of the $GSSO_3H$ reduction.
The sample was incubated anaerobically for 15 min under the conditions described in Table 2. After incubation and denaturation, the centrifugate was analyzed for GSH, $NADP^+$, and, after ion-exchange chromatography, for $GSSO_3H$ content. The control, which was identical except for omission of NADPH, was not incubated.

Component determined	μmoles
GSH formed	3.3
$GSSO_3H$ reduced	3.7
$NADP^+$ formed	3.9

However, this stoichiometry is not only fulfilled by a reaction catalyzed by S-sulfoglutathione reductase, but could also be obtained as the sum of the reactions

$$GSSO_3^- + GSH \rightarrow GSSG + HSO_3^-$$

and

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The first reaction proceeds nonenzymatically, but should in all probability be enzyme catalyzed in the cell. The second reaction is catalyzed by glutathione reductase, which is very active in rat liver.[23] If these two reactions were taking place, however, GSH should act as a cofactor in the $GSSO_3H$ reduction. Since dialysis did not diminish the activity, this did not seem to be the case. Neither did the addition of 0.6 μmole of GSH to the reaction mixture stimulate the $GSSO_3H$ reduction.

In order to differentiate between an S-sulfoglutathione reductase catalysis and a reduction involving glutathione reductase, competition experiments were also carried out (Table 6). It was found that the sum of the activities of the individual reactions of $GSSO_3H$ and GSSG exceeded that with both substrates in admixture. No conclusions can be drawn from these results, however, as $GSSO_3H$ is an inhibitor of glutathione reductase.

Table 6. Competition between $GSSO_3H$ and GSSG reductions.
Assay mixtures were prepared as outlined in Methods. In (2) 6.6 μmoles of GSSG was substituted for $GSSO_3H$, and in (3) both substrates were added. The controls did not contain NADPH and were treated with metaphosphoric acid before addition of the enzyme. Thus, (1) demonstrated the $GSSO_3H$ reducing activity, (2) the glutathione reductase activity, and (3) both activities in admixture. The GSSG reduction was assumed to be equivalent to half the GSH production (in (3) corrected for $GSSO_3H$ reduction). The reactions were continued for 10 min in a nitrogen atmosphere.

Compound determined (μmoles)	1	2	3
GSH formed	1.3	4.7	3.8
$GSSO_3H$ reduced	1.5	—	0.9
GSSG reduced	—	2.4	1.5

Furthermore, support for an enzymatic reaction not involving glutathione reductase was obtained by inhibition experiments with sulfite. Thus, 5 mM sulfite in the standard system inhibited 60 % of the activity with $GSSO_3H$, whereas an apparent activation was found when 3 mM GSSG served as the substrate. The glutathione reductase activity was measured by the sulfhydryl formation, and the apparent stimulation was probably due to concomitant sulfitolysis of GSSG (which releases GSH). It was demonstrated by the spectrophotometric assay of a purified porcine glutathione reductase (lacking S-sulfoglutathione reductase activity) that 5 mM sulfite caused a 10 % inhibition at 3 and 0.2 mM concentrations of GSSG and NADPH, respectively. The NADPH concentration is lower than that of the standard assay, however, but it was shown that increasing the NADPH concentration caused a decrease of the inhibition.

It was also found that acidic derivatives of GSH were weakly inhibitory when tested in the assay system. At a 3 mM concentration only GSO_2SH caused a significant effect. The glutathione reductase activity was not inhibited at this GSO_2SH concentration (Table 7). This conforms to our hypothesis

Table 7. Effect of acidic GSH derivatives on $GSSO_3H$ and GSSG reductions. The incubations were carried out anaerobically for 15 min as described in Methods. When glutathione reductase activity was measured, 2 mM GSSG served as the substrate. GSH derivatives (6 μmoles) were added as the sodium salts. The activities, determined as GSH formation, are expressed in relative values, where the activities without additions have been set equal to 1.

Addition	Relative activity	
	$GSSO_3H$ reduction	GSSG reduction
GSO_2H	1.00	0.91
GSO_3H	0.90	0.94
GSO_2SH	0.64	1.01

that an S-sulfoglutathione reductase should contain an ionic binding site for the thiosulfate group of $GSSO_3H$, capable of binding anionic groups of related GSH derivatives. A corresponding site should be absent in glutathione reductase. From the fact that the two reductions were affected differently by the acidic GSH analogues, it may be inferred that the $GSSO_3H$ reduction was not catalyzed by glutathione reductase.

Finally, it was established that the activity was sensitive towards *p*-hydroxymercuribenzoate and iodoacetamide (Table 8).

Table 8. Inhibition of $GSSO_3H$ reduction by "sulfhydryl reagents".
The enzyme was preincubated for 20 min with the inhibitors and NADPH, and the reaction was then started by the addition of $GSSO_3H$. In the control the enzyme was preincubated with NADPH only. Other conditions were as described in Methods. The reaction was stopped after 10 min, and the activity was determined by the electrolytic reduction method.

Inhibitor		Inhibition (%)
p-Hydroxymercuribenzoate	0.1 mM	100
	0.01 mM	63
Iodoacetamide	0.1 mM	100

We conclude that there is present in rat liver an enzymatic activity capable of reducing $GSSO_3H$ in the presence of NADPH. This activity is distinct from glutathione reductase and evidently similar to the S-sulfoglutathione reductase of pea tissues.[6]

REFERENCES

1. Waley, S. G. *Biochem. J.* **71** (1959) 132.
2. Robinson, H. C. and Pasternak, C. A. *Biochem. J.* **93** (1964) 487.
3. Sörbo, B. *Acta Chem. Scand.* **12** (1958) 1990.
4. Coletta, M., Benerecetti, S. A. and De Marco, C. *Italian J. Biochem.* **10** (1961) 244.
5. Sörbo, B. *Acta Chem. Scand.* **18** (1964) 821.
6. Arrigoni, O. and Rossi, G. *Biochim. Biophys. Acta* **46** (1961) 121.
7. Eriksson, B. *Acta Chem. Scand.* **21** (1967) 1119.
8. Ellman, G. L. *Arch. Biochem. Biophys.* **82** (1959) 70.
9. Swan, J. M. In Benesch, R. *et al.*, (Eds.), *Sulfur in Proteins*, Academic, New York 1959, p. 3.
10. Kolthoff, I. M. and Sandell, E. B. *Textbook of Quantitative Inorganic Analysis*, 3rd Ed., Macmillan, New York 1963, p. 639.
11. Calam, D. H. and Waley, S. G. *Biochem. J.* **85** (1962) 417.
12. Eriksson, B. and Sörbo, B. *Acta Chem. Scand.* **21** (1967) 958.
13. Dohan, J. S. and Woodward, G. E. *J. Biol. Chem.* **129** (1939) 393.
14. Eriksson, B. *Acta Chem. Scand.* **20** (1966) 1178.
15. Klotzsch, H. and Bergmeyer, H.-U. In Bergmeyer, H.-U., (Ed.), *Methods of Enzymatic Analysis*, 2nd Printing, Verlag Chemie, Weinheim/Bergstr. 1965, p. 363.
16. Racker, E. *J. Biol. Chem.* **190** (1951) 685.
17. Siegel, J. M., Montgomery, B. A. and Bock, R. M. *Arch. Biochem. Biophys.* **82** (1959) 288.
18. Eriksson, B. and Eriksson, S. A. *Acta Chem. Scand.* **21** (1967) 1304.
19. Sörbo, B. *Arch. Biochem. Biophys.* **98** (1962) 342.
20. Carnegie, P. R. *Biochem. J.* **89** (1963) 471.
21. Wieland, T., Pfleiderer, G. and Lau, H. H. *Biochem. Z.* **327** (1956) 393.
22. Pinto, R. E. *Biochem. J.* **79** (1961) 43.
23. Rall, T. W. and Lehninger, A. L. *J. Biol. Chem.* **194** (1952) 119.

Received August 29, 1967.